ATTACHMENT J

Published Papers on Toxicological Effects of PCBs and Other Health-Related Documents

J-1 Berkshire Medical Center. 2012. *2012 Report on Cancer.* Available from Berkshire Medical Center.

J-2 Carlson, E.A., C. McCulloch, A. Koganti, S.B. Goodwin, T.R. Sutter, and J.B. Silkworth. 2009. Divergent transcriptomic responses to aryl hydrocarbon receptor agonists between rat and human primary hepatocytes. *Toxicological Sciences* 112:257-272.

J-3 Carlson, E.A., J.D. Schell, S. Bodreddigari, T.R. Sutter, and C.H. Sutter. 2012, Species differences in PCB toxicodynamics and toxicokinetics relevant to the Aroclor 1254 reference dose. *Organohalogen Compounds* 74:1059-1062.

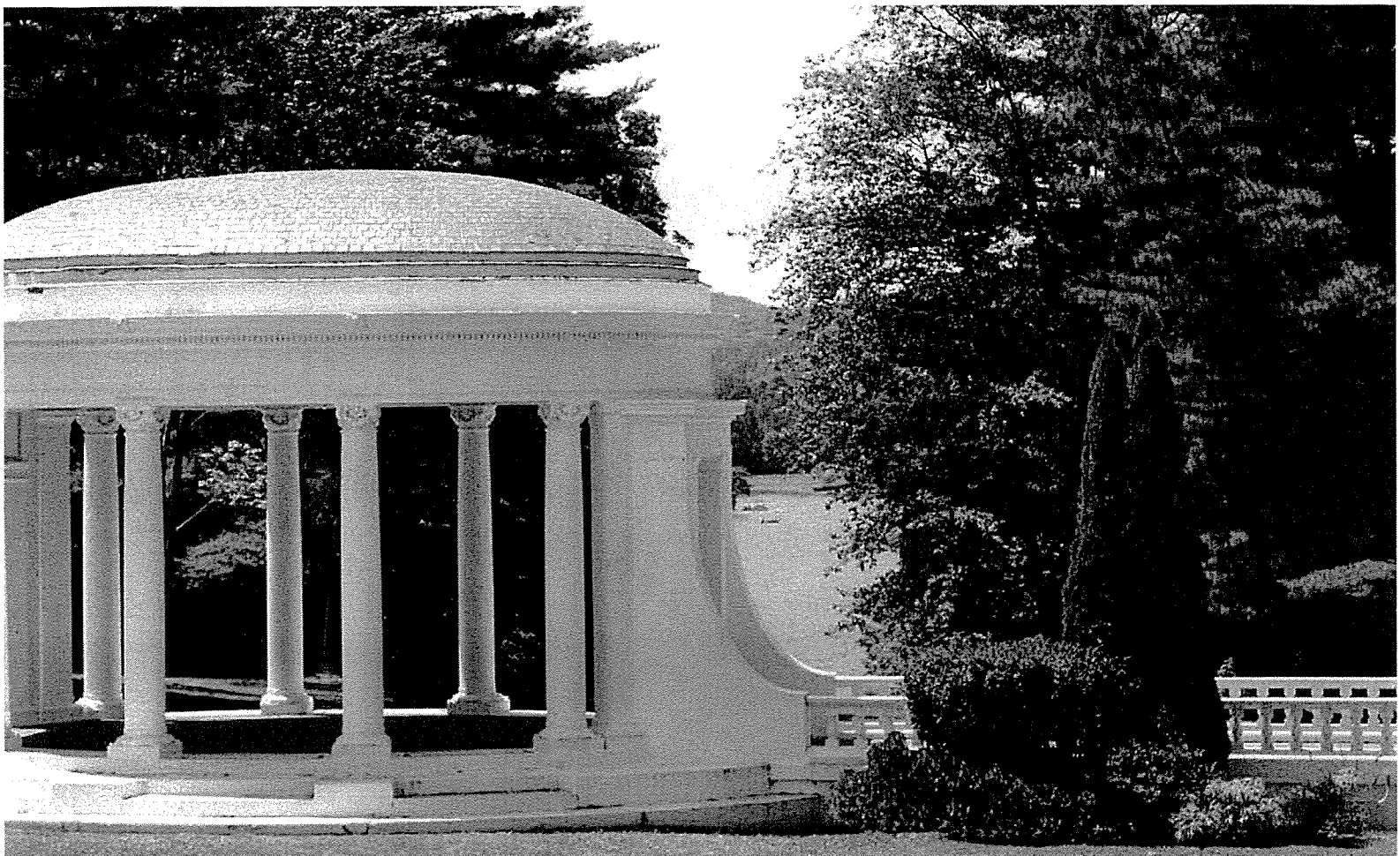
J-4 Golden, R., J. Doull, W. Waddell, and J. Mandel. 2003. Potential human cancer risks from exposure to PCBs: A tale of two evaluations. *Critical Reviews in Toxicology* 33:543-580.

J-5 Golden, R., and R. Kimbrough. 2009. Weight of evidence evaluation of potential human cancer risks from exposure to polychlorinated biphenyls: An update based on studies published since 2003. *Critical Reviews in Toxicology* 39:299-331.

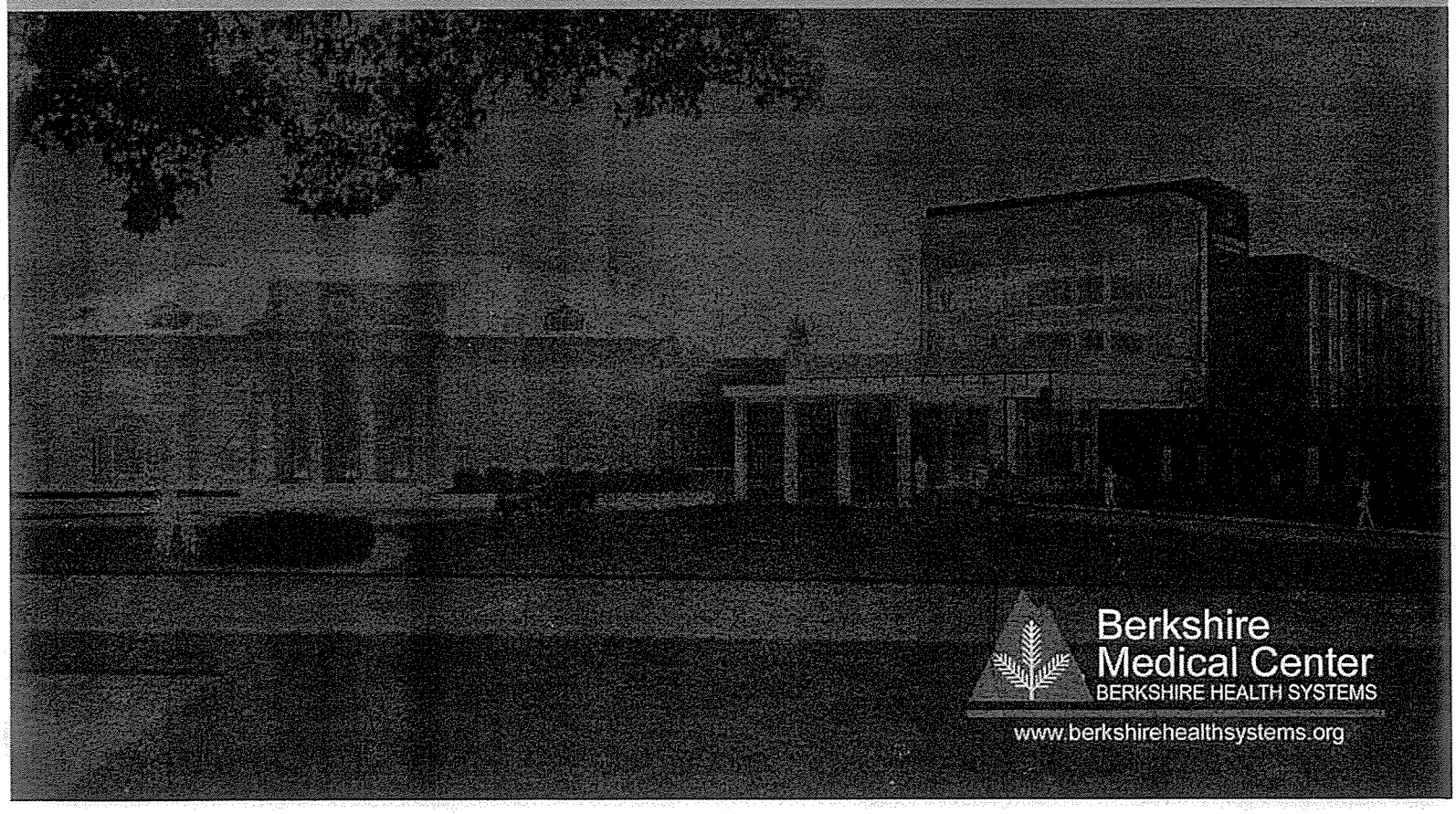
J-6 Silkworth J.B., A Koganti, K. Illouz, A. Possolo, M. Zhao, and S.B. Hamilton. 2005. Comparison of TCDD and PCB CYP1A induction sensitivities in fresh hepatocytes from human donors, Sprague-Dawley rats, and rhesus monkeys and HepG2 cells. *Toxicological Sciences*, 87:508-519.

J-7 Westerink, W.M., J.C. Stevenson, and W.G. Schoonen. 2008. Pharmacologic profiling of human and rat cytochrome P450 1A1 and 1A2 induction and competition. *Arch. Toxicol.* 82:909-921.

Attachment J-1



2012 Report on Cancer



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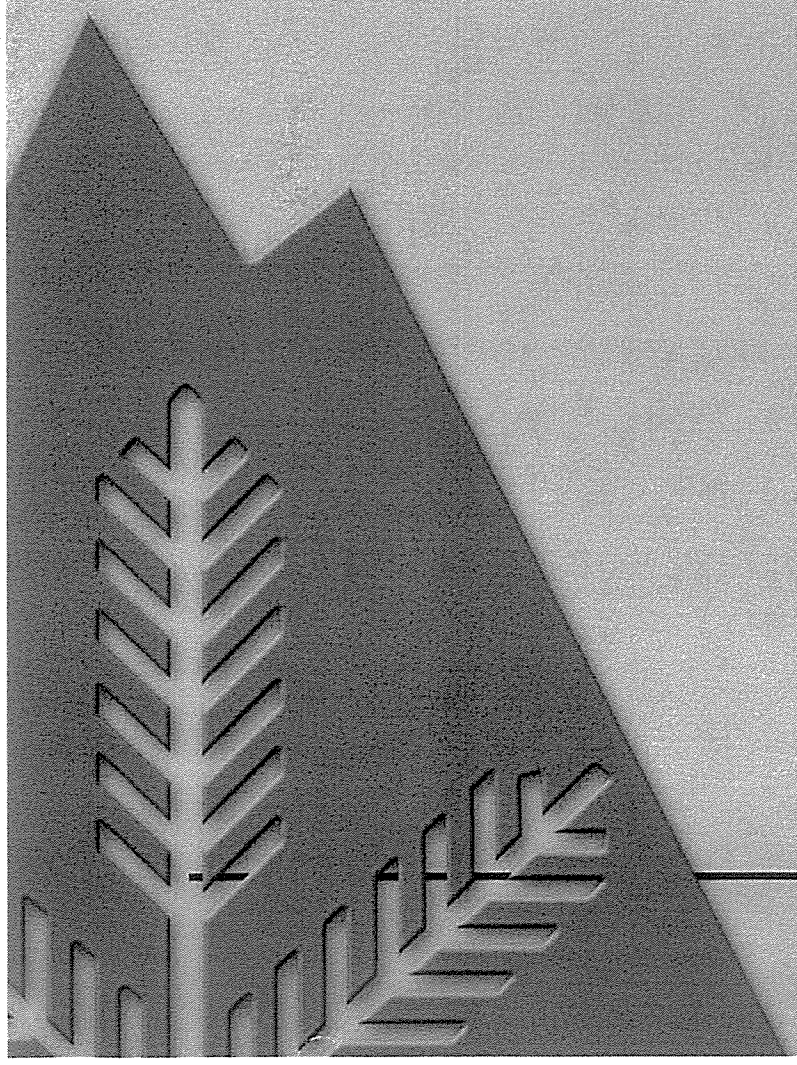
The 2012 CareChex® awards referred to in this report are based on
FFY 2008-2010 U.S. Medicare Provider Analysis and Review (MedPAR) data.



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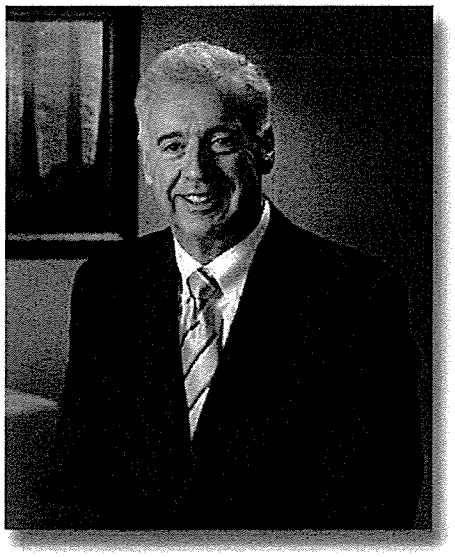
CANCER CARE 2012



from David E. Phelps,
President and CEO, Berkshire Health Systems

Berkshire Medical Center has a long history of providing a high level of quality care for those in our community affected by cancer, and this has been validated year in and year out by independent national organizations.

The nation's leading accreditation agency for cancer care, the Commission on Cancer of the American College of Surgeons, has twice recognized BMC with its Outstanding Achievement Award, an honor given only to select cancer care programs in the US. BMC's cancer program is among the Top 4% of Hospitals in the US for the treatment of cancer, according to CareChex, a leading independent healthcare ratings organization.

This level of recognition reflects the excellent outcomes we have achieved and is a blueprint for our continual commitment to providing the most advanced and skilled care possible.

This outstanding care is the result of a collaborative approach by highly skilled cancer care physicians, including medical and radiation oncologists, pathologists, radiologists, and a surgical oncologist, teamed with oncology nurses and therapists, rehabilitation specialists and wellness and mental health professionals. It is a program that focuses on all aspects of cancer, from preventive care and screening to aggressive treatment and support services for the patient and their loved ones, all aimed at enhancing survivorship.

As we move forward, we maintain a constant emphasis on measuring and improving the Quality of Cancer Care we provide and are committed to ongoing investments to ensure that we are at the forefront of cancer care not only locally, but nationally.

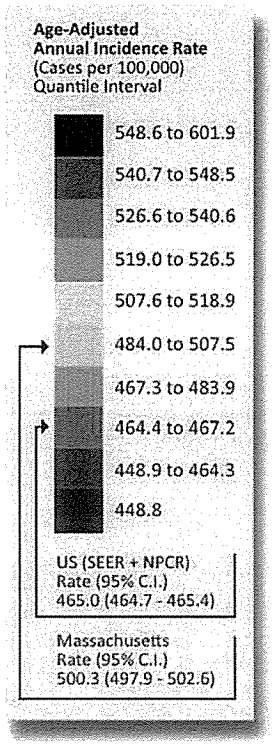
Berkshires are in mid-range of cancer rates statewide

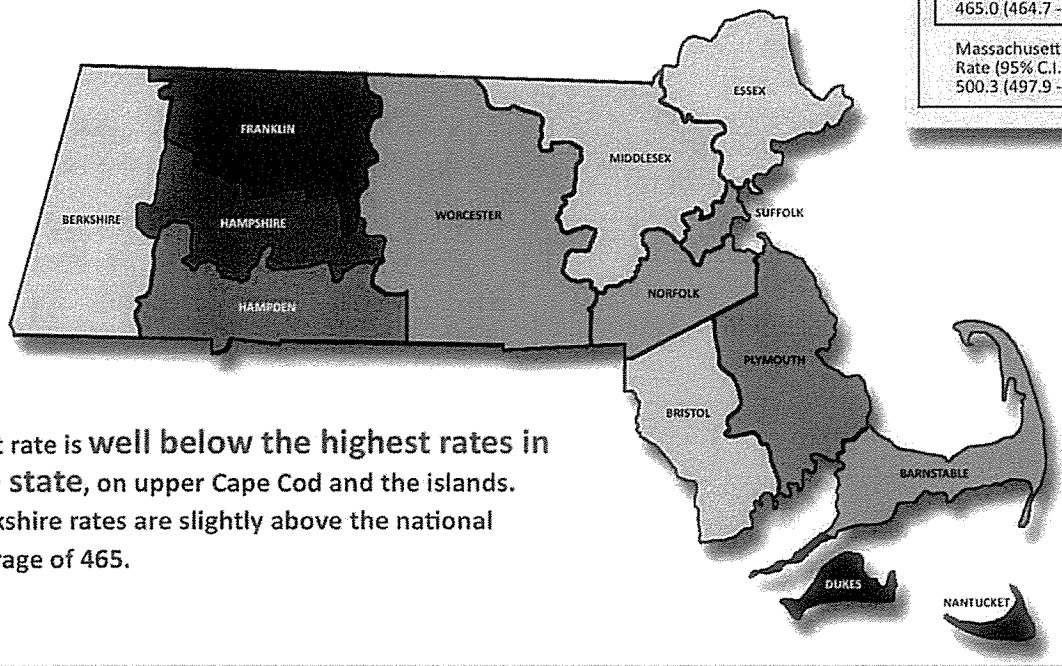
By Harvey Zimbler, M.D.
Medical Oncologist,
and **Louis Gainer,**
Quality Analyst

Though some have wondered aloud over the years whether Berkshire County and Pittsfield in particular might have a higher rate of cancer than most other parts of Massachusetts and the nation, the latest statistics from the National Cancer Institute of the National Institutes of Health actually show that is not the case, has not been for many years and there is no indication the rates will rise.

In fact, Berkshire County lands squarely in the middle range of cancer incidence rates for all counties in Massachusetts, which itself has the lowest incidence of cancer in the Northeast U.S. The county's annual incidence rate for all forms of cancer is between 507.6 and 518.9 cases per 100,000 residents.

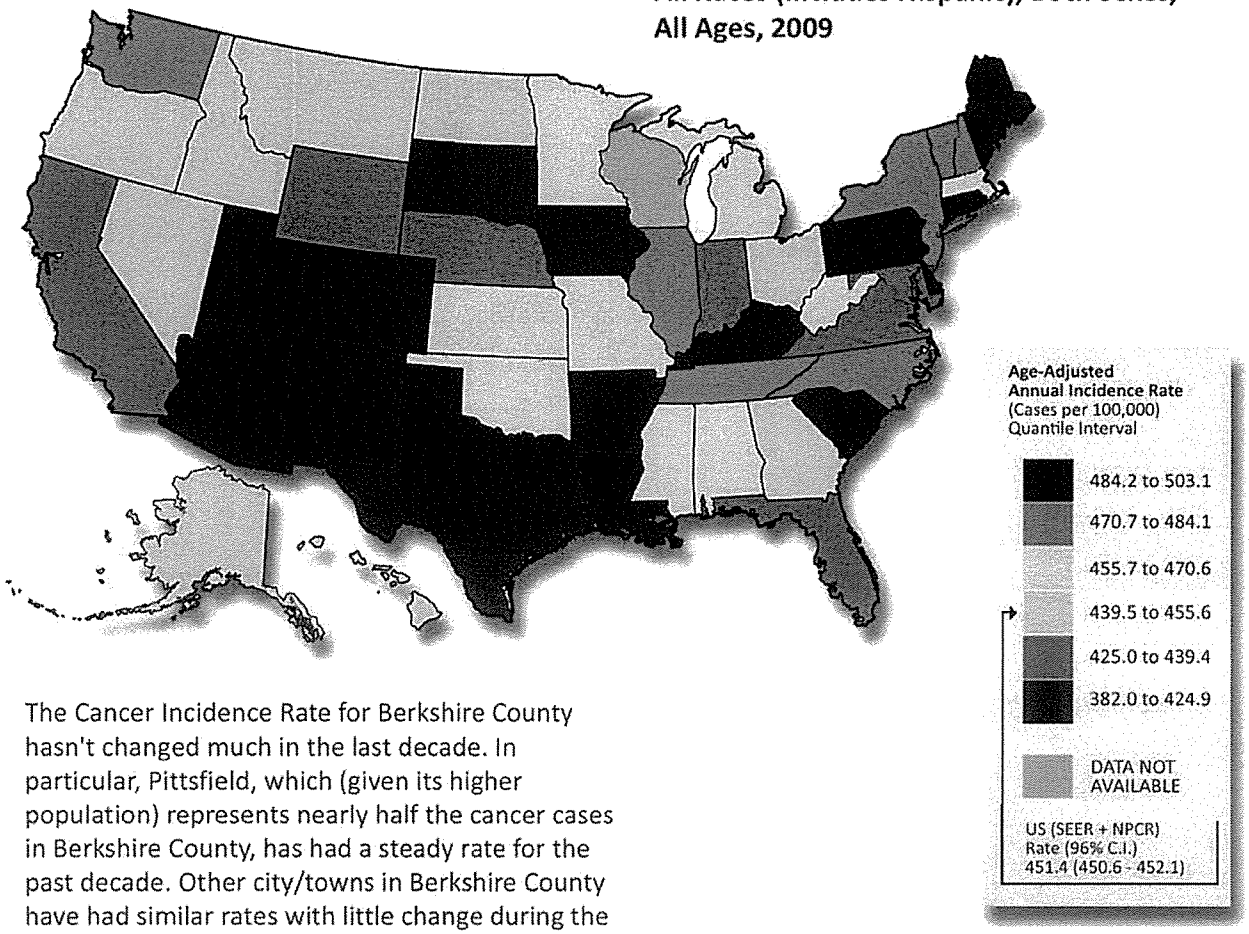
Incident Rates for Massachusetts, 2005 - 2009 All Cancer Sites, All Races, Both Sexes, All Ages





That rate is **well below the highest rates in the state,** on upper Cape Cod and the islands. Berkshire rates are slightly above the national average of 465.

Latest Incidence Rates for United States
All Cancer Sites
All Races (includes Hispanic), Both Sexes,
All Ages, 2009



The Cancer Incidence Rate for Berkshire County hasn't changed much in the last decade. In particular, Pittsfield, which (given its higher population) represents nearly half the cancer cases in Berkshire County, has had a steady rate for the past decade. Other city/towns in Berkshire County have had similar rates with little change during the last 10 years. There are no alarming indicators about cancer rates in Berkshire County.

Still, BMC continues to improve in early detection, early and aggressive execution of care plans and increasing life expectancy of cancer survivors as we fight the battle locally in the global war on cancer.

Data in this report is derived from:
The National Cancer Institute (NCI) , part of the National Institutes of Health (NIH), which is one of 11 agencies that compose the Department of Health and Human -Services (HHS) - Surveillance, Epidemiology and End Results (SEER) Program and THE COMMONWEALTH OF MASSACHUSETTS DEPARTMENT OF PUBLIC HEALTH and Massachusetts Cancer Registry (MCR) and Massachusetts Community Health Information Profile - (MassCHIP)

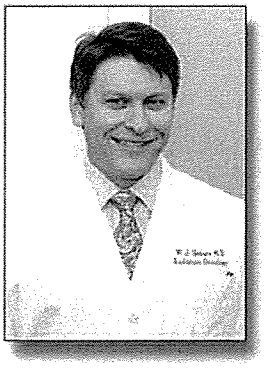
SOME TERMS EXPLAINED

(C.I.) Cancer Incidence Rate. The number of new cancers occurring in a population during a year, usually expressed as the number of cancers per 100,000 people.

95 Confidence Interval. In the world of statistics and journals of medicine, this is a generally used and accepted expression of a very high level of confidence in the data.

Age-Adjusted Rate. Age-Adjusted Population measures the rates occurring in the different age segments, e.g, over 65, and adds them to get a total rate, which allows one to compare across different communities by accounting for any variation caused by a difference in the age distribution.

5-Year Survival Rates at BMC consistently better than US averages for all leading types of cancer



By Wade Gebara, M.D., Radiation Oncologist, Chairman, Cancer Committee, and Michael DeLeo, M.D., Medical Oncologist

There's a distinct 'survival advantage' to being treated for cancer at BMC.

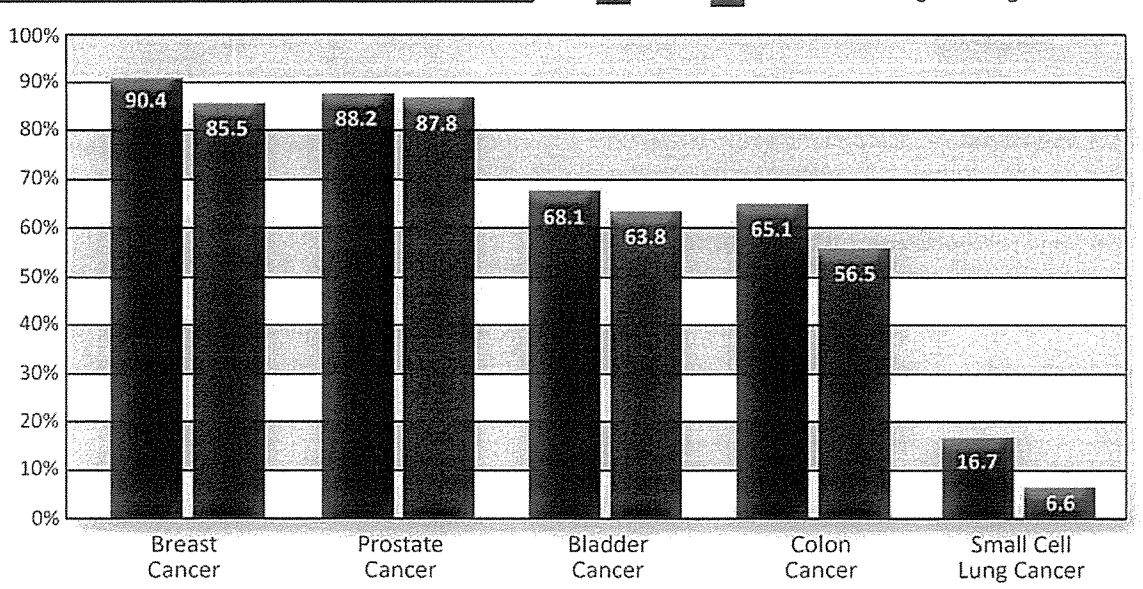
A comparison of survival data of patients treated at BMC versus the leading national cancer database for the five most frequently diagnosed cancers – breast, prostate, bladder, colon and small-cell lung cancer – reveals that our local cancer program is performing consistently better than average in all groups at the critical point of five years post-diagnosis.

Those outcomes are part of why BMC has twice received the **Outstanding Achievement Award** from the **Commission on Cancer** of the American College of Surgeons. BMC has earned that honor each time it has been eligible since the award's inception in 2004. In the most recent round, BMC's cancer program was one of only 82 in the U.S. and five in New England to be honored with this national recognition.

Along with our accreditation and awards from the Commission on Cancer, these survival outcomes reflect the high standards of care and commitment to excellence by a multi-disciplinary team of physicians, nurses and allied health professionals at BMC.

It was the CoC that compiled the five-year survival statistics:

Cancer 5-Year Survival Rates Comparing BMC and American College of Surgeons Commission on Cancer



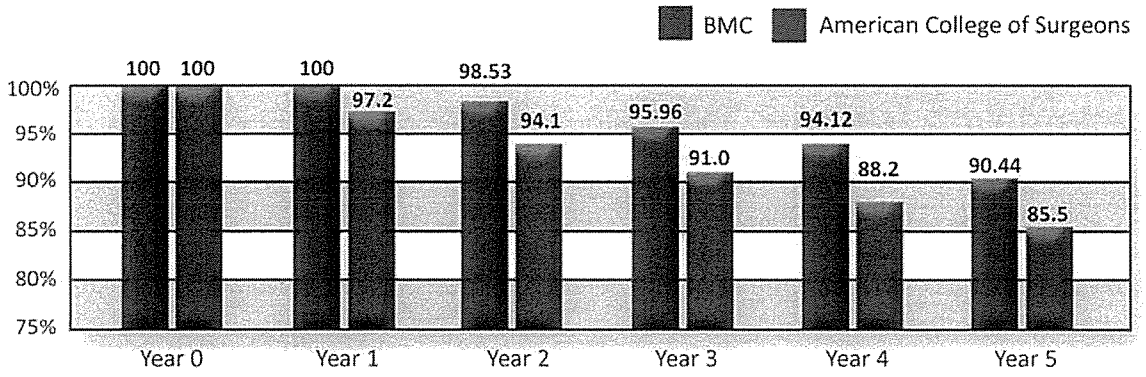
A year-by-year look at the BMC Survival Advantage

BMC's performance in cancer care also has drawn high marks from CareChex, a division of The Delta Group, the nation's premier independent rating program for hospitals. Their most recent scorecard placed BMC 140th out of over 4,500 hospitals nationwide in terms of outcomes in the treatment of malignancies, based on measures for mortality, complications and patient safety.

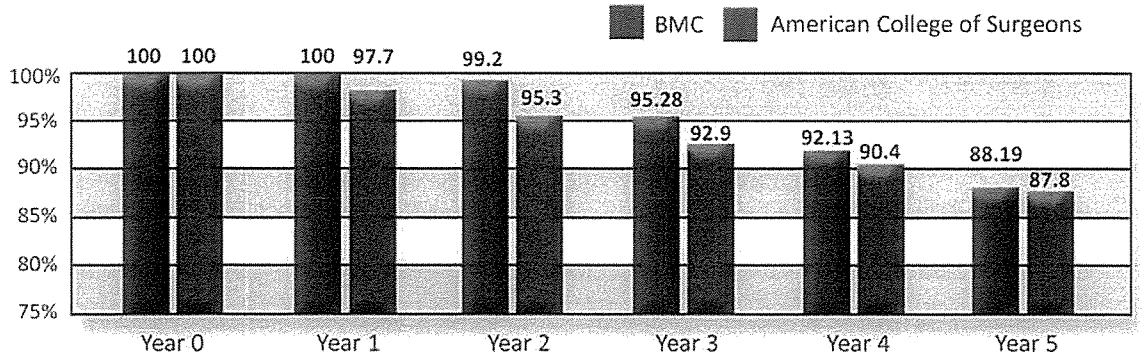
The following five disease-specific graphs compare the survival advantage of being treated at BMC versus the national average. The national average is made up of data from all 1,500 hospitals that care for more than 70% of patients with cancer in the U.S.

You will see a survival advantage for each of the first five years following diagnosis.

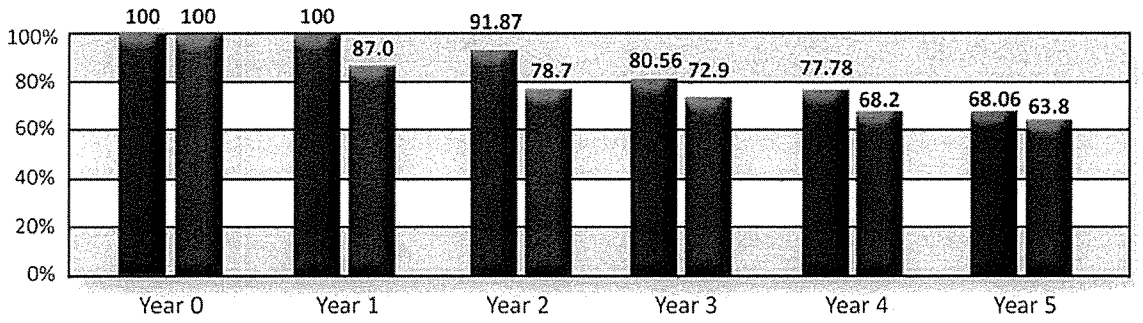
BREAST CANCER 5-Year Survival Rates (%) Comparing BMC and American College of Surgeons Commission on Cancer



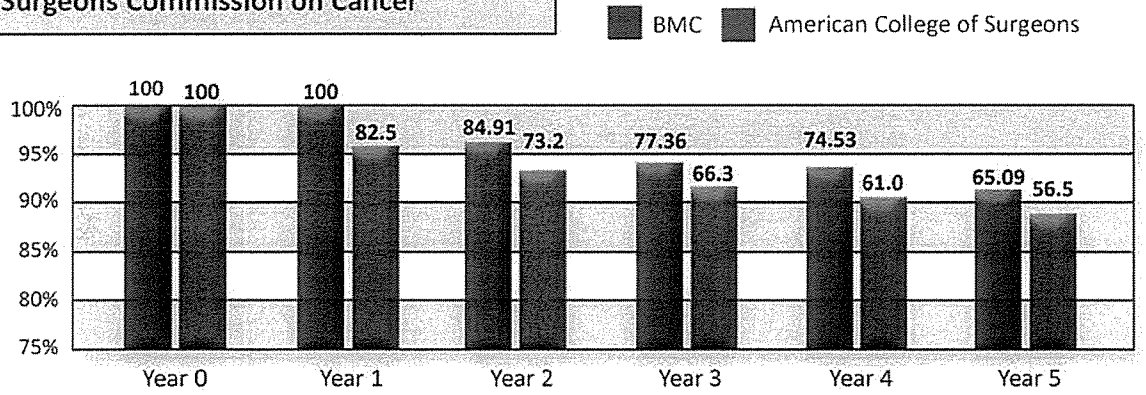
PROSTATE CANCER 5-Year Survival Rates (%) Comparing BMC and American College of Surgeons Commission on Cancer



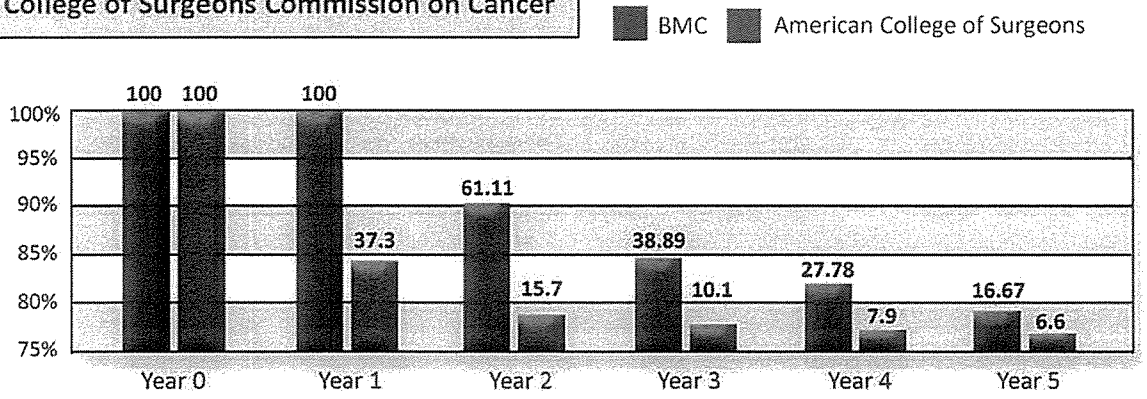
BLADDER CANCER 5-Year Survival Rates (%) Comparing BMC and American College of Surgeons Commission on Cancer



COLON CANCER 5-Year Survival Rates (%) Comparing BMC and American College of Surgeons Commission on Cancer



SMALL-CELL LUNG CANCER 5-Year Survival Rates (%) Comparing BMC and American College of Surgeons Commission on Cancer



QUALITY OF CARE

There's an essential link between the quality of cancer care and successful outcomes of treatment. While even the best of efforts don't always guarantee eradication or survival, our determination to adhere to best practices and embrace cutting-edge methods of effective, compassionate cancer care have made a genuine difference. In this year's report, we focus on our scorecard for two of the most common forms of cancer: breast and colorectal.

BHS scores high on quality care, including breast and colorectal

By Jessica Krochmal, M.D.
Pathologist, Cancer Liaison Physician

The accreditation standards of the Commission on Cancer require the BHS Cancer Committee to review and monitor the reported quality of care provided to breast and colorectal patients. Our Cancer Programs Practice Profile Report (CP3R) includes three breast measures and three colorectal measures for 2008-2010.

3 Breast Measures

Radiation therapy is administered within 1 year (365 days) of diagnosis for women under age 70 receiving breast conserving surgery for breast cancer.
RESULT: 100%

Chemotherapy is considered or administered within 4 months (120 days) of diagnosis for women under 70 with AJCC Stage 1 (breast cancers greater than 1cm in size, but not greater than 2cm, with no regional lymph node metastasis and no distant metastasis), or Stage II or III breast cancer for which estrogen and progesterone assays are negative.
RESULT: 83-100%

Tamoxifen or third generation aromatase inhibitor is considered or administered within 1 year (365 days) of diagnosis for women with AJCC Stage 1 (breast cancers greater than 1cm in size, but not greater than 2cm, with no regional lymph node metastasis and no distant metastasis), or Stage II or III breast cancer for which estrogen and progesterone assays are positive.
RESULT: 96-100%

3 Colorectal Measures

Adjuvant Chemotherapy considered or administered within 4 months (120 days) of diagnosis for patients under the age of 80 with AJCC Stage III (lymph node positive) colon cancer.
RESULT: 100%

At Least 12 Regional Lymph Nodes are removed and pathologically examined for resected colon cancer. *(Pre-operative therapy may limit the availability of lymph nodes.)*
RESULT: 89-100%

Radiation Therapy is considered or administered within 6 months (180 days) of diagnosis for patients under the age of 80 with clinical or pathologic AJCC Stage IIB (Cancer has spread beyond the colon wall into nearby organs and/or through the tissue lining the colon, or Stage III receiving surgical resection for rectal cancer.
RESULT: 75-100%

All discordant cases are reviewed and analyzed by the committee to identify trends which could lead to process improvements for our patients. BMC results have always been within the 95% confidence interval specified by the American College of Surgeons.

NOTE: While the result target is always 100%, there often are circumstances where a patient simply chooses to forego or is not a candidate for a specific treatment at a specific time due to other mitigating health or medical factors.

***The AJCC notation** in these measures refers to the staging or classification system developed by the **American Joint Committee on Cancer** for describing the extent of disease progression in cancer patients.*

BMC hits top percentiles in Northeast for critical measures of breast cancer care

By Michael DiSiena, D.O.
Surgical Oncologist

In the eyes of a leading national organization which has established a series of exacting quality standards for breast cancer care, Berkshire Medical Center ranks among the best providers in the Northeast.

The National Quality Measures for Breast Centers (NQMBC), an initiative of the National Consortium of Breast Care Centers, puts BMC at or near our goal of being in the top 10th percentile of performance in many of the key measures of quality care. We utilized the 2011 data to drive further improvements in 2012, and steps are under way to enhance quality of care in areas where we are not yet performing within our top 10th percentile goal.

About NQMBC

The National Quality Measures for Breast Centers™ Program (NQMBC™) is an interactive internet model for breast centers to track and measure quality performance in 31 separate quality indicators. The NQMBC™ Program identifies quality care measures and provides immediate access to information that allows participating breast centers to compare performance with other centers across the United States.

The NQMBC™ Program is a result of the National Consortium of Breast Centers' (NCBC) commitment to increase the quality of breast health care provided by professionals to their patients. Because the NCBC has more than 1,000 breast center members of all types and sizes, we have the opportunity to compile a secure database from which all breast centers can be compared.

Here's how BMC performed in some of the more important quality-indicator categories

Imaging Timeliness of Care:
BMC Time Between Screening / Diagnostic Mammogram: **3 Days**
BMC Time Between Diagnostic Mammogram/ Needle Core Biopsy: **2 Days**

Pathology Timeliness of Care:
BMC Time Between Initial Breast Cancer Surgery/ Pathology Results: **2 Days**
Initiatives to shorten are under way.

Biopsy to Pathology Timeliness:
BMC Time between Initial Breast Biopsy & Pathology Results: **1 Day**

Breast Conservation Surgery *(Overall Rate)*:
BMC Percent of Stage I and II breast cancer patients whose definitive surgical treatment was lumpectomy reflecting patient choice: **74%***

Complication in tram flap reconstruction:
BMC Percent of complication resulting in an unplanned surgical intervention within 4 months post-op: **0% Complication**

Breast Conservation Surgery
(Re-Excision Rate):
BMC Percent of invasive breast cancer patients who had re-excision surgery or mastectomy after initial lumpectomy. **3.57%**

**Compares favorably to the 50% standard set forth by the National Accreditation Program for Breast Centers.*

BMC Breast Cancer Care: Continuous Improvement through Independent Accreditation

In our determination to continuously improve the quality of breast cancer care at BMC, we have secured independent accreditation and seek additional accreditation from the nation's leading breast cancer care quality organizations.

We already have organized our services into a fully integrated, carefully coordinated system that encompasses multiple specialties, including surgery, radiology, pathology, nursing, medical oncology, radiation oncology, plastic surgery, physical therapy, and behavioral health. Together, those specialists provide a much higher standard of care than if they were working in isolation.

Accreditation programs raise our level of performance even higher:

In Place

BMC's Women's Imaging Center already is accredited by the American College of Radiology (ACR) as a Breast Imaging Center of Excellence. That means BMC has earned accreditation in all of the College's voluntary, breast-imaging accreditation programs and modules, in addition to the mandatory Mammography Accreditation Program.

BMC's breast imaging services are fully accredited in mammography, breast MRI, MRI-guided biopsy, stereotactic biopsy, breast ultrasound and ultrasound-guided biopsy. Peer-review evaluations, conducted in each breast imaging area by board-certified physicians and medical physicists who are experts in the field, have determined that this facility has achieved high practice standards in image quality, personnel qualifications, facility equipment, quality control procedures, and quality assurance programs.

The ACR is a national organization serving more than 32,000 diagnostic/interventional radiologists, radiation oncologists, nuclear medicine physicians, and medical physicists with programs for focusing on the practice of medical imaging and radiation oncology, as well as the delivery of comprehensive health care services.

In Process

BMC is preparing for accreditation in 2012 from the National Quality Measure for Breast Centers. The NQMBC process allows us to track quality performance data on 31 indicators and, more important, compare performance with similar centers throughout the county. Participation in this program additionally provides suggestions on how to improve and modify services to enhance the quality of care. Our goal is to complete and submit data on the 31 indicators in 2012.

Additionally, BMC has committed to follow the national guidelines for treatment and diagnosis outlined by the National Comprehensive Cancer Network.

In 2013

We are preparing for the rigorous evaluation and review of performance with the goal of obtaining accreditation in 2013 from the National Accreditation Program for Breast Centers. The program represents a consortium of national, professional organizations dedicated to the improvement of the quality of care and monitoring of outcomes of patients with diseases of the breast. This mission is pursued through standard-setting, scientific validation, and patient and professional education.

Quality Controls keep consistency of care in check for cancer treatments

Good outcomes require consistency of care – and not willing to take that for granted, Berkshire Medical Center audits the care of individual subsets of patients to ensure that the best clinical practices and guidelines are being followed at all times.

This year, for example, a study was done looking at data and procedures on neo-adjuvant or pre-operative chemotherapy for breast cancer, or specifically the use of chemotherapy to treat a cancerous tumor before surgery.

BMC studied whether patients who have locally advanced breast cancer, or who have tumors too large for a good cosmetic result were treated with neo-adjuvant chemotherapy consistent with National Comprehensive Cancer Network (NCCN) guidelines.

The Overall Finding: 100% Compliance.

Those diagnosed with local invasive breast cancer (non-inflammatory) received treatment that was 100% consistent with the NCCN guidelines.

BMC Cancer Committee Analysis/Summary:

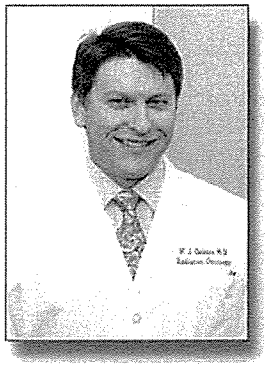
- A total of 138 patients were diagnosed with breast cancer primaries in 2011 according to the Berkshire Medical Center tumor registry database.
- Of these 138 cases,14 patients were stage III at diagnosis.
- Of these 14, 8 patients were appropriately offered pre-surgical intervention chemotherapy and six proceeded with neo-adjuvant chemotherapy. Two women chose not to the have any chemotherapy.
- Two women received preoperative treatment to downsize the malignancy for possible breast preservation.
- One of these patients was successfully able to have a partial mastectomy (to preserve the breast) while one patient required a mastectomy despite prior systemic therapy.
- Four women that received preoperative treatment had advanced disease and continued on to have a planned mastectomy after completing chemotherapy.
- Of the six women who received neo-adjuvant chemotherapy, 100% received treatment as per the NCCN guidelines. The remaining patients received treatment that was consistent with the NCCN guidelines.

Again, those diagnosed with locally advanced invasive breast cancer (non-inflammatory), received treatment that was 100% consistent with NCCN guidelines.

In addition to using our multidisciplinary team and patient conferences to manage care in real time, the Tumor Registry allows us to look back and review quality data to ensure that each and every patient gets optimal care.

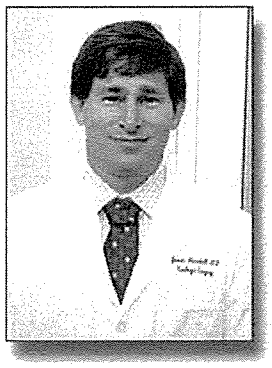
STATE of the ART SERVICES

Highly skilled professionals using today's most advanced technology in a specially designed environment means the best in cancer care at BMC, including precise diagnoses, optimal care plans and accuracy of treatment delivery. Advanced operating technology and new surgical and radiation techniques provide highly efficient procedures and faster recovery for patients. The success of our cancer care programs at BHS requires a range of services providing technology and operational support.



Technology brings high art, precision to BMC cancer care

*By **Wade Gebara, M.D.**, Radiation Oncologist, Chairman, Cancer Committee, and **Jonah Marshall, M.D.**, Urologist*



Berkshire Health Systems has invested in leading-edge technologies that bring a whole new level of precision to the targeting of tumors – a great advantage to the community of patients we serve in the Berkshires.

The Tomotherapy HI-ART System™ emits dynamically rotating 'beamlets' of radiation, each varying in intensity, to deliver radiation therapy with unprecedented precision. It is a type of Intensity Modulation Radiation Therapy, administered with computer-controlled (robotic) accelerators enabling the radiation oncologist to create treatments that can form more precisely to tumors, while minimizing dose to surrounding normal critical structures.

Our two linear accelerators treat a wide variety of cancers with targeted beams of radiation. They deliver just enough to attack the tumor, while avoiding exposure of healthy tissues. In brachytherapy, concentrated doses of radiation in small capsules or seeds are implanted inside or along the treatment site; it is particularly efficient and convenient for patients with prostate and gynecological cancer.

All of our radiation therapies are aimed at reducing local cancer recurrence.

On the surgical side, our minimally invasive daVinci®Surgical System uses surgeon-controlled robotic technology to create smaller incisions, less blood loss, shorter hospital stays and faster recovery. It is being used with great success in performing gynecologic, colorectal, prostate and other urologic, as well as thoracic surgeries.

Minimally invasive urologic oncology has exploded since the arrival of the daVinci robotic system in 2010. We have performed nearly 200 robotic cases aimed at treating cancer over the last two years. Nearly all of those cases would have required referral to an outside institution prior to 2010.

The breakdown:
62% Prostate cancer (109 cases)
30% Kidney cancer (54 cases)
8 % Bladder cancer (14 cases)

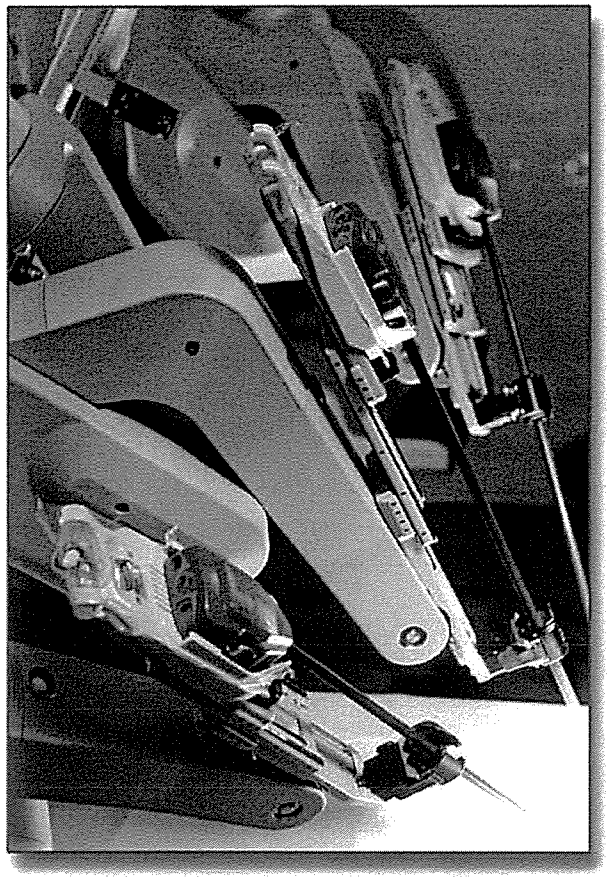
Survival rates for cancer patients undergoing robotic surgery at BMC are excellent. Cancer specific survival rates over the last 2 years for prostate cancer is 98%, kidney cancer is 98%, and bladder cancer is 86%. *(continued)*

The average length of stay in the hospital has decreased from 3.5 days to 1.2 days for prostate surgery. For kidney surgery the average length of stay has dropped from 5 days to 2.2 days.

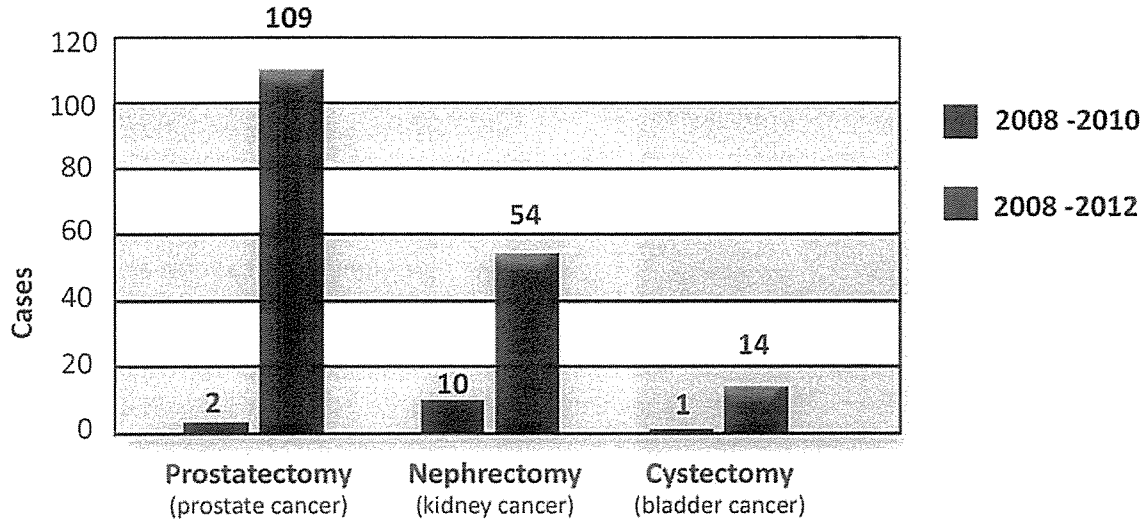
Patients undergoing major surgery no longer require an ICU stay on a routine basis and patients are instructed to walk the evening after surgery. Patients who previously had six-hour surgery are now having surgery completed in three. Patients who previously would have spent 3.5 days in the hospital now go home the morning after surgery.

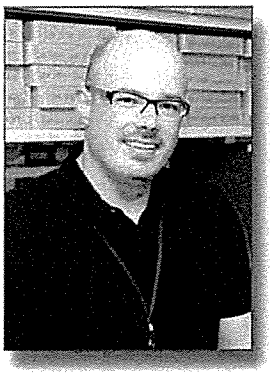
Blood transfusion rates during surgery have dropped to less than 1%.

We have developed patient care pathways to streamline nursing care and improve communication across the board. We are standardizing care, speeding recovery and eliminating errors.



Caseload before and after addition of Robotic Surgery





BHS Pathology is literally the lifeblood of cancer care in far Western Massachusetts

By Charles Abbott, M.D.,
Chairman, Pathology and Clinical Laboratories

The BHS Pathology Department forms the core diagnostic component of Cancer Care in the Berkshires, and is an active part of the cancer care team, working closely with the BHS Hematologists, Oncologists, Radiation Oncologists, Radiologists and Surgical Oncologists.

The major function of the Department of Pathology is the diagnosis of tumors and evaluation of prognostic indicators. Pathologists are physicians who specialize in diagnosing cancer through the analysis of a patient's tissue, blood or body fluids. The pathologist is responsible for making the initial cancer diagnosis, and directs all additional laboratory testing to ensure that patients receive the most individualized cancer care possible.

In addition, pathologists direct all aspects of laboratory testing and the blood bank. Although most patients rarely meet their pathologist, we are all acutely aware of the life changing ramifications of a cancer diagnosis, and we know that behind every specimen we see there is a patient with their own unique story.

The BMC laboratory is a world class facility that provides state of the art testing to patients. The laboratory is led by a team of six pathologists, all of whom have received specialty training from leading academic institutions in the U.S . The Pathology Department exceeds the American College of Surgeons Commission on Cancer standards, by providing timely, accurate, synoptic reports that allow each patient to receive the most personalized cancer care.

Historically, pathology reports were a relatively simple one-page document. Today, with advances in cancer diagnosis and treatment, pathology reports extend to many pages, and inform 70% of the decisions made about your care. This may include the specific type of chemotherapy that your cancer will best respond to, the dose of chemotherapy you receive, the type and extent of surgery that you may need, and the radiation dosing necessary. The BMC laboratory has the latest instrumentation for the morphometric, genetic and molecular evaluation of your cancer. This enables us to provide you with the most up to date diagnostic techniques to ensure you (and your family – for familial cancers) receive the most individualized care possible.

At a time of critical blood donation shortages, we are immensely proud that the BHS laboratory, through its very active blood donor program and bloodmobile, is able to collect greater than 90% of the blood we use in your cancer care from residents in the Berkshires. This enables us to ensure that blood products will be available for you if needed as you go through your cancer care.

In 2011, we collected nearly 2,600 red blood cell units from BMC donors, plus 817 from the local American Red Cross (a total of more than 3,400). **Some 3,100 units were transfused.**

CANCER PREVENTION

One of the surest ways to combat cancer is to take every possible step to prevent it from happening in the first place. While cancer still has a pervasive way of invading even the healthiest of bodies, we must remain committed to lessening those odds through good diet, exercise, healthy lifestyle choices and other preventive measures. Among the prevention programs Berkshire Health Systems provides its community is BMC Tobacco Treatment Services and our HPV Vaccination program.

Success rate of BMC stop-smoking program significantly better than national averages

By Carol McMahon, M.S.,
Master Certified Tobacco Treatment Specialist

People who quit smoking with the help of Berkshire Medical Center's Tobacco Treatment Program are at least 10 to 15 percent more likely to be successful than people who have gone through similar programs elsewhere in the US.

BMC clients have, on average, achieved a consistent success rate of 35% who continue to abstain from smoking for one year after treatment – significantly better than the 20 to 25 percent success rates seen in typical programs for behavior modification for the same duration.

What is BMC doing better? The program's coordinators say among the major differences is that BMC offers free, ongoing one-on-one counseling after completion of the multi-week course. Also, the BMC program provides clients nicotine replacement products (patches, gum lozenges) at no additional cost.

The goal of the program is to reduce the incidence of death and disease related to tobacco use by providing science-based, state of the art tobacco treatment services in the BMC treatment area for anyone wishing to change his/her tobacco use behavior. **1,099 patients were provided with tobacco counseling at BMC in 2011.**

Service providers are Certified Tobacco Treatment Specialists trained at UMass Medical School of Preventive & Behavioral Health Center for Tobacco Training & Research Counseling using US Department of Health & Human Services Clinical Practice Guidelines.

Treatment formats include both individual counseling tailored to a patient's personal needs and Group Counseling for the benefits of a mutual support environment. **Scheduling is flexible. Patients can be self-referred by contacting the Treatment office at 413.499-2602.** Most health insurance plans pay for counseling services.

BHS echoes CDC call for HPV Vaccine as cancer prevention for *all* young people

By Vicki Smith, M.D.,
Pediatrician,
Cancer Committeee

It's a strong weapon in the prevention of cancer caused by the HPV or human papilloma virus, which each year in the U.S. is responsible for 15,000 cases of cancer in females and 7,000 cases in males, including children and young adults.

HPV is a common virus that is easily spread by skin-to-skin contact during sexual activity. It is possible to have HPV without knowing it, and therefore easy to spread it to others unknowingly.

Certain HPV viruses cause cancer, including oral, cervical, vulvar, penile and anal cancer. They also cause most types of genital warts in men and women.

The HPV vaccine is safe and effective for both males and females ages 9 through 26 years.

The Center for Disease Control (CDC) recommends the HPV vaccine for:

- 11 or 12 year old boys and girls
- 13-21 year old boys and girls who did not get any or all of the three recommended doses when they were younger
- The vaccine is also recommended for gay and bisexual men and men with compromised immune systems (including HIV) through age 26, if they did not get fully vaccinated when they were younger.

CANCER SCREENING

Berkshire Health Systems provides a full range of comprehensive screening programs designed to deliver early-detection diagnosis and track progress of treatment... In this year's report, we focus on four fronts – underscoring the importance of breast and cervical screening for women, addressing the current debate over the need (or not) for male prostate screening, and the latest recommendation for the early detection of the leading cause of cancer death, lung cancer.

BHS urges breast screening

By Lisa Loring, M.D.,
Radiologist, Medical Director, Women's Imaging Center

As many as nearly 90 percent of Berkshire County women over the age of 40 have had mammography screenings over the past two years, and while that's encouraging news, BHS continues to sound the bell.

All of the nation's leading cancer and medical organizations, including the American College of Radiology, American Cancer Society, the American Medical Association and the National Cancer Institute – recommend annual mammographic screening from age 40 years.

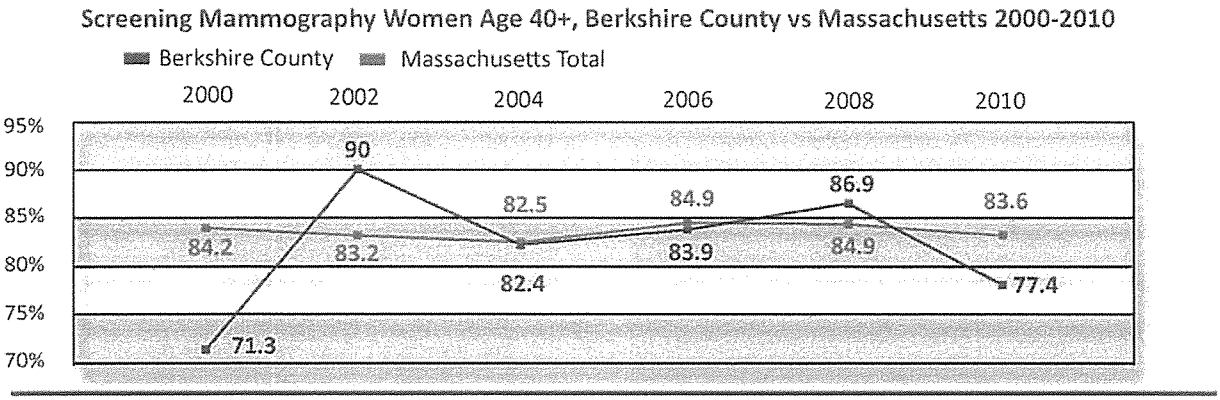
Breast Screening Recommendations

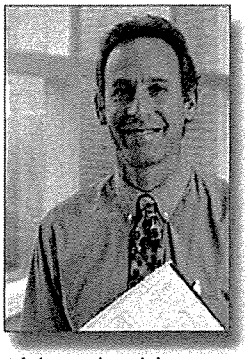
- For the general population, a baseline mammogram is recommended between the age of 35 and 40 years and annually thereafter.
- If there is a maternal history of breast cancer, screening for daughters should begin 10 years before the age at which the mother was diagnosed. For example, if mother was diagnosed at age 40, then screening should begin at age 30 for daughters.
- If there is a history of lymphoma with mantle radiation, the risk of breast cancer increases and therefore these patients too should start mammographic screening earlier. Annual screening mammography is recommended 8 years following completion of chest irradiation but not before the age of 25.

To date there are no guidelines discussing if and at what age screening should be stopped.

Cancer Screening in Berkshire County has had an average rate higher than the total for the state. For example, the percentage of women receiving mammography screenings for breast cancer in the past two years was 84.7 percent for both Berkshire County and statewide. For women ages 40 and over, the mammography screening rate was a range of 80.3 - 89.1 in Berkshire County and 83.9 to 85.5 percent statewide.

More recent surveys have indicated screening rates as high as 86-87 % in Berkshire County.
(Kaiser Family Foundation Statehealthfacts.org shows Women Age 50+ at 87.5% in 2009.)





BMC encourages regular gynecology exams and Pap smears to continue fight against cervical cancer

By Herb Kantor, M.D.,
Gynecologist, Chairman,
Maternal Child Health

Although widespread Pap smear screening has helped reduce the incidence of cervical cancer by more than 50 percent over the past 30 years, it is estimated that half of the women who are diagnosed with cervical cancer today have never had one. BHS is joining the effort to turn that trend around.

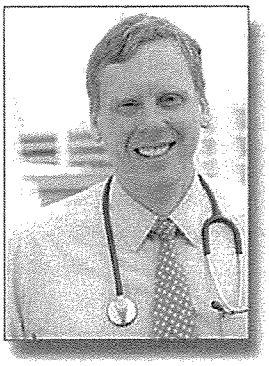
The cervical cytology test or Pap smear, named after its Greek inventor, Dr. Georgios Papanikolaou, is a simple procedure that can detect abnormal cervical cells. The Pap test allows early diagnosis and treatment so that the abnormal cells do not become cancer. Routine Pap tests help decrease the chance that abnormal cells are missed. If a Pap misses abnormal cells they may be found on the next scheduled Pap test.

Berkshire gynecologists and Berkshire Health Systems join the American College of Obstetricians and Gynecologist s (ACOG) in underscoring the importance of adhering to following recommendations for Pap smear frequency:

Pap Smear Frequency Recommendations

- First Pap smear at age 21
- Women 21 to 30 should have a Pap test every 3 years
- For women 30 to 65, a Pap test plus an HPV or human papilloma virus test every 5 years is preferred; however, a Pap test alone every 3 years is acceptable.
- Women over 65 should not be screened provided that:
  -she previously had sufficient negative screening results
  -she does not have a prior history of cervical dysplasia
  -she is not infected with human immunodeficiency virus (HIV)
  -her immune system is not weakened (for example, organ transplant patients)
  -she was not exposed to diethylstilbestrol (DES) before birth

**** Regardless of the frequency of the Pap test, physicians still recommend an ANNUAL gynecologic examination even if a Pap test is not performed at every visit.****



Importance of Colonoscopy underscored

By Jeffrey St. John, M.D., Chief, Gastroenterology

It's a minor inconvenience with a potentially life-saving benefit. Although there are several ways to screen for colorectal cancer, screening with colonoscopy has been proven consistently to decrease the risk of death from this, the second leading cause of cancer death in the U.S. and 10% of cancer deaths overall.

Because colonoscopy allows the physician performing the procedure to detect and remove pre-cancerous polyps, the procedure can eliminate that polyp's risk of becoming cancerous. Colonoscopy is a safe and relatively painless examination that most often requires less than 30 minutes to complete.

The Risks of not getting thoroughly screened for colorectal cancer are high:

Approximately 30% of people who develop colorectal cancer die of the disease.
The lifetime "risk" of developing colorectal cancer is an average risk of 5%.
The vast majority (90%) of colorectal cancer patients develop the disease after age 50.

Our recommendations reflect that urgency:

Guidelines for Colorectal Screening

- **Routine colorectal cancer screening** should begin at age 50 and continue until life expectancy in less than 10 years (or to 75-85 years of age at the latest).
- **African Americans** appear to be at increased risk of colorectal cancer and should initiate screening at the age of 45.
- **People with an increased risk of colon cancer** based on such variables as listed below will often require screening at an earlier age and have to undergo more frequent screening compared to those at average risk. You are in this higher-risk category if you have:
  -A personal history of colorectal cancer or colon polyps
  -A family history of colorectal cancer or genetic syndrome predisposing to an increased risk
  -Inflammatory bowel disease

Use of BMC Lung Nodule Clinic is among screening guidelines urged locally

By Daniel Doyle, M.D.,
Chief, Pulmonary Medicine

To closely monitor the presence or progression of lung nodules – one possible sign of early-stage lung cancer – BMC offers a weekly clinic where patients can be evaluated and referred as needed for further consultation.

Urging residents and their primary care physicians to take advantage of the service is part of BMC's outreach in communicating its overall screening guidelines for lung cancer. And there's a good reason to listen: lung cancer remains the leading cause of cancer death in the U.S. and worldwide, responsible for as many deaths as the next four cancer killers (breast, prostate, colon and pancreas) combined.

Seventy percent of patients are diagnosed with advanced disease, stage III or IV. This has prompted efforts to find an effective mechanism to find early stage disease. Multiple recent studies have examined the use of Low Dose CT scanning (LDCT) as a screening tool to identify early lung cancers.

In June of this year, the **American Cancer Society, American College of Chest Physicians** (ACCP), **American Society of Clinical Oncology** (ASCO) and the **National Comprehensive Cancer Network** collaborated on a review of this topic. That collaboration resulted in the formulation of a **Clinical Practice Guideline** issued jointly by the ACCP and ASCO and published in *Journal of the American Medical Association* 2012; 307(22): 2418-2429).

Clinical Practice Guideline: Low-Dose CT Scanning for Lung Cancer

- The target population for screening with Low-Dose CT consists of **smokers and former smokers aged 55 to 74 with > 30 pack years who currently smoke or have ceased within the past 15 years.** *Screening is not recommended for individuals with multiple severe illnesses that would prevent participating in curative treatment.*
- The effective duration of screening has not been established. Currently **annual screening** is recommended in the guidelines.
- Pre-test counseling should include a **description of the potential benefits and harms of screening.**
- Screening should be part of a coordinated program with **multiple specialists for screening and image interpretation** as well as **follow-up evaluation and treatment** similar to participants to the National Lung Screening Trial (NLST).
  This would include the presence of specialized thoracic radiologists and thoracic surgeons, pulmonologists and oncologists as participants in the program, all of whom are available in BMC's Lung Nodule Clinic.

Patients need to be aware that most insurance companies are not yet paying for screening CTs for lung cancer. That may change. Please check with your insurance company for updates.

Addressing debate over prostate screening, BMC issues Best Practice statement to physicians

By Stephen St. Clair, M.D.,
Chief, Urology

Are prostate screenings really necessary for all men? There is not yet universal agreement on the answer to that question. Current guidelines about screening for prostate cancer remain somewhat controversial. So this is intended as a best practice statement for those physicians practicing in the Berkshires in Western Massachusetts.

Different Views:

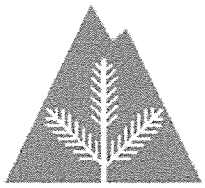
A recent report from the US Preventive Services Task Force recommended against screening for prostate cancer (PSA) for all men. An immediate response from the American Medical Association (AMA) condemned this report as shortsighted, and the AMA currently recommends that all men over the age of 50 should have a discussion with their physician regarding screening for prostate cancer.

The **American Cancer Society** (ACS) recommends that men make an informed decision with their doctor about whether to be tested for prostate cancer. Research has not yet proven that the potential benefits of testing outweigh the harms of testing and treatment. The ACS believes that men should not be tested without learning about what we know and don't know about the risks and possible benefits of testing and treatment.

(continued)

CANCER SCREENING

The **American Urologic Association** states the man who wishes to be tested for prostate cancer should have both a PSA and a digital rectal exam. A recent article in the *New England Journal of Medicine* suggests that the quality of life years benefit gained from prostate cancer screening may argue in favor of regular PSA screening. It seems clear that prostate cancer screening, based on European studies, saves lives. The question is whether the side effects from prostate cancer treatment outweigh the benefit.



BMC Recommendations

- We currently recommend that patients be counseled about the pros and cons of prostate cancer screening. Any man that would like to have a PSA obtained for prostate cancer screening should be given the opportunity. More specifically:

  -All men should receive a baseline PSA by the time they're 50. At that age, men should talk to a doctor about the pros and cons of testing to decide if testing is a right choice for them.
  -African American men and any man whose father or brother had prostate cancer before age 65 should have this talk with a doctor starting at age 40.
  -If men decide to be tested, they should have the PSA blood test with or without a rectal exam.
  -How often they are tested will depend on their PSA level.
  -Patients with significant lower urinary symptoms should also be offered the PSA blood test.
  -Regular annual screening should be left up to a decision between the patient and his doctor.
  -PSA screening should be discontinued for asymptomatic men with a normal PSA when their life expectancy is less than 10 years.

Faced not only with the difficult news of a cancer diagnosis, patients are easily overwhelmed with prior family and job commitments. Emotions range from fear, anger and guilt to confusion and frustration. Individuals struggle with financial worries and lack of certainty on how their lives will change. The impact of a new diagnosis affects the entire family. Attending various appointments becomes another full time commitment and learning the medical terminology is similar to learning a new language. These issues are compounded for those who are not fluent in English, are underinsured, lack transportation, or are a caregiver for another person.

Navigation program is the heart of cancer care – and survival

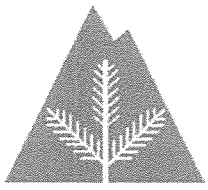
Care Navigation in the Community Setting

The nurse navigators attend various outreach events to promote awareness on the availability of screening tests for early detection of cancer.

Care Navigation at Diagnosis

Care Navigation begins with one-on- one contact with a Registered Nurse. This nurse begins the process by assessing the individual needs and identifying barriers and obstacles to receiving care. The goal is to ensure that patients receive care that is timely, efficient, equitable and patient-centered to improve outcomes.

The RN stays in contact with the patient to provide proactive solutions to any obstacles encountered. Time is spent educating patients and reinforcing information provided by the many specialists. It is important for the patient and their family feel confident in understanding why treatment is necessary so they can make informed decisions with their providers. Referrals are made to nutrition, psychosocial support, financial assistance and rehabilitation. This process promotes compliance with physician treatment recommendations improving outcomes.



Support During Treatment

Care Navigation helps to address the complexities encountered by individuals throughout the continuum. A key role of the nurse navigator is to teach the patient to identify and communicate health issues to providers for optimal symptom management.

Survivorship

After treatment has ended, care navigation continues. This is a time when patients are getting use to their new self. It is also a time to develop or encourage healthy lifestyles to help reduce the risk of a recurrence. Patients often find they are interested in attending support groups at this point. The RN navigators co-facilitate various support groups.

People Served

Formalized navigation programs are in place for those diagnosed with breast, prostate and beginning in 2012, for head and neck cancers. Total hours spent on providing services in the last 3 months of 2011 was 460 hours.

Number of patients followed in 2011:

Breast	139	Head and Neck	5
Prostate	164	Rectal	5
Lung	14	Other	4
Colon	7	TOTAL	338

The goal for 2012 is to increase the number served by 20% by adding services to lung cancer patients.

Patient Care Assistance Fund ensures access to breast care

Determined to help remove financial barriers to proper breast care – including women who won't get mammograms because they don't have enough money – BHS has established programs to offset those costs.

With healthcare reform, women have health insurance. But high deductibles may prompt them to postpone screening tests. Established in 2008, the Patient Assistance Fund offers financial assistance to those who meet eligibility requirements.

With this help, women are more likely to get their recommended exams. They also are provided with necessary breast cancer treatment-related products (wigs, bras/prosthesis and lymphedema supplies, etc.), as well as transportation to and from the screenings if needed.

Proceeds from the annual Venison and Polenta Dinner, sponsored by long-time BHS employee Drew Demairisico, have been donated to this fund. ***In 2011, over 60 individuals were provided with medically necessary items totaling $3,509.***

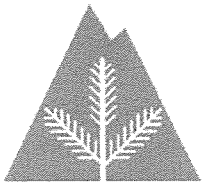
Denise Kaley Fund helps women with necessities not covered by insurance

In the maze of grant-making vehicles for cancer patients care and treatment, this fund, established by friends in honor of Denise Kaley, stands alone in helping cancer patients cover the costs of life's necessities: heating oil, car repairs, groceries, babysitting, rent, mortgage payments and medical expenses not covered by insurance, to name just a few.

The procedures are simple, direct and transparent. Three professionals from our local hospitals work closely with women with cancer in Berkshire County to help them navigate systems for support. They refer those patients who have the most urgent needs that fit the Fund's guidelines. The Fund's Advisory Committee (Barbara Bonner, Fund Advisor; Ellen Boyd and Beth Rose) reviews each case, usually on the same day the application is received and payments are made immediately. The Denise Kaley Fund is a true emergency fund that fills a painful void in financial assistance to women with cancer in Berkshire County.

Two of the professionals who make up the Fund's Community Referral Network are leading members of the BHS community: Kathy Hart, Director of Care Navigation Services at Berkshire Medical Center and Cheryl Thomson, Program Manager of Advocacy for Access at Fairview Hospital. With their help the Fund has made 27 grants totaling $11,599 since January 2011. Most grants are approximately $400-600.

While the fund is now endowed, fundraising is ongoing. As the Fund expands it will be able to be increasingly responsive to the huge needs of women in our community. BHS has made the success of the fund and its meaningful reach into the community a reality.



Direct Access program opens door to early-detection colon screenings

Among our top priorities at BMC is the early detection of colorectal cancer – the second leading cause of cancer-related deaths in the U.S. – which is "treatable and beatable" if found early.

Experts say 130,000 new colorectal cancer cases will be diagnosed this year, and most will have no known risk factors. At age 50, men and women should consult with their physicians to schedule this life-saving test. Depending on family history, some may need to have screenings before 50. And yet, in Berkshire County, only 60 percent of people over 50 have had their screening colonoscopy. We are determined to reduce these alarming trends through a comprehensive program of outreach, education and screenings.

To make scheduling easier, BMC has a Direct Access program through its Endoscopy Department. Direct Access allows patients and physicians to schedule a colonoscopy directly by calling the BMC Endoscopy Department.

A second feature of the new program is Open Access, where the patient has a pre-screening interview with an Endoscopy nurse at BMC. If the patient meets open access criteria, they can skip the previously required pre-visit to the endoscopist and go directly to BMC's Endoscopy Department on the day of procedure.

Patient Care Assistance Fund

Berkshire Medical Center has developed a special patient fund that will assist underinsured patients in need of a screening colonoscopy who cannot afford their high copayments or deductibles. The Screening Colonoscopy Patient Fund has been created through generous community donations.

The new Screening Colonoscopy Patient Fund is available for people who are insured and meet the eligibility income criteria of 400% or lower of the federal poverty level. The fund is available based on income and copayments/deductibles greater than $100.

Lung Nodule Clinic at BMC speeds diagnosis

The BMC Lung Nodule Clinic is designed to speed a timely diagnosis when a lesion has been identified on either a chest X-ray or CT scan. Surgical specialists work closely with pulmonologists and interventional radiologists to provide tissue samples to pathology for diagnosis.

If the biopsy results in a diagnosis of lung cancer, the surgeon is already familiar with the case and can immediately evaluate if surgery should be recommended. If surgery is not required, a team of medical and radiation oncologists is put together for consultations and to make treatment recommendations.

The Lung Nodule Clinic saw 36 patients from 3/30/11-12/14/11. Appointments can be made by calling Pulmonary Department at 413-447-2695.

BHS engages in Clinical Trials focused on cancer drugs, preventive treatment

BMC partner Berkshire Hematology Oncology (BHO) has been conducting clinical research trials for more than 30 years. BHO has participated in several major studies with pharmaceutical companies and national groups, working to get new drugs approved and available to advance the treatment of many cancers. Great care is taken to ensure both scientific integrity and ethical conduct in all clinical trials.

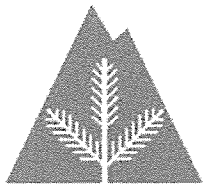
Clinical research and clinical trials can provide a meaningful opportunity for patients. Those eligible and who choose to participate are part of the worldwide pursuit of cancer treatments, not only today, but for the future.

Among the drug trials in which BHO has participated:

ATAC Study (Arimidex, Tamoxifen, alone or in combination)
The ATAC study is one of the largest clinical trials of additional breast cancer treatment that has been conducted in postmenopausal women with early breast cancer. The study included 9,366 women worldwide who were candidates for additional hormonal treatment, including 48 patients enrolled at Berkshire Hematology Oncology. After 10 years of follow-up, Arimidex was approved for treatment in this patient population.

Denosumab Study

Denosumab is a new class of medication that helps prevent bone loss in patients undergoing hormonal treatment for early breast cancer. Berkshire Hematology Oncology enrolled 16 patients of the 208 women who were included in the study that resulted in FDA approval of Denosumab.



We have also participated in two large prevention trials:

STAR Study (Study of Tamoxifen and Raloxifene)
This was one of the largest breast cancer prevention clinical trials ever conducted, enrolling 19,490 postmenopausal women who were at increased risk for breast cancer. This long-term trial was coordinated by the National Surgical Adjuvant Breast and Bowel Project, a network of cancer research professionals, and was sponsored by the National Cancer Institute.

These results helped clarify that both raloxifene and tamoxifen are good preventive choices for higher risk postmenopausal women. Randomization started in July 1999 and stopped in 2004. Results were published in 2010. BHO enrolled 72 women in this study.

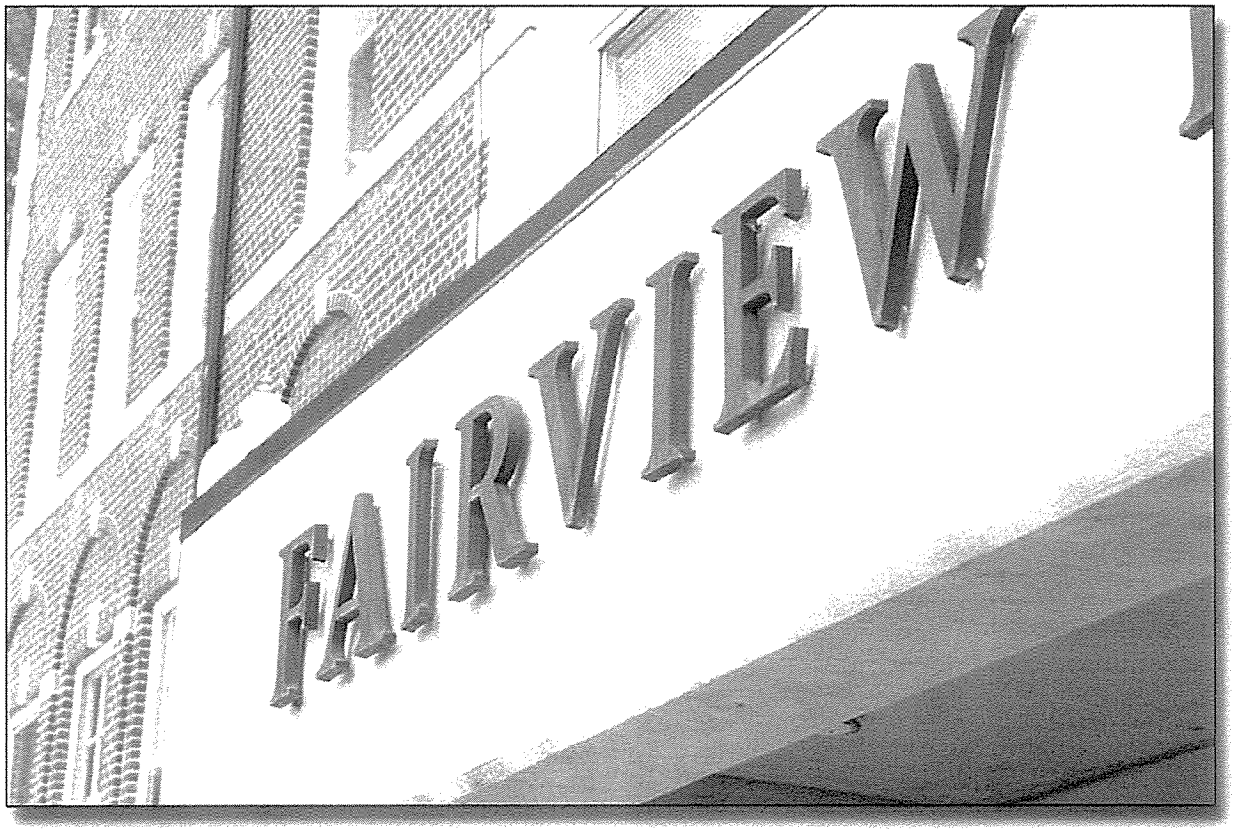
SELECT Study on Prostate Cancer Prevention

BHO was among the participants in the largest-ever prostate cancer prevention trial, which used Selenium and Vitamin E. The study was looking for good and bad results from the use of two supplements, Selenium and Vitamin E. The study was conducted by SWOG (Southwest Oncology Group) and ran from 2000 to 2008. The study ended intervention on October 2008 after definitive findings of no benefit from either supplement. BHO had enrolled 68 men to this study.

Fairview Hospital Provides South Berkshire Cancer Care

As part of Berkshire Health Systems, Fairview Hospital plays a critical role in offering primary care, primary prevention and cancer screening services. Fairview meets the needs of cancer patients with comprehensive diagnostic and testing services through the BHS Departments of Radiology and Pathology and Clinical Laboratories, which provide state-of-the-art imaging and an advanced laboratory. The physician specialists in these departments provide services at both BMC and Fairview. Fairview supports access to both medical and surgical oncology care along with support groups for women with breast cancer.

Fairview is a comprehensive acute care hospital, and as part of the Berkshire Health Systems network, provides South Berkshire patients and their families with easy access to the full range of treatments and support services for cancer patients, including on-site urology physician care.



BMC scores high in Cancer Care, Overall Medical Excellence

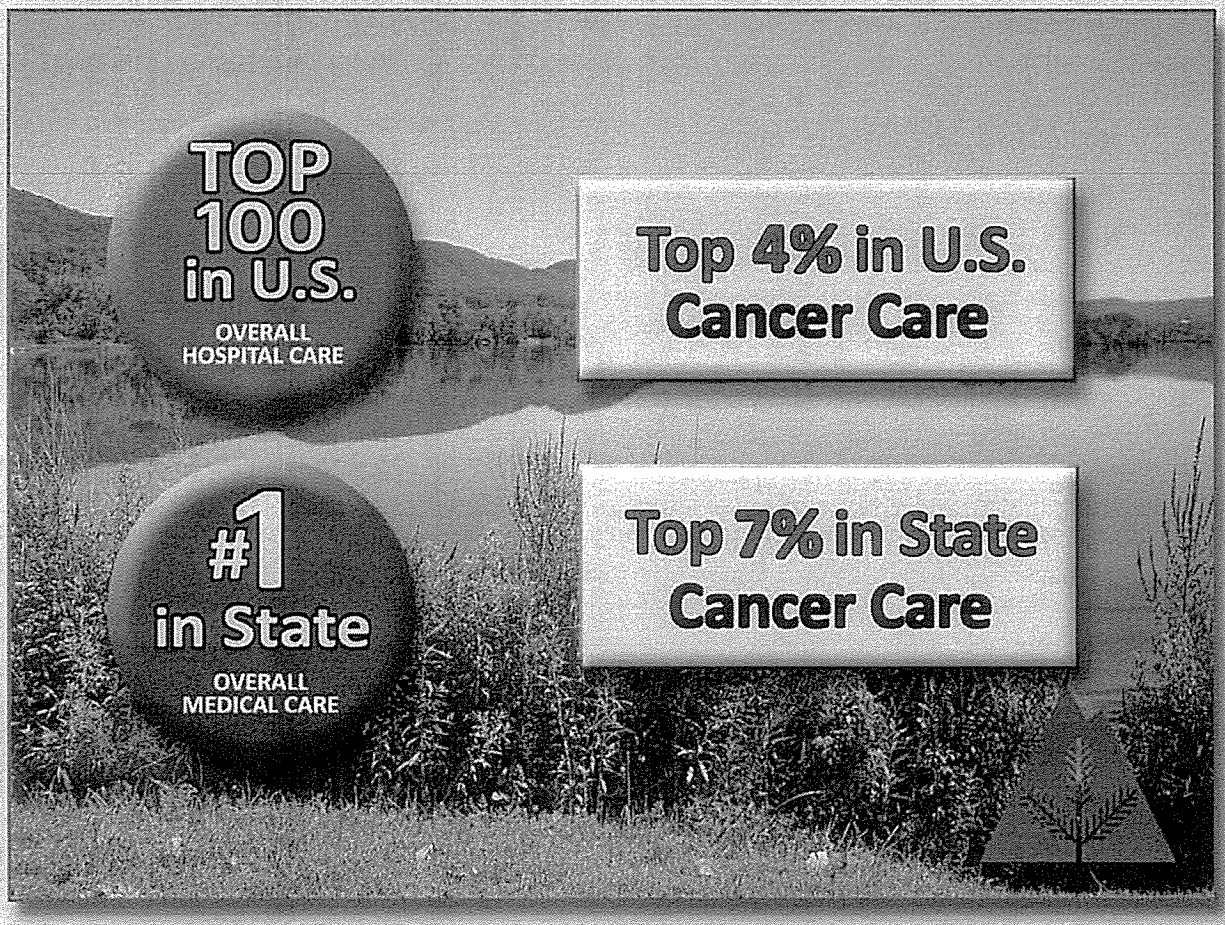
CareChex gives hospital 96.5 Score for quality of cancer services, outcomes

CARECHEX®
A Rating Service of The Delta Group

CANCER CARE

NATIONAL SCORE	RANK in U.S.	PERCENTILE
96.5	140 *(4,500 hospitals)*	≥96th

BMC has received high scores for Cancer Care and overall medical excellence by CareChex®, a medical quality rating service of The Delta Group — the nation's largest privately-held healthcare information service company. CareChex is designed to assist hospitals in improving the quality of inpatient care. Relying on both public and proprietary measures of performance, CareChex compares the quality of hospital care to national, state, and local standards using a variety of process, outcome, and patient satisfaction measures to assign a composite quality score and rating. CareChex uses peer-reviewed methodologies addressing key components of the quality of inpatient care, including core processes, complications and mortality.



Attachment J-2

TOXICOLOGICAL SCIENCES 112(1), 257–272 (2009)
doi:10.1093/toxsci/kfp200
Advance Access publication August 19, 2009

Divergent Transcriptomic Responses to Aryl Hydrocarbon Receptor Agonists between Rat and Human Primary Hepatocytes

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Received June 19, 2009; accepted August 17, 2009

Toxicogenomics has great potential for enhancing our understanding of environmental chemical toxicity, hopefully leading to better informed human health risk assessments. This study employed toxicogenomic technology to reveal species differences in response to two prototypical aryl hydrocarbon receptor (AHR) agonists 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and the polychlorinated biphenyl (PCB) congener PCB 126. Dose-responses of primary cultures of rat and human hepatocytes were determined using species-specific microarrays sharing over 4000 gene orthologs. Forty-seven human and 79 rat genes satisfied dose-response criteria for both chemicals and were subjected to further analysis including the calculation of the 50% effective concentration and the relative potency (REP) of PCB 126 for each gene. Only five responsive orthologous genes were shared between the two species; yet, the geometric mean of the REPs for all rat and human modeled responsive genes were 0.06 (95% confidence interval [CI]; 0.03–0.1) and 0.002 (95% CI; 0.001–0.005), respectively, suggesting broad species differences in the initial events that follow AHR activation but precede toxicity. This indicates that there are species differences in both the specific genes that responded and the agonist potency and REP for those genes. This observed insensitivity of human cells to PCB 126 is consistent with more traditional measurements of AHR activation (i.e., cytochrome P450 1A1 enzyme activity) and suggests that the species difference in PCB 126 sensitivity is likely due to certain aspects of AHR function. That a species divergence also exists in this expanded AHR-regulated gene repertoire is a novel finding and should help when extrapolating animal data to humans.

Key Words: **TCDD; PCB; AHR; microarray; toxicogenomics; human; relative potency.**

Modern biotechnologies (e.g., toxicogenomics) are rapidly evolving and show great promise in improving our understanding of the biological mechanisms underlying toxic responses to pharmaceuticals and environmental chemicals (National Research Council [NRC], 2007a). In addition, recent reports have endorsed the development of *in vitro* approaches for modern toxicity testing (EPA, 2009; NRC, 2007b). In fact, they highlight the need to develop better dose-response data and improved models for extrapolation across species. As described by NRC (2007b), such extrapolation modeling will have at least three components: (1) a mechanistic understanding of dose-response modeling; (2) pharmacokinetic modeling that can relate effects seen at certain *in vitro* concentrations to effective concentrations in human tissue; and (3) human data regarding elements of the same toxicity pathway. This approach certainly has the potential to improve the accuracy of modern human health risk assessment.

Current human health risk assessment approaches have a high reliance on responses observed in laboratory animals from both *in vitro* and *in vivo* models. While responses of animals and animal cells to some toxicants have been shown to be generally predictive of human health risk (e.g., certain heavy metals, alkylating agents, and polycyclic aromatic hydrocarbons), simple interspecies extrapolations are not always valid. This is particularly evident for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally related dioxin-like compounds (DLCs). Although the cellular responses to TCDD are initiated through a well-researched receptor-based mechanism, the aryl hydrocarbon receptor (AHR) pathway (reviewed by Okey, 2007), it is also well established that the potency of TCDD is not consistent across species (Henck *et al.*, 1981; Schwetz *et al.*, 1973). To date, some species/strain differences in TCDD potency have been attributed to variation in AHR gene sequence leading to differential ligand-receptor affinity. For example, divergent TCDD toxic potencies seen between responsive C57 and nonresponsive DBA mice strains are generally due to an amino acid substitution in the AHR ligand-binding domain resulting in differential AHR affinity for TCDD between these strains (Ema *et al.*, 1994; Okey *et al.*, 1989; Poland *et al.*, 1994). Interestingly, the human AHR has the very same affinity-lowering amino acid substitution as the DBA mouse leading to reduced TCDD potency for cytochrome P450 1A1 (CYP1A1) enzyme induction *in vitro* (Ema *et al.*, 1994; Harper *et al.*, 1988;

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Ramadoss and Perdew, 2004) and a much milder/incomplete TCDD toxicity in transgenic mice "humanized" with the human AHR (Flaveny *et al.*, 2009; Moriguchi *et al.*, 2003).

It is also increasingly evident that DLCs may not retain their relative potencies (REPs), as compared to the potency of TCDD, across species. Species variation in REPs may be due to a number of reasons including species differences in toxicokinetics. However, species differences in explicit AHR pharmacology (e.g., affinity and/or intrinsic efficacy) may also lead to deviating REPs. For instance, estimated REPs for mono-ortho-substituted polychlorinated biphenyls (PCBs) differ greatly between fish and rodents (Van den Berg *et al.*, 1998). Using an *in vitro* system, Hestermann *et al.* (2000a) determined that the general inability of mono-ortho PCBs to elicit embryotoxicity and induce CYP1A in various fish species is due to the exceptionally low intrinsic efficacy of these congeners to activate the AHR in fish cells.

In vitro studies using primary cells and cell lines suggest that species differences exist between humans and rodents in the REP of PCB 126 for CYP1A1 enzyme induction (Drenth *et al.*, 1998; Pang *et al.*, 1999; Silkworth *et al.*, 2005; Vamvakas *et al.*, 1996; van Duursen *et al.*, 2003; van Duursen *et al.*, 2005; Westerink *et al.*, 2008; Zeiger *et al.*, 2001). In addition to the lesser potency of TCDD, the REP of PCB 126 in human cell systems appears to be almost two orders of magnitude lower than numerous findings in rodents and rodent cell cultures (Haws *et al.*, 2006). This species discrepancy is toxicologically significant because PCB 126 is a persistent coplanar PCB, the most potent PCB AHR agonist in rodents (i.e., REP_{PCB126} is one-tenth that of TCDD), and displays a relatively high affinity for AHRs of "responsive" rodents (e.g., C57 mice and Sprague-Dawley rats) (Bandiera *et al.*, 1982; Safe *et al.*, 1985). In addition, the apparent difference in *in vitro* PCB 126 REP estimates between human and rodent is not likely due to species differences in the affinity of the AHR for PCB 126. Specifically, PCB 126 maintains a "relative affinity" (compared to TCDD) of approximately 0.02 for both human and responsive rat AHR proteins (Fan *et al.*, 2009). Furthermore, the human AHR, when expressed in the liver of transgenic humanized C57 mice, also exhibits, as seen with TCDD, the expected 10-fold lesser binding affinity for PCB 126 compared to responsive wild-type C57 mice (Flaveny *et al.*, 2009). This 10-fold lesser affinity of PCB 126 can also be easily explained by the human AHR having the same ligand-binding domain amino acid substitution as "nonresponsive" DBA mice. However, this does not explain the apparent and even exaggerated response insensitivity of human cells to PCB 126. Thus, the remarkable species difference in PCB 126 REP might be due to species differences in relative intrinsic efficacy (compared to TCDD) rather than species differences in relative AHR affinity.

It is evident that the AHR is responsible for the suite of toxicities observed in rodents exposed to TCDD and DLCs (Okey, 2007; Perdew, 2008). However, the prototypical AHR-regulated gene, *CYP1A1*, appears not to play a prominent role in some TCDD toxicities (Nukaya *et al.*, 2009; Pohjanvirta, 2009; Uno *et al.*, 2004), questioning its use as a universal "biomarker of effect." Since potencies and REPs of ligands may vary across responses mediated by the same receptor in the same cell type, termed "functional selectivity" (Kenakin, 2007; Michel and Alewijnse, 2007; Urban *et al.*, 2007), it is important to investigate and estimate the REPs of DLCs for altering AHR-regulated genes other than *CYP1A1*. Studies demonstrating human-rodent differences in the REP of PCB 126 *in vitro* have been limited to analysis of the dose-response for CYP1A1 enzyme induction and activity. Therefore, the current investigation sought to determine if the discrepancy in REP for PCB 126 observed between rat and human cell cultures for *CYP1A1* induction was also true for additional AHR-regulated genes that may play more important roles in subsequent toxicity. Genome-wide dose-responses were determined for TCDD and PCB 126 in cultures of primary hepatocytes from human and rat donors using species-specific microarrays. Species-specific TCDD 50% effective concentrations (EC50s) and PCB 126 REPs were simultaneously calculated for all genes satisfying a nonlinear mixed-effects dose-response model. By characterizing how REPs between TCDD and PCB 126 vary among the responsive genes and across species, one should gain greater insight into the human toxicological response to AHR activation. Overall, this study is consistent with the vision outlined in the recent NRC report (NRC, 2007b) to develop a greater understanding of how to use *in vitro*–derived data to predict toxicity across species.

MATERIALS AND METHODS

Chemicals. TCDD (molecular weight = 322) was obtained from Accustandard (New Haven, CT; catalog no. D404N; chemical abstract service (CAS) no. 1746-01-6; Lot no. 970401R-AC; 99.1% pure). The single contaminant was a pentachlorohydroxydiphenyl ether by gas chromatography/mass spectrometry (GC/MS). PCB 126 (molecular weight = 326.4) was obtained from Accustandard (catalog no. C-126N; CAS no. 57465-28-8; Lot no. 081699MT-AC; 99.2% pure). The single contaminant was identified as a tetrachlorobiphenyl by GC/MS.

Hepatocyte Sources. Cultures of human hepatocytes were prepared from nontransplantable human tissue acquired after informed consent for use in research by In Vitro Technologies, Inc. (IVT; Baltimore, MD). An external Food and Drug Administration-certified Institutional Review Board approved the use of human tissue for ADME-Tox research at IVT. Human donor 1 (WRG), IVT Lot MHU-L-052004, was a 41-year-old Caucasian male who died from an astrocytoma. Human donor 2 (RFA), IVT Lot FHU-L-072004, was a 56-year-old Caucasian female who died from a cerebrovascular accident. Human donor 3 (ZYZ), IVT Lot MHU-L-0730044, was a 46-year-old African-American male who died from anoxia. Donors 1, 2, and 3 of the current study correspond to human donors 3, 4, and 5 from the study of Silkworth *et al.* (2005), respectively. Serology for all human donors tested negative from human immunodeficiency virus, hepatitis B virus, and hepatitis C virus but positive for cytomegalovirus. Urinalyses and blood chemistries for all donors were within normal limits. Rat hepatocytes were isolated by IVT from six female Sprague-Dawley rats (Crl:CD(SD)IGS BR, Charles River Laboratories, Wilmington, MA) and divided into two pools of three rats each (i.e., termed Rat pool 1 and Rat pool 2). Rats were treated in accordance with the Animal Welfare Act.

Hepatocyte Cultures and Chemical Treatments. Hepatocytes were isolated according to the two-step collagenase perfusion procedure of Li *et al.* (1992). Isolated hepatocytes were counted using trypan blue exclusion to determine yield and to confirm >70% viability. Freshly isolated hepatocytes from rat or human donors were transferred into collagen-coated 75 cm^2 T-flasks at a cell density of 14×10^6 cells per flask in Plating Medium (Dulbecco's modified Eagle's medium [MEM]) supplemented with bovine serum albumin, fructose, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, sodium bicarbonate, L-glutamine [2.4mM], hydrocortisone (2.38μM), insulin [135nM], MEM nonessential amino acids [1.2%], amikacin, penicillin [200,000 U/l], streptomycin [200 mg/l], gentamycin, and Fungizone). A single flask was used for each cell type at each chemical treatment level. Cultures were placed in a 37°C/5% CO_2 incubator for 2 days before use to establish hepatocyte monolayers. Confluency was visually checked each day of the culture period and was generally 90–100%.

TCDD and PCB 126 stock solutions were prepared in dimethyl sulfoxide (DMSO) as previously described (Silkworth *et al.*, 2005). Established 48-h cell cultures were exposed to seven concentrations each of TCDD (ranging from 10^{-14} to $10^{-6.5}$M) or PCB 126 (10^{-12} to 10^{-5}M) or vehicle control (DMSO) in serum-free media for an additional 48 h at 37°C/5% CO_2. Serum-free medium was used, since serum can significantly reduce the cellular uptake of these chemicals (Hestermann *et al.*, 2000b). Exposure media was changed once at 24 h post-exposure initiation. The final DMSO concentration in the incubations (including the vehicle control) was 0.5%. There were no visible indications that any chemicals had precipitated at any of the incubation concentrations, but this was not analytically confirmed. Culture viability was assessed for each exposure group as previously described (Silkworth *et al.*, 2005). TCDD and PCB 126 did not affect culture viability at any concentration tested.

RNA Extraction and Microarray Processing. After 48 h treatment, total RNA was isolated from cells of each culture using TRIzol Reagent (Invitrogen Life Science, Carlsbad, CA). Cells were washed with ice-cold PBS and then disrupted by the addition of TRIzol Reagent followed by scraping. The 30 ml of cell solution was sent frozen at −70°C to the University of Memphis, where RNA isolation was completed. The solution was thawed at room temperature and let sit for 5 min. Total RNA was then isolated according the manufacturer's instructions. The RNA was dissolved in RNAse-free water and quantified by spectrophotometry. Quality of RNA isolations was determined using the Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA). Yields of rat RNA ranged from 59 to 200 μg. The RNA integrity numbers of all rat samples were 10. Yields of human RNA ranged from 56 to 153 μg. Again, RNA integrity numbers of all human samples were 10. One RNA sample from each exposure group was analyzed using Affymetrix Genechip technology according to the standard protocol. A single technical replicate (i.e., all procedures following RNA isolation were repeated on different days) was also generated for one PCB 126–exposed human cell culture. However, this technical replicate was only used for preliminary analyses and not included in the final modeling procedure. Human samples were analyzed using HG-U133A arrays (22,283 probe sets) and rat samples with RG-U34A arrays (8799 probe sets). The final data set utilized 40 human and 26 rat arrays following quality control (QC) analysis (see Probe Set Orthology Analysis subsection of Supplementary File 1 and QC results presented in Supplementary File 2). All raw microarray data (i.e., .CEL files) are available in the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) as series GSE14555.

Microarray Data Preprocessing. For dose-response modeling, all .CEL files (within each species) were preprocessed using the default settings of the justGCRMA function of gcrma package version 2.8.0 (Wu *et al.*, 2004) as implemented in R version 2.4.1 (R Development Core Team, 2006). This function background corrects perfect-match probe intensities using probe sequence information, $\log_2$ transforms the data, quantile normalizes across the arrays, and summarizes probe intensities via the robust multiarray average method (Irizarry *et al.*, 2003) to give an intensity value ($\log_2$ scale) for each probe set. All gcrma-processed intensity data for each probe set are available online at (www.ncbi.nlm.nih.gov/geo) as GEO series GSE14555. Probe sets were annotated with NetAffx annotation release #25 for rat chips (RG-U34A) and release #24 for human chips (HG-U133A).

Following preprocessing, fold change estimates were produced for each probe set by first back transforming the data from $\log_2$ and then dividing the probe set intensity for exposed cells by the vehicle control within each human donor/rat pool. Thus, if expression levels of both exposed and control were identical, then fold change equals one for that species-, chemical-, and subject-specific dose group. Fold change estimates for all rat and human probe sets are given in Supplementary Files 3 and 4, respectively.

REP Dose-Response Modeling. Figure 1 depicts the dose-response model selection procedure. Gene expression data were run separately through this four-step selection scheme for induced and repressed probe sets (within each species) due to different constraints required for some model parameters (described below). In step 1, probe sets that did not respond in cell cultures from at least two subjects/pools were removed using a fold change filter. For human data, in order to pass the filter, a ≥2 absolute fold change (i.e., either twofold induced or 0.5-fold repressed) in response to TCDD was necessary for at least two out of the three human donor cultures. The same filter was applied to the rat data except that ≥twofold change (induced or repressed) was needed for both rat pools in order to pass the initial filter.

Preliminary analyses revealed that for many probe sets passing the initial filter in step 1 data derived from one of the human donors failed to exhibit a fold change >1.5 for any TCDD dose (i.e., a nonresponsive human donor cell culture for that particular probe set). Inclusion of such data in downstream analyses either resulted in a failure to achieve model convergence or, if a model did converge (i.e., a model fit was achieved), relatively large within-group error standard deviations and parameter estimates with extremely wide confidence intervals (CIs) (see example in Supplementary File 5). Therefore, in step 2, any nonresponsive human donor data, within a probe set, were independently eliminated from downstream analyses. This was accomplished in a nonstringent manner for each human probe set by removing all donor-specific data if that donor cell culture did not exhibit a fold change ≥1.5 (induced or repressed) for any dose of TCDD. Thus, for many human probe sets, data from only two human donors were used for dose-response modeling. Nonresponding probe sets from rat pools were not present in the rat data set because the initial fold change filter in step 1 ensured that both pools responded at least twofold.

A modified version of the Hill equation (Hill, 1913) was employed for dose-response modeling in step 3 defined as

$$\mu(\delta) = 1 + \frac{(\alpha - 1)}{1 + 10^{\eta(\pi - \delta)}}, \quad (1)$$

where $\mu(\delta)$ is the estimated mean fold change response for a population at dose δ ($\log_{10}$M), α the maximal agonist effect (i.e., the change in height of the right asymptote), π the potency ($\log_{10}$M) at $\alpha/2$ (i.e., EC50), and η the Hill coefficient (also known as the Hillslope). The baseline expression level at $\delta = -\infty$ is equal to 1 for fold change data, allowing for this Hill model parameter to remain constant at 1.

One goal of the current study was to accurately estimate the REP_{PCB126} for all dose-response probe sets. In order to achieve this goal, dose-response curves were fit simultaneously for both TCDD and PCB 126 (the REP dose-response model) using the following indicator Equation 2:

$$\pi = \pi_{TCDD} I_{TCDD} + (\Delta\pi + \pi_{TCDD}) I_{PCB126}, \quad (2)$$

where π_{TCDD} is the estimated EC50 for TCDD (i.e., the base chemical) and $\Delta\pi$ is the relative change in EC50 (from TCDD) for PCB 126 data. The indicator functions I_{TCDD} and I_{PCB126} are equal to 1 when calculating the response of the subscripted chemical; otherwise, they are equal to 0. Therefore, if the EC50s for both TCDD and PCB 126 were identical, then the estimated $\Delta\pi$ would be equal to 0. Thus, the REP_{PCB126} can be easily derived from $\Delta\pi$ using Equation 3.

$$REP_{PCB126} = 10^{-\Delta\pi}. \quad (3)$$

In addition, the EC50 for PCB 126 could also be generated by adding the estimates for $\Delta\pi$ and π_{TCDD} together.

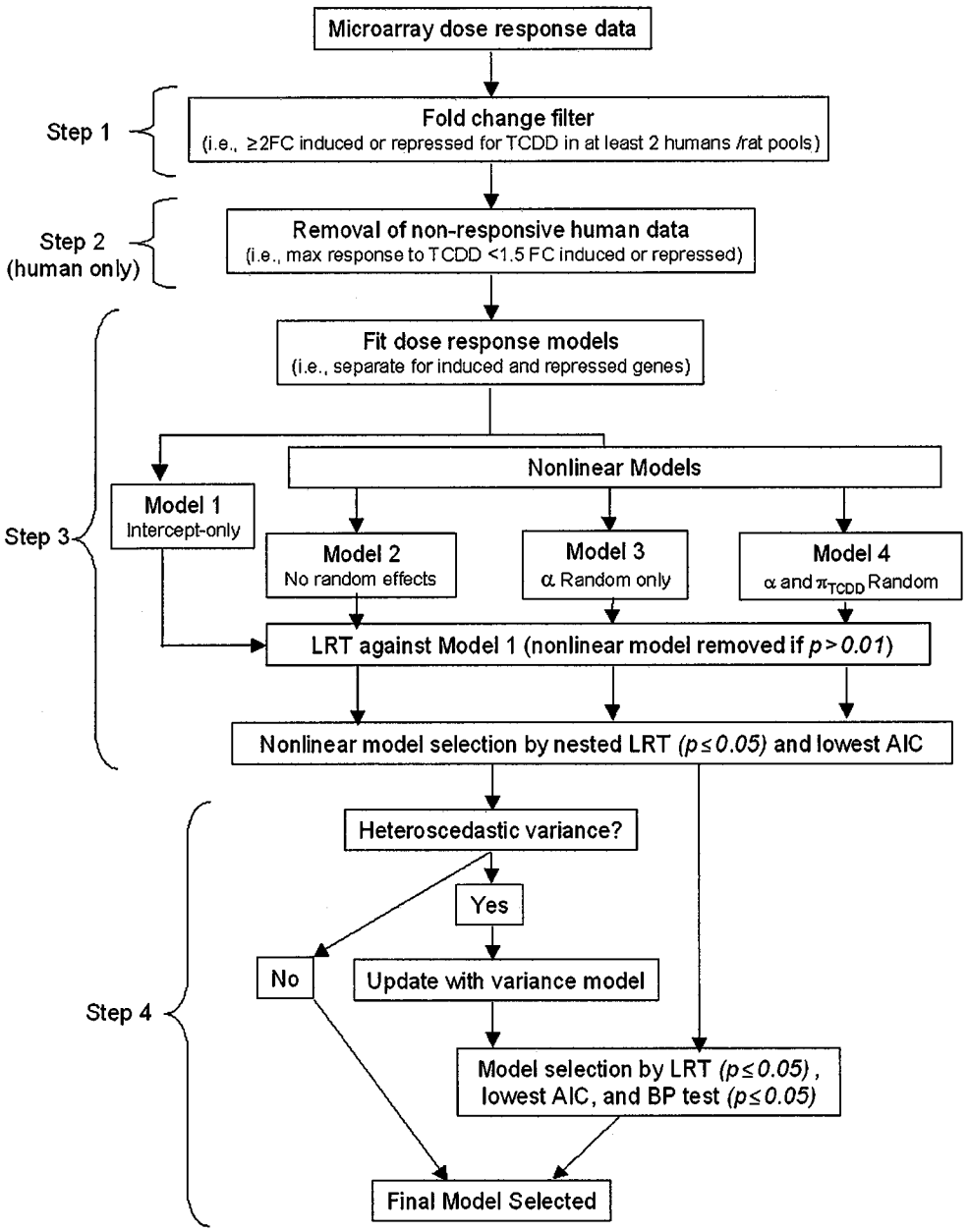

FIG. 1. Model selection scheme used to determine those genes (represented by probe sets) that satisfied dose-response criteria. Each probe set that passed an initial fold change filter in step 1 proceeded to step 2 where data for any nonresponsive human donor were removed. Step 3 tested for convergence using models 1–4 (described in the Materials and Methods section). Nonlinear models 2–4 that converged were only accepted if deemed significantly ($p \leq 0.01$) better fits than model 1 (i.e., intercept-only model) using LRTs. If multiple nonlinear models were deemed significantly better fits than model 1, the best-fit model was selected by nested LRTs ($p \leq 0.05$) and, if necessary, lowest AIC. Step 4 tested any nonlinear model selected during step 3 for homogeneity of residual variance using the BP test against heteroscedasticity (Breusch and Pagan, 1979) in step 4. The effects of incorporating a weighted power variance model (via LRT, lowest AIC, and BP tests) were then determined for any models demonstrating significant ($p \leq 0.05$) heteroscedasticity prior to selection of the final nonlinear dose-response model.

In order to maintain equal maximal agonist effects and parallel dose-response curves, which would be expected since PCB 126 is a full AHR agonist in primary hepatocytes from humans and rats (Silkworth *et al.*, 2005), both the maximal change in response α and Hill coefficient η were set not to vary between chemical congeners. However, preliminary analyses indicated that α and, in some instances, both α and π_{TCDD} could vary significantly among cell cultures derived from different human donors. Thus, it was apparent that many probe sets would require a nonlinear mixed-effects model with random effects allowing either α only or both α and π_{TCDD} to vary among cell cultures derived from different human subjects/rat pools.

Finally, four separate models (see below) were attempted during step 3 of the model selection procedure for each probe set passing the initial fold change filter.

$$\pi(\delta) = 1, \quad \text{(model 1)}$$

$$\pi(\delta) = f(\alpha, \pi_{TCDD}, \Delta\pi, \delta, \eta) + \varepsilon, \quad \text{(model 2)}$$

$$\mu(\delta)_i = f(\alpha + b_{1i}, \pi_{TCDD}\Delta\pi, \delta, \eta) + \varepsilon_i, \quad \text{(model 3)}$$

$$\mu(\delta)_i = f(\alpha + b_{1i}, \pi_{TCDD} + b_{2i}, \Delta\pi, \delta, \eta) + \varepsilon_i. \quad \text{(model 4)}$$

Model 1 represents a flat response that does not change with dose δ and has a y intercept of 1 (intercept-only model). Model 2 is a generalized nonlinear least squares model (i.e., no random effects), where each parameter in Equation 1 is explained by $f()$, and ε represents the residual error of $\mu(\delta)$. Model 3 is a nonlinear mixed-effects model with each parameter in Equation 1 explained by $f()$ whose arguments include a single random effect b_i, which represents the variability of fixed term α among the subjects/pools i. Model 4 is identical to model 3 with the exception of an additional random effect in the arguments of $f()$, where b_{1i} and b_{2i} represent the variability of fixed terms α and π_{TCDD}, respectively, among the subjects/pools i. The value of index i depended upon the number of donors/pools used in the dose-response modeling (i.e., $i = 1, \ldots,$ [2–3]).

All random effects in models 3 and 4 are assumed to be normally distributed with mean 0 and variance-covariance matrix Ψ (i.e., a general positive-definite matrix). The residual errors ε and ε_i are assumed to be independently distributed as $N(0,\sigma^2)$ and ε_i independent of any random effects.

The $EC50_{TCDD}$ estimate (i.e., π_{TCDD}) was constrained to be between -14 $\log_{10}$M and -6.5 $\log_{10}$M to ensure a maximal TCDD response was achieved within the observed dose range. The Hill coefficient (η) was constrained to be ≥ 0. Since induced and repressed probe sets were modeled separately, α was constrained to be ≥ 1 for induced probe sets and constrained between 1 and 0 for repressed probe sets. The method for estimation of model parameter starting values is given in the Model Starting Value Estimation subsection of Supplementary File 1.

Initial model selection in step 3 was conducted by testing any nonlinear models that converged (i.e., models 2, 3, and/or 4) against the intercept-only model (model 1) by likelihood ratio tests (LRTs) at a stringent significance level of $p \leq 0.01$, and the Akaike information criteria (AIC) of each model were compared (Akaike, 1974). If any nonlinear model failed to be significantly better than model 1 (i.e., either $p > 0.01$ or $p \leq 0.01$ and $AIC_{model\ 1} <$ AIC of nonlinear model), it was removed from further analyses. For the second selection round, the best fitting model was determined by nested LRTs and, if LRT was significant at the $p \leq 0.05$ level, the lowest AIC. The most parsimonious model (i.e., order of complexity was models $2 < 3 < 4$) was chosen if any LRT failed to reach the stated significance level of $p \leq 0.05$. Preliminary analyses of selected best-fit dose-response models indicated that the residual variance might not be constant with increasing/decreasing predicted response for some probe sets (e.g., the human probe set for *CYP1A1*). Methods for the variance modeling procedure used in step 4 are described in the Residual Variance Modeling subsection of Supplementary File 1.

Finally, for later derivation of EC50 and REP values, maximum likelihood estimates (MLEs; i.e., approximations of the population mean) and 95% upper and lower confidence bounds for parameters α, π_{TCDD}, $\Delta\pi$, and η were recorded for each modeled probe set following completion of the model selection scheme depicted in Figure 1. For graphing purposes and tabular data, $\Delta\pi$ was converted to the REP_{PCB126} values using Equation 3. In addition, for summary statistics, it was necessary to remove data from redundant probe sets representing the same species-specific gene. This was always accomplished in the most conservative manner. For example, when determining the geometric mean REP, redundant probe sets were removed by only using data from the probe set with the lowest $\Delta\pi$ MLE (i.e., the highest REP_{PCB126} estimate).

Various functions in the R package nlme version 3.1-88 (Pinheiro and Bates, 2004) were used for the dose modeling procedure: model 1 was fit with gls, model 2 with gnls, and models 3–4 employed the nlme function. The "method" argument for all models was set to "ML" or maximized log likelihood. The nonparametric Kruskal-Wallis rank sum and Mann-Whitney tests were performed using the kruskal.test and wilcox.test functions of the R package stats. Post hoc testing following significant ($p \leq 0.05$) Kruskal-Wallis tests utilized the kruskalmc function of the pgirmess package of R.

RESULTS

Microarray Rat/Human Ortholog Analysis

The main objective of the present study was to determine if the previously observed species difference in PCB 126 REP for CYP1A1 induction/activity also applied to genes other than CYP1A1. To accomplish this, it was necessary to identify genes that demonstrated a dose-response to both TCDD and PCB 126 in either species. It was also important to identify orthologous rat/human genes that were present on the two species-specific microarrays used in this investigation. The complete methodology for this comparative examination is given in the Probe Set Orthology Analysis subsection of Supplementary File 1. Overall, somewhere between 4158 and 4190 orthologs were shared between the two microarrays. The exact number of shared orthologs could not be determined because some human Entrez gene IDs were actually orthologous to multiple rat Entrez genes. Approximately 96% of the human orthologs identified on the rat RG-U34A array were also represented on the human HG-U133A array. Conversely, approximately 39% of the rat orthologs identified on the human HG-U133A array were also present on the rat RG-U34A array. However, this microarray gene overlap, determined by an entirely automated process, was limited by the quality/completeness of several databases and the accuracy of the microarray probe set annotation.

Preliminary Analysis of CYP1A1 Dose-Response Data

A preliminary analysis of REP dose-response curves fit by models 2–4, as well as additional models not used in the full analysis, was conducted with the HG-U133A probe set 205749_at representing the known AHR-regulated human gene *CYP1A1*. Visual inspection of dose-response data for *CYP1A1* revealed that at least a random effect allowing the α parameter to vary among individual human donor cell cultures was needed. This was statistically validated by comparing fits for model 2 (i.e., no random effects) and model 3 (α random only) by a LRT (Table 1). Although model 4 did converge, this more complex model was not proven a significantly ($p = 0.09$) better fit than the more parsimonious model 3. Other models, differing in random effects from models 3 and 4 (e.g., a model with π_{TCDD} as a random effect only), were also attempted, but these models either did not converge or were not deemed significantly better fits than model 3 as determined by nested LRTs and/or lowest AIC (data not shown).

Plotting the standardized residuals versus predicted values for the model 3 fit of *CYP1A1* clearly demonstrated a heteroscedastic trend (i.e., increasing residual variance with

TABLE 1
Model Selection Results for Human *CYP1A1* (*205749_at*)

Model[a]	Description	AIC[b]	ΔAIC[c]	*p* value[d]
1	*y* Intercept only	578	—	—
2	No random effects	559	−19	<0.0001
3	α Random (selected)	490	−69	<0.0001
4	α and π_{TCDD} Random	489	−1	0.0868

[a]Models listed in increasing order of complexity.
[b]AIC (Akaike, 1974). Lower score signifies better fit.
[c]Change in AIC from less complex model.
[d]*p* value for nested LRT against next less complex model.

increasing predicted response; data not shown), and a Breusch-Pagan (BP) test ($p = 1.7 \times 10^{-5}$) (Breusch and Pagan, 1979) confirmed this heteroscedasticity. Thus, an extended version of model 3 was attempted with a power variance function to model the variance with increasing predicted response as described by Equation 4 (see Residual Variance Modeling subsection of Supplementary File 1). This extended model provided significantly better fit than that of the equal variance model ($p < 0.001$ by LRT) with homogenous ($p = 0.7675$ by BP test) and normally distributed ($p = 0.4733$ by Pearson test) residual variance. Figure 2a depicts the donor-level dose-response for the human *CYP1A1* probe set (i.e., 205749_at) predicted by the final model chosen (i.e., model 3 extended with a power variance function) via the model selection scheme in Figure 1. For comparative purposes, the dose-response for rat *Cyp1a1* probe set E00778cds_s_at predicted by model 3 is given in Figure 2b.

The PCB 126 REP for the human *CYP1A1* probe set 205749_at was 0.001 (95% CI = 0.00047, 0.0022) and REPs for the rat *Cyp1a1* probe sets E00778cds_s_at and E00717UTR#1_s_at were 0.068 (95% CI = 0.02, 0.24) and 0.027 (95% CI = 0.0049, 0.15), respectively (Supplementary File 6). Thus, as previously determined by Silkworth *et al.* (2005), the present investigation has reproduced the robust species divergence in the REP of PCB 126 for induction of *CYP1A1* gene expression using a different technology, microarrays.

REP Dose-Response Modeling

Table 2 summarizes the results from the REP dose-response model selection scheme (Fig. 1). Altogether, 831 human probe sets and 365 rat probe sets passed the initial fold change filter in step 1. However, many probe sets were subsequently eliminated due to failure to generate any convergent dose-response nonlinear models (i.e, model 2, model 3, or model 4) in step 3 (Fig. 1). Nonlinear models were selected for a total of 57 human probe sets (45 induced and 12 repressed) and 97 rat probe sets (48 induced and 49 repressed). In this report, EC50 and REP values are estimates derived from model output parameters. Such parameter MLEs used to estimate EC50 and REPs and their 95% upper and lower confidence bounds for all rat and human probe sets successfully modeled are given in Supplementary File 6. In addition, boxplots of residuals and plots of residuals versus fitted values are presented in Supplementary Files 7 and 8 for all modeled human and rat genes, respectively. Table 3 summarizes the $EC50_{TCDD}$ (i.e., π_{TCDD}) and REP_{PCB126} estimates for the 10 most induced (i.e., largest α MLE) and 10 most repressed (i.e., smallest α MLE) human probe sets. For convenience, REP estimates are reported in Table 3, rather than $\Delta\pi$, as calculated using Equation 3. Model estimates for the 10 most induced and 10 most repressed rat probe sets are given in Table 4.

One important observation was that significant variation in predicted maximal agonist effect (i.e., α parameter) existed not only among human donor cell cultures but also between cell cultures derived from the two separate rat pools. This is clearly demonstrated by the selection of models possessing at least an α random effect (i.e., models 3 and 4) for a significant number of probe sets during step 3 (Table 2). Such variation could be due to interindividual differences in response or to technical variation. Since fresh primary human hepatocyte cultures were used, cells from the different human donors could not be cultured, exposed, and processed for RNA isolation on the same day. For consistency, experimentation on the two rat pools was similarly conducted at different times. In addition, all procedures downstream of RNA isolation (including array hybridization) were done in batches that did not overlap among human donors or between rat pools. Therefore, potential "batch effects" were primarily confounded within donors/pools.

A single technical replicate was performed on a human donor 2 sample (i.e., -12 $\log_{10}$M PCB 126 dose), where all technical processes following RNA isolation were conducted at different times on the same RNA sample. This limited analysis revealed a relatively higher correlation at both the probe level and probe set level (i.e., gcrma preprocessed) between the technical replicates compared to a biological replicate or another low-dose, donor-matched array processed in the same "batch" (data not shown). Thus, this replicate does provide some evidence that technical differences following RNA isolation may account for a limited amount of the total variation observed in this study, but it does not rule out the potential influence of technical variation introduced prior to and including RNA isolation.

Variation in cell culture response level was clearly evident among the human donors. Nonresponsive human donor data were removed for nearly 53% of the human probe sets (i.e., either induced or repressed) during step 2. Although there did not appear to be a trend as far as which human donor cell culture was identified as nonresponsive in step 2, the cell culture for human donor 2 was identified as nonresponsive for 42% of all human probe sets subsequently modeled in step 3. In addition, for many probe sets where donor 2 data were not removed in step 2, the general lower responsiveness of the cultures derived from human donor 2, similar to that seen for

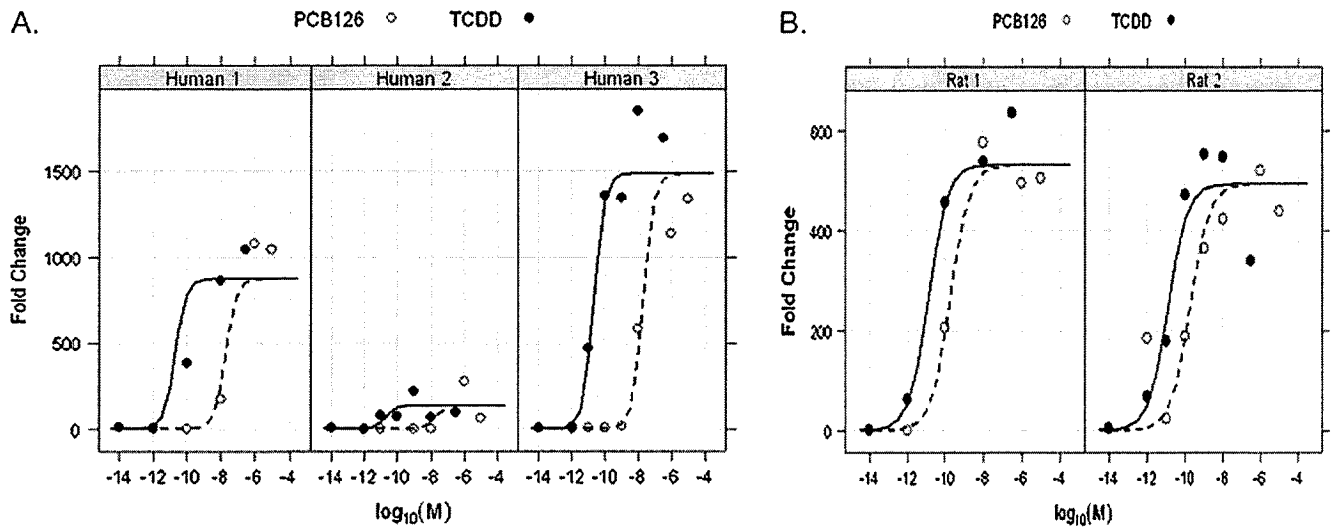

FIG. 2. Dose-response models for probe sets representing cytochrome P450 1A1 (CYP1A1) gene expression. Fold change estimates for each of three treated human donors (A) and each of two treated rat pools of two rats each (B) are shown (PCB 126 = open circles, TCDD = closed circles). The responses from human HG-U133A *CYP1A1* probe set 205749_at and the rat RG-U34A *Cyp1a1* probe set E00778cds_s_at are plotted against the nominal culture concentration ($\log_{10}$M). Nonlinear individual responses predicted by each species-specific dose-response model are represented by solid and dashed lines for TCDD and PCB 126, respectively.

human *CYP1A1* (Fig. 2a), appeared to be driving the selection of models possessing at least an α random effect in step 3. All modeled human probe sets where a nonresponsive donor was removed in step 2 are clearly marked in Supplementary File 6. Removal of donor-specific data added potential bias to this study toward finding more genes displaying a dose-response to both TCDD and PCB 126. Implementation of the step 2 procedure (Fig. 1) resulted in the successful modeling of 20 additional human probe sets that failed to generate convergent models if step 2 was not conducted (data not shown). Furthermore, for many probe sets with a nonresponsive donor that were modeled without step 2 implementation, subsequent removal of the nonresponsive donor data resulted in lower within-group error standard deviations and parameter estimates with relatively narrower CIs (e.g., see Supplementary File 5).

Since databases are constantly being updated, probe sets were curated using Entrez gene IDs to account for redundant probe sets, update gene names, and correct the initial annotations for the chips used. Overall, 47 distinct human and 79 distinct rat genes were identified. But, in highlighting the importance of this curation, three rat probe sets (J03524_s_at, J02669_s_at, and U10697_s_at) had each been

TABLE 2
Summary of the Model Selection Scheme Results Depicted in Figure 1

		Human		Rat	
Step[a]	Description[b]	Induced	Repressed	Induced	Repressed
1	Number of probe sets passing fold change filter	461	370	155	210
2	Percentage of probe sets for which only two human donors were modeled	60.5	43.5	NA[c]	NA
3	Model 2 selected probe sets	20	9	23	48
	Model 3 selected probe sets	17	2	25	1
	Model 4 selected probe sets	8	1	0	0
	Total number of probe sets modeled in step 3	45	12	48	49
4	Percentage for which the variance model was selected	48.9	0.0	39.6	42.9

[a]Steps 1–4 as depicted in Figure 1.
[b]The effect of various steps in the model selection scheme.
[c]Step 2 was only conducted for human gene expression data.

TABLE 3
Model Summaries for Top 10 Induced and Top 10 Repressed Human Probe Sets[a]

Direction	Probe set	Gene symbol	TCDD EC50[b]	PCB 126 REP[c]
Induced	205749_at	*CYP1A1*	−10.7 (−11.0, −10.4)	0.001000 (0.00047, 0.0022)
	202436_s_at	*CYP1B1*	−9.3 (−9.8, −8.9)	0.000920 (0.00038, 0.0022)
	202437_s_at	*CYP1B1*	−9.4 (−9.9, −8.9)	0.000600 (0.00022, 0.0016)
	202435_s_at	*CYP1B1*	−9.4 (−9.9, −8.9)	0.005900 (1.3E-05, 2.7)
	207609_s_at	*CYP1A2*	−10.3 (−11.2, −9.3)	0.000700 (0.00024, 0.0020)
	205623_at	*ALDH3A1*	−9.5 (−10.3, −8.7)	0.004100 (0.00063, 0.027)
	207608_x_at	*CYP1A2*	−10.5 (−11.2, −9.8)	0.001400 (0.00061, 0.0032)
	219255_x_at	*IL17RB*	−9.1 (−9.4, −8.8)	0.001500 (0.00042, 0.0051)
	201798_s_at	*FER1L3*	−8.7 (−8.9, −8.5)	0.000480 (0.00029, 0.00079)
	201195_s_at	*SLC7A5*	−9.6 (−10.5, −8.8)	0.000730 (9.1E-05, 0.0059)
Repressed	205649_s_at	*FGA*	−8.9 (−13.1, −4.8)	0.001300 (1.8E-05, 0.092)
	213920_at	*CUTL2*	−9.6 (−10.6, −8.6)	0.000066 (3.1E-06, 0.0014)
	206643_at	*HAL*	−9.4 (−10.1, −8.6)	0.000910 (8.5E-05, 0.0096)
	206002_at	*GPR64*	−10.6 (−13.2, −8.1)	0.000001 (2.8E-11, 0.0096)
	203661_s_at	*TMOD1*	−11.1 (−12.1, −10.0)	0.002800 (0.00012, 0.062)
	201010_s_at	*TXNIP*	−12.9 (−15.8, −10.2)	0.000002 (7.9E-10, 0.0049)
	201669_s_at	*MARCKS*	−12.4 (−15.3, −9.4)	0.000054 (6.2E-08, 0.047)
	206340_at	*NR1H4*	−8.5 (−9.7, −7.3)	0.001200 (5.8E-05, 0.026)
	222217_s_at	*SLC27A3*	−10.5 (−11.7, −9.3)	0.001800 (0.000035, 0.095)
	219718_at	*FLJ10986*	−9.5 (−11.3, −7.8)	0.000044 (4.2E-07, 0.0047)

[a]Probe sets with the 10 highest and 10 lowest MLEs for the α model parameter (fold change).
[b]EC50 $\log_{10}$M (95% confidence bounds) derived from the MLE for the π_{TCDD} model parameter (EC50).
[c]REP (REP) with 95% confidence bounds derived from the MLE $\Delta\pi$ parameter (REP) using Equation 3 of the Materials and Methods section.

mapped to multiple genes due to a lack of probe specificity and/or poor rat gene annotation. After such correction, a total of 75 distinct rat expression measures were successfully modeled for REP and used for downstream analyses of model parameters.

Having identified a set of responsive gene probe sets for both species, we could then test the initial hypothesis that there are genes, in addition to CYP1A, for which the PCB 126 REP for human cells is also far less than 0.1 (i.e., the approximate mean value observed in mainly rodent studies as summarized by Haws *et al.* 2006). For each model, a one-tailed hypothesis test was conducted to test whether the $\Delta\pi$ parameter was significantly greater than 1 (i.e., $REP_{PCB126} < 0.1$), and results were recorded in Supplementary File 6. For human models, 77.5% (i.e., 44 out of 57 probe sets) possessed REP estimates significantly ($p \leq 0.05$) lower than 0.1. Only 17.5% of rat modeled probe sets (i.e., 17 out of 97) had REP estimates significantly ($p \leq 0.05$) lower than 0.1.

The distributions of species-specific PCB 126 REP estimates are given as Tukey boxplots in Figure 3. In order to remove the influence of redundant probe sets, the geometric mean REP was conservatively represented for each species using only the lowest $\Delta\pi$ MLE (i.e., highest REP estimate) for each distinct gene expression measurement. The resulting geometric mean REPs were 0.0022 (95% CI = 0.001, 0.005) and 0.057 (95% CI = 0.03, 0.1) for human and rat genes, respectively. Wilcoxon-Mann-Whitney tests on the $\Delta\pi$ parameters found that the median human REP was significantly different from the median rat REP ($p = 1.6 \times 10^{-10}$) and the median REP estimates from either species were significantly ($p \leq 0.05$) less than 0.1.

Figure 4 depicts the relationship between the estimated EC50s for TCDD and PCB 126 of all successfully modeled probe sets within each species. Redundant probe sets representing the same gene were included in Figure 4 in order to more accurately portray the full range of estimates obtained. The PCB 126 EC50 values were calculated by adding MLEs of model parameters π_{TCDD} and $\Delta\pi$ for each probe set. Clearly, the majority of $EC50_{PCB126}$ estimates for human probe sets are found at concentrations greater than -8 $\log_{10}$M, while most of rat probe sets had $EC50_{PCB126}$ estimates at concentrations less than -8 $\log_{10}$M. This aspect of the species differences in sensitivity to PCB 126 is not fully appreciated when just examining REPs since, as a ratio, it disguises the underlying EC50s.

Cross-Species Comparison of Modeled Genes

Successfully modeled genes were screened to identify rat/human orthologs using the getHOMOLOG function of the R package annotationTools version 1.8.0 as described in detail in Supplementary File 1. Briefly, Entrez gene IDs prescribed to each probe set of interest were used to query the Homologene database for any associated orthologous genes for the opposing species. Forty-five distinct rat genes were identified as potential orthologs to the 47 modeled human genes. Human orthologs were identified for 62 out of 79 rat genes modeled. In this specific case, the failure to identify rat orthologs to two of the modeled human genes was due to microarray annotation error and

TABLE 4
Model Summaries for Top 10 Induced and Top 10 Repressed Rat Probe Sets[a]

Direction	Probe set	Gene symbol	TCDD EC50[b]	PCB 126 REP[c]
Induced	E00778cds_s_at	*Cyp1a1*	−10.9 (−11.3, −10.5)	0.068 (0.02, 0.24)
	E00717UTR#1_s_at	*Cyp1a1*	−11.6 (−12.3, −10.9)	0.027 (0.0049, 0.15)
	E01184cds_s_at	*Cyp1a2*	−10.9 (−11.7, −10.0)	0.047 (0.0039, 0.56)
	M33312cds_s_at	*Cyp2a1*	−10.4 (−11.2, −9.5)	0.035 (0.0038, 0.33)
	rc_AI176856_at	*Cyp1b1*	−10.1 (−10.6, −9.6)	0.038 (0.0068, 0.22)
	M26127_s_at	*Cyp1a2*	−11.2 (−11.6, −10.7)	0.010 (0.0025, 0.042)
	X83867cds_s_at	*Cyp1b1*	−10.0 (−10.6, −9.3)	0.050 (1.2E-05, 258)
	K00136mRNA_at	*LOC494499*	−11.0 (−11.7, −10.2)	0.016 (0.0019, 0.14)
	J02669_s_at	*Cyp2a1/Cyp2a2*	−10.8 (−11.8, −9.8)	0.014 (0.00089, 0.23)
	U90448_at	*Cxcl5*	−9.9 (−10.8, −9.0)	0.140 (0.013, 1.5)
Repressed	J00692_at	*Acta1*	−11.8 (−12.3, −11.3)	0.010 (0.0022, 0.044)
	D89375_s_at	*Sult1b1*	−10.8 (−11.6, −9.9)	0.680 (0.074, 6.3)
	M33550cds_s_at	*Cyp2c40*	−10.3 (−11.3, −9.4)	0.390 (0.025, 6.2)
	S77900_g_at	*Myl9_predicted*	−12.4 (−13.4, −11.5)	0.006 (0.00047, 0.08)
	M83107_at	*Tagln*	−11.9 (−12.5, −11.3)	0.010 (0.0048, 0.025)
	M18335_f_at	*Cyp2c7*	−10.7 (−11.5, −9.9)	0.370 (0.041, 3.2)
	M14775_s_at	*Cyp2c7*	−11.3 (−12.0, −10.5)	0.049 (0.0048, 0.5)
	M83107_g_at	*Tagln*	−11.8 (−12.3, −11.3)	0.010 (0.0024, 0.042)
	J03786_s_at	*Cyp2c40*	−10.3 (−11.0, −9.6)	0.210 (0.023, 1.9)
	M31031mRNA_f_at	*Cyp2c7*	−10.9 (−11.5, −10.2)	0.140 (0.024, 0.79)

[a]Probe sets with the 10 highest and 10 lowest MLEs for the α model parameter (fold change).
[b]EC50 $\log_{10}$M (95% confidence bounds) derived from the MLE for the π_{TCDD} model parameter (EC50).
[c]REP with 95% confidence bounds derived from the MLE $\Delta\pi$ parameter (REP) using Equation 3 of the Materials and Methods section.

incomplete rat genome annotation. Although similar annotation problems resulted in a failure to identify human orthologs for some rat genes (e.g., UDP-glucuronosyltransferase isoforms), a number of modeled rat genes lack clear human orthologs (e.g., rat *Mug1/2* and some cytochrome P450 isoforms).

Only 24 of 45 identified human/rat orthologs modeled for human REP (i.e., ~53%) were represented by probe sets on the rat RG-U34A array. Yet, 57 of the 62 human/rat orthologs modeled for rat REP (i.e., ~92%) were represented by probe sets on the human HG-U133A array. Thus, orthologous probe sets for the vast majority of rat genes demonstrating dose-response to TCDD and PCB 126 were also present on the human HG-U133A array yet failed to yield dose-response models. Conversely, many human modeled genes did not have a rat ortholog on the RG-U34A array. Wilcoxon-Mann-Whitney tests were performed, within each species, to detect significant differences between median REP estimates for orthologs shared across arrays versus those responsive genes that were not shared between the arrays. Differences (i.e., $p \leq 0.05$) were not detected for either species using these tests, indicating that the clear species difference in REP estimates seen in Figure 3 is consistent regardless of whether the responsive orthologs were present on both species-specific arrays or not.

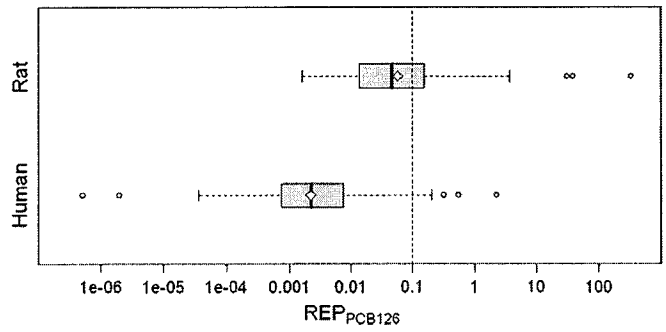

FIG. 3. Standard Tukey boxplots summarizing the geometric mean PCB 126 REP (REP_{PCB126}) MLEs predicted for 47 human and 75 rat genes. Only "distinct" genes were used in the summarization by conservatively representing each gene with the redundant probe set possessing the highest REP_{PCB126} estimate. Note that the x-axis is in $\log_{10}$ scale. Center black line of each box represents the median value, open diamonds are the geometric means, and hinges are the first and third quartiles. Whiskers extend to the most extreme data point no more than 1.5 times the interquartile range away from the box. Open circles represent statistically determined outliers.

Only five orthologous genes (represented by 16 probe sets) were commonly modeled in both rat and human: rat *Cyp1a1* (i.e., E00717UTR#1_s_at and E00778cds_s_at) and human *CYP1A1* (i.e., 205749_at), rat *Cyp1b1* (i.e., rc_AI176856_at and X83867cds_s_at) and human *CYP1B1* (i.e., 202435_s_at, 202436_s_at, and 202437_s_at), rat *Cyp1a2* (i.e., E01184cds_s_at and M26127_s_at) and human *CYP1A2* (i.e., 207608_x_at and 207609_s_at), rat *Nqo1* (i.e., J02679_s_at) and human *NQO1* (i.e., 201468_s_at), and rat *Hal* (i.e., M58308_at) and human *HAL* (i.e., 206643_at). Interestingly,

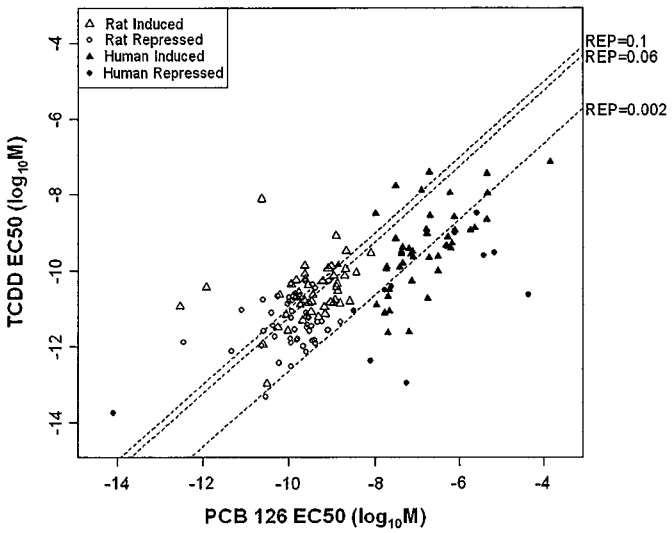

FIG. 4. Relationship between the estimated EC50s for TCDD and PCB 126 for all successfully modeled probe sets. Note that more than one probe set in this figure may represent the same species-specific gene since redundant probe sets were included. The units for both axes are $\log_{10}$ Molar. Symbols differentiate data for induced (triangles) and repressed (circles) human (solid symbols) and rat (open symbols) probe sets. The EC50s for PCB 126 were derived by adding the MLEs for the π_{TCDD} and $\Delta\pi$ model parameters. Diagonal dashed lines represent locations of REP_{PCB126} values of interest (identified at the upper right on outside of plot).

the *HAL* ortholog was induced in rat cells and repressed in human cells by both TCDD and PCB 126.

The TCDD and PCB 126 dose-responses of each of the four orthologous genes induced in both species were modeled simultaneously to generate cross-species REP estimates (compared to rat TCDD) for human TCDD (i.e., rat TCDD EC50 divided by human TCDD EC50) and human PCB 126 (i.e., rat TCDD EC50 divided by human PCB 126 EC50). This cross-species model also generated a rat TCDD EC50 and an "intraspecies" REP estimate for rat PCB 126 (i.e., rat TCDD EC50 divided by rat PCB 126 EC50). Complete methodology for cross-species REP modeling is given in the Cross-Species Dose-Response Modeling subsection of Supplementary File 1. Probe sets representing the *HAL/Hal* orthologs were not modeled for cross-species REP since the divergent direction of gene expression change between species would require a significantly more complex model. Table 5 displays the cross-species REP model parameter estimates for each of the modeled orthologous genes identified as induced in both species.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Enrichment

Since few orthologs were found in common between species among the modeled genes, all modeled probe sets were screened for any enriched process or functional associations (i.e., gene ontology [GO] categories or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways) that may be shared between the two species. This could provide insight into the common genetic pathways of toxicity. The full methodology for GO and KEGG enrichment analysis is given in Gene Ontology and Pathway Analysis subsection of Supplementary File 1. Both induced and repressed probe set lists were analyzed together within each species, and either the RG-U34A or HG-U133A array served as the background for rat or human probe sets, respectively. Tables 6 and 7 summarize the terms significantly ($p \leq 0.05$) enriched in human and rat probe set lists, respectively. Only three enriched terms were shared by both species: the GO molecular function term "unspecified monooxygenase activity" (GO:0050381) and KEGG pathways "tryptophan metabolism" (00380) and "metabolism of xenobiotics by cytochrome P450" (00980).

Figure 4 demonstrates that *in vitro*–derived potencies and PCB 126 REPs vary somewhat across individual genes both within and between species. Variation in the mean TCDD EC50s among enriched categories was also seen for both species (Tables 6 and 7). The difference in the mean TCDD EC50 of the most and least sensitive category in either species was slightly greater than 10-fold. There was also variation in mean rat REP estimates across significantly enriched functional categories (Table 7). For instance, the difference between mean REPs for the KEGG pathways "arachidonic acid metabolism" and "caffeine metabolism" is approximately 10-fold; yet, these two categories possess approximately the same TCDD EC50 (i.e., -10.9 $\log_{10}$M). Conversely, little variation was observed in mean PCB 126 REP estimates for enriched human pathways (Table 6), with the possible exception of the slightly higher mean REP for the KEGG pathway "bile acid biosynthesis" which also possessed a comparatively higher mean TCDD EC50 (i.e., -8.9 $\log_{10}$M). Overall, data from the current *in vitro* study demonstrated that human REP estimates did not vary widely across significantly enriched gene functional categories. However, considerably more variation in REP for enriched categories was observed for rat data.

Incorporation of Chemical-Specific Dose-Response Models

In order to elucidate genes that were potentially responsive to either TCDD or PCB 126 in a chemical-specific manner and to capture important genes potentially missed when both chemicals were modeled simultaneously, models representing a single dose-response curve were attempted separately for each chemical (i.e., chemical-specific dose-responses). A similar model selection scheme to that depicted in Figure 1 was employed except that the initial fold change filter required a twofold change in at least two human subjects/rat pools for only the chemical of interest (i.e., the PCB 126 fold changed filtered probe set list differed from that used for REP modeling). Application of this modeling procedure using human probe sets generated additional models, not previously modeled for REP, for seven TCDD (i.e., one induced and six repressed) and 12 PCB 126 (i.e., 11 induced and 1 repressed)

TABLE 5
Cross-Species REP Models for Responsive Rat-Human Orthologs

Ortholog	Rat TCDD EC50[a]	Model REP estimates[b]		
		Rat TCDD EC50/human TCDD EC50	Rat TCDD EC50/rat PCB126 EC50	Rat TCDD EC50/human PCB126 EC50
CYP1A1	−11.1 (−11.6, −10.5)	0.27 (0.066, 1.1)	0.057 (0.011, 0.29)	0.00021 (0.000047, 0.0009)
CYP1B1	−10.0 (−10.5, −9.6)	0.28 (0.089, 0.87)	0.055 (0.009, 0.33)	0.00046 (0.000089, 0.0023)
CYP1A2	−10.9 (−11.1, −10.6)	0.11 (0.050, 0.26)	0.020 (0.0085, 0.046)	0.00004 (0.000011, 0.00014)
NQO1	−11.4 (−11.8, −10.9)	0.02 (0.00044, 0.88)	0.018 (0.0055, 0.061)	0.00007 (7.5E-10, 6.8)

[a]EC50 $\log_{10}$M (95% confidence bounds) derived from the MLE for the π_{TCDD} model parameter (EC50).
[b]REP with 95% confidence bounds derived from the MLE $\Delta\pi$ parameters (REP) as indicated using Equation 3 of the Materials and Methods section.

dose-response genes. Four TCDD-only (i.e., one induced and three repressed) and 17 PCB 126-only (i.e., 4 induced and 13 repressed) models were also obtained for rat genes. Supplementary File 9 contains all output data for both human and rat probe sets successfully modeled for chemical-specific dose-responses.

Figure 5 depicts the distribution of EC50s for TCDD and PCB 126 for all modeled rat and human genes including genes that responded in a chemical-specific manner. The probe set with the lowest EC50 was used to conservatively represent any redundant probe sets. Figure 5 clearly demonstrates that most human genes do not reach the EC50 level until the PCB 126 concentration exceeds −8 $\log_{10}$M, whereas the majority of rat genes reach their EC50 levels at TCDD and PCB 126 concentrations well below −8 $\log_{10}$M. Perhaps coincidently, the human TCDD response range appears to resemble the rat PCB 126 response range rather than that of rat TCDD. Except for the five orthologous genes, each treatment group represents different genes. Nevertheless, it is informative to note that, for those genes that did respond in a dose-dependent fashion, the treatments could be differentiated. The nonparametric Kruskal-Wallis rank sum test indicated significant differences ($p = 2.2 \times 10^{-16}$) among median EC50s of the experimental groups, and post hoc multiple comparison testing determined that the median rat EC50 for PCB 126 and human EC50s for TCDD and PCB 126 were significantly different ($p \leq 0.05$) from the median rat EC50 for TCDD. Furthermore, the median $EC50_{PCB126}$ for human and rat were significantly different ($p \leq 0.05$). Thus, this single chemical modeling procedure provided little additional evidence of divergent, chemical-specific genomic responses, in either species, as would be expected for these two full agonists of the AHR.

TABLE 6
Functional Class Enrichment for REP Modeled Human Genes

Category	Term ID	Term	# Genes	*p* Value[a]	TCDD EC50[b]	REP_{PCB126}[c]
Biological process	GO:0032787	Monocarboxylic acid metabolic process	5	9.0E-03	−9.5	0.0034
	GO:0048729	Tissue morphogenesis	3	1.7E-02	−8.8	0.0072
Molecular function	**GO:0050381**	**Unspecific monooxygenase activity**	**3**	**4.5E-03**	**−10.2**	**0.0020**
	GO:0004030	Aldehyde dehydrogenase [NAD(P)+] activity	2	2.8E-02	−9.7	0.0036
	GO:0004029	Aldehyde dehydrogenase (NAD) activity	2	3.7E-02	−9.7	0.0036
KEGG pathway	**hsa00380**	**Tryptophan metabolism**	**5**	**7.3E-05**	**−10.0**	**0.0025**
	hsa00980	**Metabolism of xenobiotics by cytochrome P450**	**4**	**2.0E-03**	**−10.0**	**0.0024**
	hsa00120	Bile acid biosynthesis	3	8.0E-03	−8.9	0.0138
	hsa00340	Histidine metabolism	3	1.1E-02	−9.6	0.0023
	hsa00071	Fatty acid metabolism	3	1.5E-02	−9.6	0.0049
	hsa00280	Valine, leucine, and isoleucine degradation	3	1.6E-02	−10.4	0.0011
	hsa00053	Ascorbate and aldarate metabolism	2	3.6E-02	−9.7	0.0036
	hsa00641	3-Chloroacrylic acid degradation	2	5.0E-02	−9.7	0.0036

Note. Categories in bold font were enriched in both human and rat gene lists.
[a]Modified Fisher exact *p* value (EASE score). Significance cutoff was set at $p < 0.05$ and a minimum of two genes/category.
[b]Geometric mean TCDD EC50 estimate ($\log_{10}$M) for each gene in that category.
[c]Geometric mean PCB 126 REP estimate (REP_{PCB126}) for each gene in that category. Redundant probe sets represented by highest REP_{PCB126} estimate.

TABLE 7
Functional Class Enrichment for REP Modeled Rat Genes

Category	Term ID	Term	# Genes	p Value[a]	TCDD EC50[b]	REP_{PCB126}[c]
Biological process	GO:0006953	Acute-phase response	5	5.4E-04	−10.7	0.053
	GO:0001936	Regulation of endothelial cell proliferation	3	6.5E-03	−11.3	0.020
	GO:0002526	Acute inflammatory response	5	1.3E-02	−10.7	0.053
	GO:0031960	Response to corticosteroid stimulus	4	1.4E-02	−11.0	0.047
	GO:0007517	Muscle development	6	1.9E-02	−11.8	0.037
	GO:0009310	Amine catabolic process	4	2.4E-02	−11.2	0.019
	GO:0042537	Benzene and derivative metabolic process	2	2.5E-02	−11.1	0.105
Molecular function	**GO:0050381**	**Unspecific monooxygenase activity**	**10**	**4.6E-09**	**−10.8**	**0.101**
	GO:0005506	Iron ion binding	13	2.0E-05	−10.8	0.084
	GO:0004867	Serine-type endopeptidase inhibitor activity	7	4.9E-05	−11.1	0.030
	GO:0015020	Glucuronosyltransferase activity	3	2.0E-02	−10.7	0.129
	GO:0042379	Chemokine receptor binding	3	4.1E-02	−10.2	0.194
	GO:0008009	Chemokine activity	3	4.1E-02	−10.2	0.194
KEGG pathway	**rno00980**	**Metabolism of xenobiotics by cytochrome P450**	**11**	**5.6E-09**	**−10.8**	**0.142**
	rno00591	Linoleic acid metabolism	5	5.8E-04	−10.9	0.200
	rno00232	Caffeine metabolism	4	6.4E-04	−10.9	0.022
	rno00380	**Tryptophan metabolism**	**5**	**2.5E-03**	**−11.1**	**0.036**
	rno00590	Arachidonic acid metabolism	5	8.7E-03	−10.9	0.264
	rno00150	Androgen and estrogen metabolism	4	3.4E-02	−10.6	0.110
	rno00040	Pentose and glucuronate interconversions	3	3.4E-02	−10.7	0.129

Note. Categories in bold font were enriched in both human and rat gene lists.
[a]Modified Fisher exact p value (EASE score). Significance cutoff was set at $p < 0.05$ and a minimum of two genes/category.
[b]Geometric mean TCDD EC50 estimate ($log_{10}M$) for each gene in that category.
[c]Geometric mean PCB 126 REP estimate (REP_{PCB126}) for each gene in that category. Redundant probe sets represented by highest REP_{PCB126} estimate.

DISCUSSION

Previous investigations using *in vitro* systems have clearly demonstrated that human cells are less sensitive than rodent cells to PCB 126–induced CYP1A1 activity (Drenth *et al.*, 1998; Silkworth *et al.*, 2005; Vamvakas *et al.*, 1996; van Duursen *et al.*, 2003; van Duursen *et al.*, 2005; Westerink *et al.*, 2008; Zeiger *et al.*, 2001). This species difference in sensitivity is beyond the well-described approximately 10-fold species difference in AHR affinity for TCDD (Ema *et al.*, 1994; Harper *et al.*, 1988; Ramadoss and Perdew, 2004). Competitive AHR-binding assays have indicated that the species difference in PCB 126 REP cannot be explained by species variation in PCB 126 relative affinity (i.e., compared to TCDD) for the AHR (Fan *et al.*, 2009; Flaveny *et al.*, 2009) and points toward a species discrepancy in PCB 126 relative intrinsic efficacy to activate the AHR. Since some toxicities attributed to TCDD and DLCs may be independent of the CYP1A1 enzyme (Nukaya *et al.*, 2009; Pohjanvirta, 2009; Uno *et al.*, 2004), the present investigation utilized two species-specific microarrays, sharing over 4000 rat/human orthologs, to generate dose-response models for genes responding to both TCDD and PCB 126. Thus, the goal of this study was to determine if the relative insensitivity of primary human hepatocyte cultures to PCB 126–induced *CYP1A1* gene expression could be extended to other responsive genes.

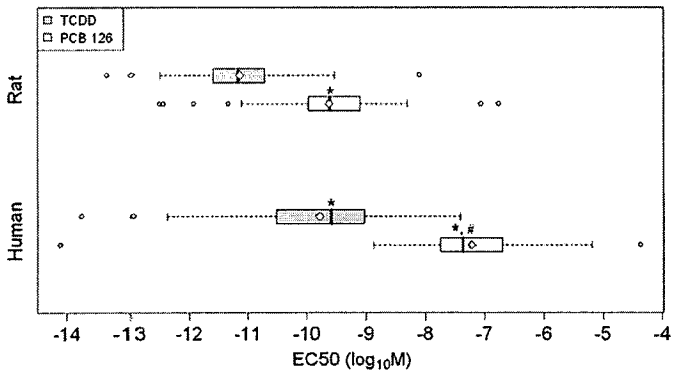

FIG. 5. Standard Tukey boxplots summarizing the geometric mean species-specific EC50 MLEs for TCDD and PCB 126. Redundant probe sets of the same gene were conservatively represented by the probe set with the lowest EC50 estimate. Note that the x-axis is in log_{10} Molar scale. Center black line of each box represents the median value, open diamonds are the geometric means, and hinges are the first and third quartiles. Whiskers extend to the most extreme data point no more than 1.5 times the interquartile range away from the box. Open circles represent outliers. Asterisks and pound (#) indicate statistical differences ($p \leq 0.05$) from unmarked EC50 estimates.

The relatively large variation in the maximal agonist effect (i.e., α parameter) among human donors and even between rat pools for some, but not all, probe sets made modeling dose-response quite challenging. However, the use of within-donor/pool fold change values and the implementation of mixed-effects

models allowed for the generation of numerous dose-response models in either species. Despite this accomplishment, for many human probe sets, only two out of three donors displayed a robust dose-response while the third donor failed to respond >1.5-fold at any dose measured. This resulted in either a lack of model convergence or, if a model was generated, an extremely poor fit. Thus, implementation of the nonresponsive human donor removal procedure (step 2, Fig. 1) not only addressed the poorly fit models in an entirely automatic manner (i.e., not requiring additional visual inspection) but also allowed for the generation of 20 additional dose-response models. This additional procedure did add potential bias to this analysis toward finding more human dose-response genes; a bias not inconsistent with the major goal of this study.

It is tempting to speculate that the large variation in maximal agonist effect observed among the human donors/rat pools was due entirely to interindividual differences in susceptibility to TCDD and PCB 126 (i.e., biological variation). Indeed, previous microarray investigations using primary human hepatocytes have observed large interindividual variations in both the basal expression hepatic enzymes (Slatter *et al.*, 2006) and xenobiotic-induced gene expression (Goyak *et al.*, 2008). In addition, since the rat pools were made from tissue of entirely different outbred animals, there is no reason to believe that these cultures would result in identical maximal responses. However, due to several aspects of the experimental design used in the current study, owing primarily to the utilization of fresh human hepatocytes, such conclusions regarding maximal response cannot be definitively made. Although a single technical replicate did suggest that technical aspects following RNA isolation may not have greatly influenced the data, this did not rule out technical differences due to date of RNA isolation and cell culture among donors and between rat pools. However, incorporation of within-donor/pool fold change estimates and mixed-modeling procedures to account for individual-level variation did allow us to successfully model many genes displaying dose-response in this study. Because the goal of our study was to estimate PCB 126 REPs, the real source of maximal agonist effect variation was not of primary interest as long as it could be accurately modeled. Particularly, since the height of the right asymptote of any one dose-response curve is not generally transferable across studies or end points, let alone species, making it more or less pharmacologically irrelevant in comparative studies.

The major goal of this study was to estimate species-specific PCB 126 REPs for all genes responding in a dose-response manner to both TCDD and PCB 126. The median PCB 126 REP estimate for 47 modeled human genes was significantly ($p \leq 0.05$) lower than both the median REP estimate of 0.1 found in the Haws *et al.* (2006) database (derived mainly from rodent studies) and the median REP for the 79 rat genes modeled in the current study. This lower median human REP is in line with a previous human primary hepatocyte–derived REP estimate of 0.003 for ethoxyresorufin-*O*-deethylase (EROD) activity (Silkworth *et al.*, 2005). Importantly, Silkworth *et al.* (2005) utilized primary hepatocytes derived from five human donors, including all three human donors used in the present study. Several other investigations using human cells/cell lines have also demonstrated PCB 126 REP estimates well below 0.1 (Drenth *et al.*, 1998; Pang *et al.*, 1999; Vamvakas *et al.*, 1996; van Duursen *et al.*, 2003; Westerink *et al.*, 2008; Zeiger *et al.*, 2001). The current genomic rat REP estimate was within an order of magnitude uncertainty of the Haws *et al.* (2006) median PCB 126 REP estimate of 0.1 and similar to previous REP estimates using rat primary hepatocyte EROD activity (Chen and Bunce, 2004; Silkworth *et al.*, 2005; Zeiger *et al.*, 2001). Therefore, the relative insensitivity of primary human hepatocytes to PCB 126 could be extended to genes other than *CYP1A1*. Conversely, the relative "sensitivity" of primary rat hepatocyte cultures to PCB 126 also extends to genes other than *Cyp1a1*.

So, why do *in vitro* PCB 126 REP estimates vary between rat and human? Although recent studies have suggested that human and responsive rodent AHRs share approximately the same relative affinity (compared to TCDD) for PCB 126 (Fan *et al.*, 2009; Flaveny *et al.*, 2009), there could conceivably be some aspect of initial receptor binding and occupancy related to species differences in PCB 126 REP that these studies have failed to quantitate. Perhaps, species differences in AHR structure affect some other aspect of receptor function downstream of initial ligand binding such as transactivation (e.g., Ramadoss and Perdew, 2005) or interaction with other signal transduction pathways. Finally, recent evidence of species differences in ligand-specific AHR-coactivator interactions (Zhang *et al.*, 2008) might explain the relatively weaker PCB 126 REP in human cells.

Conceivably, the more relevant question to address is why PCB 126 is so potent in the rat. The median rodent-derived PCB 126 REP given in the Haws *et al.* (2006) database is within a half order of magnitude of median REPs for several polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Haws *et al.*, 2006); yet, PCB 126 has a much weaker affinity for the rat AHR than 2,3,7,8-tetrachloro dibenzofuran (TCDF) (~68-fold difference), 1,2,3,7,8-pentachloro dibenzodioxin (PeCDD) (~53-fold difference), and 2,3,4,7,8-pentachloro dibenzofuran (PeCDF) (~16-fold difference) (Fan *et al.*, 2009). Furthermore, the affinity of the human AHR for PCB 126 is at least 50-fold weaker than these same DLCs measured by Fan *et al.* (2009), but in contrast to the rat, previous studies using human HepG2 cells expectedly estimate relatively higher REPs for 1,2,3,7,8-PeCDD (i.e., ~0.75; Lipp *et al.*, 1992) and 2,3,7,8-TCDF (i.e., ~0.09; Wiebel *et al.*, 1996) compared to PCB 126 (i.e., ~0.002; Silkworth *et al.*, 2005). All such data clearly demonstrate a peculiarly high intrinsic efficacy for PCB 126 to activate the rat AHR compared to human, over and above that expected for this class of ligand based solely upon receptor affinity.

A major finding of the current investigation was that the species-specific REP estimates generated for human and rat cell

cultures were derived from relatively nonoverlapping sets of genes (i.e., only five orthologs were modeled in both species). Furthermore, only one GO category and two KEGG pathways were similarly enriched between species for TCDD/PCB 126 dose-response, complicating any attempt to translate REP estimates for most functional categories across species. Interestingly, Rowlands *et al.* (2007) found extremely limited gene overlap between rat and human primary hepatocytes (i.e., *CYP1A1*, *CYP1A2*, *CYP1B1*, *TIPARP*, and *IL17RB*) using microarrays following exposure to TCDD, TCDF, or 4-PeCDF. Common TCDD-responsive gene sets were also quite limited (<40 genes) in two *in vivo* comparative studies using more closely related rat and mouse strains (Boutros *et al.*, 2008; Boverhof *et al.*, 2006). Since many of the toxic responses attributed to TCDD and DLCs appear to be shared between rat and mouse, these aforementioned *in vivo* rodent studies suggest a very limited repertoire of genes are directly involved in eliciting AHR agonist toxicity.

It is important to address the fact that most of the genes demonstrating dose-responses to both TCDD and PCB 126 in the present study were not shared between species. Failure to identify more commonly responsive human-rat orthologs may partly be technical in nature due to the incomplete overlap of genes represented on the two different microarrays, where only about half of the human genes modeled for REP had orthologs on the rat RG-U34A array. For instance, the well-described TCDD-responsive gene *TIPARP* (Ma *et al.*, 2001) was modeled for human PCB 126 REP in the current study, but the rat *Tiparp* ortholog is not represented by the RG-U34A array. However, this technical issue does not address why human-derived dose-response models were not generated for 52 of the 57 orthologs modeled for rat REP that were also present on the human HG-U133A array. Furthermore, the two species-specific microarrays employed in the current study shared over 4000 rat/human orthologs; yet, only five orthologs were modeled for dose-response in both species. Previous cross-species genome-wide screens for phylogenetically and positionally conserved dioxin response elements (DREs) (Sun *et al.*, 2004) and a preliminary gene promoter analysis of TCDD/PCB 126–responsive orthologs in the current study (data not shown) have clearly indicated that most core DRE sequences may not be well conserved between rat and human orthologs or even between rat and mouse orthologs. Furthermore, it appears that the human and rodent AHRs may differ greatly in their ability to recruit certain coactivator proteins (Flaveny *et al.*, 2008; Zhang *et al.*, 2008). Species differences in coactivator recruitment are likely related to divergent AHR structure and might explain the discrepancy in genes responding in the present investigation. In addition, divergent AHR functioning may explain why approximately four times more induced genes than repressed genes were seen for human cells compared to a relatively even ratio of induced/repressed genes seen for the rat cultures of the present study and in previous *in vivo* rat studies (Boutros *et al.*, 2008; Boverhof *et al.*, 2006). Although the exact mechanism for repression genes by TCDD is unknown, it appears that the AHR is required for the downregulation of at least some genes (Ovando *et al.*, 2006), and repression may involve AHR cross talk with other signal transduction pathways (Patel *et al.*, 2009).

One expectation of the current investigation, and of modern genomics in general, was that genes displaying clear dose-responses might be relatable to *in vivo* toxicities. Comparison of rat *in vitro* gene expression changes modeled in the current study with hepatic gene expression following acute exposure of rats to TCDD and Aroclor 1254 (Silkworth *et al.*, 2008) has defined a set of *in vivo-in vitro* transferable rat genes including *Cyp1a1*, *Cyp1a2*, *Cyp2a1/2*, *Cyp1b1*, *Cyp2b3*, *Nqo1*, *Ugt1a6*, *Ugt1a7*, *Igfbp1*, *Igf1*, *Mettl7b*, and *Tgfb1i4*. Many of the same responsive rat genes identified in both the study of Silkworth *et al.* (2008) and the present study were also differentially expressed at 13 weeks of exposure of female SD rats to TCDD or PCB 126 (Vezina *et al.*, 2004) and at 6–168 h following single oral gavage of male SD rats with TCDD (Fletcher *et al.*, 2005). Despite these aforementioned *in vitro-in vivo* similarities, a gene-by-gene comparison of the *in vitro* rat gene list obtained in the current study with the previously mentioned rat *in vivo* studies along with *in vivo* rat-mouse comparative studies by Boutros *et al.* (2008) and Boverhof *et al.* (2006) does reveal that many responses observed in the rat liver *in vivo* may not be predicted by *in vitro* responses. Differences in gene lists between *in vivo-in vitro* studies most likely involve a multitude of factors. For instance, Dere *et al.* (2006) compared *in vivo-in vitro* genomic responses to TCDD in mouse liver/mouse hepatoma cells and found exposure system differences that may have been either artifacts of cell culture (i.e., cell cycle genes) or secondary *in vivo* responses previously found to be well downstream of primary AHR signaling (Hayes *et al.*, 2007). Also, in some cases, the comparability of the present *in vitro* study with these *in vivo* investigations is limited due to the use of different animal strains, genders, and array platforms. Overall, one cannot expect primary cultures of a single cell type (e.g., hepatocytes) to entirely mimic the *in vivo* response of a complex organ such as the liver. Clearly, more information is needed to determine which gene expression changes observed in these previous *in vivo* studies demonstrate robust dose-responses and are related to actual toxic events. Nonetheless, the initial events eliciting *in vivo* toxicity of TCDD and DLCs most likely involves AHR activation of gene expression. Therefore, at least some of the potency and REP estimates made in the current *in vitro* study, which were entirely consistent with AHR regulation, will likely be relevant to AHR-mediated toxicity *in vivo*.

Mechanistic studies in various animal models, ranging from fish to birds and rodents, have determined that TCDD toxicity is mediated by the AHR. Although the genes modeled for rats and humans in this study were generally not the same, both sets were consistent with AHR regulation, that is, they responded in a dose-response manner to both TCDD and PCB 126.

Therefore, the REP estimates for these genes may be relevant to potential TCDD and PCB 126 hepatotoxicity in humans. Although the liver is perhaps the most sensitive target organ in the rat to TCDD and DLC toxicity, evidence in highly exposed humans suggests that human liver might not be a sensitive target organ for AHR-mediated toxicity. Since the skin is clearly the most overtly responsive human target organ to TCDD and DLC toxicity, future studies in our laboratory will focus on determining *in vitro* REPs for the most toxic DLCs in rodents (i.e., PCB 126, PCDDs, and PCDFs) using normal human epidermal keratinocytes.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

General Electric Company.

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Attachment J-3

SPECIES DIFFERENCES IN PCB TOXICODYNAMICS AND TOXICOKINETICS RELEVANT TO THE AROCLOR 1254 REFERENCE DOSE

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Introduction

Chemical-Specific Adjustment Factors (CSAFs) [1] and Data-Derived Extrapolation Factors (DDEFs) [2] can replace default uncertainty factors (UFs) for interspecies extrapolation (UF_A) and intraspecies variability (UF_H) commonly employed in the derivation of reference dose (RfD) human health risk values. For development of DDEFs, the default UFs are sub-divided into toxicokinetic (TK) and toxicodynamic (TD) components, representing 3-fold each (i.e., $\sim 10^{1/2}$). DDEFs for TK and TD are estimated using a variety of techniques from pharmacokinetic or biologically-based dose response models to relatively simple ratios quantitating animal-to-human sensitivity or human-to-human variability. Here, we generate potential DDEFs to replace the UF_A currently employed in the chronic oral RfD for the polychlorinated biphenyl (PCB) mixture, Aroclor 1254 [3]. The current Aroclor 1254 RfD is set by the US EPA at 2×10^{-5} mg/kg-d, based upon an oral-dosing study of rhesus monkeys (*Macaca mulatta*) [4,5]. The critical effects include various dermal lesions and decreased antibody response. The lowest dose employed (i.e., 0.005 mg/k-d) served as the LOAEL and this dose was divided by a composite UF of 300 to achieve the RfD. Of particular interest, the UF_A was reduced to 3 from the default of 10 due to, "... similarities in toxic responses and metabolism of PCBs between monkeys and humans and the general physiologic similarity between these species." [3] In this study, we analyze the necessity for a 3-fold UF_A in extrapolation to a human safe dose level. We also present new *in vitro* data which directly compares sensitivity of human and rhesus keratinocytes to induction of a biomarker for an early key event in the presumed mode of action (MOA). Following the DDEF guidelines, a quantitative extrapolation factor (EF) to replace the UF_A is derived.

Methods

Aroclor 1254, lot no. 122-078, was the same material used in several published studies [6,7]. The calculated dioxin toxic equivalency (TEQ) for the Aroclor lot used was ~21ppm (~70% due to PCB 126). Sources and purity analyses for the other chemicals have been described previously [8]. Neonatal foreskin normal human epidermal keratinocytes (NHEKs), purchased from Lonza (Walkersville, MD), were grown in keratinocyte-SFM (Invitrogen, Carlsbad, CA). Confluent fifth passage NHEKs were incubated in complete media (50 µg/ml bovine pituitary extract; 5 ng/ml EGF) for 48 h, changed to basal media (no supplements) for 24 h and then treated with chemicals in basal media for the time indicated or for 48 h in the dose-response studies [8]. Rhesus eyelid keratinocytes were purchased from Lonza. Confluent second passage rhesus keratinocytes were incubated in KBM Gold (Lonza) with the provided supplements, consisting of BPE, EGF, insulin hydrocortisone and epinephrine for 48 h, changed to basal keratinocyte –SFM for 24 h and then treated with vehicle (0.7% DMSO) or chemicals in keratinocyte-SFM for 48 h. Total mRNA was isolated using RNA Stat-60 (Tel-Test). Real-time PCR was carried out using the Roche LightCycler 480 and the LC480 SYBR Green I Master kit. Actin was used as the reference for sample normalization. Human primers for *CYP1A1* and *ACTIN* have been described previously [8]. The following primers (5'-3') were used for rhesus mRNAs: *CYP1A1*, ATCCCCCACAGCACCACAAGAGAC and TGCCCAAGCCAAAGAGAATCACCT; *ACTIN*, GCTGGCCGGGACCTGACTGACTA and CCGCCGTGGCCATCTCCTG. Relative quantification of the mRNA was determined using the calculated efficiencies and the previously described method [9]. Threshold modeling procedures are described in detail previously [8], except the new interspecies threshold model used here contrasts dose response data between human and rhesus for TCDD, PCB 126, and Aroclor 1254. Guidelines outlined in the EPA external review DDEF draft were followed to assess interspecies differences in TK and TD and to estimate interspecies TD EFs (i.e., EF_{AD}) [2]. Briefly, critical effects were identified and information regarding the MOA was assembled. Dose response models were constructed for the initial key event in the hypothesized MOA using *in vitro* cell cultures derived from the relevant target tissue. Species differences in TD

components were then calculated from ratios of EC50s or thresholds. These ratios served as candidates for an EF_{AD} to replace the TD portion of the UF_A, and adjusted RfDs were generated.

Results and Discussion

Mode of Action. Dermal lesions seen in rhesus orally-dosed with Aroclor 1254 grossly resemble signs of polychlorinated dibenzofuran (PCDF) poisoning observed during the Yusho and Yu-Cheng events [10]. These same lesions have been induced in rhesus with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [11], PCDFs [12], other Aroclors [13], and single co-planar PCB congeners [14]. Histologically, the rhesus dermal lesions exhibit pathologies highly similar to those of human chloracne induced by dioxins and PCDFs. Conserved histopathology includes: involution and/or disappearance of sebaceous glands; keratinization of the epidermal layer; and, sebaceous gland metaplasia [10]. It is widely-accepted that chloracne is specifically the consequence of sufficient exposure to potent and efficacious "dioxin-like" aryl hydrocarbon receptor (AHR) agonists [10]. AHR activation has been verified in human chloracne by demonstration of strong expression of the sensitive biomarker *CYP1A1* in lesions [15]. Due to the specificity of this response, a single MOA /mechanism is highly probable. Since both the CSAF and DDEF guidelines explicitly state that information regarding the entire MOA is not necessary to develop alternatives to default UFs [1,2], we focused on the initial key event in the MOA for this critical effect, i.e., activation of the AHR pathway. Furthermore, there is a general scientific consensus implicating AHR activation as the initial key event for all "dioxin-like" toxicities [16]. This includes the "dioxin-like" immune suppression observed in the critical study for the Aroclor 1254 RfD. The key events downstream of AHR activation in the chloracne MOA have yet to be fully elucidated, but likely include: induction/repression of AHR-regulated genes in the target tissue; altered cellular differentiation; and, aberrant proliferation of keratinocytes [10].

Toxicokinetics. As defined by the US EPA, "[t]oxicokinetics is concerned with delivery of the biologically active chemical to the target tissue of interest." [2] The UF_A used for the Aroclor 1254 RfD was 3, reduced from the default of 10 partially based upon the assumption that rhesus and humans metabolize PCBs in a similar manner [3]. However, unique PCB congener profiles were apparent in rhesus tissues obtained from the critical study [17], suggesting considerable TK differences may exist between rhesus and other species. Based upon the fact that the MOA for the critical effect specifically involves "dioxin-like" toxicity, we will focus on potential TK differences for the most potent "dioxin-like" PCB congeners. Although the exact congener make-up of the Aroclor employed in the critical study is not known, analyses of the dioxin toxic equivalency (TEQ) of various Aroclor 1254 lots have determined that the co-planar congener PCB 126 makes up the majority of the TEQ [18]. Unfortunately, analytical methods were likely lacking at the time of the critical study to reliably detect this congener in tissue samples from exposed animals. Similarly, since the use of Aroclor 1254 was greatly reduced in the early 1970s, measurements of PCB 126 in workers at the time they were most highly exposed to Aroclor 1254 could not be made. However, some information may be gleaned from what is known about TCDD TK in monkeys, rodents, and humans. In rodents, TCDD TK models predict that basal and AHR-induced levels of hepatic Cyp1a2 are important for TCDD tissue distribution due to Cyp1a2-mediated hepatic sequestration of TCDD [19]. A similar phenomenon has been observed in rodents exposed to PCBs 126 and 169 [20]. In Cyp1a2 knockout mice, more TCDD is distributed to extra-hepatic tissues (e.g., skin) and knockouts exhibit increased sensitivity to some toxic endpoints [21]. Hepatic sequestration of TCDD appears to be lacking in *Macaca spp.* [22], possibly explaining their increased sensitivity to some toxic endpoints (e.g., dermal /developmental /reproductive effects), but not others (e.g., hepatotoxicity). Recent studies have revealed that macaque *CYP1A2* may be under the process of becoming a pseudogene [23]. Thus, macaques may lack a functional CYP1A2 protein entirely. This is in stark contrast to humans where CYP1A2 is the third most abundant CYP in the liver and hepatic CYP1A2 induction has been observed in humans highly exposed to TCDD and PCDFs [24]. Various "dioxin-like" compounds bind with comparable affinity to rat and human CYP1A2 including TCDD and PCB 126 [25]. Furthermore, TCDD and PCB 126 are capable of inducing *CYP1A2* expression to varying levels in human hepatocytes, although higher concentrations were required to achieve induction levels comparable to rat cells [26].

Toxicodynamics. As defined by the US EPA, "[t]oxicodynamics describes the critical interaction of the active chemical moiety with the target site and the ensuing sequence of events leading to toxicity." [2] The rhesus monkey is highly sensitive to the toxic effects of dioxins and "dioxin-like" compounds. For example, ingestion of PCB-containing caulk was the suspected culprit in inducing severe "dioxin-like" toxicity and high mortality in rhesus monkeys housed in two separate facilities [27,28]. This ultra-sensitive phenotype can be at least partially

explained by the occurrence of the same AHR ligand binding domain amino acid substitution in rhesus as in ultra-sensitive mouse and rat strains. This AHR genotype results in increased AHR affinity and subsequent toxicity for TCDD in rodents, and has not been identified in any human AHR sequence evaluated to date [29,30]. Activation of the AHR pathway is the initial key event for the critical effects behind the Aroclor 1254 RfD. Previous investigations in our laboratory directly compared the *in vitro* response of human and rhesus hepatocytes for induction of the AHR activation biomarker, CYP1A1, following incubation with TCDD, PCB 126, or Aroclor 1254 [6]. We found that human hepatocytes were at least 2 orders of magnitude less sensitive to PCB 126- and Aroclor 1254-mediated CYP1A1 induction than rhesus cells. In this study, we expand upon previous work in hepatocytes to look at interspecies responses of keratinocytes, a sensitive cell type for one of the critical effects cited in the RfD. New dose response data for rhesus keratinocytes exposed to TCDD, 1,2,3,6,7,8-HxCDF, PCB 126, or Aroclor 1254, and human keratinocyte data previously described in Sutter *et al.* [8], are depicted in Figure 1. In addition, cells from human donors 1 and 5 were exposed to Aroclor 1254. For donor 1, Aroclor 1254 slightly induced CYP1A1 (< 1% of the maximal response achieved by TCDD) at only 10 and 30 μM. Although cells from donor 5 failed to respond to Aroclor 1254 at any concentration tested, they were responsive to TCDD, HxCDF, and PCB 126. Rhesus/human EC50 ratios for TCDD, HxCDF, and PCB 126 were 0.034, 0.038, and 0.00087, respectively. The interspecies ratio for PCB 126 is likely over-estimated because this congener acted as a partial agonist in human cells and not rhesus. Since we were unable to obtain a convergent model for human cells exposed to Aroclor 1254, we decided to focus on the bottom of the dose response curve by $\log_{10}$-transforming the response data and developed an interspecies threshold model similar to our previous threshold model. Figure 2A depicts the model used to directly estimate rhesus/human threshold ratios for TCDD and Aroclor 1254. TCDD and PCB 126 were also modeled simultaneously for rhesus (n=1) and human (n=4) donors (Figure 2B). Rhesus /human threshold ratios were 0.00068 (95% CI; 0.0004-0.0011) for Aroclor 1254 and 0.000095 (95% CI; 0.000048-0.00018) for PCB 126.

Figure 1

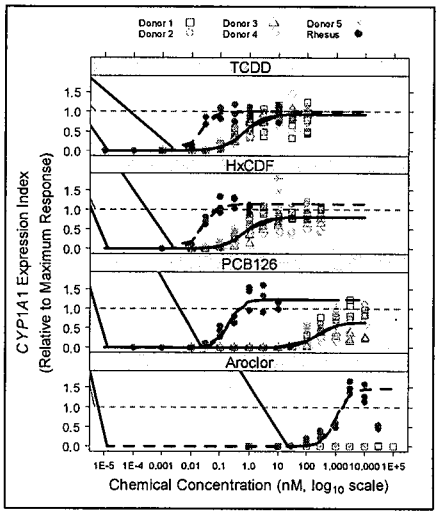
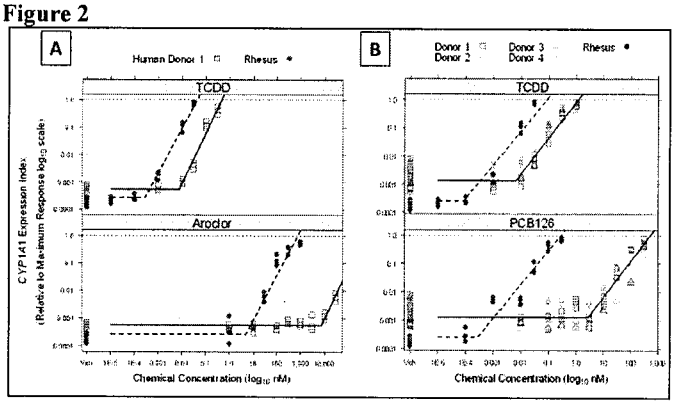


Figure 2



New RfD Estimation. Following the DDEF guidelines [2], we have examined the critical effects for the Aroclor 1254 RfD and gathered qualitative and quantitative information relevant to the UF_A. AHR activation was determined to be the initial key event in the MOA for the critical effect. In regards to interspecies TK differences, it is clear there is no basis for an assumption that the average human will accumulate the most potent "dioxin-like" congeners present in Aroclor 1254 (i.e., PCB 126) at the target tissue to a greater extent than rhesus monkeys. However, quantitative data are lacking, so we have elected to take a conservative approach and leave the TK portion of the UF_A at a default of 3. On the other hand, clear species differences for TD were found in keratinocytes and hepatocytes for a sensitive biomarker of the initial key event in the MOA for the critical effect. Using the most conservative estimate of EF_{AD} (i.e., the upper bound threshold ratio for Aroclor 1254 of 0.0011),

an adjusted RfD was calculated as follows: 2.0×10^{-5} mg/kg-d ÷ 0.0011 (EF_{AD}) = **1.8×10^{-2} mg /kg-d.** Thus, after adjustment for only interspecies TD differences, the safe dose from chronic oral exposure to Aroclor 1254 is ~900 fold higher than that suggested by the current RfD in IRIS. This finding is consistent with negative epidemiological studies of capacitor workers highly exposed to Aroclor 1254, with body burdens often exceeding those of the monkeys used in the critical study [31, 32]. Although "dioxin-like" AHR activation (i.e., CYP1A1/2 induction) has been described in PCDD- and PCDF-exposed humans exhibiting chloracne [15, 24], there is no evidence that this key event was produced in Aroclor 1254-exposed capacitor workers. Thus, the EF_{AD} derived here is consistent with the US EPA draft DDEF guidance [2] that states, "[q]uantitatively, DDEF values for UF_A components might be less than 1 if humans are less sensitive."

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Attachment J-4

Critical Reviews in Toxicology, 33(5):543–580, 2003

ISSN: 1040-8371
DOI: 10.1080/10408440390242414

Potential Human Cancer Risks from Exposure to PCBs: A Tale of Two Evaluations

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ABSTRACT: In 1999 the Agency for Toxic Substances and Disease Registry (ATSDR) released a Draft Toxicological Profile for Polychlorinated Biphenyls (PCBs). In reviewing the potential human carcinogenicity of PCBs, ATSDR (1999) concluded that "The weight of evidence does not support a causal association for PCBs and human cancer at this time." Just 1 year later, in an updated Toxicological Profile for Polychlorinated Biphenyls (PCBs), the conclusions of another analysis (ATSDR, 2000) on whether exposure to PCBs might represent a carcinogenic risk to humans had dramatically changed to "Overall, the human studies provide some evidence that PCBs are carcinogenic" and "some of these studies provide meaningful evidence that PCBs are carcinogenic in humans." Because this is a substantially different conclusion than that reached only one year previously, it raises a number of questions that must be considered particularly since "weight of evidence" has a precise meaning in the context of evaluating a body of epidemiological data.

The present review addresses the additional scientific data that became available between the ATSDR 1999 and 2000 evaluations that was of a magnitude to offset the weight of evidence from numerous epidemiological studies that exposure to PCBs was not causally associated with human cancer to a conclusion only 1 year later that there was now "meaningful evidence" that PCBs posed a carcinogenic risk to humans. Also of interest are the criteria upon which this conclusion is based and the distinction between "weight of evidence" and the newer descriptors of "some evidence" and "meaningful evidence."

However, as shown in this review, only one relevant study was published between the ATSDR 1999 and 2000 evaluations and the results of this study were unequivocally supportive of the 1999 conclusion. Because of the continuing controversy surrounding this issue, in this review, all relevant epidemiological data on PCBs are summarized and subjected to another weight of evidence evaluation. This critical review is based on the most recent guidelines (U.S. EPA, 1999a, 2003) for conducting weight-of-evidence evaluations on a body of epidemiological data. Applying a weight-of-evidence evaluation to the PCB epidemiological studies can only lead to the conclusion that there is no causal relationship between PCB exposure and any form of cancer, thereby confirming the conclusions of ATSDR (1999).

Also considered is the methodology and logic used by ATSDR (2000) that resulted in overturning the weight of evidence conclusions concerning the human carcinogenicity of PCBs in ATSDR (1999). This issue may have public health and policy implications. It seems appropriate that unbiased evaluations of a body of data, even of controversial issues such as the potential human carcinogenicity of PCBs, be conducted in a transparent manner following applicable guidelines. The dramatic differences between the conclusions of ATSDR (1999) and ATSDR (2000) do not appear to be consistent with this process.

KEYWORDS: PCBs, cancer, humans, weight-of-evidence.

INTRODUCTION

The issue of whether exposure to polychlorinated biphenyls (PCBs) might pose a cancer risk to humans has been widely debated. While PCBs are carcinogenic to rats (Mayes et al., 1998; ATSDR, 2000), there are growing questions concerning the relevance of these findings to humans.[1] However, for PCBs, there are more than 40 studies on human populations that have investigated possible associations between exposure to PCBs and increased risk of cancer. These studies fall into two large groupings: (1) studies of cohorts with occupational exposure to PCBs as a result of manufacturing or use of PCB-containing products and (2) studies of cohorts with environmental exposure to PCBs designed to investigate possible associations with breast or endometrial cancer. The robustness of the human studies combined with the uncertainty surrounding the relevance of the animal data to humans makes it even more critical to evaluate the available epidemiological data in the most rigorous manner. Recognizing the importance of critically assessing a body of epidemiological data, the U.S. Environmental Protection Agency (U.S. EPA, 1999a, 2003) recently revised its cancer risk assessment guidelines.[2] Included in this guidance is a discussion of criteria for assessing the adequacy of epidemiologic studies, criteria for causality, assessment of evidence of carcinogenicity from human data, and weight-of-evidence analysis. These guidelines are essentially echoed by the International Programme for Chemical Safety (IPCS, 1999).

In 1999 the Agency for Toxic Substances and Disease Registry (ATSDR, 1999) released a *Draft Toxicological Profile for Polychlorinated Biphenyls (PCBs)*. In reviewing the potential human carcinogenicity of PCBs, ATSDR concluded that "The weight of evidence does not support a causal association for PCBs and human cancer at this time." The five studies cited as the basis for this conclusion were Bertazzi et al. (1987), Brown (1987), Sinks et al. (1991, 1992), and Yassi et al. (1994). However, additional studies are reviewed in the text, including Brown and Jones (1981), Gustavsson et al. (1986, 1997), Shalat et al. (1989), Loomis et al. (1997), and Bahn et al. (1976).

In addition, ATSDR (1999) also notes: "A number of epidemiology studies have examined the possibility of PCBs causing cancer. Several reviews have shown that most of the epidemiological studies have been inconclusive or have not shown an association between PCBs and cancer (Cogliano, 1998; Danse et al., 1997; Kimbrough, 1995; Longnecker et al., 1997; Smith, 1997; Swanson et al., 1995;

[1]While the precise mechanism by which PCBs cause liver tumors in rodents is unknown, an accumulating body of data suggests that the mode of action may not be relevant to human exposure situations. PCBs are not considered to be genotoxic (ATSDR, 2000) and there is no evidence that PCBs react with DNA in vivo (Whysner et al., 1998). Mode-of-action studies suggest PCBs induce GSTP-positive foci and inhibit gap junctions (Ruch and Klaunig, 1986; Swierenga et al., 1990; Kolaja et al., 2000; Santomauro et al., 1999; Kato et al., 1998; Bager et al., 1997; Kang et al., 1996; Fitzgerald, 1990). A recent study conducted on serially acquired tissue sections from the study by Mayes et al. (1998) suggests that hepatic iron accumulation produces oxidative damage and enhanced cell proliferation resulting in proliferation of spontaneously initiated cells (Whysner and Wang, 2001). In addition, the formation of glutathionylated estrogen quinines and the generation of reactive oxygen species (ROS) have also been reported in the same PCB-exposed rats (Brown et al., 2001). These various lines of inquiry, all pointing to a mode of action for PCBs consistent with both a clear threshold as well as the possibility that rodent tumors might occur by a mode/mechanism of action not likely to occur in humans, may well undermine reliance on the rodent tumor data for human cancer risk assessment. This will place even greater emphasis on human epidemiological data in order to determine if PCBs pose a carcinogenic risk to humans. While the extent to which animal studies may yield data not relevant for human risk assessment (e.g., the alpha-2u-globulin association with renal neoplasia in male rats [U.S. EPA, 1991; Hard et al., 1993; Dietrich, 1995]) may be a matter of scientific debate for other modes of action, there is little doubt that this will become more common. The more recent experience with chloroform is another example of mode of action data influencing the risk assessment process (Golden et al., 1997). There are likely to be more examples of this phenomenon as toxicological studies elucidate modes of action for other chemicals. The most recent Cancer Risk Assessment Guidelines (U.S. EPA, 2003) explicitly embrace the concept that mode of action data be considered in the risk assessment process. The science of toxicology, therefore, provides a powerful tool that permits relevant animal data for some chemicals to be appropriately used for risk assessment while at the same time determining where it may be inappropriate to rely on animal data for other chemicals. In this latter situation, a rigorously conducted weight of evidence analysis of available epidemiological studies is the only way to determine if such chemicals pose a carcinogenic risk to humans.

[2]Both U.S. EPA (1999a) and U.S. EPA (2003) Cancer Risk Assessment Guidance are referred to in this review since only U.S. EPA (1999a) was available at the time ATSDR (2000) was prepared.

Vater et al., 1995; Ward et al., 1997). Each new study involves a longer latency for each cohort, allowing the possibility that cancer mortality will become significant. Fish eaters continue to be studied. In all of these studies, it is difficult to account for the presence of other toxic compounds invariably present."

Just 1 year later, in an updated *Toxicological Profile for Polychlorinated Biphenyls (PCBs)*, the conclusions of another analysis on whether exposure to PCBs might represent a carcinogenic risk to humans had dramatically changed. ATSDR (2000) concluded that "Overall, the human studies provide some evidence that PCBs are carcinogenic" and "some of these studies provide meaningful evidence that PCBs are carcinogenic in humans." Because this is a substantially different conclusion than that reached only 1 year previously, it raises a number of questions that must be considered. The conclusion of ATSDR in 1999 was that the "weight of evidence does not support a causal association for PCBs and human cancer at this time." The phrase "weight of evidence" has a precise meaning in the context of evaluating a body of epidemiological data. Both the U.S. EPA (1999a, 2003) and the IPCS (1999) have described in detail the process by which a weight of evidence evaluation is undertaken. In essence, this involves evaluating all relevant data and judging how well they collectively satisfy the main causation guidelines (i.e., strength of association, consistency of association, specificity of association, dose-response, temporality, and biological plausibility).

When an agency such as ATSDR concludes that "The weight of evidence does not support a causal association for PCBs and human cancer at this time," it can only be assumed that this conclusion was, in fact, the result of an assessment that followed well-established guidelines. Indeed, as described in ATSDR (1999), this conclusion was based on a review of at least 10 studies that had investigated whether occupational exposure to PCBs was associated with increased risk of cancer. ATSDR (1999) also acknowledged numerous other reviews of essentially the same data all of which reached a similar conclusion. The available database relied on by ATSDR (1999) as well as the cited reviews encompass approximately 18 studies, most of which have investigated possible associations between occupational exposure to PCBs and increased risk of cancer in humans.

It is therefore of interest to ascertain what kind of additional scientific data became available between the ATSDR (1999) evaluation and the ATSDR (2000) evaluation that was of a magnitude to offset the weight of evidence from 18 epidemiological studies. Of equal interest are the criteria on which this conclusion is based and the distinction between "weight of evidence" and the newer descriptors of "some evidence" and "meaningful evidence." The U.S. EPA (1999a, 2003) risk assessment guidelines only recognize a weight-of-evidence approach for evaluating a body of epidemiological data and do not mention these other categories/descriptors. Interestingly, the weight of evidence concept is not used at all in ATSDR (2000), as it might pertain to the potential for PCBs to cause human cancer. This too is critical since the U.S. EPA (1999a, 2003) and IPCS (1999) have not changed their weight-of-evidence evaluation criteria.

The first aspect of this inquiry concerns the kind of new data that would "tip the scales" from a conclusion by ATSDR in 1999 that exposure to PCBs was not causally associated with human cancer to a conclusion by ATSDR in 2000 that there was now "meaningful evidence" that PCBs posed a carcinogenic risk to humans. One would presume that in order to cause a change of this magnitude, one or more positive studies must have been published after the ATSDR (1999) evaluation was completed. However, as demonstrated in this review, this is not the case. The only relevant study published between the 1999 and 2000 PCB evaluations was by Kimbrough et al. (1999). In the review that follows, all of the data available for both the ATSDR 1999 and 2000 evaluations are summarized and subjected to another, independent weight-of-evidence evaluation. This evaluation is based on the most recent guidelines (U.S. EPA, 1999a, 2003) for conducting weight of evidence evaluations on a body of epidemiological data.

While breast cancer studies are not extensively considered as part of this review, it is important to briefly acknowledge that almost 30 studies conducted over the past two decades have addressed the concern that PCBs might be associated with an increased risk of breast cancer. These studies fall into several different categories. The early studies (e.g., Wassermann et al., 1976; Unger et al., 1984; Falck et al., 1992; Dewailly et al., 1994) were each based on fewer than 20 cases of breast cancer, their results were subject to considerable chance variation, and often known risk factors for breast cancer were not taken into account. In addition, the data in all of these studies were derived from active cases of breast cancer in which disease-induced weight loss may have contributed to spurious elevations of PCBs in either breast tissue or serum. Reviews of the literature (Key and Reeves, 1994; Adami et al., 1995) based on the data available up until about 1994

concluded that there was no association between environmental exposure to PCBs and increased risk of breast cancer.

Virtually none of the numerous large studies (Hunter et al., 1997; Hoyer et al., 1998; Helzlsouer et al., 1999; Dorgan et al., 1999; Wolff et al., 2000; Demers et al., 2000; Zheng et al., 2000; Moysich et al., 2002; Gammon et al., 2002; Lopez-Carrillo et al., 2002; Demers et al., 2002; Woolcott et al., 2001; Ward et al., 2000; Holford et al., 2000; Stellman et al., 2000; Millikan et al., 2000) conducted after the earlier reviews were undertaken have detected a statistically significant increased risk of breast cancer associated with PCB exposure. Many of these studies were prospective in design, thereby eliminating the possible confounding effects of disease-related alterations in PCB serum levels. While a few studies have reported a significant association between PCBs (or select PCB congeners) and breast cancer in some population subgroup (e.g., postmenopausal women who had never lactated), the results from these studies either have not been replicated or are based on women with invasive breast cancer, thereby casting doubt on the reported association due to the unknown effects of invasive breast cancer on PCB levels in breast adipose tissue (Liljegren et al., 1998). In a comprehensive review of environmental risk factors and breast cancer, Laden and Hunter (1998) concluded, "In summary, most of the recent large studies have not found evidence of increased breast cancer risk associated with blood levels of DDE or total PCBs. The possibility that a positive association might be limited to women with particular reproductive characteristics [e.g., women who have never breast fed, as observed by Moysich] should be examined carefully in the large ongoing studies. Nevertheless, it appears that these environmental exposures are unlikely to be responsible for rising breast cancer rates."

Similarly, one of the most recently reported studies (and one of the largest) concluded that "the results do not support the hypothesis that DDE and PCBs increase the risk of breast cancer as encountered through environmental exposure." (Zheng et al., 2000). Likewise, the recent study by Demers et al. (2000) concluded that "taken together, results from six large epidemiological studies reported during the last 2 years, including our own, provide little indication that organochlorine exposure is a risk factor for [breast cancer]." In a more recent review, Laden et al. (2001) summarized the results of five of the largest studies, noting, "Combined evidence does not support an association of breast cancer risk with plasma/serum concentrations of PCBs or DDE. Exposure to these compounds, as measured in adult women, is unlikely to explain the high rates of breast cancer experienced in the northeastern United States."

Finally, perhaps the strongest and most convincing evidence against any association between PCBs and increased risk of breast cancer is provided by the occupational cohort studies that are consistently negative with respect to any increased risk of these types of cancer. Adami et al. (1995) conducted a meta-analysis of every occupational study that included a cohort of women in order to assess the relationship between PCB exposure and breast cancer. This analysis demonstrated that long-term, high-level occupational exposure to PCBs is not associated with increased risk of breast cancer (summary observed/expected ratio = 0.84; 95% CI 0.50–1.33). In general, the studies have reported a deficit in breast cancer incidence. Subsequent to the analysis by Adami et al. (1995) in a study of a PCB-exposed capacitor worker cohort, Kimbrough et al. (1999) obtained SMRs for breast cancer for 2544 female hourly workers and 1078 female salaried workers of 82 (95% CI 53–121) and 104 (95% CI 38–226), respectively. Overall, the results from the epidemiologic studies do not support a causal relationship between PCBs and breast cancer.

Also considered here are the methodology and logic used by ATSDR (2000), which resulted in overturning the weight-of-evidence conclusions concerning the human carcinogenicity of PCBs in ATSDR (1999). It appears that established guidelines were followed in ATSDR (1999) but abandoned in ATSDR (2000). This issue may have public health and policy implications.

The weight of evidence review of the relevant data that follows is intended to be as transparent as possible. The potential human carcinogenicity of PCBs is a controversial and emotional subject; however, in a sense this is precisely why the guidelines established by the U.S. EPA (1999a, 2003) as well as the IPCS (1999) for conducting these kinds of evaluations are so critical. In conducting the evaluation, the following key aspects of these guidelines are paramount:

- Analyzing the contribution of evidence from a body of human data requires examining available studies and weighing them in the context of well-accepted criteria for causation (U.S. EPA, 1999a).
- A judgment is made about how closely they satisfy these criteria, individually and jointly, and how far they deviate from them. Existence of

temporal relationships, consistent results in independent studies, strong association, reliable exposure data, presence of dose-related responses, freedom from biases and confounding factors, and high level of statistical significance are among the factors leading to increased confidence in a conclusion of causality (U.S. EPA, 1999a).

- A clear exposure-response relationship (e.g., increasing effects associated with greater exposure) strongly suggests cause and effect, especially when such relationships are also observed for duration of exposure (e.g., increasing effects observed following longer exposure times) (U.S. EPA, 2003).
- An inference of causality is strengthened when a pattern of elevated risks is observed across several independent studies. The reproducibility of findings constitutes one of the strongest arguments for causality. If there are discordant results among investigations, possible reasons such as differences in exposure, confounding factors, and the power of the study are considered (U.S. EPA, 2003).
- Generally, the weight of human evidence increases with the number of adequate studies that show comparable results on populations exposed to the same agent under different conditions (U.S. EPA, 1999a).
- The analysis takes into account all studies of high quality, whether showing positive associations or null results, or even protective effects.
- In weighing positive studies against null studies, possible reasons for inconsistent results should be sought, and results of studies that are judged to be of high quality are given more weight than those from studies judged to be methodologically less sound (U.S. EPA, 1999a).
- In assessing the human data within the overall weight of evidence, determination about the strength of the epidemiologic evidence should clearly identify the degree to which the observed associations may be explained by other factors, including bias or confounding (U.S. EPA, 2003).

After reviewing the available data, several of these guidelines are revisited in order to determine the extent to which the weight of evidence on PCBs fulfills the causation criteria.

The studies cited by ATSDR (1999) are summarized next to illustrate that the conclusions reached by ATSDR (1999) accurately reflect the weight of evidence. This brief review also reflects the consensus conclusions of the numerous reviews cited in this same document that PCBs do not pose a human cancer risk. For completeness, several other studies, not cited by ATSDR (1999) but supportive of their conclusion, are also included in this evaluation.

DESCRIPTION AND ASSESSMENT OF OCCUPATIONAL STUDIES OF PCB-EXPOSED COHORTS[3]

In this section, all relevant human cancer mortality studies of PCB-exposed occupational populations are reviewed and their strengths and weaknesses are assessed in the context of a weight-of-evidence evaluation as recommended by the U.S. EPA (2003). Studies are grouped together when a single cohort of workers has been studied more than once. The results of such follow-up studies, reflecting a greater length (latency) of time for the expression of disease endpoints, particularly cancer, offer a significant advantage over other studies which may not have sufficient latency or years of observation. Also reviewed are a few cancer incidence reports, involving small cohorts, that have been cited by the U.S. EPA and/or ATSDR in their various reviews of PCBs and human cancer. The available studies have been organized into two categories:

- Groups of two or more studies in which the cancer mortality in a single cohort was investigated more than once. These groups are arranged in chronological sequence starting with the earliest study.
- One-time-only cohort studies of PCBs and risk of cancer. These studies are addressed in order of cohort size, from largest to smallest.

A few studies that are often cited in reviews of potential associations between exposure to PCBs and increased risk of cancer are not included in this review. Because of unresolved confounding by other potential carcinogens, the results of these studies cannot be integrated into a weight-of-evidence evaluation of the potential carcinogenic risks posed by PCBs. In particular, this is the situation with respect to all of the Yusho and Yu-Cheng data, which have been reported in several publications.

The cancer mortality studies from Yusho and Yu-Cheng involved cohorts heavily exposed to polychlorinated dibenzofurans in addition to PCBs, and it is generally agreed that attributing any outcome to PCBs is highly problematic and probably incorrect. It is now recognized that most (or all) of the

[3]Three nonoccupational (i.e., population-based) studies are also included in this section.

effects observed in these two poisoning incidents were caused by the ingestion of PCDFs. The Halogenated Organics Subcommittee of the U.S. EPA Science Advisory Board (1997) concluded that "Recent studies indicate that the major etiologic agents in Yusho were polychlorinated dibenzofurans rather than polychlorinated biphenyls.... Thus, a discussion of the human health effects of polychlorinated biphenyls should not use 'Yusho' as an example. Industrial exposure data more accurately reflect human health effects." Similarly, ATSDR (1999) concluded that "The effects from these incidents are not reviewed in this profile because CDFs appear to be the main causal agent."

Following the reviews of individual studies, the totality of the data is analyzed using a weight-of-evidence approach to determine the strength of the evidence concerning the potential human carcinogenicity of PCBs and the extent to which the data fulfill the causation criteria.

Groups of Studies Reflecting Follow-Up of a Single Cohort

Brown and Jones (1981), Brown (1987), Nicholson (1987), Taylor (1988), Kimbrough et al. (1999), Kimbrough et al. (2003)

This grouping of studies spanning more than 20 years reflects an ongoing investigation of a single population of workers exposed to PCBs. These studies included workers from two capacitor plants who have been studied two or more times. Due to some confusion surrounding this cohort, the worker groups studied are summarized in Table 1.

Brown and Jones (1981)

Brown and Jones (1981), the first study in this series, is a National Institute for Occupational Safety and Health (NIOSH) retrospective cohort mortality study of 2567 workers in two plants where PCBs were used in capacitor manufacture. Plant No. 1 (actually two separate plant sites owned by the General Electric Company and located in upstate New York within about 1 mile of each other, i.e., Site A and Site B in Table 1) is the same facility studied later by Nicholson (1987), Taylor (1988), and Kimbrough et al. (1999, 2003). Plant No. 2 is located in Massachusetts and owned by another company (i.e., Aerovox Corp.). The cancer mortality at this plant was not included in the studies by Nicholson (1987), Taylor (1988), or Kimbrough et al. (1999, 2003).

All workers included in the Brown and Jones (1981) study were employed for at least 3 months in areas of the plants where PCBs were used and received heavy exposure to PCBs. Exposures were to Aroclors 1254, 1242, and 1016. The workers were also exposed to other chemicals, including trichloroethylene, trichlorobenzene, toluene, methyl isobutyl ketone, and epoxide stabilizers. An industrial hygiene survey was performed at both plants in the spring of 1977. In Plant No. 1, at a time when PCB usage was almost phased out, the time-weighted average (TWA) PCB air concentrations in personal samples ranged from 24 to 393 $\mu g/m^3$ and for area samples ranged from 3 to 476 $\mu g/m^3$. In Plant No. 2, the PCB air concentrations ranged from 170 to 1260 $\mu g/m^3$ for personal air samples and from 50 to 810 $\mu g/m^3$ for area samples. Measurements made at Plant No. 1 in 1975 indicated air levels ranging from 260 to 1160 $\mu g/m^3$ in areas

TABLE 1
Worker Cohorts Studied by Brown and Jones (1981) through Kimbrough et al. (2003)

	Plant #1		Plant #2
	General Electric		Massachusetts company (Aerovox Corp.)
Study	Site A	Site B	
Brown and Jones (1981)	Yes	Yes	Yes
Brown (1987)	Yes	Yes	Yes
Nicholson (1987)	Yes	Yes	No
Taylor et al. (1988)	Yes	Yes	No
Kimbrough et al. (1999)	Yes	Yes	No
Kimbrough et al. (2003)	Yes	Yes	No

where large capacitors were produced and 360–2000 $\mu g/m^3$ at Plant No. 2.

For the total worker population studied in Brown and Jones (1981), all-cause mortality was lower than expected [observed (O) = 163 vs. expected (E) = 182.4] as was all cancer mortality (O = 39 vs. E = 43.8). There were no increases above expected in numbers of deaths from cancer of the intestine, stomach, pancreas, respiratory system, breast, and lymphatic or hematopoietic systems. Excess mortality was noted for rectal cancer (O = 4 vs. E = 1.19; SMR = 336, 95% CI 92–860) and liver cancer (O = 3 vs. E = 1.07, SMR = 280, 95% CI 58–820), although neither of these was statistically significant. The only statistically significant difference in observed versus expected deaths occurred in females from Plant No. 2 for cancer of the rectum (O = 3 vs. E = 0.50, SMR = 336, $p < .05$).

There was no apparent association between latency period and frequency of cancer deaths. The authors considered the results of the study to be inconclusive due to the short observation period and small number of deaths. They also observed that previously reported findings by Bahn et al. (1976) of increased mortality due to malignant melanoma and cancer of the pancreas among workers exposed to PCBs in a petroleum refinery were not corroborated.

Brown (1987)

In a follow-up of Brown and Jones (1981), Brown (1987) added 7 more years of observation and did not alter the criteria for selecting study members. Twenty-one additional workers were found to meet the selection criteria and were added to the cohort for a total of 2588. The number of deaths in the cohort increased during this period from 163 to 295. Thus, this study provides a larger database and, therefore, a more statistically reliable evaluation of this multiple manufacturing site cohort.

Brown (1987) reported that, for the entire cohort, total mortality and total cancer mortality were less than expected. Total cancer at Plant No. 1 was significantly less than expected (O = 18 vs. E = 31). The total cohort SMR for rectal cancers decreased from 336 to 211, although the SMR for rectal cancer at Plant No. 2 remained higher than at Plant No. 1, but it was no longer statistically significant. However, Brown (1987) reported a significant excess when liver, biliary, and gallbladder cancers were grouped together in the cohort, due to the fact that two additional cancers in that diagnostic grouping were observed in the 7-year follow-up period (O = 5 vs. E = 1.9, SMR = 280, $p < .05$).

As described by Brown (1987), of the liver/gallbladder/biliary cancer deaths, only one of the five deaths was possibly attributable to primary hepatic carcinoma. Of the remaining four carcinomas, two (and possibly three) were bile-duct cancers and one was an apparent metastasis from the gallbladder. The fifth cancer was from an unknown primary site. Thus, the statistical elevation reported may be nothing more than the combined classification of liver tumors, gallbladder tumors, and biliary tumors.[4] However, there is no evidence that the etiology or occurrence of these three tumor types is similar; for example, alcoholic cirrhosis and hepatitis virus are risk factors for liver cancer, but not for biliary or gallbladder tumors (DeVita et al., 1993). In addition, as pointed out in ATSDR (2000), if the metastatic liver cancer is not included in the analysis, the SMR for the combined liver/biliary tract/gallbladder cancer in the whole cohort loses statistical significance.

There is no dose-response or latency relationship between PCB exposure and the observed cancer elevation. Of the 5 liver/gallbladder/bile duct cancers, 4 occurred in workers who had worked at the plant 1.5 years or less. One case (i.e., primary site unknown) occurred after only 0.3 years of exposure. The lack of a dose-response or latency relationship between PCBs and the observed elevations in these three types of cancers suggests that the actual etiologic agent is not likely to be PCBs.

Brown (1987) concluded that his work provided only "limited information" for associating PCBs with cancer of the liver and related anatomic sites because: (1) misclassification of the cause of death is quite possible for cancers in this category; (2) most of the cancers were not of the type expected based on rat feeding studies; and (3) the study failed to demonstrate reasonably expected patterns of dose response and latency. In light of these confounding factors and limitations, ATSDR (1999) concluded that "the liver cancer cannot be unequivocally attributed to PCB exposure." The U.S. EPA also determined that this study was inconclusive because no dose-response relationship was apparent and the number of cancers in the cohort was small (U.S. EPA, 1999a).

The update by Brown (1987) prompted a laudatory editorial by Silbergeld (1987) concerning "The

[4]Based on animal carcinogenicity data, only liver cancer would be a biologically plausible consequence of PCB exposure.

Fullness of Time in Epidemiology." Silbergeld, noting the inconclusive results from Brown and Jones (1981) due to the small number of deaths and short period of observation, and the finding 7 years later of an increased incidence of liver and biliary cancer, opined that "one reason for the difference is simply the effect of the passage of time to increase the frequency of measurable events." This concept is explicitly acknowledged in ATSDR (1999): "Each new study involves a longer latency for each cohort, allowing the possibility that cancer mortality will become significant." With respect to this statement it is also obvious that longer latency would similarly allow for the possibility that initially reported increased cancer mortality would become insignificant (see below).

Nicholson (1987)

This study investigated cancer mortality in 788 employees (459 males and 329 females) at the two capacitor manufacturing facilities in upstate New York also studied by Taylor (1988) and Kimbrough et al. (1999, 2003). However, the cohort in Nicholson (1987) was defined differently than in Brown (1987) and 521 of the 769 individuals in Nicholson (1987) were not included in Brown (1987).

Although Nicholson (1987) was not published in a peer-reviewed journal, it did receive extensive peer review prior to its release. This study was prepared for the Workers' Compensation Board of the Ontario Ministry of Labour. The report was written by the Industrial Disease Standards Panel (IDSP) of the ministry and was unanimously accepted.

The cohort in this study was specifically selected to enhance the likelihood of detecting latent cancers among workers with long exposure histories—the criteria for inclusion were employment beginning before 1954 for a period of at least 5 years. Of the 769 individuals who qualified, 188 were deceased and the death certificates were obtained for all of these individuals. At each of the two facilities studied, Aroclors 1254 and 1242 were used prior to 1970; Aroclor 1016 and, occasionally, Aroclor 1221 were used after that date. Industrial hygiene surveys in 1977 indicated that at certain job locations PCB airborne exposure levels ranged from 300 to 1000 $\mu g/m^3$. These exposure measurements were used to compare groups of workers with respect to PCB exposure and cause of death.

The numbers of deaths attributed to all causes and all cancers were less than expected. Nicholson (1987) analyzed the data from this cohort in two ways: (1) all workers in the cohort, and (2) the cohort less the 249 workers who were also in the Brown cohort. The Brown cohort had been selected for workers with direct dermal (and inhalation) exposure to PCBs. In the subgroup of 521 workers not common to the Brown cohort (and therefore not directly exposed to PCBs), leukemias were in excess (O = 4 vs. E = 1.28). However, leukemias were not in excess in the full cohort of 721 workers, indicating that leukemia was less than expected in the group directly exposed to PCBs. Nicholson (1987) also reported that no excess cancer deaths occurred in male or female workers having 30 or more years since first exposure; that is, no deaths were observed in the "30 or more year" category. The inconsistency between the separate Brown (1987) and Nicholson (1987) groups suggests that the reported positive findings were due to multiple comparisons or chance. Nicholson (1987) concluded that "neither the results of the full study nor of the subgroup... indicated any cancer risks." He also noted that "continued follow-up of this group, as well as others with identified exposure, is clearly warranted."

Taylor (1988)

Taylor (1988) studied workers at the same two upstate New York General Electric facilities that had been previously studied by Brown and Jones (1981), Brown (1987), and Nicholson (1987). The Taylor (1988) study is the second largest occupational study of workers highly exposed to PCBs (only Kimbrough et al., 1999, and Kimbrough et al., 2003, described later, are larger). Taylor (1988) showed no significant increases in any type of cancer mortality. Although the study was not published in a peer-reviewed journal, it did receive extensive peer review. The study was initiated by Dr. Taylor while on assignment from NIOSH to the New York State Department of Health (NYSDOH). The study was performed in collaboration with NYSDOH scientists, and was completed at Harvard University as Dr. Taylor's PhD thesis. Thus, the study underwent extensive review and critique by other epidemiologists.

Taylor (1988) involved a cohort of 6292 workers (3601 males and 2691 females) employed for at least 3 months during the period 1946–1976 at 2 GE capacitor manufacturing facilities (the Hudson Falls and Fort Edward capacitor plants). The PCB exposure history of these workers is described in three

previous reports (Brown and Jones, 1981; Brown, 1987; Nicholson, 1987).

Exposure in the cohort was characterized as either direct or indirect. The direct exposure group was further categorized into three subgroups: low—air contact only; medium—air contact plus occasional dermal contact; and high—air contact plus frequent dermal contact. A fourth subgroup, the indirect exposure group, included employees who worked in offices and manufacturing areas where PCBs were not used. Employee exposure monitoring indicated that the air concentrations of PCBs in the work areas of both the direct and indirect exposure groups exceeded concentrations external to the plant. Geometric mean serum PCB levels in the direct exposure group (302 ppb) exceeded levels in the indirect exposure group (61 ppb) by fivefold and in the nonexposed reference subjects (16 ppb) by almost 20-fold.

Taylor (1988) found no increase in cancer mortality or in overall mortality compared to national rates. Deaths due to malignant melanoma, lymphatic cancers, or the combination of liver, gallbladder, and biliary cancers were not significantly elevated, and brain cancers were well below the expected value. PCB exposure was shown to be negatively associated with cancer mortality (all types combined) and lung cancer (the only cancer outcome with numbers of cases sufficient to permit a regression analysis). There were also no statistically significant associations between cumulative PCB exposure levels and all cancer combined, lung cancer, or colorectal cancer. The author concluded that "Total mortality was less than expected for both males and females. No effect of PCB exposure on overall cancer rates was observed."

Due to the size of the cohort, this study had 2.2 times as many person-years of observation and approximately 1.7 times as many total deaths as the then existing next largest study conducted to date, namely, Brown et al. (1987). The author's final caveat is noteworthy: "Follow-up of this cohort in future years will be possible and will give a more secure evaluation of the PCB hazard."

Kimbrough et al. (1999)

This study, the largest occupational study ever conducted on a population of workers heavily exposed to PCBs, found no association between PCB exposure and deaths from cancer or any other disease (Kimbrough et al., 1999). Of all the studies that have investigated potential associations between exposure to PCBs and increased risk of cancer, this study has the longest latency. It should also be noted that Kimbrough et al. (1999) represents the fifth study of this group of PCB exposed workers. The other studies that investigated cancer mortality in these workers are Brown and Jones (1981), Brown (1987), Nicholson (1987), and Taylor (1988).

The cohort in Kimbrough et al. (1999) consisted of 4,062 men and 3,013 women who worked between 1946 and 1977 at two GE capacitor manufacturing facilities (the Hudson Falls and Fort Edward capacitor plants). The PCB exposures consisted primarily of Aroclors 1254, 1242, and 1016. Aroclor 1254 was phased out beginning in 1954, Aroclor 1242 was no longer used after 1971, and from 1971 to 1977 Aroclor 1016 was used at these two plants.

Jobs at the two facilities were classified as high exposure and low exposure. High-exposure jobs were those experiencing direct dermal contact and inhalation exposure to PCBs while filling, impregnating, repairing, or moving PCB-filled capacitors. As late as 1975, the PCB air levels in the filling/impregnation areas ranged from 227 to 1500 $\mu g/m^3$. The low-exposure jobs were those where employees had inhalation exposures limited to the background levels within the plant. In 1977, after PCB use had ended, the PCB concentrations in the high-exposure areas ranged from 170 to 576 $\mu g/m^3$ and the PCB concentrations in the low/background exposure areas ranged from 3 to 50 $\mu g/m^3$.

The average follow-up time for the workers was 31 years, providing a sufficiently long latency period from which to determine whether there was a statistically significant increase in mortality due to cancer or other causes. The cohort was followed through 1993, providing 120,811 person-years of observation for men and 92,032 person-years of observation for women. There were 763 (19%) deceased males and 432 (14%) deceased females. Death certificates were available for 98.5% of the decedents and only 1.3% of the cohort was lost to follow-up. Standardized mortality rates (SMRs) were calculated using both U.S. and local county mortality rates.

Among all of the workers, including those classified as having the highest PCB exposure, there were no statistically significant increases in deaths due to cancer or any other disease. There were also no statistically significant increases in cancer or other mortality associated with length of employment or latency. Among the male hourly workers, no statistically significant elevations occurred in the six a priori cancers of interest (i.e., cancers reported as significantly, but not consistently, elevated in

earlier studies—rectum, liver, brain, melanoma, gastrointestinal and hematopoietic); most SMRs were below 100. These negative results are of particular interest because 42% of these hourly male workers had worked at least some portion of their tenure in a high-exposure job.

When considering the cancers of a priori interest, there also were no statistically significant elevations among hourly female workers or among the salaried workers of either sex. While the SMR for intestinal cancer in hourly women was almost significant, the analysis by latency and length of employment indicated no pattern consistent with a work-related exposure. Only the women with 5–10 years of employment had a significantly elevated SMR; trend analysis, however, showed no increase, but rather a decrease in the observed over the expected by length of employment. For the grouping of liver, biliary, and gallbladder cancer, 4 cancer deaths were observed while 4.7 were expected in the hourly workers of the cohort for an SMR of 85. For the entire cohort (salaried plus hourly), 5 deaths from liver cancer were observed whereas 6.2 cancer deaths were expected (for an approximate SMR of 81).

The mortality experience of workers who worked in a high-exposure job for at least 1 day was also evaluated. This subgroup consisted of 1268 hourly male workers with 37,739 person-years of observation and 362 hourly female workers with 10,584 person-years of observation. Among the men, there was a significant deficit in the category "all causes of death," and the category "all cancer deaths" was almost significantly less than expected (SMR = 77; 95% CI = 57–101). Among the smaller female group, these two categories did not deviate from that expected. When the criterion for inclusion in the high-exposure job category was increased to include those employed in such jobs for at least 180 days, the 723 hourly male workers with 22,217 person-years of follow-up (an average of 31 years of latency) had SMRs for both "all causes" and "all cancers" that were still less than expected (87 and 82, respectively). Among the 184 hourly female workers in this category (with 5783 person-years of follow-up, and an average of 31 years of latency), the SMRs for both categories were also less than expected.

When the inclusion criterion was changed to include only those employed for at least 1 year in a high exposure job, the number of hourly employees who satisfied this definition was reduced to 479 men (15,181 person-years of follow-up, an average latency interval of 32 years) and 122 women (4047 person-years of follow-up, an average latency of 33 years). Among this group of men, the SMRs for "all causes" and "all cancers" were 89 (95% CI = 74–107) and 73 (95% CI = 46–109), respectively. Among the women of this highly exposed subgroup, the SMRs for "all causes" and "all cancers" were 81 and 61, respectively. For the workers in the two longest high exposure categories (i.e., either 180 days or 1 year employed in a high exposure job), the authors concluded that "there was no consistent trend across the length-of-employment categories and/or latency categories in either males or females that would suggest an association between PCB exposure and increased mortality."

The death rate due to all types of cancer combined was at, or significantly below, the expected level. Based on national death rates, 158 and 136 deaths were expected among the hourly male and female workers, respectively, and 128 and 150 deaths were observed; neither difference was significant.

Kimbrough et al. (2003)

Kimbrough et al. (2003) conducted a 5-year update of their 1999 mortality study (Kimbrough et al., 1999) on 7075 PCB exposed capacitor workers that now included 1654 deaths and 235,984 person-years of observation with follow-up through 1998. This study represents the sixth time that the mortality experience of capacitor workers employed at the two New York GE facilities has been examined by several investigators over the past 20 years, including Brown and Jones (1981), Brown (1987), Nicholson et al. (1987), Taylor (1988), and Kimbrough et al. (1999). As in their first study (Kimbrough et al., 1999), this study also included all hourly and salaried workers employed for at least 90 days between January 1946 and June 1977 at two capacitor manufacturing plants in upstate New York. Approximately 1840 workers were employed for less than 90 days and were eliminated from the cohort, leaving respectively 2984 and 2544 male and female hourly workers and 1078 and 469 male and female salaried workers in the study cohort. Hourly males and females contributed 95,691 and 84,287 person-years of observation, respectively, and salaried male and female workers contributed 38,154 and 17,852 person-years of observation, respectively. Follow-up time for the cohort ranged from 34 to 40 years with a mean follow-up time of 37 years. Vital status was determined through 1998, with considerable effort taken to establish the

vital status of workers not identified as deceased, working, or receiving a pension. Expected numbers of deaths were based on mortality rates for the U.S. population for the years 1940–1998. In addition, the cohort was also compared to the mortality experience of the state of New York (excluding New York City).

As in their previous study (Kimbrough et al., 1999) among all of the workers, including those classified as having the highest PCB exposure, no statistically significant increases in deaths due to cancer were found. There were also no statistically significant increases in cancer or other mortality associated with length of employment or latency.

None of the sporadically observed statistically significant excesses in cancer mortality reported in the literature were observed in the total cohort in this study including cancer of the liver and biliary tract (SMR = 89; 95% CI 18–260 and SMR = 103; 95% CI 21–299 in male and female hourly workers), pancreas (SMR = 124; 95% CI 64–216 and SMR = 102; 95% CI 43–200 in male and female hourly workers), rectum (SMR = 100; 95% CI 27–254 and SMR = 142; 95% CI 38–362 in male and female hourly workers), non-Hodgkin's lymphoma (NHL)[5] (SMR = 121; 95% CI 57–222 and SMR = 114; 95% CI 48–224 in male and female hourly workers), malignant melanoma (SMR = 124; 95% CI 45–270 and SMR = 117; 95% CI 24–341 in male and female hourly workers)or all cancers combined (SMR = 98; 95% CI 84–112 and SMR = 110; 95% CI 94–126 in male and female hourly workers). The only significant finding was for connective tissue neoplasms in salaried female workers (O = 2, E = 0.20; SMR = 956, 95% CI 115–3451) as previously reported (Kimbrough et al., 1999). However, due to the wide confidence interval and because one of the connective tissue tumors was a pericytoma, a lesion of borderline malignancy, this finding is questionable.

Because duration of employment is a useful surrogate for cumulative PCB exposure, data were also analyzed based on employment of 6 months or more or 1 year or more. In total, 1268 highly exposed workers who were engaged in jobs with direct dermal and inhalation exposure to PCBs and who experienced long-term repeated exposures did not exhibit any excess cancer mortality. The 479 hourly male workers employed in high-exposure jobs for 1 year or more (mean time 4 years, range 1–22 years) represent a very highly PCB-exposed group of workers. There were no significant increases in mortality from all cancers (SMR = 81; 95% CI 56–113) or any of the specific cancers noted earlier.

These results of this study with the addition of 5 more years of follow-up fail to demonstrate a causal association between occupational exposure to PCBs and excess cancer mortality. As with their previous study (Kimbrough et al., 1999), if any study were going to demonstrate an association between exposure to PCBs and increased mortality from cancer, this would be the study. With over 7000 PCB-exposed capacitor workers that now include 1654 deaths, almost 236,000 person-years of observation with follow-up through 1998 representing a mean follow-up of 37 years, the results of this study represent the most rigorous assessment to date of possible associations between occupational exposure to PCBs and increased risk of cancer.

Bertazzi et al. (1982, 1987); Tironi et al. (1996)

These three studies represent a longitudinal series of mortality investigations of workers in an Italian capacitor manufacturing plant in which different PCB mixtures were used from 1946 to 1980. Only the study by Bertazzi et al. (1987) was considered in the ATSDR (1999) review. The final study, Tironi et al. (1996), involved a cohort of 3,656 workers, making it the third largest study of highly PCB-exposed workers.

Bertazzi et al. (1982)

Bertazzi et al. (1982) presented the initial findings of this series of mortality studies. The cohort consisted of 1310 workers (1020 females and 290 males) who were employed at the facility for at least 6 months. Between 1954 and 1978, there were 27 deaths with 14 due to cancer. There was a statistically significant increase in total cancers among males and in all cause mortality among females compared to local population mortality rates (SMR = 241; 95% CI 109–320). The authors recognized that the small number of deaths in this initial investigation of the cohort limited the significance of their findings: "At the time the study was conducted [the workers] had not aged enough for mortality to be informative about possible long term effects due to PCBs. The small numbers of deaths

[5]Present ICD coding practices preclude the ability to create SMRs specific for non-Hodgkin's lymphoma only; difficulties with disease nomenclature and coding by "lymphatic and other hematopoietic cancers" make it difficult to assess NHL across studies.

which occurred . . . prevented any meaningful stratification by type of exposure, duration of exposure and latency." The results of Bertazzi et al. (1982) must, therefore, be considered inconclusive.

Bertazzi et al. (1987)

In the Bertazzi et al. (1987) update, the cohort selection criteria were changed from 6 months of employment to only 1 week of employment with inclusion of both production and nonproduction workers in the cohort. The new cohort had 2100 members (544 men and 1556 women) with mortality experience followed from 1946 to 1982. Sixty-four deaths (3% of the cohort) were reported, 30 in men and 34 in women. Mortality due to all cancers (O = 14 vs. E = 5.5 national, SMR = 253; 95% CI 144–415 and E = 7.6 local, SMR = 183; 95% CI 104–300) and cancer of the gastrointestinal tract (O = 6 vs. E = 1.7 national, SMR = 346; 95% CI 141–721 and E = 2.2 local, SMR = 274; 95% CI 112–572) were significantly increased among male workers. The latter finding was based on grouping together cancers of the stomach, liver, biliary tract, and pancreas. This procedure is highly questionable, given the likelihood that these widely disparate cancers have different etiologies and risk factors. Deaths from hematologic neoplasms and from lung cancer were also elevated in men, but the results were not statistically significant. Overall mortality in females was significantly increased above local rates (O = 34 vs. E = 16.5), due primarily to accidents. Total cancer deaths (O = 12 vs. E = 5.3, SMR = 226; 95% CI 123–385) and deaths from hematologic neoplasms (Hodgkin's disease and lymphosarcoma) (O = 4 vs. E = 1.1, SMR = 377; 95% CI 115–877) were also significantly elevated over local rates.

The results of the Bertazzi et al. (1987) study are limited by several factors, including the small number of cancer cases observed, the limited latency period, the lack of a pattern or trend when the data are analyzed by duration of exposure, and the fact that some deaths occurred in males with low potential for direct PCB exposure (Kimbrough, 1987; ATSDR, 1998). However, the major problem in the study design was the 1 week minimum period of employment required for inclusion in the study cohort without evidence of direct workplace exposure to PCBs. This makes it difficult to conclude that excess cancer cases are attributable to PCB exposures rather than to other factors. The study also did not show a dose-response relationship or any direct relationship between latency and any disease endpoint. The authors' overall conclusion was that their study "did not permit a causal association to be either proved or dismissed." The U.S. EPA considered the results of the study inconclusive because of lack of a dose-response relationship and the small number of cancers in the cohort (U.S. EPA, 1999a).

Tironi et al. (1996)

Tironi et al. (1996) is a follow-up study of the same workers examined by Bertazzi et al. (1982, 1987). The mortality rate was studied for the period 1954–1991. This study was the first on this cohort to note the exceptionally high air concentrations of PCB to which the workers were exposed; concentrations of 54% chlorinated PCBs ranged from 5200 to 6800 $\mu g/m^3$ in 1954, and were reduced to 48 to 275 $\mu g/m^3$ in 1977. Exposures persisted until 1982 even though PCB usage ended in 1980.

The Tironi et al. (1996) cohort consisted of all workers in every department of the plant, including administration, who worked in the plant for a minimum of 1 week in the period 1946–1982. The focus of the study was on female workers because most of the capacitor manufacturing work was performed by women. A total of 1556 women were included in the study, resulting in 44,328 person-years of observation with a 99% participation rate. Local mortality data were used for comparison.

In this study, none of the previously reported excesses of cancer (which had been based upon small numbers of deaths) was evident. The only significant excesses reported for women were for all causes of mortality where 47 deaths were observed versus 34.4 expected, a number that was driven by excess deaths due to accidents and traumas (ICD8 codes 800–999), with 12 deaths observed versus 3.7 expected. Total deaths due to malignancies were 19 observed versus 16.1 expected, a result that was not statistically significant. For men, there were no statistically significant excesses for any classification of death. Total deaths due to malignancies were not significantly elevated (20 observed vs. 18.4 expected).

While slight elevations of cancer of the "GI tract" and of the "Lymphatic and hematopoietic system" were observed, neither was statistically significant, in contrast to the earlier and more limited report of Bertazzi et al. (1987), with fewer cancer deaths and less stable relative risk estimates. Moreover, as only 39 total cancer cases were evaluated

in Tironi et al. (1996), the SMRs still cannot be considered stable estimates of the risk experienced by this cohort. Two examples of this are the relative risks associated with the "lymphatic and hematopoietic system" cancer in women and the "GI tract" cancers in men. When comparing the SMRs reported by Bertazzi et al. (1987) to those in the present study, the risk of "lymphatic and hematopoietic system" cancer in women dropped from 377 to 144, while the risk of "GI tract" cancers in men decreased from 274 to 195.

Collectively, the results of Bertazzi et al. (1981, 1987) and Tironi et al. (1996) illustrate that follow-up is the best way to determine if highly unstable outcomes in preliminary studies due to small numbers of deaths are still present after the passage of additional time. The inability of Tironi et al. (1996) to confirm previously reported statistically significant increases in certain cancers suggests that the initial reports did not reflect causal associations.

Gustavsson et al. (1986); Gustavsson and Hogstedt (1997)

Gustavsson et al. (1986)

Gustavsson et al. (1986) analyzed cancer incidence in 142 Swedish male capacitor manufacturing workers employed for at least 6 months between 1965 and 1978 who were exposed to a 42% chlorine PCB mixture, providing a mean exposure duration of 6.5 years and a median latency period of 13 years. The vital status was determined for all 142 persons. The airborne PCB concentration measured in 1973 was 100 μ/m^3, but exposures were likely higher in prior years.

There were 21 deaths from all causes, while 22.12 were expected using national mortality rates. Of these deaths, 7 were from cancer versus 5.39 expected, an excess that was not statistically significant. A subgroup of 19 individuals with higher exposures than the rest of the cohort (capacitor fillers and capacitor repairmen) was analyzed separately and there was no increase in mortality or cancer incidence in this high-exposure subgroup.

Gustavsson and Hogstedt (1997)

Gustavsson and Hogstedt (1997) followed up on Gustavsson et al. (1986) by expanding the cohort to include all capacitor workers at the plant regardless of nationality (241 male workers) and adding 11 years of follow-up for cancer incidence and 9 years for mortality. Of these 241 male workers, 157 were alive, 56 had died, and 28 had emigrated. The total cohort was divided into two exposure groups. The "low" exposure group consisted of 170 individuals (71% of the cohort) who had always performed a low exposure job. The 71 "high" exposure workers consisted of anyone exposed to intermediate or high exposure jobs for at least six months. While there were two cases of liver cancer, no statistically significant increase in mortality was found for any tumor type.

The authors noted that their study was small and acknowledged that the two cases of liver cancer were insufficient to draw any conclusion regarding the relationship between liver cancer and PCB exposure. The study provides no information regarding other chemicals to which the workers had been exposed, and did not control for any risk factors, including alcohol consumption. The small size of the cohort and the inability to rule out the possible contribution of alcohol, other chemicals, or hepatitis virus to the two cases of liver cancer suggests that this study does not demonstrate a causal association between exposure to PCBs and cancer.

Studies of Cohorts Investigated Only Once

Loomis et al. (1997)

Loomis et al. (1997) conducted a mortality study of 138,905 men who worked for at least 6 months between 1950 and 1986 at 5 electrical power companies in the United States. Women were excluded because they rarely worked in jobs that involved PCB exposure. From the 1930s to 1978, PCBs were used in both electrical capacitors and transformers as flame-resistant dielectric fluids. Capacitor fluids were essentially pure PCB fluids (Aroclor mixtures), while transformer fluids typically contained Aroclors mixed with various percentages of chlorinated benzenes (normally about 30% to 60% of the transformer fluid). Unless flame resistance was a requirement, most transformers used mineral oil instead of chlorinated compounds because mineral oil had superior electrical properties and was less expensive. Thus, according to a U.S. EPA contractor, less than 5% of the transformers produced in this country during the period

that PCBs were used were actually filled with PCBs (Durfee, 1976; ATSDR, 1999).[6]

For the total cohort, Loomis et al. (1997) reported that all-cause mortality and total-cancer mortality did not correlate with estimated PCB exposure. Moreover, for the entire cohort, no type of cancer correlated with estimated PCB exposure. Several causes of death, including death from liver/biliary cancer, were seen at levels lower than expected, and several of these results were statistically significantly lower.

However, when Loomis et al. (1997) examined relative risk by occupational category, in one subgroup of 210 mechanics who had worked 0–5 years there was a statistically significant association between estimated PCB exposure and melanoma (RR = 2.57; 95% CI 1.06–6.20). No association was seen among 215 other workers with greater estimated PCB exposure, nor was there an association among groups of workers in other job categories assumed to be associated with PCB exposure (electrician, lineman, cable splicers, laborers, and material handlers). No association was found between estimated total career exposure to PCBs and melanoma.

Loomis et al. (1997) also divided the cohort into 5-, 10-, and 20-year lag periods and analyzed each of these lag periods by 3 categories of total career exposure. Of the nine categories (three lag periods by three levels of exposure), three yielded statistically significant findings for an association between estimated PCB exposure and melanoma. However, these findings are based on a small number of cases: one death due to melanoma in the highest exposure category and two and eight deaths in the other two categories of exposure. There also appears to be an apparent discrepancy between the results of this procedure and the results of analyses based on duration of exposure that show no increased risk even after the longest durations of exposure. Therefore, it is important to evaluate the authors' speculation, that is, that with more than a 10- to 20-year follow-up to allow for this latency interval, other studies should confirm these results. As described in other studies in this review, the largest high-exposure studies [with PCB exposures almost certainly higher than in Loomis et al. (1997)] do not support this hypothesis.

The results of Loomis et al. (1997) are also questionable because PCB exposure was estimated and not measured. Because none of the five companies from which the cohort was selected had conducted any measurements of workplace exposures or evaluated employees' historical chemical exposure levels, a panel of experts at each company (industrial hygienists, safety personnel, and long-term workers) was asked to estimate exposure levels. Each panel was asked to assess the potential for exposure to PCBs, various solvents (acetone, 1,1,1-trichloroethane, carbon tetrachloride, and Varsol), wood preservatives, and sunlight for each of the 28 job categories into which the cohort subjects had been placed. These subjective assessments, by job category and decade, were then used to assess PCB exposure for each company. As part of this subjective assessment, the employees' frequency and duration of exposure to various chemicals (including PCBs) were estimated. Based on the exposure matrices derived in this manner, the cumulative hours of potential exposure to each chemical were estimated for each worker by summing the product of the frequency and duration of exposure over a work history. For sunlight exposure, jobs were classified as involving primarily indoor or outdoor work, but these exposure estimates were made for only three of the companies. Sunlight exposure was estimated for the two other companies.

The authors acknowledge that "the quality of our information on exposure to sunlight, a potentially important confounder, is of some concern." While occupational exposure to sunlight was estimated, the authors collected no information about exposure to sunlight during leisure time, which is a risk factor for melanoma. The authors note that "a strong association of melanoma with recreational exposure to sun could distort our results, if that exposure were differential by level of exposure to PCBs."

While the results of this study on increased risk of melanoma are similar to those reported by Sinks et al. (1992) (see later discussion), they are not consistent with the results of nine other studies involving workers with higher levels of PCB exposure (Brown and Jones, 1981; Brown, 1987; Nicholson, 1987; Taylor, 1988; Kimbrough et al., 1999, 2003;

[6]According to ATSDR (1999), "By 1976, only 5% of the transformers produced in the United States were filled with PCBs, accounting for 30% of the Monsanto Chemical Company's domestic sales; however, 95% of the capacitors produced in the United States were filled with PCBs, accounting for 70% of the company's domestic sales (IARC, 1978)." Moreover, "in 1981, an estimated 131,200 transformers containing PCBs were in service in the United States, representing approximately 1% of all operational transformers."

Bertazzi et al., 1982, 1987; Tironi et al., 1996).[7] The significant melanoma findings of the Loomis cohort were for workers with a latency of 10–20 years. By comparison, the entire cohort of the Nicholson (1987) study consisted of workers with 5 years of exposure and more than 10 years of latency, and there was no increased mortality from melanoma. Similarly, in Kimbrough et al. (1999, 2003), there was no increased mortality from melanoma in the most highly exposed group of employees, of which 98% had 10 or more years of latency and 85% had 20 or more years of latency. Thus, studies with substantially higher exposures to PCBs do not confirm the findings of Loomis et al. (1997).

Sinks et al. (1992)

Sinks et al. (1992) conducted a retrospective cohort mortality analysis of 3588 workers (2742 men and 846 women) who were employed for at least 1 day at a capacitor-manufacturing plant between 1957 and 1977. Aroclor 1242 was used in this plant through 1970, and Aroclor 1016 was used from 1970 to 1977. Because PCB fluids were heated in ovens, significant fumes were released when the oven doors were opened. About 10% of the workforce was directly involved with capacitor production. In 1977, PCB serum measurements of the capacitor workers were five times greater than those of administrative personnel and 20 times greater than those of individuals living in the community. PCB serum measurements were reported in the cross-sectional clinical study of Smith et al. (1982).

Mortality from all causes and from all cancers was less than expected. A significant increase in mortality rate was observed for malignant melanoma (8 observed vs. 2 expected), and death rates from brain and nervous-system cancers were nonsignificantly elevated over expected rates. No excess deaths were observed from cancers of the rectum, lung, or liver, biliary tract, and gallbladder, or from hematopoietic malignancies.

Although the melanoma increase was significant, there was no dose-response relationship in terms of level of exposure to PCBs, duration of employment or latency. Sinks et al. (1992) specifically note that "the risk for malignant melanoma did not vary by duration of employment, time since first employment, or estimated cumulative exposure." The failure to find dose or latency relationships argues against PCB exposure as the probable cause of the observed elevation.

Sinks et al. (1992) also noted that the skin cancer excesses are not consistent with findings from similar studies, that is, Brown and Jones (1981), Brown (1987), Nicholson (1987), Taylor et al. (1988), and Bertazzi et al. (1982, 1987). Malignant melanoma was not significantly elevated in four other capacitor-manufacturing cohort studies conducted subsequent to Sinks et al. (1992), including Kimbrough et al. (1999, 2003), Tirioni et al. (1996), and Gustavsson and Hogstedt (1997).

Other limitations of the study include the lack of evaluation of exposures to other chemicals, the relatively short latency period, and the small number of deaths within the cohort. Sinks et al. (1992) concluded that the results of their study should not be interpreted as demonstrating a causal relationship between PCBs and malignant melanoma. The U.S. EPA (1999a) found Sinks et al. (1992) inconclusive because no dose-response relationship was apparent and the number of cancers in the cohort was small. ATSDR (2000) noted that one and probably three cases of melanoma should have been excluded from the analysis since one case was diagnosed 2 months prior to employment and the other two cases had worked at the plant for 1 month.

Thus, even though both Loomis et al. (1997) and Sinks et al. (1992) suggest that there is increased risk of melanoma in PCB-exposed workers, both studies fail to show a dose response and are inconsistent with numerous other studies. Of the 16 studies conducted on PCB-exposed workers, these are the only 2 that report an increased risk of melanoma.

Yassi et al. (1994)

This study examined cancer mortality in males who worked between 1947 and 1975 at a transformer-manufacturing plant in Canada. However, only 27,000 gallons of PCB-containing transformer fluid were ever used at this plant, and this volume was used in only 85 of the more than 51,000 (0.17%) transformers built between 1956 and 1975. Consequently, this study is predominantly one of exposure to mineral oil transformer fluids and other chemicals used at the plant rather than to PCBs.

[7]Both the Nicholson (1987) and Kimbrough et al. (1999, 2003) cohorts include workers whose PCB exposures were likely to be at least an order of magnitude greater, on average, than those of the utility workers in Loomis et al. (1997). Smith et al. (1982) found that total PCB levels in PCB-exposed capacitor manufacturing workers were about ten times higher than levels in utility company workers involved in the maintenance and repair of PCB-containing transformers.

Other potential exposures included cutting oils, solvents, and welding fumes.

The mortality analysis was conducted on all workers with confirmed employment of more than 6 months and for all transformer assembly workers with 6 months or greater employment. The only statistically significant finding was for pancreatic cancer with an SMR of 7.64 (95% CI 3.29–15.06) among all workers ($N = 8$) and 12.90 (95% CI 2.59–37.70) among workers ($N = 3$) in "transformer assembly." There was only one death attributed to liver/gall bladder for the entire cohort, and this was not a significant finding.

Yassi et al. (1994) concluded that consideration of latency "increased the likelihood that these deaths were related to workplace exposures." The highest mortality for pancreatic cancer was for those who had begun work before 1960 (5 deaths, SMR = 676). For those who entered the workforce between 1960 and 1969, the SMR was 154, and no pancreatic cancers occurred in those who began work after 1970. However, the study presented no SMR analysis by latency. Several of the 11 pancreatic cancer cases either died shortly after being hired or worked at the plant for a short period of time, thus weakening any association between latency and the appearance of pancreatic cancer. In addition, since the hire dates for more than 20% of the workforce were unknown, the validity of a latency analysis, even had such an analysis been conducted, would be highly uncertain (Wong, 1995). Moreover, since potential exposures to PCBs were not chronologically determined, it is impossible to attribute the decreasing SMRs for pancreatic cancer to any particular exposure.

The authors provided occupational histories of the 11 pancreatic cancer deaths in the cohort and a 12th case for which pancreatic cancer was a contributory cause of death. Among these 12 cases, 6 cases had 2 or less years of possible exposure (as measured by employment duration) and the employment histories of 2 more were uncertain due to incomplete information on hire and termination dates. In reviewing this study, ATSDR (1999) noted that the correlation between exposure time and pancreatic cancer appears spurious because in the group with the highest SMR only three cancers were reported, and two of these were in individuals who worked at the plant for 1 year or less. One of these individuals died within 1 year after leaving the plant. Wong (1995) and ATSDR (1999) are in agreement that these two employees had neither sufficient exposure duration nor latency for the cancers to be attributed to mineral oil or PCB exposure. Thus, there is little evidence of a dose-response relationship in this study.

ATSDR (1998) noted other "severe limitations" to the Yassi et al. (1994) study, including the fact that employees were exposed to chemicals other than PCBs and no medical histories of the employees were provided. Wong (1995) also identified additional limitations including incompleteness of the cohort ascertainment, inadequate data for vital status determinations, lack of basic employment and exposure information (such as hire date and/or departure date), inconsistency in numbers presented, small sample size in most analyses, and lack of detailed analyses by exposure variables. In responding to the comments by Wong (1995), Yassi et al. (1995) did not refute any of the issues pertaining to latency.

Yassi et al. (1994) appeared to question the meaning of their results, particularly as they relate to PCB exposure, observing that "while this study was originally undertaken because of concerns regarding PCB exposure, company records indicated that a vast majority of their transformers contained mineral oil (not PCB) rather than Askarel (containing PCB oil)." The authors concluded that "the finding of excess pancreatic cancer in this transformer manufacturing cohort (exposed to petroleum oil and other solvents used in electrical manufacturers) is consistent with the literature, although the specific causative agent remains unclear." In addition to these problems, potential confounding factors such as smoking and alcohol consumption (Gold and Goldin, 1998; Zheng et al., 1993; Fuchs et al., 1996) were not considered. Finally, of all the studies conducted on PCB-exposed workers, no other study has reported an increased risk of pancreatic cancer.

Greenland et al. (1994)

Greenland et al. (1994) conducted a case-control study of cancer mortality as related to various chemical exposures at a transformer assembly facility. This study was undertaken to address preliminary reports of excess cancer mortality associated with employment at this facility. Subjects included those who had been employed at the facility before December 31, 1984, who died between 1969 and 1984, and for whom death benefits claims had been filed. The study included 512 cases and 1202 controls. The cases were study subjects who died of all types of cancers. The controls died of conditions other than cancer, excluding diseases of the

digestive system, genitourinary diseases, diseases of the blood and blood-forming organs, mental disorders, and ill-defined conditions. There were 107 noncancer deaths excluded from the control group because their diagnoses were possibly associated with the exposure under study.

More than 250 chemicals and classes of chemicals were identified as being used at the plant. From this list of more than 250 chemicals, 30 were identified that were potentially either carcinogenic or mutagenic. The authors focused on seven of these chemicals or classes of chemicals based on assumed carcinogenic potential, amount of the material used, and the number of operations in which the material was used. The chemicals/classes of chemicals were: Pyranol (mixture of approximately 45% to 80% PCBs and trichlorobenzene); trichloroethylene; benzene; mixed solvents; asbestos; synthetic resins; and machining fluids. Thus, individuals included in the study were potentially exposed to many chemicals other than PCBs. Exposures were estimated for various jobs in the plant through interviews with plant workers, including some management personnel. For Pyranol, exposure categories were no exposure, indirect exposure (chemical present in the workplace, but worker did not perform a task which involved direct exposure), and direct exposure.

Preliminary analyses indicated a statistically significantly elevated odds ratio (OR) between Pyranol exposure and lymphomas (defined by Greenland et al. as all lymphosarcomas and reticulosarcomas combined) (OR = 3.26). However, this finding was not significant when evaluated by the Mantel test for dose response and was discounted by the authors. The authors concluded that the only unequivocal positive association found involved synthetic resins (containing asbestos) and lung cancer. However, smoking histories were not available to shed light on this association.

Hoppin et al. (2000)

This study investigated the relationship between pancreatic cancer and serum concentrations of DDT, DDE, PCBs (11 congeners), HCB, *trans*-nonaclor, and five other organochlorine compounds. The cohort consisted of 113 nonoccupationally PCB-exposed cases of pancreatic cancer and 82 matched controls. Interviews with each case and control included information on possible occupational and chemical exposures, tobacco use, diet, and medical history. Statistical analysis was limited to chemicals that were detected above the method detection limit in greater than 50% of samples, resulting in analysis of data on only DDE, PCBs, HCB, and *trans*-nonaclor. All values were lipid adjusted, which was particularly important since there were significant differences between cases and controls for cholesterol, triglycerides, and total lipids due to disease-related cachexia.

There was a statistically significant dose-response trend between increasing total PCB concentrations and risk of pancreatic cancer in the highest tertile of PCB concentrations (360 ng/g lipid) with an OR of 4.2 (95% CI 1.9–9.4). Congener-specific analyses demonstrated significantly increased risk for the highest tertile of PCB congener 153 (OR = 3.0; 95% CI 1.4–6.6) and congener 180 (OR = 8.4; 95% CI 3.4–21). The significance of these findings is unclear and was questioned by the authors, who noted that "due to cachexia among pancreatic cancer patients, the possible effect of wasting on organochlorine levels, and PCBs in particular, it is difficult to predict and complicates the interpretation of our findings." Recognizing this problem (particularly since 55% of cases reported weight loss prior to diagnosis), a sensitivity analysis was performed in an attempt to account for several different hypothetical bioconcentration scenarios by assuming 10%, 25%, and 40% bioconcentration of PCBs as a consequence of reduced serum lipids resulting from weight loss due to pancreatic cancer. This analysis revealed a significant association between pancreatic cancer and the highest tertile of total serum PCBs under hypothetical conditions of 10% bioconcentration (OR = 3.1; 95% CI 1.4–7.0) and no significant associations with any other assumed bioconcentration factor. The fact that there were no statistically significant associations between pancreatic cancer risk and assumed lipid bioconcentrations of 25% and 40%, even at the highest tertiles of exposure, is not surprising given that total lipids were 26% less in cases than controls. Thus, after accounting for the documented differences between cases and controls with respect to disease induced weight loss, resulting in diminished serum lipid levels and the subsequent effects on serum PCB concentrations, there is little, if any, evidence for an association between PCBs and increased risk of pancreatic cancer. Because of the profound effects of disease-induced weight loss and its consequent effects on serum lipids and, therefore, the measured concentrations of lipophilic chemicals, studies such as this have little value in determining potential causal relationships. A case-control study of pancreatic cancer and a biomarker such as serum PCB levels that

can be affected by cachexia is inappropriate. Hoppin et al. (2000) attempted to account for this by statistical methods even though such an approach has never been validated.

Zach and Musch (1982)

Mortality rates were reported for 89 workers involved in the manufacture of PCBs in a plant in Illinois. No industrial hygiene data were available to quantify PCB exposure levels. In addition, all workers were also exposed to other chemicals including tri- and tetrachlorobenzene and biphenyl. Analysis of the cause of death in the 30 deceased members of this cohort revealed no statistically significant increase in mortality from all cancers or any cancer type, including no deaths from liver cancer, pancreatic cancer, or melanoma.

Rothman et al. (1997)

In a nested case-control study, 74 non-Hodgkin's lymphoma (NHL) cases were drawn from a large prospective cohort study involving more than 25,000 adults from Maryland. The researchers' a priori hypothesis was that the incidence of NHL would be associated with increasing serum concentrations of DDT. No association for this exposure was found. However, in examining the data, a dose-response relationship was found between NHL and lipid-corrected serum PCB concentrations. Rothman et al. (1997) noted: "These results should be regarded as hypothesis-generating. Before causal inferences can be made about exposure to PCBs and increased risk of non-Hodgkin lymphoma, our findings require replication and the biological plausibility of the association needs further investigation." The authors also noted that occupational studies had documented PCB serum levels of at least one order of magnitude greater than those found in their cases and no association was found between NHL and PCBs in those studies. Thus, Rothman et al. (1997) concluded that: (1) "it is possible that confounding was present in our studies—i.e., that an unrecognized risk factor was associated with PCB concentrations and, more strongly, with risk of non-Hodgkin lymphoma"; (2) "the inconsistency between our findings and those from studies of PCB-exposed occupational cohorts needs to be explained"; and (3) "the biological plausibility of this association requires further investigation."

It is indeed difficult to reconcile the findings of Rothman et al. (1997) with the lack of any increase in NHL in more heavily exposed cohorts. The large occupational cohort studies of workers heavily exposed to PCBs, none of which have reported an association between increased NHL and exposure to PCBs, should be regarded as demonstrating that the hypothesis of Rothman et al. (1997) that PCB exposure is a risk factor for NHL is incorrect.

Bahn et al. (1976, 1977); NIOSH (1977)

In two letters to the editor, Bahn et al. (1976, 1977) reported on the incidence of tumors in workers at a New Jersey petrochemical facility where Aroclor 1254 had been used from 1949 to 1957. In the first letter, Bahn et al. (1976) reported that a significantly increased incidence of malignant melanomas was observed among research and development workers (2 of 31) and refinery personnel (1 of 41). It should be noted that these letters to the editor are case reports, were not peer reviewed, and involve an extremely small cohort.

The first Bahn (1976) letter was followed 5 months later by a letter from Lawrence (1977), who pointed out another important reason why no conclusions can be drawn from Bahn (1976): "Bahn et al.... fails to address the very important issue of exposure to other, perhaps carcinogenic compounds.... One of these, epoxide 206, a particularly effective scavenger, was found to have a 'pronounced carcinogenic effect' in an animal skin-painting experiment.... It is impossible to draw any inferences from the Bahn study concerning adverse effects of PCB without information on other chemicals to which the group may have been exposed." The comment from Lawrence (1977) prompted a brief reply by Bahn et al. (1977) that acknowledged that the extent of the workers' exposure to other chemicals was not known.

NIOSH (1977) provides some limited information on its independent investigation of the Bahn cohort. The discussion of the Bahn cohort in NIOSH (1977) comprises only two short paragraphs. NIOSH (1977) stated that it observed 8 cancers in the study population versus 5.7 expected. Three of these tumors were melanomas and two were pancreatic cancers. The incidence of these tumor types was reported to be significantly higher than expected, although no data were presented. NIOSH described this study as "preliminary," and noted that "PCB exposure histories were based on recollections of two company employees." NIOSH (1977) also stated that "The expected cancer rates

were based on US population data rather than on a rate for the locality of the petrochemical facility.... A substantial change has occurred in the cohort since release of the preliminary report by Bahn and her coworkers, and it seems likely that the findings on this new cohort will differ significantly from those of the preliminary study. The final report is not yet available."

The implications of these preliminary reports are suspect due to small cohort size and the fact that the workers in this facility were exposed to numerous other chemicals. According to the U.S. EPA (1999a), the results of NIOSH (1977) are inconclusive. Consequently, the preliminary findings of Bahn et al. and NIOSH (1977) are inadequate for use in a weight-of-evidence assessment concerning a causal relationship between exposure to PCBs and cancer.

Hardell et al. (1996)

Hardell et al. (1996) conducted a case-control study of 27 patients with non-Hodgkin's lymphoma (NHL) to determine if there was a difference between cases and controls in adipose tissue levels of several organochlorine compounds including PCDDs (7 congeners), PCDFs (10 congeners), PCBs (34 congeners), HCB, and DDE. Instead of determining serum levels of these compounds, a 2- to 10-g sample of abdominal adipose tissue was removed under local anesthesia for analysis. There were no significant differences in total PCB levels between the NHL cases and controls. However, there were significant differences between cases and controls for selected PCB congeners including 156, 157, 182/187, 171, 172/192, 170/190, 189, 202, 201, 194, and 208, with cases having higher levels than controls. There were no differences between cases and controls in adipose tissue concentrations of any of the other compounds measured. Hardell et al. (1996) concluded that "since immunosuppression is an established risk factor for NHL, our results are of interest in the etiology of NHL but need to be confirmed in larger studies." Hardell et al. (1996) attempt to support their hypothesis concerning immunological impairment by stating that occupational exposure to PCBs has been associated with this effect. However, they do not reference credible data in support of their statement citing two studies involving Yusho and Yu-Cheng cohorts poisoned by a heat degraded mixture of PCBs containing PCDFs. Neither of these studies supports the idea that PCBs per se are immunosuppressive. Other than these reports, there is no evidence to support an association between exposure to PCBs and immunosuppression in humans (ATSDR, 1999). In addition, none of the many studies on heavily exposed occupational cohorts have reported an association between PCBs and NHL.

Finally, while Hardell et al. (1996) selected cases prior to their beginning treatment for NHL, there is no information concerning the length of time between the onset of symptoms and diagnosis. This makes it difficult to rule out a disease-induced process that resulted in a differential distribution of organochlorines in adipose tissue. Consequently, this kind of study is incapable of determining if elevated adipose tissue levels of PCBs are the result of disease-induced redistribution of lipophillic chemicals.

WEIGHT-OF-EVIDENCE ASSESSMENT OF THE EPIDEMIOLOGICAL CANCER DATA FROM COHORTS OCCUPATIONALLY EXPOSED TO PCBS

As evidenced by the preceding review, there is a substantial number of epidemiology studies of workers occupationally exposed to PCBs. At least 10 of these studies provided the basis for the conclusion by ATSDR (1999) that "the weight of evidence does not support a causal association for PCBs and human cancer at this time." In order to establish a basis for evaluating additional studies, as well as studies not available when ATSDR (1999) reached their conclusion, this section provides a weight-of-evidence assessment of all of the studies reviewed earlier.

The weight-of-evidence approach must be used to draw conclusions from a variety of data that are not entirely consistent. The best guidelines for accomplishing this can be found in the U.S. EPA's recent (1999, 2003) *Guidelines for Carcinogen Risk Assessment*, which unequivocally embrace this philosophy. These guidelines are based on the principles established by Hill (1965) and commonly referred to as the causation criteria.

An objective weight-of-evidence evaluation of epidemiological studies requires (a) the observation of a specific endpoint(s) and (b) the application of the principal causation criteria [i.e., strength of association, consistency of association, dose-response relationship, temporally correct association, specificity of the association, and biological

plausibility (coherence with existing information)] to determine whether a causal relationship can be inferred between exposure to a particular agent and an increased risk of some disease.

As discussed in detail next, applying a weight-of-evidence evaluation to the PCB epidemiological studies can only lead to the conclusion that there is no causal relationship between PCB exposure and any form of cancer While a few studies suggest that occupational exposure to PCBs might be associated with an increased risk of some types of cancer, these are generally isolated findings that could not be confirmed in follow-up studies.

In reviewing the U.S. EPA *Proposed Guidelines for Carcinogenic Risk Assessment*, the U.S. EPA Science Advisory Board (SAB) (1997) had the following observation concerning the use of statistical significance:

It is important, in evaluating the overall evidence for an effect from epidemiological studies, to rule out chance as a possible explanation for an observed association. The degree to which chance should account for an observed association can be evaluated by calculating the statistical significance of the association.

Any time the null hypothesis is rejected, thereby resulting in a statistically significant finding, a "burden of proof" concerning that particular finding must then be met. In the evaluation that follows, all of the studies reporting a statistically significant finding of increased mortality[8] from a specific cancer are first assessed as a group to determine which studies and effects should be included in a weight-of-evidence evaluation. This approach ensures that studies and outcomes are not afforded more weight than is warranted in a final weight-of-evidence evaluation. Following evaluation of these studies, each statistically significant finding is systematically judged against each of the causation criteria to determine the extent to which they are fulfilled.

Ignoring study size, design, potential confounding factors, and other possible methodological problems, there are 13 studies that report one or more statistically significant associations between exposure to PCBs and cancer (Bahn et al., 1976, 1977; NIOSH, 1977; Bertazzi et al., 1982, 1987; Brown and Jones, 1981; Brown, 1987; Hardell et al., 1996; Loomis et al., 1997; Rothman et al., 1997; Sinks et al., 1992; Hoppin et al., 2000; Yassi et al., 1994).

Three "studies" reporting statistically significant findings were not peer reviewed or otherwise subjected to independent scientific scrutiny before publication (Bahn et al., 1976, 1977; NIOSH, 1977). In fact, they are two letters to the editor (Bahn et al. 1976, 1977) and a "criteria document" that contains a two-paragraph follow-up on the workers described by Bahn et al. (NIOSH, 1977). These should be afforded little or no weight in a weight-of-evidence evaluation of the possible human carcinogenicity of PCBs.

Moreover, as noted earlier, this initial report suffers from additional defects, including exposure to chemicals other than PCBs and failure to control for sunlight exposure (see later discussion). In addition, the preliminary findings reported by NIOSH in 1977 concerning a possible association between exposure to PCBs and pancreatic cancer have never been published.

Removing these findings from the weight of evidence from the 13 studies reporting a statistically significant finding of increased disease mortality (or incidence) leaves 10 studies that went through a peer-review process (Brown and Jones, 1981; Brown, 1987; Bertazzi et al., 1982, 1987; Hardell et al., 1996; Loomis et al., 1997; Rothman et al., 1997; Sinks et al., 1992; Hoppin et al., 2000; Yassi et al., 1994). In the two studies that reported an increased incidence of melanoma (Loomis et al., 1997; Sinks et al., 1992), potential exposure to sunlight was not adequately accounted for. This is particularly the case for leisure-time exposure to sunlight. In neither of these studies was there any consideration of the anatomic location of the melanoma; the case for sunlight as a possible confounder is strengthened if the melanoma is in an anatomic location that would receive sun exposure. Conversely, the case for some other etiologic factor is enhanced if the melanoma is in an anatomic location not likely to be exposed to the sun. This could have been done by Sinks et al. (1992), since interviews were conducted with the next of kin of all melanoma cases. It would have been more difficult for Loomis et al. (1997) to accomplish this. There is a vast literature demonstrating that exposure to sunlight is the single most important risk factor for the development of malignant melanoma. Reviews of the epidemiology of melanoma stress the importance of this factor, particularly since leisure-time exposure to sunlight has increased so dramatically (Serraino et al., 1998; Elwood and Jopson, 1997; Berwick and Halpern, 1997; Elwood and Koh, 1994;

[8]Three studies (Hardell et al., 1996; Rothman et al., 1997; Hoppin et al., 2000) report on cancer incidence.

Goldstein and Tucker, 1993; Nelemans et al., 1992). Thus, it is important for studies with a melanoma finding to account for exposure to sunlight.[9]

Therefore, it is inappropriate to place much weight on the findings of Sinks et al. (1992) and Loomis et al. (1997) [or the Bahn et al. (1976) case reports and the NIOSH (1977) report], in a weight-of-evidence evaluation of the possible carcinogenicity of PCBs. This is particularly true given that malignant melanoma was not significantly elevated in any other capacitor-manufacturing cohort study (including Brown and Jones, 1981; Brown, 1987; Nicholson, 1987; Taylor et al., 1988; Kimbrough et al., 1999, 2003; Bertazzi et al., 1982, 1987; Tironi et al., 1996).

The study by Yassi et al. (1994), reporting a significant association between PCB exposure and pancreatic cancer, is of dubious value in a weight-of-evidence evaluation given the fact that workers were minimally exposed to PCBs. Consequently, this study is essentially one of exposure to mineral oil transformer fluids rather than to PCBs. Commenting on Yassi et al. (1994), ATSDR (1999) noted that "the role of PCBs, if any, is unclear due to severe limitations such as exposure to other chemicals and the fact that no medical histories of the workers was provided." In addition, it should also be noted that the reported SMR of 764 is too high to be plausible. If this were a correct estimate of pancreatic cancer risk resulting from PCB exposure, excess pancreatic cancer should have been seen in other studies with far greater exposure to PCBs. However, Yassi et al. (1994) is the only study to report this finding.

Hoppin et al. (2000), a nonoccupational exposure study, reported an association between serum levels of PCBs and increased risk of pancreatic cancer. This study involved a substantial number of patients with disease-induced weight loss. After accounting for the documented differences between cases and controls with respect to weight loss, resulting in diminished serum lipid levels and the subsequent effects on serum PCB concentrations, there was essentially no evidence for an association between PCBs and increased risk of pancreatic cancer.

The results of the many studies conducted on workers with substantially higher exposure to PCBs must be afforded greater weight in a weight-of-evidence determination of a potential association between exposure to PCBs and increased risk of pancreatic cancer. In none of the studies of workers highly exposed to PCBs and studied several times (i.e., Brown and Jones, 1981; Brown, 1987; Nicholson, 1987; Taylor et al., 1988; Kimbrough et al., 1999, 2003; Bertazzi et al., 1982, 1987; Tironi et al., 1996; Gustavsson et al., 1986; Gustavsson and Hogstedt, 1997) or a single time (Loomis et al., 1997; Sinks et al., 1992) has an increase in pancreatic cancer ever been reported.

The studies by Hardell et al. (1996) and Rothman et al. (1997), each of which reported a significant association between PCBs and non-Hodgkin's lymphoma (NHL), have methodological limitations. The case group in Hardell et al. (1996) consisted of only 27 NHL patients. In this small group, certain PCB congeners in adipose tissue (measured after the diagnosis of disease) were reportedly elevated. Since none of the large occupational cohort studies, with much greater PCB exposures than in the Hardell et al. (1996) study, has reported a significant association with NHL, it would be unwarranted to conclude that exposure to PCBs is causally related to NHL.

The study by Rothman et al. (1997) involved a large prospective cohort in which 74 cases of NHL developed. Although the authors concluded that there was an increased risk of NHL associated with elevated serum levels of PCBs that were measured well before a diagnosis of disease, they were explicit that the results of their study were "hypothesis-generating" and should not be part of any causal inference. The large worker cohort studies can be viewed as having conducted the hypothesis testing proposed by Rothman et al. (1997) and have ruled out a link between PCB exposure and NHL. None of these studies involving workers with known exposure to PCBs, in many cases at least an order of magnitude higher than the upper quartile in Rothman et al. (1997), have reported an increased incidence of NHL.

As discussed earlier, Brown (1987) updated the study of a PCB-exposed cohort that had originally been evaluated in 1981 (Brown and Jones, 1981). In the first study, the only statistically significant finding was increased mortality from rectal cancer in females from one of the two plants studied. The authors considered the results of the study to be inconclusive due to the short observation period and small number of deaths. In the Brown (1987) follow-up, there was a statistically significant excess risk of cancer when liver, biliary tract, and gallbladder

[9]It should be noted that none of the many occupational studies involving other cohorts exposed to PCBs have accounted for exposure to sunlight. Had any of these studies reported an association between PCBs and increased risk of melanoma, they would all be subject to this same criticism.

cancer were grouped together, although mortality from rectal cancer was no longer significantly elevated. However, there is no evidence that the etiology or occurrence of these three tumor types is similar. In addition, as noted by ATSDR (2000), even this finding is no longer statistically significant if the one case of metastatic liver cancer is removed from this grouping. Brown (1987) concluded that his work provided only "limited information" associating PCBs with liver and other related cancers. Part of the reason for this was that alcohol consumption and hepatitis viruses B and C, potentially confounding variables, could not be addressed in this study. This could be important since alcoholic cirrhosis secondary to excessive alcohol consumption and hepatitis viruses B and C are recognized risk factors for liver cancer (Lau and Lai, 1990; Yoshihara et al., 1998; Chen et al., 1997). This is likely the reason that ATSDR (1999), in reviewing Brown (1987), concluded that "the liver cancer cannot be unequivocally attributed to PCB exposure." The U.S. EPA also determined that this study was inconclusive because no dose-response relationship was apparent and the number of cancers in the cohort was small (U.S. EPA, 1999a).

Following the passage of an additional 10 and 15 years of time, workers from Plant No. 1 studied by both Brown and Jones (1981) and Brown (1987) were once again studied by Kimbrough et al. (1999, 2003). There was no excess mortality from rectal cancer as reported by Brown and Jones (1981). In addition, while this was not the plant at which excess mortality from liver/biliary tract/gallbladder cancer was reported by Brown and Jones (1987), mortality from these cancers was not increased in either of these studies. In fact, there were no excesses of any type of cancer, including melanoma, NHL, gastrointestinal tract, pancreas, or all cancer combined. As noted by Kimbrough et al. (1999), "neither overall cancer mortality nor numbers of any of the a priori cancers of interest previously reported as being elevated were elevated in this cohort." Additionally, as most recently reported by Kimbrough et al. (2003), "We again failed to find any significant excess mortality in the a-priori cancers of concern or in any other cancers in the total cohort or in the highly exposed portion of the cohort. These results expand on our previous observations and as before the data fail to demonstrate any causal association between occupational PCB exposure and excess cancer mortality or any other causes of death." Longer term follow-up, as exemplified by the extensive studies of Kimbrough et al. (1999, 2003), has now empirically demonstrated that there are none of the excesses in cancer mortality as initially reported by Brown and Jones (1981) or Brown (1987).

Based on very small numbers, Bertazzi et al. (1982, 1987) reported a statistically significant increase in all cancers combined in male workers, and Bertazzi et al. (1987) also reported a significant increase in all cancer in female workers, although the statistical significance of all of these increases had disappeared by the time Tironi et al. (1996) studied the same cohort nine years later. The length of potential exposure to PCBs in at least 7 of the 26 cases was 1 year or less, thus weakening any potential association with PCBs. Bertazzi et al. (1997) also reported a significant increase in cancer of the gastrointestinal tract in male workers; however, this was only achieved as a result of grouping together cancers of the stomach, liver, biliary tract, and pancreas. In addition, based on a total of four cases, Bertazzi et al. (1987) also reported a significant increase in hematologic cancer in females, although of these four cases, two had potential exposure to PCBs of 0.2 and 0.7 years, respectively. These findings in both males and females had disappeared by the time Tironi et al. (1996) studied the same cohort 9 years later.

From the preceding summary, it is evident that that none of the studies that initially suggested an association between exposure to PCBs and increased risk of cancer can be relied on as supporting the hypothesis that PCBs are carcinogenic to humans. Were these initial studies the only data on which to base a decision concerning the potential carcinogenicity of PCBs to humans, one would have to conclude that there was insufficient evidence for the human carcinogenicity of PCBs and base all regulatory decisions on the animal data. However, this is not the case. Even the initial "positive" results reported by Brown and Jones (1981) and Brown (1987) are useful because it now appears that these findings were chance events that could not be confirmed in other large studies, including Taylor (1988) and, more recently, Kimbrough et al. (1999, 2003). Thus, the studies by Brown and Jones (1981) and Brown (1987), together with the four studies that could not confirm these findings (Nicholson, 1987; Taylor et al., 1988; Kimbrough et al., 1999, 2003), are the best sources of information with which to assess the potential cancer risk of PCBs.

The series of studies by Bertazzi et al. (1982, 1987) and Tironi et al. (1996) also demonstrate the importance of study follow-up. Based on very small numbers of deaths, Bertazzi et al. (1982) reported an increased incidence in the category of

TABLE 2
Extent to Which the Causation Criteria Are Satisfied by the Available Data on PCBs

Causation criterion and disease endpoints	Comment	Criteria fulfilled
Strength of the association		
Liver/biliary/gallbladder cancer	With ATSDR (2000) acknowledgment of no significant association between PCBs and liver/biliary/gallbladder cancer in Brown et al. (1987), no studies show a significant association with occupational exposure to PCBs.	No
Melanoma	Significant associations reported by Loomis et al. (1997) and Sinks et al. (1992) confounded by an inability to account for exposure to sunlight, the most important risk factor for this disease, which weakens to an unknown extent the reported associations with PCBs.	No
Rectal cancer	Significant increase only in Brown and Jones (1981); no longer significant at first follow-up (Brown et al., 1987).	No
Pancreatic cancer	Significant increase only reported in a single study (Yassi et al., 1994); association with PCBs was highly problematic. The magnitude of the elevated risk is not plausible, or pancreatic cancer would be more common, particularly in more heavily occupationally exposed workers.	No
Non-Hodgkin's lymphoma (NHL) and other hematologic cancers	Significant association with NHL in two studies (Hardell et al., 1996; Rothman et al., 1997); Hardell et al. incapable of determining if association a consequence of exposure or the disease. The increase in Hodgkin's disease and lymphosarcoma in Bertazzi et al. (1987) had disappeared by the time the cohort was studied 9 years later by Tironi et al. (1996).	No
Gastrointestinal tract cancer	The significant increase in GI tract cancer (i.e., the combined incidence of stomach, liver, biliary tract and pancreatic cancer) reported by Bertazzi et al. (1987) disappeared by the time the cohort was studied 9 years later by Tironi et al. (1996). The strength of the association was undermined by combining different types of cancers into the category of GI tract cancer.	No
All cancers	Significant increase in all cancers combined in Bertazzi et al. (1982, 1987) had disappeared when cohort studied 9 years later by Tironi et al. (1996).	No
Consistency of the association		
Liver/biliary/gallbladder Cancer	Association between PCB exposure and liver/biliary/gallbladder cancer only reported in a single study (Brown, 1987). Increased liver cancer or the combined liver/biliary/gallbladder cancer grouping not reported in 11 other studies of heavily exposed occupational cohorts.	No
Melanoma	This finding reported in two studies of workers exposed to PCBs (Loomis et al., 1997; Sinks et al., 1992). Melanoma not reported in 10 other studies of heavily exposed occupational cohorts.	No
Rectal cancer	Elevated rectal cancer only reported in a single study (Brown and Jones, 1981) and this finding no longer significant on follow-up of the same cohort (Brown, 1987; Nicholson, 1987; Taylor et al., 1988; Kimbrough et al., 1999, 2003). Not reported in 7 other studies.	No

(Continued on next page)

TABLE 2
Extent to Which the Causation Criteria Are Satisfied by the Available Data on PCBs (Continued)

Causation criterion and disease endpoints	Comment	Criteria fulfilled
Pancreatic cancer	Increased pancreatic cancer only observed in a single occupational study (Yassi et al., 1994); pancreatic cancer not reported in 12 other studies.	No
NHL and other hematologic cancers	NHL reported in two studies, one in which it was impossible to determine if the association was due to PCBs or the disease itself (Hardell et al., 1996), and the other in which the authors advised against using the data for causal inference (Rothman et al., 1997). NHL not reported in 8 other studies on occupational cohorts far more heavily exposed to PCBs. A report of an increase in Hodgkin's disease and lymphosarcoma (Bertazzi et al., 1987) in females had disappeared by the time the cohort was studied 9 years later by Tironi et al. (1996)	No
Gastrointestinal tract	Elevated GI tract cancer only observed in a single study (Bertazzi et al., 1987); this finding no longer significant on follow-up of the same cohort 9 years later (Tironi et al., 1996). GI tract cancer not reported in 9 other studies on occupational cohorts heavily exposed to PCBs.	No
All cancers	Significant increase in all cancers combined reported by Bertazzi et al. (1982, 1987) not confirmed on follow-up of the same cohort (Tironi et al., 1996) or in 9 other studies of occupational cohorts heavily exposed to PCBs.	No
Dose-response		
Liver/biliary/gallbladder cancer	Liver/biliary/gallbladder cancer only observed in one study (Brown 1987) and the data failed to demonstrate a dose-response relationship; 12 other studies of workers exposed to PCBs at equivalent levels failed to find increased risk of liver/biliary/gallbladder cancer at any exposure level.	No
Melanoma	Sinks et al. (1992) analyzed the data for dose response and failed to find such a relationship. Loomis et al. (1997) established a D-R relationship with some estimated PCB exposures and durations and melanoma based on very small numbers of cases. The failure to adequately consider exposure to sunlight may have had an unknown confounding effect on the D-R relationship reported for PCBs.	No
Rectal cancer	Rectal cancer was observed in a single study and an analysis of this data by length of employment failed to support a dose-response relationship (Brown and Jones, 1981). This finding was no longer statistically significant when the same cohort was studied six years later (Brown et al., 1987).	No
Pancreatic cancer	Pancreatic cancer was observed in a single occupational study in which exposure to PCBs, if it occurred at all, was de minimus (Yassi et al., 1994); no dose-response relationship was reported; questionable relevance of dose response in Hoppin et al. (2000) in active cases of pancreatic cancer; 11 other studies with greater PCB exposure have not reported increased pancreatic cancer.	No

TABLE 2
Extent to Which the Causation Criteria Are Satisfied by the Available Data on PCBs (Continued)

Causation criterion and disease endpoints	Comment	Criteria fulfilled
NHL and other hematologic cancers	Hardell et al. (1996) reported an association between selected PCB congeners and NHL, but there was no analysis for dose response. Rothman et al. (1997) reported a dose-response relationship between NHL and lipid-corrected serum PCB concentrations, but questioned this result, noting that occupational studies in which PCB serum levels were at least an order of magnitude greater had not observed an association between NHL and PCBs.	No
Gastrointestinal tract cancer	Elevated mortality from GI tract cancer reported by Bertazzi et al. (1987); 3 of the 6 total cases had PCB exposure for 1 year or less, suggesting little, if any, potential for a D-R relationship; this result was no longer significant on follow-up of the same cohort 9 years later (Tironi et al., 1996).	No
All cancers	An elevated incidence in all cancers combined reported by Bertazzi et al. (1982, 1987); this finding had disappeared 9 years later on follow-up of the same cohort (Tironi et al., 1996). The results of Bertazzi et al. (1982) were based on so few deaths (8 males and 6 females) that no dose-response analysis was possible. The results in Bertazzi et al. (1987) were also based on very small numbers (14 males and 12 females) and, of this total, 7 were exposed to PCBs for < 1 year, suggesting little, if any, potential for a D-R relationship.	No
Temporality		
Liver/biliary/gallbladder cancer Melanoma Rectal cancer Pancreatic cancer NHL and other hematopoietic cancer Gastrointestinal tract cancer All cancers	While it is likely that some exposure to PCBs occurred in every study in which a significant association was reported prior to the onset or death from a particular cancer, the other causation criteria must be fulfilled before it is possible to conclude that such exposures were causal. In some studies with exposure inclusion criteria of only 1 week of employment or when cancers occurred within 6 months to a year of employment the temporality criteria may be in question.	Yes
Specificity of association		
Liver/biliary/gallbladder cancer Melanoma Rectal cancer Pancreatic cancer NHL and other hematopoietic cancer Gastrointestinal tract cancer All cancers	For PCBs, the totality of the data demonstrates a striking pattern of nonspecificity of association with no instances where an increase in a particular cancer reported in a study associated with exposure to PCBs were predictive of results in another study of workers exposed to PCBs. In short, none of the studies reporting isolated significant associations between occupational exposure to PCBs and any type of cancer can be used to predict a similar outcome in another study. The lack of specificity over all of the data undermines a causal association between exposure to PCBs and increased risk of cancer.	No

(Continued on next page)

TABLE 2
Extent to Which the Causation Criteria Are Satisfied by the Available Data on PCBs (Continued)

Causation criterion and disease endpoints	Comment	Criteria fulfilled
Coherence with existing information (biological plausibility)		
Liver/biliary/gallbladder cancer Melanoma Rectal cancer Pancreatic cancer NHL and other hematopoietic cancer Gastrointestinal tract cancer All cancers	Because liver cancer is caused by PCB exposure in animal studies, it can be hypothesized that cancer of the liver might be associated with occupational exposure to PCBs. However, the finding of excess liver cancer per se has never been reported; rather the finding by Brown (1987) of an excess of liver/billiary/gallbladder cancer has only been reported once, suggesting that liver cancer is not a likely consequence of PCB exposure. Moreover, in this study, only one of five cases of liver cancer originated in the liver.	No
	For malignant melanoma, because PCBs have caused dermal effects in some animal studies and chloracne in some human exposure situations, some might argue that it is biologically plausible that melanoma could be a consequence of exposure to PCBs. However, there is no biological basis for inferring any relationship between chloracne and melanoma. Skin cancer of any kind has never been reported as a consequence of exposure to PCBs in any animal carcinogenicity study.	No
	For other isolated findings (i.e., rectal, pancreatic, GI, and hematological cancers) there is no biological basis for inferring that these cancers might be etiologically associated with exposure to PCBs. None of these cancers has been observed in the many animal carcinogenicity studies conducted on PCBs. With respect to reports of NHL associated with environmental exposures to PCBs, because this has never been reported following occupational exposures, there is no biological basis to explain how a relatively small exposure to a chemical could cause NHL while a much larger exposure would not produce a greater incidence of the same disease.	No
	Assessment of biological plausibility as this criterion might pertain to PCBs is also complicated when known risk factors for some reported endpoints are not adequately accounted for or, in some cases, not considered at all [e.g., melanoma (sunlight), pancreatic cancer (smoking, alcohol, diabetes, pancreatitis), and liver cancer (alcohol, hepatitis)].	

all cancers in males. With 6 years of follow-up (even though the exposure inclusion criteria were changed from 6 months to 1 week), Bertazzi et al. (1987) reported that the only statistically significant findings were increases in all cancers and gastrointestinal tract cancer in males and all cancers and hematologic cancers in females. With an additional 9 years of follow-up, Tironi et al. (1996) did not observe any statistically significant increases in any cancer category or sex, including all cancers, gastrointestinal tract cancer, or any hematological cancers.

Clearly, if exposure to PCBs caused the small excesses of cancer reported in earlier studies [e.g., Brown and Jones (1981), Brown (1987), Bertazzi et al. (1982), and Bertazzi et al. (1987)], there should be greater evidence of this effect years later as more workers in these cohorts aged and died. The fact that none of these initial findings could be confirmed on follow-up suggests that the initial reports were chance findings. Such findings are not unusual when dealing with small numbers, but are not observed with exposures to chemicals that are widely accepted as human carcinogens. This holds true for every chemical exposure known to be associated with increased risk of cancer in humans (NTP, 2000). In addition to elevations of specific endpoints that are statistically significant, one of the primary reasons that the epidemiological data for these substances are so compelling is that the studies are generally consistent with one another and collectively fulfill the causation criteria.

Either individually or collectively, none of the studies initially categorized as "positive" for some type of cancer lend credible support to the notion that PCBs are carcinogenic to humans. Moreover, the largest and most robust studies, having the benefit of even longer latency periods on follow-up, are consistent in failing to find any association between PCB exposure and human cancer. It is also worthwhile to note that in neither of the large cohorts in which follow-up evaluations were conducted (i.e., Brown and Jones, 1981, through Kimbrough et al., 2003, and Bertazzi et al., 1981, through Tironi et al., 1996) was melanoma, pancreatic cancer, or NHL ever reported as associated with exposure to PCBs. The final findings from these two large cohorts, each studied in detail three or more times, should be afforded the most weight in any determination of the likelihood that occupational exposure to PCBs might increase the risk of cancer. Additional support for the proposition that occupational exposure to PCBs does not increase the risk of cancer are the large studies by Sinks et al. (1992) and Loomis et al. (1997). As already noted, while neither accounted adequately for exposure to sunlight neither of these studies reported significant associations with rectal cancer, liver cancer, gastrointestinal tract cancer, pancreatic cancer, NHL or other hematological cancers, or all cancers combined. Thus, there are sufficient studies available to conclude that PCBs are not carcinogenic to humans at the range of exposures that has been experienced by humans, thereby confirming the validity of the ATSDR (1999) conclusion that "The weight of evidence does not support a causal association for PCBs and human cancer at this time."

In Table 2, the causation criteria are systematically applied to all effects that have been reported to be statistically associated with exposure to PCBs by one or more studies. As previously described, a weight-of-evidence assessment using these causation criteria requires assessment of the following criteria: strength of association; consistency of association; dose-response relationship; temporally correct association; specificity of the association; and coherence with existing information (biological plausibility). Whether these criteria are satisfied by the available data on each of the relevant effects is addressed in Table 2. The seven statistically significant findings assessed next are malignant melanoma, liver/biliary/gallbladder cancer, rectal cancer, pancreatic cancer, NHL and other hematologic cancers, gastrointestinal tract cancer, and all cancers combined.

CONCLUSION

Based on the weight-of-evidence analysis just described, the most plausible conclusion is that PCBs do not pose a carcinogenic risk to humans at human exposure levels. While some selected data at first glance may appear to support the idea that PCBs might be associated with an increased risk of cancer, a critical review of all available studies indicates otherwise; that is, occupational exposure to PCBs is not associated with an increased risk of cancer. The significant associations for NHL and pancreatic cancer reported in several cancer population-based studies (i.e., Hardell et al., 1996; Hoppin et al., 2000; Rothman et al., 1997) are not plausible given the consistent lack of similar findings in more heavily exposed occupational cohorts. The extent to which the totality of data on statistically significant associations reported in one or more studies fulfill the causation criteria necessary to establish a causal association between exposure to PCBs and increased risk of cancer is summarized in Table 3.[10]

PCBs have been regulated as possible human carcinogens based, in part, on an interpretation of

[10]Note that in terms of the GI tract line in Table 3, a significant increase in intestinal metaplasia in F344 rats was reported by Ward (1985), but this has not been observed in any other of the many animal carcinogenicity studies of PCBs.

TABLE 3
Extent to Which the Causation Criteria are Satisfied by the Totality of the Available Data on Specific Cancer Endpoints .

Cancer endpoint	Causation criteria					
	Strength of association	Consistency of association	Temporally correct association	Dose-response relationship	Specificity of association	Biological plausibility*
Liver	No	No	Yes	No	No	Yes
Rectal	No	No	Yes	No	No	No
Pancreatic	No	No	Yes	No	No	No
Melanoma	No	No	Yes	No	No	No
NHL	No	No	Yes	No	No	No
GI tract	No	No	Yes	No	No	No
All cancers	No	No	Yes	No	No	No

*Assuming that, based on animal studies, the only plausible site is the liver.

the epidemiological data that such data are "limited" with respect to showing a causal association. In all cases the few endpoints that are the basis for this conclusion could not be confirmed in follow-up studies of the same cohort or are offset by an inability to confirm the finding in other cohorts. To base a regulatory conclusion on the initial findings in a series of studies of the same cohort is a disincentive to conduct follow-up studies if the results of such studies are essentially ignored. Fortunately, a process and a set of commonsense rules have been developed in order to objectively evaluate a body of data to determine its collective weight. This process has been followed carefully in the weight-of-evidence assessment that demonstrates that PCBs do not pose a carcinogenic risk to humans.

A final observation concerning the positive findings from the studies evaluated in this review is in order. These studies have reported elevations in at least nine kinds of cancer (i.e., liver/biliary/gallbladder, pancreas, NHL, melanoma, rectal, and GI tract) as well as all cancers combined. Very few chemical carcinogens are multisite carcinogens, and even for these chemicals, the results are highly consistent between studies. Clearly the animal data for PCBs do not support the idea that PCBs are a multisite carcinogen. Similarly, the cohorts followed over time in several studies also tend to refute a conclusion that PCBs are a multisite carcinogen.

As additional support for the conclusions reached in this report, it should be noted that other independent reviews of the same data have reached essentially the same conclusions. After reviewing the available human epidemiological data (with the exception of Kimbrough et al., 1999), ATSDR (1998), concluded, "The weight of evidence does not support a causal association for PCBs and human cancer at this time."

Similarly, Kimbrough (1988) concluded, "Thus far, no conclusive adverse effects have been demonstrated in people who carry body burdens of PCBs from environmental exposure to trace amounts of PCBs.... Even workers with exposures two orders of magnitude greater than environmental exposures show no convincing health effects.... Thus, despite positive laboratory animal data and except for chloracne, exposure to PCBs has led to no convincing, clinically demonstrable, chronic health effects in humans."

In a more recent update, Kimbrough (1995) reached a similar conclusion, stating that "in the opinion of [this] author, the available evidence for cancer... is inconclusive." A recent review of the PCB occupational studies by the American Council on Science and Health concluded that "cancer has not been correlated with levels of PCB exposure and, therefore, cannot be attributed to PCB exposure" and that "no conclusive evidence exists that background levels in the general population, or even the very high levels that occurred among some occupational groups, resulted in acute or carcinogenic effects." (Danse et al., 1997).

In a recent meta-analysis of the data on the possible relationship between occupational exposure to PCBs and cancer, Longnecker et al. (1997) observed that "overall, data on occupational PCB exposure and cancer risk are inconclusive." Longnecker et al. (1997) reviewed many of the studies addressed in the present review (with the obvious exception of Kimbrough et al., 1999, 2003; Tironi et al., 1996; Loomis et al., 1997). Included in the Longnecker et al. (1997) review is a compilation of the

mortality data from eight different studies concerning cancers at selected sites (rectum, liver/biliary, pancreas, skin, breast, prostate, kidney, brain, and lymphoma). The site-specific observed and expected mortality findings among PCB workers were tabulated with a composite SMR calculated from the totals. The SMRs ranged from 0.9 to 1.8; however, none of the SMRs was statistically significant. The two largest studies published since this meta-analysis (i.e., Kimbrough et al., 1999, 2003) only serve to enforce the conclusions of Longnecker et al. (1997).

Finally, it is worth repeating some of the U.S. EPA (1999a, 2003) recommendations concerning a weight-of-evidence evaluation of a body of epidemiological data. Several of these recommendations are listed next followed by an assessment of how well the weight of evidence concerning PCBs and the analyses set forth in this review fulfill each one.

- "Analyzing the contribution of evidence from a body of human data requires examining available studies and weighing them in the context of well-accepted criteria for causation. A judgment is made about how closely they satisfy these criteria, individually and jointly, and how far they deviate from them."

  All of the relevant data for PCBs have been systematically weighted against the causation criteria. When viewed in their totality, the available studies supports the conclusion that exposure to PCBs does not pose a carcinogenic risk to humans.

- "Existence of temporal relationships, consistent results in independent studies, strong association, reliable exposure data, presence of dose-related responses, freedom from biases and confounding factors, and high level of statistical significance are among the factors leading to increased confidence in a conclusion of causality."

  All of these factors have been considered in reaching the conclusion that the weight of evidence does not demonstrate that exposure to PCBs is a carcinogenic risk to humans. In particular, the totality of the data demonstrates no strong association, no dose response, inconsistent results, and confounding.

- "An inference of causality is strengthened when a pattern of elevated risks is observed across several independent studies. The reproducibility of findings constitutes one of the strongest arguments for causality. If there are discordant results among investigations, possible reasons such as differences in exposure, confounding factors, and the power of the study are considered."

  In assessing all available data, the lack of consistency of findings across independent studies is striking, particularly the inability to confirm initial findings when the same cohort is studied again. The lack of any pattern of elevated risks is further evidence of lack of consistency.

- "A clear exposure-response relationship (e.g., increasing effects associated with greater exposure) strongly suggests cause and effect, especially when such relationships are also observed for duration of exposure (e.g., increasing effects observed following longer exposure times)."

  In assessing all available data, there is a lack of a dose-response relationship in the findings from the numerous studies evaluated in this review. The failure of the data to fulfill this important criterion suggests that there is no cause and effect relationship between exposure to PCBs and increased risk of cancer.

- "Generally, the weight of human evidence increases with the number of adequate studies that show comparable results on populations exposed to the same agent under different conditions. The analysis takes into account all studies of high quality, whether showing positive associations or null results, or even protective effects."

  All studies, whether showing positive associations or null results, have been systematically considered and collectively these studies lead to the conclusion that exposure to PCBs does not pose a carcinogenic risk to humans. In particular, the multiple studies on cohorts followed over time do not confirm that PCBs are a carcinogenic hazard to humans.

- "In weighing positive studies against null studies, possible reasons for inconsistent results should be sought, and results of studies that are judged to be of high quality are given more weight than those from studies judged to be methodologically less sound."

  In reviewing the totality of the data, possible reasons for inconsistent results have been systematically addressed, and results of studies that are of high quality have been given more weight than those from studies that are methodologically less sound. This has resulted in a determination that exposure to PCBs does not pose a carcinogenic risk to humans.

- "Generally, no single factor is determinative. For example, the strength of association is one of the causal criteria. A strong association (i.e., a large relative risk) is more likely to indicate causality than a weak association. However, finding of a large excess risk in a single study must be balanced against the lack of consistency as reflected by null results from other equally well designed and well conducted studies. In this situation, the positive association of a single study may either suggest the presence of chance, bias or confounding, or reflect different exposure conditions."

The isolated results from all studies that reported a statistically significant increase in cancer at any site were initially accepted on face value. These results were then "balanced against the lack of consistency as reflected by null results from other equally well designed and well conducted studies." This systematic process leads to the conclusion that exposure to PCBs does not pose a carcinogenic risk to humans.

ATSDR (2000) PCB EVALUATION

The unequivocal findings of the study by Kimbrough et al. (1999) (and now supplemented by the results of Kimbrough et al., 2003) clearly and unambiguously add to the ATSDR (1999) conclusion that the "weight of evidence does not support a causal association for PCBs and human cancer at this time." No additional data were available when ATSDR (2000) reached a seemingly opposite conclusion that "Overall, the human studies provide some evidence that PCBs are carcinogenic" and "some of these studies provide meaningful evidence that PCBs are carcinogenic in humans." Because of the public health implications of how these different conclusions were reached, some possible explanations are explored here.

One possible explanation is that the conclusion in ATSDR (1999) was incorrect and that the weight of evidence does, in fact, support a conclusion that there was a causal association between exposure to PCBs and human cancer. However, ATSDR (1999) cited numerous reviews that questioned this conclusion and the results of the weight of evidence review in the present document are in agreement with this conclusion. In addition, the Draft 1999 Toxicological Profile was subjected to internal ATSDR review by the Health Effects Review Committee as well as external peer review. Consequently, there is no basis for assuming that the ATSDR (1999) conclusions were inaccurate.

A possible alternative explanation might be that the criteria for establishing causality changed between 1999 and 2000 such that evaluations of a body of epidemiological data no longer were based on weight of evidence, but rather on different criteria. This would imply that terms such as "some evidence" and "meaningful evidence" have now been recognized as an acceptable way to summarize a complex body of epidemiological data to reach conclusions concerning causality. However, this is not the case either, and the U.S. EPA (1999a, 2003) *Cancer Risk Assessment Guidelines* and IPCS (1999) *Principles for the Assessment of Risks to Human Health from Exposure to Chemicals* remain as the standard by which causality is established. Furthermore, nowhere in ATSDR (2000) is there an explanation of what is meant by "meaningful evidence." Rather, this descriptor appears to convey an impression that the conclusion rests on the arbitrary selection of only those studies that support the notion that exposure to PCBs represents a carcinogenic risk.

Thus, it is unknown (and unexplained) why the largest negative study ever conducted did not strengthen the ATSDR (1999) conclusion that the weight of evidence does not support a causal association for PCBs and human cancer. Assuming that the conclusion by ATSDR in 1999 was scientifically based (and there is no reason to assume otherwise), one can only speculate on the reasons for reaching a contrary conclusion. A brief analysis of the logic underlying the ATSDR (2000) reevaluation is presented next.

As noted by ATSDR (2000):

"Because no individual study indicated a statistically significantly increased risk of primary liver/biliary tract/gallbladder cancer, Nicholson and Landrigan (1994) combined the results from the various studies available at the time by summing observed and expected cases. Based on a total of 8 observed and 2.8 expected cases from studies of capacitor manufacturing workers from three cohorts (Bertazzi et al., 1987; Brown, 1987b; Brown and Jones, 1981; Gustavsson et al., 1986), statistically significant increases were found for liver/biliary tract/gallbladder (SMR = 285, $p = .008$) and for biliary tract/gallbladder separately ($p < .05$, SMR not reported). Although the Nicholson and Landrigan (1994) analysis is based on combined results from cohorts having different durations and levels of exposure, latencies, and follow-up, and did not include data from the most recent studies

(Gustavsson and Hogstedt, 1997; Kimbrough et al., 1999a), it provides an indication that PCBs are associated with cancer of the liver, biliary tract, and/or gallbladder in humans." (p. 304)

Inexplicably, ATSDR (2000) did not mention the results of the study by Loomis et al. (1997).

As summarized by ATSDR (2000), Nicholson and Landrigan (1994) based their analysis on liver/biliary/gallbladder cancer data from three cohorts (Brown and Jones, 1981; Brown, 1987; Bertazzi et al., 1987; Gustavsson et al., 1986) by summing observed (O = 8) and expected (E = 2.8) incidences to yield an SMR = 285 ($p = .008$). Assuming that this approach is valid, the only way to determine if the totality of data support the conclusion that exposure to PCBs is associated with cancer of the liver/biliary tract/gallbladder in humans is to use all available data. Additional data since 1994 on liver/biliary tract/gallbladder cancer, and certainly available when the ATSDR (2000) evaluation was undertaken, include Kimbrough et al. (1999) (O = 5, E = 6.2), Gustavsson et al. (1996) (O = 1, E = 0.51), and Loomis et al. (1997) (O = 67, E = 91.8). Using all of this data in conjunction with the data summarized by Nicholson and Landrigan (1994) (i.e., O = 81, E = 101.3) yields SMR = 79, a result that is not statistically significant. Clearly, using this approach endorsed by ATSDR (2000), the totality of the data does not provide an indication that PCBs are associated with cancer of the liver, biliary tract, and/or gallbladder in humans.

In a similar analysis for melanoma based on mortality data from five cohorts (O = 9, E = 3.7), Nicholson and Landrigan (1994) calculated a significant ($p = .014$) SMR of 243. Additional relevant data since 1994, including Kimbrough et al. (1999) (O = 12, E = 8.1) and Loomis et al. (1997) (O = 116, E = 112), yield new totals (O = 137, E = 124) with an SMR of 110. Including all the data does not demonstrate a significant effect of PCBs on malignant melanoma.

Table 4 presents the results of a similar SMR analysis (using all available data) for liver/biliary/gallbladder cancer and melanoma as well as the other cancer sites reviewed earlier. Based on our calculations, none of these cancers are significantly elevated when SMRs using all available data are tabulated as suggested by Nicholson and Landrigan (1994). While not explicitly stated, it appears, given the emphasis in ATSDR (2000) on the results from using the above methodology for liver/biliary/gallbladder cancer, that this may be the underlying basis for "meaningful evidence." However, it is of interest (and perhaps even ironic) that this methodology, although not endorsed by or following any recognized guidelines and using all available data, still yields the same conclusion as ATSDR (1999) as well as the results of the present review—that is, that the weight of evidence does not support a causal association between exposure to PCBs and increased risk of cancer.

TABLE 4
Summary SMRs for Cancer Endpoints of Potential Concern

Endpoint	Observed	Expected	SMR
Liver/biliary/ gallbladder	80	103	78
Melanoma	137	124	110
Rectal cancer	139	160.1	87
Pancreatic cancer	274	317.7	86
NHL	215	206.1	104
Gastrointestinal tract	757	872	87
All cancers	779	874	89

While ATSDR (2000) concludes that some mortality studies suggest that occupational exposure to PCBs is associated with melanoma, no attempt is made to systematically review the data according to any accepted guidelines (e.g., U.S. EPA, 1999a, 2003; IPCS, 1999). Since melanoma is significantly associated with occupational exposure to PCBs in only 2 of 19 studies (i.e., Sinks et al., 1992; Loomis et al., 1997), these must serve as the sole basis for a causal association. In reviewing the eight cases of melanoma reported by Sinks et al. (1992), ATSDR (2000) concluded that one and possibly two others should not have been included in the analysis. In addition, it also acknowledged that there was no relationship between melanoma and latency or duration of employment or any indication of a dose response. Sinks et al. (1992) concluded that the results of their study should not be interpreted as demonstrating a causal relationship between PCBs and malignant melanoma.

With respect to the study by Loomis et al. (1997), there was no increased risk of melanoma in the total cohort (SMR = 1.04) and while there was a significant association in one subgroup of workers with 0–5 years of exposure, there was no association in other workers with greater exposure and no association between estimated total career exposure to PCBs and melanoma. Based on a small number of cases, there were 3 significant associations when the cohort was divided into 5-, 10- and 20-year lag

periods and analyzed by 3 categories of total PCB exposure. Loomis et al. (1997) acknowledge that "the quality of our information on exposure to sunlight, a potentially important confounder, is of some concern." While occupational exposure to sunlight was estimated, there was no information about exposure to sunlight during leisure time, which is the most well-established risk factor for melanoma. Finally, Loomis et al. (1997) speculated that with more than a 10- to 20-year follow-up to allow for this latency interval, other studies should confirm their results. Kimbrough et al. (1999, 2003), with PCB exposures almost certainly higher do not support this hypothesis.

The conclusions in ATSDR (2000) with respect to melanoma are, "Considering the limitations of the Bahn et al. (1976, 1977) and Loomis et al. (1997) studies, the apparent relationships to PCB exposure are more uncertain than in the Sinks et al. (1992) study, although the data from the three studies collectively suggest an association." While it may be correct to conclude that "some" studies suggest an association between exposure to PCBs and melanoma, this ignores the totality of the data as well as the guidelines from the U.S. EPA (1999a, 2003) on evaluating a body of epidemiological data:

All studies that are considered to be of acceptable quality, whether yielding positive or null results, or even suggesting protective carcinogenic effects, should be considered in assessing the totality of the human evidence. Conclusions about the overall evidence for carcinogenicity from available studies in humans should be summarized along with a discussion of uncertainties and gaps in knowledge. Conclusions regarding the strength of the evidence for positive or negative associations observed, as well as evidence supporting judgments of causality, should be clearly described. In assessing the human data within the overall weight of evidence, determination about the strength of the epidemiologic evidence should clearly identify the degree to which the observed associations may be explained by other factors, including bias or confounding. [emphasis added]

Had the U.S. EPA (1999a, 2003) guidelines been followed, the only logical conclusion that could have been reached was that the weight of evidence does not support a causal association for PCBs and melanoma. Particularly for melanoma, given the inability of any studies to adequately account for exposure to sunlight, the most well-established risk factor for this disease, there is no scientific basis for a conclusion that some studies (i.e., 1 out of 19) suggest an association with PCBs. The failure of any of the studies of cohorts studied more than once (e.g., Brown and Jones, 1982, through Kimbrough et al., 2003, and Bertazzi et al., 1982, through Tironi et al., 1996) and almost certainly more heavily exposed to PCBs appears to support this.

In ATSDR (2000) a puzzling conclusion is that "Occupational mortality data indicate that exposures to PCBs during capacitor manufacturing and repairing were associated with . . . intestinal cancer." It is assumed that "associated with" implies that one or more studies reported a significant association between exposure to PCBs and increased risk of intestinal cancer. However, for this disease endpoint, none of at least 19 studies conducted on cohorts exposed to PCBs have reported a significant association between exposure to PCBs and increased risk of intestinal cancer. This includes cohorts studied more than once (i.e., Brown, 1982, through Kimbrough et al., 2003, and Bertazzi et al., 1982, through Tironi et al., 1996), which would be expected to show such an association if exposure to PCBs was, in fact, causal of intestinal cancer. The citing by ATSDR (2000) of intestinal cancer as a disease of potential concern from exposure to PCBs appears to be the antithesis of a weight of evidence conclusion. In this case, a disease endpoint is singled out even while it is acknowledged that no occupational studies have reported intestinal cancer to be significantly elevated.

While minimally relied on in reaching conclusions about associations between exposure to PCBs and increased risk of liver cancer, it is, nevertheless, curious that studies of Yusho and Yu-Cheng exposures or contaminated fish consumption are even mentioned in ATSDR (2000), particularly with respect to PCBs and human cancer. The Yusho and Yu-Cheng poisoning exposures involved simultaneous exposures to PCBs as well as furans and any reliance on these data contradicts a previous position by ATSDR (1999): "The effects from these incidents are not reviewed in this profile because CDFs appear to be the main causal agent." Furthermore, the Japanese government recently concluded that PCDFs and not PCBs as had been previously believed were the causative agent responsible for the symptoms of the rice oil poisoning incident.Given the almost universal acceptance that Yusho or Yu-Cheng data are not relevant for assessing potential adverse effects of PCBs, it is unwarranted that these essentially irrelevant data were brought back into the evaluation of the potential carcinogenic effects of PCBs in ATSDR (2000) after they

had been eliminated from consideration in ATSDR (1999).

With respect to contaminated fish consumption, any reliance on such data is unwarranted since, as acknowledged by ATSDR (2000) for stomach cancer, "the effect cannot be definitely attributed to PCBs because consumption included smoked fish and PCBs were not the only contaminants in the fish." The same would hold true for any effects reported to be associated with consumption of contaminated fish. It is inappropriate to include the results of this study by Svensson et al. (1995) in an assessment of the possible associations between exposure to PCBs and risk of cancer. This study involved fish consumption and associations between total TEQ and the endpoints measured. Since the TEQ is a composite measure of PCBs, TCDD, and TCDF, this study is incapable of contributing any information on possible associations between PCBs and increased risk of cancer. Neither "PCB" nor "polychlorinated biphenyl" is even mentioned by Svensson et al. (1995) in the discussion section of this study.

One last issue bears comment. It is beyond dispute that conducting large epidemiology studies is an expensive, difficult, and time-consuming undertaking. Due to their complexity, all studies are fraught with methodological and interpretative problems. Typically, the authors of large epidemiology studies are the first to recognize the problems inherent in their own studies and acknowledge them in the discussion of their results. Every study is subject to criticisms pertaining to cohort selection, confounding, bias, exposure assessment, multiple comparisons, etc. Clearly, some studies are better than others because of larger cohorts, longer latency, more accurate exposure information, or more complete follow-up. This is explicitly recognized in the U.S. EPA (1999a, 2003) guidelines with the recommendation that more weight be given to better studies.

Given these recognized limitations, the criticisms in ATSDR (2000) pertaining to the various studies are puzzling. After discussing the studies by Brown and Jones (1981) and Brown (1987), there is one sentence describing limitations for both studies. After discussing the studies by Bertazzi et al. (1987) and Tironi et al. (1996), there is one sentence describing limitations for both studies. There are no limitations noted for the studies by Gustavsson and Hogstedt (1997) and Gustavson et al. (1996) and one sentence each in describing the limitations of Sinks et al. (1992) and Loomis et al. (1997). However, for the study by Kimbrough et al. (1999), more than half a page is devoted to describing the limitations with a conclusion that all of the negative findings are suspect.

Most (or perhaps all) of the various criticisms of the study by Kimbrough et al. (1999) would apply to an even greater extent to all of the other studies that comprise the body of epidemiological studies of worker cohorts occupationally exposed to PCBs. However, this study is the fifth to follow up the same group of workers, is the largest of its kind, and encompasses the longest latency period of any such study conducted to date. The results from most of the earlier studies were based on fewer numbers of deaths and far less latency. Consequently, it appears unwarranted to criticize one study for problems that plague all previous studies to an even greater degree.

Clearly, the reasons for the substantial changes in the conclusions concerning the human carcinogenicity of PCBs from ATSDR (1999) to ATSDR (2000) are unknown. However, it does seem appropriate to suggest that unbiased evaluations, even of controversial issues such as the potential human carcinogenicity of PCBs, be conducted in a transparent manner following applicable guidelines. The dramatic differences between the conclusions of ATSDR (1999) and ATSDR (2000) is not consistent with this process.

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Attachment J-5

Critical Reviews in Toxicology, 2009; 39(4): 299–331

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REVIEW ARTICLE

Weight of evidence evaluation of potential human cancer risks from exposure to polychlorinated biphenyls: An update based on studies published since 2003

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Abstract

Drawing on all data available in 2003, the WoE of the human epidemiological data for polychlorinated biphenyls (PCBs) demonstrates that exposure to a mixture of PCBs (i.e. Aroclors) did not pose a cancer risk to humans (Golden et al. (2003). This evaluation was based on criteria established by the US Environmental Protection Agency (EPA) as well as on a different methodology used by the Agency for Toxic Substances and Disease Registry (ATSDR). Subsequently, at least 15 more studies on the potential cancer risks (both incidence and mortality) of PCBs have been published. All studies published since 2003 are critically reviewed using the criteria established by the EPA (2005) and ATSDR (2000). None of the studies published since 2003 change the conclusions drawn by Golden et al. (2003): "that the weight of evidence does not support a causal association for PCBs and human cancer". This conclusion pertains to all cancers combined, as well as to the various cancers that have been sporadically reported in the occupational cohort mortality studies. With respect to breast cancer risk, the WoE is compelling that environmental exposure to PCBs is not etiologically implicated in breast-cancer risk. This conclusion is supported by the consistently negative findings for increased breast-cancer mortality in occupational studies, which now involve almost 9,000 women occupationally exposed to PCBs. Similarly, the incidence studies in which PCB background levels are reported to be associated with increased risk of non-Hodgkin's lymphoma or prostate, testicular, and intestinal cancer are not corroborated by occupational cohort studies with PCB exposures far in excess of environmental exposures. The most likely explanation for these discordant findings is discussed in this review. Finally, the recent elucidation of the mode of action by which PCBs promote liver tumors in rats, combined with the demonstration that none of the key events in the mode of action occurred until substantial tissue accumulation of total PCBs had occurred, casts further doubt that PCB exposure at environmental or occupational levels poses a carcinogenic risk to humans. The dramatic differences between rodents and humans in sensitivity to PCB-mediated induction of *CYP1A1* suggests that even occupational exposures to PCBs have never resulted in PCB body burdens approaching the levels required to initiate the sequence of events involved in the promotion of liver tumors in rodents.

Keywords: *Cancer; humans; polychlorinated biphenyls (PCBs); polymorphisms; weight of evidence*

Introduction

Whether exposure to polychlorinated biphenyls (PCBs) might pose a cancer risk to humans remains controversial. In 1999, the Agency for Toxic Substances and Disease Registry (ATSDR) released the *Draft Toxicological Profile for Polychlorinated Biphenyls*. In reviewing the potential human carcinogenicity of PCBs, the ATSDR (1999) concluded that "the weight of evidence does not support a causal association for PCBs and human cancer at this time". However, 1 year later, in an updated toxicological profile for PCBs, the ATSDR changed its mind, stating that "overall, the human studies provide some evidence that PCBs are carcinogenic" and that "some of these studies provide meaningful evidence that PCBs are carcinogenic in humans" (the ATSDR, 2000). The only study published after the ATSDR (1999) draft evaluation and before the ATSDR (2000) final evaluation is by Kimbrough et al. (1999). This study, the largest occupational study at the time of a

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(Received 22 April 2008; revised 13 June 2008; accepted 22 June 2008)

ISSN 1040-8444 print/ISSN 1547-6898 online © 2009 Informa UK Ltd
DOI: 10.1080/10408440802291521

population of workers heavily exposed to PCBs, found no significant associations between PCB exposure and deaths from any cancer or any other disease. Of all the studies that had investigated potential associations between PCB exposure and increased risk of cancer, this study had the longest latency. It also confirmed the results of four previous studies of this cohort of PCB-exposed workers. This study clearly added to the weight of evidence (WoE) against a causal association between PCB exposure and human cancer. Nevertheless, in the 2000 version of the *PCB Toxicological Profile*, the ATSDR did not cite Kimbrough et al. (1999) as further support for its previous conclusion that "the weight of evidence does not support a causal association for PCBs and human cancer at this time". Instead, the ATSDR abandoned the WoE approach in favor of another methodology not endorsed or used by any regulatory agency. As detailed in the comprehensive review by Golden et al. (2003), this methodology was based on a book chapter by Nicholson and Landrigan (1994), in which the summed observed and expected cancer mortality rates (for men and women combined) from several capacitor-worker studies were tested for statistical significance. This 1994 analysis provided the basis for the ATSDR's (2000) conclusion that there was "some" or "meaningful" evidence of an association between human PCB exposure and cancer, even though the ATSDR did not determine if that conclusion held when all relevant pre-1994 and post-1994 data were combined in this way. As shown in Golden et al. (2003), none of the cancers highlighted by the ATSDR (2000) was significantly elevated when standardized mortality ratios (SMRs) using all available data were tabulated as suggested by Nicholson and Landrigan (1994).

The ATSDR (2000) evaluation of PCBs is the latest government review of the potential human carcinogenicity of PCBs. However, the conclusions of this review were clearly not based on a WoE review using all data available at the time, or on the guidelines now recommended by EPA (2005). It should also be noted that while the ATSDR does not seem to have a document summarizing WoE guidelines, this concept is embraced on their website (www.atsdr.cdc.gov/HEC/CSEM/pediatric.appendixf.html). Thus, the conclusions of the ATSDR (1999) as confirmed in Golden et al. (2003) stand as the most recent evaluations regarding the human carcinogenicity of PCBs based on the WoE. It does not appear that any other government agency is planning to address this issue in the near future.[1]

Given that it has been almost 8 years since the last government assessment of the human carcinogenicity of PCBs, and 5 years since the last private assessment of this issue, as well as the fact that additional relevant studies have been published in the interim, a re-evaluation of the issue seems warranted. A re-evaluation also seems appropriate since the EPA has finalized revised guidelines for carcinogen risk assessment (EPA, 2005). Some key highlights from these guidelines should be noted. In discussing the assessment of evidence of carcinogenicity from human data, the EPA (2005) states: "All studies that are considered to be of acceptable quality, whether yielding positive or null results, or even suggesting protective carcinogenic effects, should be considered in assessing the totality of the human evidence. Conclusions about the overall evidence for carcinogenicity from available studies in humans should be summarized along with a discussion of uncertainties and gaps in knowledge". Further, the guidelines suggest that conclusions regarding the strength of the evidence for positive or negative associations, as well as evidence supporting judgments of causality, should be clearly described. In assessing the human data within the overall WoE, the guidelines stress that determinations about the strength of the epidemiologic evidence should clearly identify the degree to which observed associations may be explained by other factors, including bias or confounding.

The EPA guidelines also address statistical considerations, stating that "the analysis should apply appropriate statistical methods to ascertain whether the observed association between exposure and effects would be expected by chance". In particular, the issue of combining statistical evidence across studies is addressed as follows: "Meta-analysis is a means of integrating the results of multiple studies of similar health effects and risk factors. This technique is particularly useful when various studies yield varying degrees of risk or even conflicting associations (negative and positive). It is intended to introduce consistency and comprehensiveness into what otherwise might be a more subjective review of the literature. *The value of such an analysis is dependent upon a systematic review of the literature that uses transparent criteria of inclusion and exclusion*" (emphasis added).

In discussing evidence for causation, the EPA (2005) guidelines embrace the well-known criteria described by Sir Bradford Hill (1965), including strength of association, consistency of association, biological plausibility, temporality, and biological gradient (i.e. exposure-response relationship). The systematic application of these criteria is often referred to as a WoE evaluation. These 'causation criteria' were explicitly followed in the review by Golden et al. (2003). These guidelines are embraced not only by the EPA (2005), but also by the ATSDR and International Programme on Chemical Safety (IPCS, 1999). The Food and Drug Administration (FDA, 1998) also applies similar guidelines for assessing the efficacy of new drugs that embrace virtually all of these criteria. This is not surprising, since the role of FDA is to determine if a new drug 'causes' a beneficial health effect (i.e. whether it has efficacy).

The present review is intended to evaluate the WoE from 2003 onwards regarding the relationship, if any, between human exposure to PCBs and cancer. While the WoE evaluation process necessarily involves scientific judgment, the goal of the present review (as well as that of the previous review) is to be as transparent as possible. This includes considering all available studies, applying clear weighting procedures (e.g. follow-up studies vs. one-time-only

studies), evaluating confounding factors, and clearly describing study deficiencies. Studies were identified using PubMed searches both initially and throughout this evaluation, as a number were published while this paper was in preparation. Using the EPA (2005) guidelines as a foundation, this review will separately address five factors: (a) incidence studies reporting associations between environmental PCB exposure and four types of cancer; (b) updates of previously reported PCB occupational cohort mortality studies; (c) a new PCB occupational cohort mortality study; (d) an assessment of the WoE linking environmental and occupational PCB exposure to breast-cancer risk; and (e) the methodology using observed and expected mortality to compute a summary SMR, because of its endorsement by the ATSDR (2000).

General population exposure incidence studies on specific types of cancer

A number of incidence studies published after 2003 have reported the association of four types of cancer (i.e. prostate, testicular, colon, and non-Hodgkin's lymphoma [NHL]) with environmental exposure to PCBs. These studies are reviewed below. However, because it is difficult to directly compare incidence data with mortality data, and also because none of these cancers have been reported as significantly elevated in occupational mortality studies (see below for additional discussion of prostate cancer), the WoE is evaluated separately for each type of cancer.

Prostate cancer

Ritchie et al. (2003)

This small pilot study investigated the relationship between organochlorine pesticides, PCBs, and prostate cancer in 58 prostate cancer patients and 99 controls. On the basis of self-reported chemical exposures, it does not seem that any cases had been occupationally exposed to PCBs or any other organochlorine compounds. Gas chromatography was used to measure 30 PCB congeners and 18 organochlorine pesticides in serum. The magnitude of association was assessed by multiple logistic regression analysis. After adjustment for age, body mass index, and a history of prostatitis, oxychlordane and PCB 180 were associated with a significantly increased risk of prostate cancer. For PCB 180, the risk of prostate cancer was statistically significantly increased (Odds ratio [OR] = 3.13, 95%CI 1.33–7.34) at the intermediate serum level (0.009–0.041 μg/g), but not significantly increased (OR = 1.47, 95%CI 0.58–3.73) at the highest serum level (> 0.041 μg/g). Consequently, the findings provided no evidence of a dose–response relationship. All stages of disease were represented in the 58 cases in this study (i.e. Gleason II, III, III–IV and IV—severity scores designed to measure disease progression). This fact suggests that increased levels of organochlorine compounds in cancer cases cannot be unequivocally associated with disease incidence, since it may represent redistribution from lipid stores due to disease-induced weight loss or treatment-related effects, both of which are known to affect PCB serum levels (Gammon et al., 1996; Baris et al., 2000). The description of patient characteristics did not seem to account for this possibility. As noted by the authors, this study was small and designed to generate hypotheses. Furthermore, there was no significant association between total PCB (ΣPCB; all 12 congeners) and risk of prostate cancer. It is difficult, if not impossible, to determine the etiological role that might be played by a single PCB congener (e.g. PCB 180) and, indeed, no biologically plausible explanation is offered by the authors. Since no dose–response relationship was demonstrated and many comparisons were made, this finding may represent a chance observation.

Ritchie et al. (2005)

This study was an extension of the Ritchie et al. (2003) study, which explored associations between the same 58 cases of prostate cancer and different groupings of 30 PCB congeners. These groupings were based on known chemical and biological properties of PCBs, including degree of chlorination (i.e. low, moderate, or high), enzyme induction properties and biological action (i.e. estrogenic or neurotoxic, antiestrogenic [Ah-receptor agonists], and enzyme-inducing phenobarbital-type cytochrome P-450). Some of these groupings have been used to assess potential risk of breast cancer (e.g. weakly estrogenic PCBs); however, there is little, if any, biological basis for assuming that any of them might be etiologically associated with prostate cancer. Two PCB groupings, moderately chlorinated and phenobarbital-type inducers, were reported as having identical significantly increased ORs and trends for prostate cancer (OR = 2.44, 95%CI 1.01–5.90, $p = 0.043$). However, neither of these findings was significant when the serum values were lipid adjusted.

The associations reported by Ritchie et al. in 2005 and 2003 are inconsistent with the results of several large occupational cohort studies, in which exposure to PCBs was substantially greater than the environmental exposures of the 58 cases in this study (see comment below). There is no evidence of any increased mortality risk of prostate cancer in the overall results of any of these studies. None of these studies are mentioned by Ritchie et al. (2003, 2005).

Hardell et al. (2006a)

This was an incidence study involving 58 cases of prostate cancer and 20 controls. Adipose-tissue samples were collected from cases and controls and analyzed for 37 PCB congeners), polybrominated diphenyl ethers, chlordane, dichlorodiphenylethylene (DDE), hexachlorobenzene (HCB), and lipid content. Prostate specific antigen (PSA) level was also measured. All Gleason-score stages of disease were represented. As with previous incidence studies of this type, the authors investigated associations between a number of PCB-congener groupings (e.g. estrogenic, lower-chlorinated, moderately chlorinated, higher-chlorinated, enzyme-inducing, and toxicity equivalent [TEQ] groupings) and various PSA categories (< 4, 4–10, > 10, < 16.5,

and >16.5). There were no significant differences between cases (PSA <16.5 or >16.5) and controls in adipose-tissue concentrations of ΣPCB. However, there was a significant difference ($p=0.005$) between cases with PSA>16.5 and controls in tissue concentrations of PCB 153. In addition, there were some seemingly random statistically significant findings in cases with PSA>16.5 and congener groupings: enzyme-inducing PCBs (OR=4.97, 95%CI 1.25–19.8), lower-chlorinated PCBs (OR=3.75, 95%CI 1.01–13.9), known and predicted phenobarbital-type inducers (OR=4.97, 95%CI 1.25–19.8). There were also seemingly random groupings in cases with PSA>10: enzyme-inducing PCBs (OR=3.52, 95%CI 1.06–11.7) and known and predicted phenobarbital-type inducers (OR=3.52, 95%CI 1.06–11.7). Disease-induced weight loss was considered as a possible explanation for higher concentrations in cases than controls, but there were no significant differences in body mass index at the time of disease diagnosis or one year before diagnosis. Adjustments for age were also considered in the analysis. There was no significant difference in ΣPCB (ng/g lipid) between cases (mean 1,087, min–max 230–2,574) and controls (mean 1,121, min–max 447–4222). With respect to PCB 153, other than its ubiquitous presence and ease of detection, there was no biological basis offered by Hardell et al. (2006a) for why this congener, or any of the congener groupings used in this study, might play an etiological role in the development of prostate cancer. The fact that ΣPCB was not associated with increased risk at any PSA level, as well as the wide confidence intervals for the few significant findings, suggests that the data were unstable and that observed associations could have been chance observations.

Weight of evidence evaluation for prostate cancer

On the basis of the key causation criteria (i.e. strength of the association, consistency of the association, dose-response relationship, temporality, and specificity of the association), the WoE from occupational cohort mortality studies does not demonstrate that exposure to PCBs is a risk factor for prostate cancer. While not all of the studies reported prostate cancer as a separate cause of death, there were at least seven that do. In those studies (i.e. three single and four follow-up studies involving a total of more than 9,000 male workers) in which prostate cancer was listed as a specific cause of death, no SMRs in the cohort were significantly elevated for occupational exposure to PCBs (Gustavsson and Hogstedt, 1997; Kimbrough et al., 1999, 2003; Charles et al., 2003; Ruder et al., 2006; Prince et al., 2006a, 2006b). Given that these studies involved workers subject to high-dose occupational exposure to PCBs, it is biologically implausible that the background PCB-related prostate-cancer cases in the incidence studies by Ritchie et al. (2003, 2005) or Hardell et al. (2006) were etiologically associated with exposure to PCBs. The study by Charles et al. (2003; reviewed below with mortality studies), which found no significant association between occupational exposure to PCBs and increased risk of prostate cancer, adds to the WoE that PCBs are not etiologically associated with prostate-cancer risk.

As reported by Ritchie et al. (2003) and Hardell et al. (2006), PCB 180, PCB 153, and various PCB congener groupings were significantly associated with increased incidence of prostate cancer. If any of these findings of an association between PCBs (single congener or congener grouping) and increased risk of prostate cancer were causal for prostate cancer, it is reasonable to expect an even greater effect would have been reported in occupational mortality studies, all of which involved substantially greater exposure to both PCB 153 and PCB 180, as well as to the PCB congeners in the various groupings used by Richie et al. (2003) and Hardell et al. (2006). Furthermore, the congeners associated with prostate cancer in the Ritchie et al. (2003) and the Hardell et al. (2006) studies differed, and the confidence intervals were wide, suggesting either an observation made by chance or a consequence of multiple comparisons. It should also be pointed out that these studies are similar in concept to the early breast-cancer studies. That is, both sets of studies measured serum PCB levels in active cancer cases where serum levels can be affected by weight loss or by chemotherapy, thereby resulting in spurious associations (Gammon et al., 1996; Baris et al., 2000). The results of the early breast-cancer studies were invalidated in large part by an abundance of prospective data (i.e. analysis of PCBs in serum collected well before disease diagnosis) that demonstrated no association between PCB body burdens and increased risk of breast cancer.

It is often difficult to determine whether the criterion of biological plausibility can be applied to a particular disease outcome because of a lack of relevant data. However, prostate-cancer data suggest that it is biologically implausible that exposure to PCBs is etiologically implicated in this disease. In what is generally recognized as the definitive PCB cancer bioassay, male and female Sprague–Dawley rats were exposed for 24 months to PCB Aroclors 1016, 1242, 1254, and 1260 (Mayes et al., 1998, 1999). While the results showed increases in hepatic tumors, there were striking decreases in extra-hepatic tumors, including a marked decrease in prostate tumors from all Aroclors tested. Consequently, the animal data suggest that it is not biologically plausible that PCBs are a risk factor for prostate cancer.

It is also important to consider how one (or a few) congeners in a complex mixture would be etiologically responsible for causing a particular disease. With PCBs, it is now clear that some congeners induce mixed-function oxidases (MFOs), while other congeners suppress MFOs (Brown et al., 2007). Given the agonist–antagonist properties of PCB mixtures, the only biologically plausible metric is ΣPCB, since only this metric 'integrates' the mixture effects of induction and suppression. Although both Ritchie et al. (2003, 2005) and Hardell et al. (2006) implicated individual PCB congeners or groupings of PCB congeners in prostate-cancer risk, ΣPCB was not significantly associated

with prostate cancer in either study. In addition, there is no biological basis (and none is suggested by the authors) for assuming that the moderately chlorinated congeners, enzyme-inducing congeners, or phenobarbital-type congeners might have an etiological link with prostate cancer. Once again, it should be noted that no occupational cohort study reports an association between exposure to PCBs (i.e. all congeners) and increased risk of prostate cancer. In all of these studies, individuals were exposed to PCB 153 and 180, as well as to the PCB congeners that make up any of the various groupings reported as associated with prostate cancer. Finally, as reviewed in greater detail below for NHL and breast cancer, it is becoming increasingly clear that disease risk may be influenced by metabolic gene variants, some of which may coincidentally affect the metabolism, excretion, or retention patterns of PCBs, thereby leading to non-causal associations between slightly elevated PCB concentrations (all within the normal population background range) and increased risk. For prostate cancer, this includes genetic polymorphisms of a number of genes, many of which are involved in PCB metabolism (Yang et al., 2006; Daly, 2006; Fukatsu et al., 2004; Suzuki et al., 2003; Tanaka et al., 2002; Murata et al., 2001). In individuals with one or more of these polymorphic genes, background levels of PCBs (including individual congeners) might be minimally elevated (or decreased) leading to spurious associations with disease risk. Consequently, the WoE suggests that exposure to PCBs is not associated with increased risk of prostate cancer.

Testicular cancer

Hardell et al. (2003, 2004, 2006b)

While increased mortality from testicular cancer has never been associated with exposure to PCBs, Hardell et al. (2003, 2004, 2006b) report that prenatal exposure might be a risk factor for testicular cancer as a consequence of *in utero* exposure to PCBs. The hypothesis is that testicular cancer is initiated during the fetal period by exposure to weakly estrogenic chemicals. The Hardell et al. (2003, 2004, 2006b) studies all report on the same 58 cases of testicular cancer (seminoma representing ≈30–40% of all cases and non-seminoma representing ≈60% of all cases) and 61 age-matched controls, as well as 44 case and 45 control mothers. Blood was collected from all study participants between 1997 and 2000 and frozen for later analysis for 37 PCB congeners, DDE, HCB, and chlordane. Since the mean age of case and control men at the time they provided blood samples was approximately the same (31 years), it seems that blood was collected from mothers about 30 years after they were pregnant with their sons. The means, medians, and ranges of ΣPCB in case and control mothers were 859, 792, and 236–2114 ng/g lipid and 592, 563, and 141–1193 ng/g lipid, respectively, and were significantly different ($p = .0006$). The reproductive histories of case and control mothers were assessed by questionnaire and, as indicated in Hardell et al. (2003), there were no significant differences between cases and controls in duration of breast feeding or birth order. There were no significant differences between case and control men in total serum PCB levels or various congener groupings. However, case mothers had significantly increased serum concentrations of ΣPCB (OR = 3.8, 95%CI 1.4–10.0), as well as significantly increased concentrations of PCB congeners in two groups: enzyme-inducing PCBs (OR = 2.6, 95%CI 1.03–6.50) and TEQ congeners (OR = 3.3, 95%CI 1.3–8.4). Estrogenic PCBs were not significantly different in case and control mothers (OR = 2.4, 95%CI 0.95–6.00). Adjustments were made for age and body mass index in the analyses.

Weight of evidence evaluation for testicular cancer

The Hardell et al. (2003, 2004, 2006b) studies are based on the hypothesis that *in utero* exposure to maternal levels of PCBs, probably acting as weakly estrogenic substances, initiates the onset of testicular cancer as a result of this property. The authors cite a number of papers supporting the theory that *in utero* exposure to weakly estrogenic chemicals might be a contributing factor for testicular cancer (e.g. Sharpe and Skakkebaek, 1993; Skakkebaek et al., 2001), even though their results showed no association between estrogenic PCB congeners and testicular cancer. However, Hardell et al. failed to cite the key paper from the same authors that largely retracted this hypothesis. In 1993, Sharpe and Skakkebaek put forth the "estrogen hypothesis" as a biologically plausible explanation for an apparent increase in male reproductive-tract disorders, including testicular cancer. They speculated that *in utero* exposure to a number of weakly estrogenic compounds could explain this phenomenon. In revisiting this hypothesis, Sharpe (2003) noted that "... all of the identified environmental estrogens' possess weak or very weak intrinsic estrogenic activity when measured by conventional in vitro and in vivo assays for estrogenicity... Based on estrogenic potency, human exposure to the most potent environmental estrogens would need to be at least 1000-fold higher than this level for adverse effects relevant to the human male to be induced, and such levels of exposure are remote". Other reviews have also concluded that the "estrogen hypothesis" as it pertains to testicular cancer etiology (i.e. excess estrogen during gestation) is likely invalid (Hsieh et al., 2002; Dieckmann et al., 2001).

With respect to two other PCB congener groupings (enzyme inducing and TEQ) associated with testicular cancer, there is no biological basis for speculating that *in utero* exposure to either grouping plays an etiological role in the development of testicular cancer. Indeed, Hardell et al. did not provide any citations to studies that might support a hypothetical etiological role in testicular cancer for either enzyme-inducing PCBs or TEQ congeners. It is questionable that the testes are a site for the development of cancer due to PCB exposure, given that Mayes et al. (1998) reported no increase in testicular cancer even at the highest doses of Aroclors 1016, 1242, 1254, and 1260 in a chronic cancer bioassay (although this study only involved

postnatal exposure). The Aroclors used include all the congeners in the various groupings assessed by Hardell et al. (2003, 2004, 2006). In addition, there is little reason to believe that the testes are a target organ for *in utero* PCB exposure, with the exception of animal studies involving exposures that are orders of magnitude greater than any possible human environmental exposure (ATSDR, 2000). Finally, the hypothesis that *in utero* exposure to weakly estrogenic chemicals might be etiologically associated with increased risk of testicular cancer is also refuted by the extensive human data on diethylstilbestrol, which has thousands of times more estrogenic activity than PCBs. In the most recent follow-up of diethylstilbestrol-exposed cohorts, 3,613 men whose *in utero* exposure to diethylstilbestrol was known were assessed from 1978 to 1994. Testicular cancer incidence in diethylstilbestrol-exposed men was not significantly elevated when compared with non-diethylstilbestrol-exposed controls (Strohsnitter et al., 2001).

Because of the questionable biological plausibility of the Hardell et al. findings compared with the results of the effects of *in utero* exposure to diethylstilbestrol, no causal association between maternal PCB serum levels and testicular cancer in later life can be assumed. PCB serum levels in mothers whose sons later develop testicular cancer may be hypothetically relevant if they were determined during the pregnancy of the child who later developed testicular cancer. However, PCB levels determined 30 years later are clearly of questionable relevance.

It is also difficult to explain or account for how or why serum PCB levels in case mothers remained elevated (albeit still in the normal range) 30 years after giving birth to sons who later developed testicular cancer, or even to determine whether their serum levels were elevated during their pregnancies. The inference that the minimal difference in serum PCB levels between case and control mothers was causal for testicular cancer is not biologically plausible, particularly as a consequence of the weakly estrogenic properties of some PCB congeners. Not addressed by Hardell et al. (2003, 2004, 2006b) is the fact that, while some PCB congeners are weakly estrogenic, other congeners are weakly anti-estrogenic, thereby further reducing the net estrogenic effects of the mixture. Hardell et al. (2006b) speculate that a biological peculiarity (e.g. polymorphism of *CYP* enzymes) might have altered the metabolism of PCBs in case mothers, thereby slightly raising background serum levels above levels in controls. If this were the case, the minimally elevated PCB levels in mothers whose sons developed testicular cancer would not necessarily be etiologically linked to the disease. This would be similar to the *CYP1A1* polymorphism that has been reported to be associated with increased risk of breast cancer in conjunction with similarly elevated PCB serum levels. However, as described below, this finding is not causal, but rather the result of other factors that produce this seemingly enigmatic finding. Consequently, until the findings of Hardell et al. (2003, 2004, 2006b) are confirmed in a more rigorous prospective study, the results of a single questionable study are insufficient to conclude that *in utero* exposure to PCBs is a risk factor for testicular cancer.

Since the Hardell et al. (2003, 2004, 2006b) studies report on a single cohort, the data are insufficient in themselves to establish a potential causal association between PCB exposure and testicular cancer because they cannot fulfill the WoE consistency criterion. Moreover, given the lack of any plausible etiological role for PCBs in the development of testicular cancer, the results of these studies can only be considered as hypothesis generating pending completion of a long-term prospective study. This is the only way to determine if *in utero* exposure is etiologically linked with the development of testicular cancer. However, given the present, very low environmental PCB exposures of the general population, such a study may not be feasible. While none of the occupational cohort studies have reported an increase in testicular-cancer mortality, this is not surprising given that the incidence of testicular cancer peaks between the ages of 30 and 35 years and would probably not be picked up in a mortality study of older workers.

Finally, the key premise of this study—that *in utero* exposure to weakly estrogenic substances is a risk factor for testicular cancer—is refuted by the most recent data from studies of diethylstilbestrol-exposed cohorts, which demonstrate that testicular cancer is not significantly increased following *in utero* exposure to this potent estrogen. In fact, Hardell et al. specifically looked at possible associations between weakly estrogenic PCB congeners and testicular cancer risk and found none. While there is no known plausible explanation for the findings reported by Hardell et al. (2004), the available evidence suggests that PCBs are not a risk factor for increased incidence of testicular cancer.

Intestinal cancer

Howsam et al. (2004)

In an incidence study, Howsam et al. (2004) reported an association between colorectal-cancer risk in both men and women and serum levels of several individual PCB congeners, particularly as a function of mutations at the K-ras and p53 genes. In this study, lipid-corrected serum levels of seven PCB congeners (28, 118, 52, 101, 138, 153 and 180) were determined along with those of other organochlorine compounds (HCH, HCB, DDT and DDE). All measurements were conducted in active cases of colorectal cancer (57 men and 43 women) and compared with those from a control population similar to the cases in all respects, except that the controls consumed significantly less ethanol. The authors do not address this issue.

Howsam et al. (2004) reported significant associations between colorectal cancers and exposure to PCB 28 (less than the limit of detection) and between both K-ras wild-type and mutated-form colorectal cancers and exposure to PCB 28 (less than the limit of detection): OR = 2.78, 95%CI 1.24–6.25 and OR = 2.83, 95%CI 1.13–7.06, respectively).

The authors also reported a significant association between exposure to PCB 118 (highest tertile) and K-ras wild-type colorectal cancer (OR=2.27, 95%CI 1.04–4.96). In addition, PCB 118 (highest tertile) was significantly associated in cases with mutated p53 (OR=2.79, 95%CI 1.22–6.37)

In discussing how these results might be biologically plausible, Howsam et al. (2004) describe CYP1A and CYP2B enzyme induction by PCB 118 and PCB 28, even though their respective toxicity equivalency factors are 0.0001 and 0.0000 (van den Berg et al., 2006). It should also be noted that there is no evidence that CYP1A1 or CYP2B1 is induced by PCB exposure, even in heavily exposed workers (Brown and Lawton, 2001). Howsam et al. (2004) also imply that exposure to these congeners might be responsible for K-ras or p53 mutations, but provide no evidence that either congener can produce this effect. Not considered or discussed was the possible effect of chemotherapy, which is known to affect serum PCB levels in cancer patients (Gammon et al., 1996; Baris et al., 2000). In addition, from the way the cases are described, it is not possible to determine if they were all colon cancer cases or if some were rectal cancer. This makes it difficult to compare these results with those from studies that reported separate data for colon and rectal cancer.

Weight of evidence evaluation for intestinal cancer

A significant association between occupational exposure to PCBs and rectal-cancer mortality was reported by Brown and Jones (1981), although this finding had disappeared by the first follow-up of this cohort (Brown, 1987). The workers assessed in the Brown and Jones (1981) and Brown (1987) studies were included in the Kimbrough et al. (1999, 2003) studies and the Prince et al. (2006a, 2006b) studies. In none of these studies was an association found between PCB exposure and mortality from rectal cancer. These studies involved prolonged occupational exposure to all PCB congeners, including the seven individual PCB congeners selected for investigation by Howsam et al. (2004).

In the numerous occupational cohort mortality studies, a statistically significant increase in intestinal cancer in women has been reported once in the total cohort by Prince et al. (2006a, 2006b), and a similar, although non-significant increase was reported by Kimbrough et al. (1999, 2003). The workers in the Kimbrough et al. (1999, 2003) studies were included in the Prince et al. (2006a, 2006b) studies, as were the workers studied by Brown and Jones (1981) and Brown (1987). The authors of these reports emphasized the lack of evidence of an exposure–response trend for intestinal cancer even in the most highly exposed workers. No increased mortality from intestinal cancer has been seen in any of the other PCB occupational mortality studies. Howsam et al. (2004) considers virtually none of these studies, which involved exposure to the same PCB congeners—at substantially higher levels—and all the other PCB congeners in the Aroclors to which the workers were exposed.

Other than their persistence and ease of detection, Howsam et al. (2004) do not explain why only seven PCB congeners were considered in their study and why no results based on total PCBs were presented. It is now known that a mixture of PCB congeners (i.e. Aroclors) has both tumor-promoting and tumor-inhibiting properties, so an analysis limited to individual congeners cannot account for these interactive effects (Brown et al., 2007). The failure by Howsam et al. (2004) to provide analyses based on ΣPCB makes it impossible to account for such effects. Finally, the authors' hypothesis that mono-ortho PCBs, as a consequence of their dioxin-like properties, might cause intestinal cancer is not borne out by the dioxin epidemiological data. Considering the little information provided by Howsam et al. (2004) and the extensive occupational cohort studies, which include greater exposure to all PCBs, including mono-ortho PCBs, the WoE suggests that PCBs are not causally associated with an increased risk of intestinal cancer.

Non-Hodgkin's lymphoma

De Roos et al. (2005)

On the basis of previous reports suggesting an association between environmental exposure to PCBs and increased incidence of NHL, De Roos et al. (2005) investigated a subset of participants from a case–control study of NHL conducted by the National Cancer Institute. Cases included 1,321 patients newly diagnosed with NHL and 1,057 controls. Cases and controls were selected from four different SEER (Surveillance Epidemiology and End Results) reporting regions. Plasma from 100 cases and 100 controls was analyzed for 36 non-coplaner PCB congeners, coplaner PCB congeners, dioxins, furans, and 13 organochlorine pesticides. Care was taken to collect blood samples only from patients who had not undergone chemotherapy, since this is known to affect PCB serum levels (Baris et al., 2000).

Primary emphasis was placed on investigating associations between individual PCB congeners and NHL. Of the non-coplaner PCB congeners, two were significantly associated with NHL at the highest plasma concentration quartile: PCB 180 (OR=3.5, 95%CI 1.34–9.15) and PCB 194 (OR=2.68, 95%CI 1.04–6.90). PCB 156 was not significantly associated with NHL at the highest plasma concentration quartile (OR=2.7, 95%CI 0.97–7.50). However, all three congeners showed significant trends with exposure, albeit with wide confidence intervals. When data were analyzed by total non-coplaner PCBs, the association with NHL was not significant (OR=1.85, 95%CI 0.67–5.14) nor was the trend with exposure ($p=0.24$).

None of the coplanar PCBs were significantly associated with NHL. When stratified by degree of chlorination, the highest quartile of highly chlorinated PCBs was significantly associated with the presence of NHL (OR=2.68, 95%CI 1.04–6.90) with a significant trend ($p=0.04$). However, it seems that this group only contained a single detectable congener (PCB 194). The data were not analyzed on the

basis of ΣPCB. Finally, there was no significant association or trend between PCB TEQ and NHL, even in the highest quartile of exposure.

Engel et al. (2007a)

In this prospective study involving three cohorts of individuals with NHL, specific PCB congeners were evaluated for associations with disease incidence (Engel et al., 2007a). The Janus cohort consisted of ≈87,600 Norwegian men and women. This group comprised 190 individuals who had developed NHL before 1999. The entire cohort had provided blood samples during a routine health examination between 1972 and 1978. Serum from these samples was frozen and later analyzed for DDE and 36 PCB congeners. The CLUE I cohort consisted of 23,938 residents of Washington County, MD, USA who had participated in the Campaign Against Cancer and Stroke in 1974. At this time, a blood sample was collected and the serum was separated and stored for later analysis. The serum samples from NHL cases were ultimately analyzed for DDE and 28 PCB congeners. The cases from this group were 74 individuals who had developed NHL between January 1975 and May 1994. The Nurses Health Study began in 1976 and involved 121,700 registered nurses who completed health-related questionnaires every 2 years. Between 1989 and 1990, 32,826 participants provided a blood sample from which serum was separated and stored. The samples from NHL cases were ultimately analyzed for DDE and 21 PCB congeners. In total, 33 women were diagnosed with NHL between the date they provided a blood sample and May 1994. Because the three cohorts differed in the timing of blood-sample collection, types of samples analyzed, and analytical methods, exposure–disease associations were assessed separately. All measurements were lipid corrected.

The basic finding from the Engel et al. (2007a) study was evidence of statistically significant correlations between increased risk of NHL and high serum concentrations of PCB congeners 118, 138, and 153, with a concentration–response trend most apparent in the CLUE I cohort. There was a significant concentration–risk trend in the CLUE I cohort, but not in the Janus cohort, suggesting an association between ΣPCB and increased risk of NHL. In all three cohorts, concentration-risk correlations were strongest in the periods closest to the time that blood samples were collected, with weaker and non-significant correlations in later periods. Table 1 illustrates the ORs and trends based on quartiles (or tertiles for the Nurses Health Study) of concentrations of PCB 118, 138, and 153, ΣPCB, and DDE during the earlier follow-up periods. As shown in Table 1, while there are a number of significant findings regarding concentration quartiles, the confidence intervals for most are very wide, which substantially undermines the precision of the estimates. Concentration of PCB congener 180 was related to significantly increased risk of NHL in the Janus cohort, but not in the other two cohorts. It should be noted that the median levels (ng/g lipid) for either PCB congeners or ΣPCB in the various concentration quartiles were approximately doubled from the lowest to the highest quartiles in all cohorts. In addition, the median PCB levels in NHL cases were only a few percent higher than in controls.

Thus, the increased risks of NHL associated with being in the highest quartile (or tertile) of PCB concentrations, reported as ORs in the range 2.5–14.2 (Table 1), were associated with lipid-based ΣPCB concentration increases of only about 1 ppm on a lipid basis and less for individual congeners. The observation of a statistical association between two parameters does not indicate which parameter (or its covariant) is the cause and which is the consequence. In this case the observed correlations indicate that either (a) the adipose PCB concentrations were increasing the risk of NHL or else (b) NHL, or its underlying biochemical or physiological preconditions, were coincidentally also causing small decreases in PCB clearance rates, thereby resulting in slightly increased serum concentrations.

Arguing for the first alternative, Engel et al. (2007a) and others have suggested mediation of NHL by PCB-induced immunosuppressive effects. The biggest problem of this interpretation, however, is that there were very small differences between PCB serum concentrations in cases and controls. To overcome this finding would require the potency of PCBs as inducers of NHL to be enormous, with 1 ppm in serum lipids causing several-fold increases in NHL incidence. This is at odds with data for occupationally exposed workers with PCB serum concentrations up to hundreds of times larger. There were no significant increases in NHL in these cohorts (Table 2).

The second alternative, however, is fully compatible with the data in Tables 1 and 2. Variations in chronic PCB concentrations within populations sharing a common background exposure are heavily influenced by variations in PCB clearance rates. Human PCB clearance is known to be largely metabolic (Brown, 1994) and mediated by P450 cytochromes that are constitutive rather than PCB-induced (Brown and Lawton, 2001). For example, in healthy humans, the mean clearance half-times for PCB congeners 118, 138,

Table 1. Odds ratios (ORs) for risk of non-Hodgkin's lymphoma in relation to quartiles and tertiles of individual PCB congeners, total PCBs (ΣPCBs) and dichlorodiphenylethylene (DDE).

Exposure	Janus study OR (95% CI)	CLUE I study OR (95% CI)	Nurses Health Study OR (95% CI)
PCB 118	5.3 (1.5–18.8)[a]	13.0 (1.6–106.8)[a]	3.3 (0.9–12.4)[a]
PCB 138	2.5 (0.9–7.1)[b]	7.8 (1.8–34.6)[b]	3.8 (1.1–13.6)[a]
PCB 153	3.6 (1.3–9.9)[b]	2.7 (0.7–9.8)[a]	3.2 (0.9–11.8)
ΣPCBs	2.9 (1.0–8.2)[a]	14.2 (2.2–91.0)[b]	4.7 (1.2–18.9)[a]
DDE	4.3 (1.2–15.0)[a]	2.1 (0.7–6.3)	2.0 (0.7–6.1)

[a]$P_{trend} < 0.05$; [b]$P_{trend} < 0.005$. Adapted from Engel et al. (2007a)

Table 2. Observed and expected cancer mortality from lymphatic, hematopoietic cancer and non-Hodgkin's lymphoma in male and female workers occupationally exposed to PCBs.

	Lymphatic and hematopoietic cancer			Non-Hodgkin's lymphoma[b]					
Study (*n*)	Observed	Expected[a]	SMR (95% CI)	Observed	Expected[a]	SMR (95% CI)	Sex of participants	ICD codes used	D-R trend analysis
Tironi et al., 1996 (1,556)	4	2.3	1.77 (0.48–1.53)	NR	NR	NR	Female	ICD 200-202	NR
Loomis et al., 1997 (138,905)	439	532	0.82 (0.75–0.91)	69	90	0.77 (0.6–0.97)	Male	ICD 200-208; 200;	NR
				176	170	1.04 (0.89–1.2)		202–203	
Kimbrough et al., 2003 (7,075)	25	25	1.0	NR	NR	NR	Male and female	ICD 200, 202, 203	NR
Ruder et al., 2006 (3,569)	20	19	1.08 (0.7–1.7)	9	7.3	1.23 (0.6–2.3)	Male and female;	ICD 200-208; 200, 202	NR
Prince et al., 2006a (2,588)	22	20	1.09 (0.68–1.65)	10	7.6	1.31 (0.63–2.41)	Male and female	ICD 200-208; 200, 202	No significant trend
Prince et al., 2006b (14,458)	99	94	1.05 (0.85–1.28)	35	36	0.98 (0.68–1.36)	Male and female	ICD 200-208; 200, 202	No significant trend

Note: ICD, International Classification of Diseases; NR, not reported; SMR, standardized mortality ratio.

[a]When only observed reported expected approximated by O/SMR.

[b]When non-Hodgkin's lymphoma (ICD 200 and 202) is reported separately or if ICD coding permits an approximation of likely cases.

and 153 are 5.9, 7.9, and 12.8 years, respectively (Brown, 1994); congener-specific and intra-individual variations in clearance rates over the decades of exposure involved can have sizeable effects on residual PCB concentrations. In the data shown in Table 1, the differences in congener and ΣPCB concentrations in the individuals who subsequently developed NHL were modest—generally under 20%. However, the methodology used in the calculations seems to have transformed small differences between the mean PCB levels in the patients and non-patients into large values for the apparent OR. This occurred because the overall distributions of PCB levels in the entire populations were quite narrow, meaning that those for PCB levels in the patient and non-patient sub-populations would have been narrower still. As a result, even a modest shift between the mean values for the PCB distributions in the patient versus non-patient sub-populations means that the upper quartile of the entire distribution can contain mostly patients and the lower quartile mostly non-patients. This means that the ratio of patient counts in the two quartiles (i.e. OR) can be quite high, and the apparent potency of the PCBs (ORs divided by the very small difference between upper and lower quartile PCB levels) can be enormous.

Thus, the surprising values for the ORs and the implied potency as indicated by the data in Table 1 are fully consistent with the second alternative—that the minor increase in PCB levels in the NHL population is simply a biochemical marker for a rather small decrease in the activity of the constitutive PCB-metabolizing P450 cytochromes, which in turn leads to a slight increase in PCB concentration, although still in the normal range.

The control of constitutive P450 expression in humans does not seem to have attracted much study; however, from rat and mouse studies it is now well established that both the constitutive P450s and the insulin-like growth factors (IGFs; IGF 1 and IGF 2) are regulated in parallel by neuroendocrine factors, including the level and timing of growth-hormone release from the pituitary, and that such factors can increase the expression of some P450s while decreasing that of others (Waxman and O'Connor, 2006). Such findings suggest that increased risk of NHL may be related to some type of neuroendocrine signaling that leads to increased production of growth factors such as growth hormone and the IGFs, along with a net suppression of P450 activity (Keller et al., 2005; Mauras et al., 1988). This interpretation is consistent with several reports indicating that polymorphic variants of certain *CYP* genes, including those involved in PCB metabolism, can have the effect of slightly altering background PCB serum levels, (see below in the discussion of breast cancer) resulting in non-causal associations between minimally elevated PCB levels and disease. In this regard, it has been reported that NHL risk may be associated with a number of metabolic gene variants in *CYP1B1* (De Roos et al., 2006), *CYP17A1* (Skibola et al., 2005), GST (Kerridge et al., 2002), and *CYP2E1* (Soucek et al., 2002). Consequently, the first alternative interpretation described above for the data in Table 1—that trivial PCB concentrations increase NHL risk—can be unequivocally rejected on the basis of the data summarized in Table 2.

In attempting to explain the data in Table 1, which are consistent with previously reported case-control incidence studies of NHL (Hardell et al., 2001; De Roos et al., 2005), and also why the concentration-risk correlations were strongest in the period closest to the time that blood samples were collected, Engel et al., (2007a) suggested an etiological role for immunosuppression. In support of this role, they cited the immunosuppressive effects of anti-rejection drugs used

in organ transplants (a median time from transplant to NHL of 1–5 years) and AIDS-related lymphoma (a median time from HIV infection to NHL diagnosis of 6–8 years). Engel and colleagues then noted that many of the PCB congeners associated with increased risk of NHL in this study have been proposed to be immunotoxic (citing Wolff et al., 1997), to alter immune function (citing Tryphonas, 1994), and that the Epstein–Barr virus may potentiate the effects of PCBs (citing Rothman et al., 1997 and Hardell et al., 2001), so that any role of PCBs in the etiology of NHL may be mediated through immunotoxic mechanisms (citing Vineis et al., 1992). However, there is no evidence to support PCB-induced immunotoxicity, particularly clinical immunosuppression in humans, as an explanation for the reported findings. Wolff et al. (1997), in a non-peer reviewed letter, propose that PCB congeners be grouped into categories according to their biological function. While PCB 118 and 138 are proposed for Group 2 (potentially antiestrogenic and immunotoxic), PCB 153 is in Group 3 (phenobarbital-like, CYP1A/B inducers, persistent) and is not suggested to be immunotoxic. No data are cited in support of the possibility that PCBs have the ability to clinically suppress the immune system in humans, particularly at background exposure levels.

The Tryphonas (1994) review of the immunotoxicity of PCBs summarizes information available up to the date of that study, including the hypothesis that the immunotoxic effects of some dioxin-like PCBs are likely mediated via the AhR and that other PCBs would be antagonistic to the effects of individual congeners. No data reviewed by Tryphonas (1994) suggest that PCBs have been associated with clinical immunosuppression in humans. While the immunosuppressive effects of anti-rejection drugs used in organ transplants and HIV or AIDS are well established as risk factors for NHL, the suggestion that background PCB levels in blood would have similar effects is unwarranted and unsupported by any data. If this were the case, it is reasonable to expect that workers occupationally exposed to PCBs would have demonstrated not only increased mortality from NHL, but would also have exhibited clinical findings consistent with immunosuppression. No such findings have been reported. Finally, the review by Vineis et al. (1992) does not mention PCBs as possible etiological factors in the development of NHL.

Cocco et al. (2008)

Finally, in a recent case–control study involving cohorts from Spain, France, and Germany with a total of 174 cases of NHL, plasma samples were collected and analyzed for 17 organochlorine pesticides, HCB, chlordane, aldrin, dieldren, endrin, mirex, DDT, and 9 PCB congeners (28, 52, 101, 118, 138, 153, 1709, 180, and 194; Cocco et al., 2008). Results were presented according to the major NHL subtypes (diffuse large B-cell lymphoma and chronic lymphatic leukemia), and the various PCB congener groupings typically used (pseudo-estrogens 28, 52, and 153, highly chlorinated anti-estrogens 170, 180, and 194, phenobarbital-inducing 101, 153, and 180, mixed PC/MC-inducing 118, 138, and 170, and immunotoxic PCBs 138, 153, 180). Risk of NHL (or any subtype) was not significantly increased with exposure to any individual PCB congener, congener grouping, or ΣPCB, nor were there any significant trends based on PCB plasma levels.

Weight of evidence evaluation for NHL

The study by De Roos et al. (2005) begins by saying that "Several studies have suggested a role of the polychlorinated biphenyls (PCB) in development of non-Hodgkin's lymphoma". Cited in support of this statement are three studies: Hardell et al. (1996), Rothman et al. (1997), and Laden et al. (2000). The studies by Hardell et al. (1996) and Rothman et al. (1997), each of which reported a significant association between PCBs and incidence of NHL as a consequence of environmental exposure, have methodological limitations, as reviewed by Golden et al. (2003). Subsequently, Engel et al. (2007a) also reported significant associations between selected PCB congeners and increased risk of NHL. The study by Laden et al. (2000) is an abstract and, without any details, cannot be afforded any weight. Moreover, a similar incidence study by several of the same authors (Quintana et al., 2004) found no association between NHL and total adipose-tissue PCB concentration (SMR = 1.05, 95%CI 0.63–1.76 and SMR = 1.08, 95%CI 0.40–2.92 for 1–3 ppm and >3 ppm, respectively). Since none of the large occupational cohort studies, with much greater PCB exposures than any of these studies, have reported a significant association between PCB exposure and NHL incidence (Table 2), it is unwarranted to conclude that environmental exposure to PCBs is causally related to NHL. In addition, it is biologically implausible that PCBs would play a role in NHL etiology, since no lymphohematopoietic malignancies of any kind have been reported in any of the high dose PCB bioassays conducted in rodents (e.g. Mayes et al., 1998).

Neither De Roos et al. (2005) nor Engel et al. (2007a) offer a biologically plausible explanation for why certain PCB congeners (i.e. those with higher degrees of chlorination such as 118, 138, or 153) would be etiologically implicated in increased risk of NHL when higher exposure to the same congeners in occupational studies does not result in significantly increased risk of NHL. The suggestion by Engel et al. (2007a) that environmental exposure to PCB congeners 118, 138, or 153 produces immunosuppression similar to that produced by known risk factors for NHL (i.e. immunosuppressant drugs or HIV/AIDS) is not biologically plausible, nor are any data cited suggesting this capability. All the occupational studies (many of which involve extensive follow-up of the same cohorts) involved prolonged occupational exposure to all PCB congeners, including the same individual PCB congeners selected for analysis by De Roos et al. (2005) and Engel et al. (2007a). While De Roos et al. (2005) acknowledge the possibility of chance associations due to multiple comparisons, they do not discuss their findings in the context of the lack of similar findings

in occupational studies. Engel et al. (2007a), however, acknowledge the discrepancy between the lack of findings in occupationally exposed cohorts and the associations reported as a consequence of environmental exposures in the general population, suggesting that this needs further exploration before conclusions can be drawn. Finally, the suggestion that PCB exposure closer to disease onset may be more etiologically important than exposure more distant from disease onset, is not supported by the occupational cohort data. If this hypothesis were correct, the initial studies reported by Brown (1981) and Bertazzi et al. (1981) should have detected this 'early onset' effect, but neither study reported such an effect. This hypothesis is also inconsistent with the fact that there is a latency period between the onset of exposure and cancer induction. Therefore, cancer latency should be longer at lower exposure and certainly greater than 1–2 years.

All studies suggesting an association between environmental blood levels of PCBs and an increased risk of NHL were reviewed by Engel et al. (2007b). This review noted the contrast between these studies and the occupational cohort mortality studies that have shown no association between exposure to PCBs and increased risk of NHL. The authors also noted that risk of NHL (in the environmental exposure studies) is elevated primarily in the time period closest to sample collection and disease diagnosis suggesting consistency with other established risk factors for NHL (i.e. intentional clinical immunosuppression following organ transplants and HIV/AIDS). However, there is no evidence, and indeed none is offered by Engel et al. (2007b), that exposure to PCBs (particularly at background levels) induces clinical immunosuppression comparable to that produced by immunosuppressive drugs or HIV/AIDS. Furthermore, comprehensive reviews of PCB immunotoxicity do not document the type of immunosuppression known to be associated with increased risk of NHL (ATSDR 2000). If PCBs were capable of producing this degree of immunosuppression, it should have been observed in the numerous studies on PCB-exposed workers.

Engel et al. (2007b) also acknowledge the possibility that PCB levels measured in the blood of individuals diagnosed with NHL may reflect not only cumulative environmental exposure, but also endogenous processes that might affect storage, dilution, or elimination. However, on this key point Engel et al. (2007b) acknowledge neither the growing body of evidence on gene variants of the types likely to minimally affect PCB metabolism and thereby background serum concentrations nor the association between additional gene variants and risk of NHL, some of which could also affect PCB metabolism and serum concentrations, as described above.

Finally, as previously noted, there is no evidence that occupational exposure to PCBs is associated with increased risk of NHL. Table 2 summarizes the available occupational studies of PCB-exposed workers. While it is difficult to directly compare all studies because of the manner in which data on various lymphohematopoietic malignancies are reported (i.e. ICD codes), it is clear that occupational exposure to PCBs is not associated with increased mortality from NHL or with lymphohematopoietic malignancy in general. If the results reported by Engel et al. (2007a) are attributable to immunosuppression from background PCB levels in blood leading to the development of NHL, one might logically expect to see much larger effects from PCB blood levels—effects approximately 100 times greater. The conspicuous lack of such effects at substantially greater exposure levels suggests that PCBs are not etiologically linked to NHL.

The hypothesis that either ΣPCB or certain PCB congeners may contribute to increased NHL risk cannot be reconciled with the fact that much greater occupational exposures to ΣPCB or the same individual congeners have not been demonstrated to lead to an increased risk of NHL. In addition, the large study by Cocco et al. (2000), demonstrating no association between increased risk of NHL and ΣPCB, individual congeners, or groupings of congeners, adds to the WoE suggesting that PCBs have no role in NHL etiology. There also seems to be an unexplained inconsistency in the associations with individual PCB congeners, with De Roos et al. (2005) reporting significant associations between and trends with NHL and PCB 156, 180, and 194, Engel et al. (2007a) reporting significant associations and trends for PCB 118, 138, and 153, and Cocco et al. (2000) reporting no associations with any of these congeners. This would suggest that factors other than a causal association (e.g. metabolic differences between different study populations leading to non-causal differential serum concentrations of PCB congeners) are a more likely explanation for the reported findings. In other words, the findings in all of the studies reporting significant associations between background levels of different PCB congeners and increased risk of disease are most plausibly explained as resulting from NHL rather than causing it. Consequently, at present, the WoE suggests that PCBs are not a risk factor for NHL. Finally, since the effects of different congeners vary among the studies, the association between NHL and specific congeners may have occurred by chance.

New occupational-exposure cohort mortality studies since 2003

The studies reviewed below were all published in or after 2003, most of them occupational mortality studies. Following a presentation of the results of each study, a comment is presented if issues exist pertaining to interpretation of the data in the context of a WoE evaluation. It should be noted that these workers had much higher PCB exposures than the general population, as they produced capacitors and transformers that were filled with PCBs from about 1938, 1946, and 1951 to 1977 in the different plants studied. No exposure information is available for the workers from these plants in the early years, since analytical methods for PCB measurements were not available until the late 1960s. For example, in several plants, PCB area and personal air levels

in 1975 ranged 227–1,500 μg/m³, while in 1977, when PCBs were being phased out, levels had dropped to 170–576 μg/m³, with air levels of 3–50 μg/m³ in areas where PCBs were not used. Reductions in air concentrations resulted after the filling operation of capacitor canisters had been automated and ventilation systems had been improved (Kimbrough et al., 2003). Similar air levels were also found in other plants studied (Brown and Jones, 1981). Wolfe et al. (1982) reported in 290 self-selected employees PCBs serum levels on a wet-weight basis that ranged from 6 ng/mL to 2,530 ng/mL (ppb) for the lower chlorinated congeners and from 1 ng/mL to 546 ng/mL for the higher chlorinated PCB congeners, whereas the general population had average PCB serum levels of 5–7 ng/mL. Lawton et al. (1985a, 1985b) found similar high levels of serum PCBs in a cohort of 190 workers. Since these studies were performed when PCBs were being phased out, it is reasonable to assume that earlier PCB levels were even higher.

Charles et al. (2003)

Charles et al. (2003) conducted a mortality study to investigate an association between occupational exposure to electromagnetic fields or PCBs and prostate cancer among US electric-utility workers. As part of the rationale for this study, the authors note that PCBs are among the environmental factors that have been suggested as possible causes of prostate cancer, although no relevant citations to any literature are provided. While the hypothetical etiological role of melatonin is mentioned, no relevant data (e.g. either from occupational mortality studies or animal studies) are provided suggesting that PCBs might play a plausible etiological role in the development of prostate cancer.

Participants were current and former employees of five large US electricity companies. Data on participants had been collected during 1987–1994, with mortality of the cohort followed up to 1988. This nested case-control study consisted of 387 cases of prostate cancer and 5 controls for each case. PCB exposure was estimated based on an analysis of job/exposure potential by a panel of industrial hygienists and others familiar with the use of PCBs in the electric utility industry—actual PCB measurements were not taken. While workers categorized in the highest 10% of electromagnetic-field exposure were twice as likely to die from prostate cancer as those exposed to electromagnetic fields at lower levels, the OR for PCB exposure and prostate-cancer mortality was not significant (OR = 1.47, 95%CI 0.97–2.24) after adjustment for suspected confounding factors. When exposure to PCBs was stratified according to cumulative exposure, there was no significant association with prostate cancer mortality even at the highest exposure level (i.e. > 2800 h, OR = 1.16, 95%CI 0.78–1.74). There was also no significant increase in prostate cancer after a 5-year lag period at the highest total PCB exposure (OR = 1.14, 95%CI 0.76–1.71). Finally, as noted by the authors, "several studies have investigated or reviewed the incidence of other cancers in workers exposed to PCBs, and our literature review found no consistent evidence that occupational exposure to PCBs was related to an increase in mortality from one or more cancers".

Mallin et al. (2004)

This was a mortality study of 2,885 white workers employed between 1944 and 1977 at an electrical capacitor manufacturing plant where PCBs and chlorinated naphthalenes were used as dielectric fluids and various other chemicals were also used. Because PCBs were not used at this plant between 1944 and 1952, there was substantial exposure to chlorinated naphthalenes alone for 8 years and somewhat lesser exposure to these chemicals through to 1977. Since there were no measurement of PCB or chlorinated-naphthalene levels in the plant, it is not possible to associate findings exclusively with either group of chemicals. It is important to note that 20% of the cohort worked only 3 months or less at this facility between 1944 and 1977; an additional 30% of the cohort worked at the facility for only 60–90 days during this period. Consequently, fully 50% of the entire cohort worked at this facility for significantly less than 1 year. Additionally, 32% of the entire cohort (19% of men and 40% of women) worked at this facility before PCBs were introduced. Moreover, as noted by the authors "males were more likely to have been exposed to PCBs than females, and females were more likely to have been exposed to chlorinated naphthalenes than males".

After adjustment for age and gender, SMRs for total mortality and all-cancer mortality were similar to expected rates for both men and women. For women, the only significant finding in the total cohort was for liver and biliary cancer (SMR = 2.27, 95%CI 1.04–4.31). In women employed 10 or more years, there was a significant increase in liver and biliary cancer (based on four cases; SMR = 6.2, 95%CI 1.70–15.92). Intestinal cancer was significantly elevated in women employed for 5–9 years (SMR = 3.69, 95%CI 1.19–8.62), but not 10 or more years. In men, stomach cancer (SMR = 2.2, 95%CI 1.03–4.27) and thyroid cancer (SMR = 15.2, 95%CI 3.14–44.50) were significantly elevated in the total cohort, although there were no significant effects on stomach-cancer when analyzed by duration of exposure. When analyzed by duration of employment, liver and biliary cancer in men (based on three cases) was significantly increased for 1–4 years' employment (SMR = 6.02, 95%CI 1.24–17.59) but not for 5–9 years' or > 10 years' employment. Consequently, there was no evidence for a significant exposure-response mortality trend for stomach, liver and biliary, or intestinal cancer in either women or men in the cohort.

A separate analysis was conducted on a subset of workers who worked anytime during 1952–1977 or 5 or more years during 1952–1977, which is the time period that PCBs were used in this facility (even though many of these workers began work at this facility well before 1952). There was a significant increase in stomach cancer (SMR = 2.82, 95%CI 1.13–5.80) in women who worked any time within 1952–1977 and intestinal cancer (SMR = 2.25,

95%CI 1.03–4.27) and liver and biliary cancer (SMR = 5.57, 95%CI 1.52–14.25) in women who worked 5 or more years in 1952–1977. However, it is important to note that most of the workers in this sub-analysis were also exposed to chlorinated naphthalenes, with women far more likely to have been exposed than men. For example, since at least four of the five cases of liver/biliary cancer in this sub-cohort were exposed to chlorinated naphthalenes prior to the introduction of PCBs, the meaning of this association is uncertain.

Comment

While data were analyzed and presented on the basis of 5 or more years' employment during 1952–1977, it seems that many of, or perhaps all, the cancer cases in this study were individuals employed prior to 1952—that is, during the time when chlorinated naphthalenes were the only dielectric fluid used. Consequently, it is not possible to ascertain whether findings reported from 1952 to 1977 could have been attributable to exposures prior to 1952. For example, with respect to liver and biliary cancer in women, of the nine cases, four worked at this facility for 60 days or less, thus undermining an association with a chronic occupational exposure at this facility. Additionally, of the nine cases, all but one worked at the facility during the time when chlorinated naphthalenes were in use. Chlorinated naphthalenes have been associated with substantial hepatic toxicity, including cirrhosis of the liver (a recognized precursor for liver cancer) in both men and women (World Health Organisation [WHO], 2001), and cirrhosis was significantly more common in men in the total cohort. Therefore, it is not possible to conclude that any of the effects reported were due to PCBs alone, chlorinated naphthalenes alone, or a combination of these or other chemicals. Given the documented hepatotoxicity of chlorinated naphthalenes, it is not possible to rule out a possible etiologic role in the increased liver and biliary cancer in women. It should also be noted that exposure to chlorinated naphthalenes has been associated with increased mortality from cancers of the stomach, rectum, trachea, esophagus, bronchus, and lung after relatively short exposure to these compounds for most of the cohort (Ward et al., 1994). Because of the mixed nature of the exposures in the Mallin et al. (2004) study (particularly to chlorinated naphthalenes during 1944–1952), it is impossible to attribute the findings exclusively to PCBs. Consequently, the strength of the associations reported in this study is undermined by probable confounding from exposure to other chemicals with recognized hepatotoxicity. However, even with the confounding due to the presence and probable previous exposure to chlorinated naphthalenes, the lack of a significant exposure-response trend for any of the reported cancers (i.e. stomach, liver and biliary, or intestinal) in either women or men in the cohort undermines an association with any exposure.

Finally, the probable confounding by substantial exposures to chlorinated naphthalenes in Mallin et al. (2004) makes it impossible to determine the extent to which PCBs might have played an etiologic role in the reported results. While the authors describe the results of a small 1994 study on PCB serum levels in 60 former workers, there is no discussion of or any attempt to characterize potential exposure to or effects from chlorinated naphthalenes. Since 32% of the cohort (i.e. 918 employees) worked at this facility in 1944–1951, with exposure exclusively to chlorinated naphthalenes, the mortality experience of these workers would have permitted a determination of potential effects without confounding by simultaneous exposure to PCBs. The authors missed the opportunity to evaluate this confounder and it is not known why this was not done, as there are many cohort studies with far less than 918 individuals, particularly since the average latency in these workers was on the order of 40–50 years.

Prince et al. (2006a, 2006b)

Two overlapping studies by Prince et al. (2006a, 2006b) are follow-ups of cohorts from two electrical capacitor plants in New York (Plant 1) and one in Massachusetts (Plant 2) in the US. Workers from these plants were studied by Brown and Jones (1981) and Brown (1987). Plant 1 workers were also studied by Kimbrough et al. (1999, 2003) in separate follow-ups. The Prince et al. studies are reviewed separately below, followed by a comment on their reported findings.

As reported by Brown and Jones (1981), excess mortality (in men and women combined) was observed for rectal cancer (SMR = 336, 95%CI 92–860) and liver and biliary cancer (SMR = 280, 95%CI 58–820), although neither finding was statistically significant. The only statistically significant finding occurred in women (based on three cases) from Plant 2 for rectal cancer (SMR = 336, $p < 0.05$). In the follow-up study (Brown, 1987), the total-cohort SMR for rectal cancers decreased from 336 to 211, and although the SMR for rectal cancer in women at Plant 2 remained higher than at Plant 1, the difference from normal values was no longer statistically significant. However, there was a significant excess when liver, biliary, and gall-bladder cancers were grouped together, although one case was metastatic disease and should not have been counted as a primary liver cancer. These studies are reviewed in detail in Golden et al. (2003).

In the study by Prince et al. (2006a), mortality was updated through to 1998 for 2,572 workers. These workers were employed for at least 90 days in electrical-capacitor manufacturing at Plant 1 and 2 and were selected because they held jobs identified as having the highest and most direct exposure to PCBs. These workers had been studied earlier by Brown and Jones (1981) and Brown (1987). There was a significant excess in the total cohort (Plants 1 and 2 combined) when liver, biliary, and gall-bladder cancers were grouped together (SMR = 2.11, 95%CI 1.05–3.77), but rectal cancer was not significantly increased (SMR = 1.47; 95%CI 0.54–3.21). One of the liver cancers of a female worker in Plant 2 was metastatic disease rather than a primary liver cancer (Brown, 1987). The only other significant

finding was in women from both plants combined, where mortality from intestinal cancer was significantly higher than normal (SMR = 1.89, 95%CI 1.21–2.82). Also of interest was the finding of a statistically significant decrease in breast-cancer mortality in the total cohort (SMR = 0.59, 95%CI 0.33–0.98). Three duration-of-employment groupings were used as a proxy for exposure to permit comparisons with results from previous studies of this cohort (i.e. employment for < 5, 5–9, and > 10 years). Cancer mortality was not associated with duration of employment for any of the reported increases noted above or for breast or prostate cancer, myeloma, or NHL.

Prince et al. (2006b) is a much larger cohort mortality study of 14,458 workers who had worked ≥90 days at Plants 1 or 2. Estimated cumulative PCB exposure was assessed using a semi-quantitative job-exposure matrix. Plant-specific PCB air-concentration data were used to assign values to the combined qualitative inhalation and dermal exposure ratings (high, medium, low, background). In the total cohort, no cancers of *a priori* interest (i.e. all cancers, cancer of the liver, intestine, stomach, breast, prostate, brain, melanoma or NHL) were significantly elevated. However, there was a statistically significant increase in intestinal cancer mortality in women (SMR = 1.31, 95%CI 1.02–1.66), and a statistically significant decrease in mortality from cancers of the trachea, bronchus, and lung in men (SMR = 0.78, 95%CI –0.65 to 0.93). There was also a significant increase in myeloma mortality in all workers (SMR = 1.85, 95%CI 1.23–2.67), in men (SMR = 2.31, 95%CI 1.32–3.76), and in all Plant 1 workers (SMR = 2.02, 95%CI 1.13–3.34).

When the data were analyzed by internal cumulative exposures (i.e. exposure-response for no lagging, 10-year lagging, and 20-year lagging) the trends for no lag and 10 year lag were statistically significant for total cancers and stomach cancer in men. There was also a significant trend with no lag for "other/unspecified parts of the uterus" and significant trends for no lag, 10-year lag, and 20-year lag for prostate cancer. For cancer of the liver and biliary passages or gall-bladder there was a significant exposure-response trend with a 20-year lag, but not with no lag or a 10-year lag. There were no significant exposure-response trends for cancer of the intestine, rectum, breast, brain, melanoma, myeloma, or NHL.

Comment

In the introductions of both of the Prince et al. studies, which are clearly focused on occupational exposure and mortality risks, the authors describe the goals of the studies by citing previously reported mortality excesses for a variety of cancers, either in these cohorts or in cohorts from other studies. In particular they note "other *a priori* outcomes of interest", citing examples that either were not confirmed in follow-up studies (none of which were cited) or had never been reported in an occupational-exposure study. For example, they noted increased all-cancer mortality by citing Bertazzi et al. (1987), but omitted Tironi et al. (1996), who did not confirm this in a follow-up study of the same cohort. They noted NHL, citing two non-occupational incidence studies by Rothman et al. (1997) and Hardell et al. (2001), even though NHL has never been significantly elevated in an occupational mortality study, and breast cancer, citing a single, largely irrelevant incidence study (Falck et al., 1992), while ignoring the fact that breast-cancer mortality has never been significantly elevated in any occupational cohort study and in fact was found to be significantly decreased in Prince et al. (2006a).

On the basis of 11 cases (some of which may have been metastases), mortality from liver, biliary, and gall-bladder cancers (as a group) was found to be significantly elevated in the total cohort in Prince et al. (2006a), but there was no exposure-response trend. In Prince et al. (2006b), on the basis of 21 cases (some of which may have been metastases), there was no significant elevation in mortality in the total cohort, although a significant trend with a 20-year lag was found. As detailed in Golden et al. (2003), there is no biological basis for grouping cancers of the liver, biliary passages, and gall bladder together, given that each has different etiologies and risk factors (DeVita et al., 1993). In agreement with this interpretation, it is noteworthy that in the International Agency for Research on Cancer (IARC, 2000) publication on the pathology of digestive system tumors, tumors of the liver and intrahepatic bile ducts are addressed in one chapter, while tumors of the gall-bladder and extrahepatic bile ducts are considered in a different chapter. It should also be noted that Brown (1987; Table 8) provided details of the liver or gall-bladder and biliary-tract cancer deaths on the basis of an analysis of death certificates and pathology reports, revealing that some of the cancers had metastasized from other sites. Given the uncertainty surrounding this issue, it is not unreasonable to expect that Prince et al. (2006a, 2006b) would have discussed this or conducted a sensitivity analysis similar to what was done by many of the same authors for brain cancer (e.g. Ruder et al., 2006). This could be important if the same proportion of the cases reported by Brown (1987) were questionable as to their origin in the liver, as in the present studies. As noted by the ATSDR (2000), if the metastatic liver cancer from Brown (1987) is not included in the analysis, the SMR for the combined liver-biliary-gall-bladder cancers in the whole cohort is no longer statistically significant. Finally, as acknowledged by Prince et al. (2006b), ethnic differences in Plant 2 as a consequence of a large population of workers of Cape Verdean and Portuguese descent (who have a recognized increased risk of liver cancer) may further confound any potential association with PCBs.

There also seems to be a disparity between the two studies by Prince et al. (2006a, 2006b). In the smaller study of the most highly exposed workers, when the SMRs for selected causes of death were stratified by duration of employment (i.e. a proxy for exposure), none of the trends for any cancer were statistically significant, including all cancers (as a group), cancer of the intestines, liver, biliary passages, or gall-bladder, breast, prostate, NHL, and myeloma. However, in the larger cohort study, there was a significant

mortality trend for all cancers and stomach cancer in men with no lag and 10-year lag, for liver, biliary-tract, and gall-bladder cancer with 20-year lag, and for prostate cancer with no lag, 10-year lag, and 20-year lag. Since the subset of workers in the smaller study (Prince et al., 2006a) was the most highly exposed, it is difficult to reconcile these differences even after recognizing the greater statistical power of the larger study. Since mortality from all cancers and liver, biliary, and gall-bladder, prostate, or stomach cancer were not elevated in the cohort, it is puzzling as to how these findings should be interpreted. One possible explanation is the conundrum of multiple comparisons. For example, in Table 3, there are three lagged analyses for 15 cancer outcomes with three exposure categories compared with the reference group for each relative risk (RR) estimate. Excluding the trend tests, this means that there are 3×15×3 comparisons in this table (a total of 135 RR estimates), which, on the basis of a 95%CI approach, means that approximately seven estimates should be significant. Setting aside the two significant trends for all cancers combined (since there is no biologically plausible basis for assuming that exposure to PCBs would be a cause of every kind of cancer), there are seven significant trends. In addition, while the RR estimates based on the internal analysis in Prince et al. (2006b) show a trend for increased cancer mortality with exposure, the external analysis shows no increase in prostate-cancer mortality. Since both of these findings can't be correct, and given the fact that no other studies (including Prince et al., 2006a) have corroborated this finding, it seems reasonable to conclude that prostate cancer is not a consequence of exposure to PCBs.

In their conclusion, Prince et al. (2006a) note that "Our results are consistent with previous studies of this cohort which also reported significantly elevated mortality from liver cancers which was not found to be associated with length of employment." However, while liver, biliary, and gall-bladder cancer was questionably elevated (see above) in the Plant 2 cohort, as reported by Brown (1987), it has never been elevated in the Plant 1 cohort, despite several follow-up studies (e.g. Kimbrough et al., 1999, 2003). With respect to the significant finding of increased levels of intestinal cancer in women in the total cohort, as noted by Prince et al. (2006a), this finding was probably due to intestinal-cancer deaths among women at Plant 2 in the updated time period, and may have been influenced by the known increased incidence for intestinal cancer in the Northeastern portion of the US, which is also greatly influenced by ethnicity (Howe et al., 2001; Schottenfeld et al., 1996). In addition, neither of the Prince et al. studies (2006a or 2006b) reported a significant exposure-response trend for intestinal cancer. As this is the only report of significantly increased mortality from intestinal cancer in the total cohort in any of the numerous studies of occupationally exposed workers, and was not significant when stratified by either duration of employment or cumulative exposure, it is not plausible that this isolated finding was a consequence of exposure to PCBs.

Ruder et al. (2006)

The study by Ruder et al. (2006) is an update of Sinks et al. (1992), which reported significantly increased mortality from melanoma and non-significant increases in brain cancer. The present study added 14 years of latency to the cohort of 3,643 capacitor workers (833 women and 2,706 men). In the overall cohort, the only significant finding was increased mortality from melanoma (SMR=2.43, 95%CI 1.1–4.6). However, there was no evidence of a dose-response relationship for melanoma, with the highest mortality in the lowest tertile of exposure (SMR=3.72, 95%CI 1.2–8.7) and no significant excess in the middle and highest tertiles. Similarly, when analyzed by estimated cumulative exposure to PCBs, only in the lowest cumulative-exposure group (<11,000unit/days) was there a significant increase (SMR=3.72, 95%CI 1.2–8.7). Cumulative exposure in the middle (11,000–89,999unit/days) and highest (>90,000unit/days) groups was not significantly associated with increased mortality from melanoma.

It should also be noted that one of the cases of melanoma was diagnosed two months prior to employment at this facility and one case originated in the gall-bladder and should not have been coded as a skin tumor. Exclusion of these cases could have changed the mortality figures (Sinks et al., 1989). In addition, there was no information on exposure to sunlight, the major risk factor for this disease.

On the basis of 12 cases, brain-cancer mortality was not found to be significantly elevated in the cohort (SMR=1.91, 95%CI 1.0–3.3). A sensitivity analysis of brain-cancer deaths indicated that two cases were likely to be metastases, and that, after omitting these two deaths, the already non-significant risk was further decreased (SMR=1.59, 95%CI 0.8–2.9). No other cancers, including lymphatic and hematopoietic (including NHL), rectal, pancreatic, prostate, breast, and liver, biliary-tract, or gall-bladder, were significantly elevated in this cohort. As noted by the authors, "...melanoma mortality was not associated with estimated cumulative PCB exposure, and for brain cancer, the association between mortality and estimated PCB cumulative exposure did not demonstrate a clear dose-response relationship".

Yassi et al. (2003)

This study by Yassi et al. (2003), while not mentioning PCBs, is interesting nevertheless, as it is a follow-up of a previous study (Yassi et al., 1994) of the same cohort, which did consider PCB exposure. Yassi et al. (2003) is an extension of the same authors' analysis of cancer incidence and mortality in a cohort of 2,222 men who worked at a transformer-manufacturing plant with extensive use of mineral-oil transformer fluid and minimal exposure to PCBs. In the first study, despite the fact that exposure was predominantly to mineral oils, it was concluded that PCBs played some etiological role in the significantly increased mortality from pancreatic cancer observed in the study. This study had numerous limitations, which are described in detail in

Golden et al. (2003). As in the previous study, there was a statistically significant increased risk of pancreatic cancer. However, unlike in the previous study, Yassi et al. (2003) makes no mention of PCBs as playing any etiological role in the development of pancreatic cancer or any other cancers. Instead, as noted by the authors, "This study contributes further evidence to the growing body of literature indicating the carcinogenic properties of mineral oils used in occupational settings, in particular those used prior to 1970s." As Yassi et al. (1994) was the only study that implied an association between PCB exposure and increased risk of pancreatic cancer, the determination by Yassi et al. (2003) that PCBs played no role in disease etiology is further confirmation that pancreatic cancer is not causally linked with exposure to PCBs.

Pavuk et al. (2004)

In this population-based, cross-sectional (i.e. ecological) study, Pavuk et al. (2004) measured serum levels of 15 PCB congeners and three organochlorine pesticides (DDT, DDE, and HCB) in residents of two districts in eastern Slovakia. The population considered 'exposed' lived in an area with extensive environmental contamination from a former PCB production plant. This population was compared with a population matched on the basis of geography, but with low serum levels of the compounds studied. The age-adjusted geometric means for the sum of 15 measured PCB congeners (as well as DDT and DDE) were statistically significantly higher in subjects from the exposed area versus the background area.

The study compared cancer incidence in these two areas from 1985 to 1994. Standardized incidence ratios and 95% CIs for each area and cancer type were calculated. In exposed (but not background-area) men, there was a significant increase in the incidence of cancer of the tongue, pharynx, and lung. In exposed (but not background-area) women, there was a significant increase in the incidence of cancer of the lip and stomach. In background (but not exposed) women, there was a significant increase in the incidence of cancer of the kidney and thyroid.

The authors speculate that their results "....raise the possibility that high environmental exposure to organochlorines in the [exposed] district may be associated with higher rates of certain cancers, particularly stomach and lung cancer". However, it is well established that ecologic studies, by their very nature, are incapable of establishing causal associations (Gordis, 2000; Rothman and Greenland, 1998). Given the large number of carefully conducted cohort studies (particularly follow-up studies) on occupationally exposed populations, it would be inappropriate to use the results of Pavuk et al. (2003) in a WoE evaluation. At best, ecologic studies are hypothesis-generating studies. The fact that none of the cancers reported by Pavuk et al. (2003; i.e. tongue, pharynx, and lung in men and lip and stomach in women) have ever been reported in the occupational cohort studies suggests that they are most likely not etiologically associated with exposure to PCBs. Finally, it may be noteworthy that most of the cancers reported by Pavuk et al. (2003) have been associated with smoking and alcohol consumption. While a superficial analysis suggests that both populations were similar in this regard, ecologic studies cannot account for these potential confounding factors.

Bosetti et al. (2003; review)

As noted in Golden et al. (2003), as well as in the ATSDR (1999), numerous independent reviews of the PCB occupational cohort studies have concluded that the WoE does not support a causal association between exposure to PCBs and cancer. Bosetti et al. (2003) is another such review that reaches a similar conclusion. This review was conducted with support from the Italian Association for Cancer Research and the Italian League Against Cancer. The review considers many of the studies summarized in Golden et al. (2003) and relies on a methodology similar to that endorsed by the ATSDR (2000): summing the observed and expected mortality figures from studies which report effects on liver, gall-bladder, and biliary-tract, lymphatic and hematopoietic, and breast cancers, and all cancers combined. The authors came to the following conclusions: "Overall, no excess for all cancer mortality was observed in the six studies providing information (573 cancer deaths versus 630.4 expected, corresponding to a standardized mortality ratio (SMR) of 91). Among neoplasms potentially related to PCB exposure, there were 12 deaths from liver cancer compared with 9.5 expected (SMR = 126). No excess was found for cancers of the breast (40 observed versus 47.4 expected, SMR = 84) and of the lymphatic and haematopoietic system (51 observed versus 53.2 expected, SMR = 96). Therefore, studies on occupational exposure to PCBs do not show any excess in all cancer mortality, or in mortality for specific cancer sites of interest."

Although Bosetti et al. (2003) obviously could not consider the relevant post-2003 studies, it nevertheless adds to the list of independent analyses of the available data by suggesting that occupational exposure to PCBs is not etiologically associated with increased risk of cancer. While relying on essentially the same methodology as used by the ATSDR (2000), Bosetti et al. (2003) considered all available data in support of their conclusions, while the ATSDR (2000) did not.

Weight of evidence evaluation for occupational cohort mortality studies published after 2003 (Excluding breast-cancer related studies)

As detailed by Golden et al. (2003), the WoE, considering the mortality studies published at that time, did not support a causal association between exposure to PCBs and increased risk of cancer. This conclusion was based on a detailed analysis of the extent to which each of the causation criteria were satisfied by all the available data on PCBs at that time (see Table 2 in Golden et al., 2003).

As described above, the six occupational mortality studies published since that time are by Charles et al. (2003), Prince et al. (2006a, 2006b), Ruder et al. (2006), Yassi et al. (2003), and Mallin et al. (2004). Only Mallin et al. (2004) studied a cohort not previously investigated. In addition, there are several incidence studies that have reported associations between PCBs and several types of cancer. As discussed above, the incidence studies do not change the WoE conclusion reached in 2003: that PCBs are not causally associated with increased risk of cancer (prostate, testicular, intestine, or NHL). The lack of causality in these studies is underscored by the fact that none of the cancers reported in these incidence studies have been found to be significantly elevated in the occupational cohort mortality studies. The following narrative considers whether the six most recent mortality studies are consistent with the conclusion reached in 2003, or if that conclusion should be modified.

As discussed above, the findings in the study by Mallin et al. (2003) are substantially confounded by both prior exposure to chlorinated naphthalenes and simultaneous exposure to chlorinated naphthalenes and PCBs. Thus, the increase in cancer mortality reported is difficult, if not impossible, to associate with PCB exposure. The principal findings of this study—that liver and biliary cancer and digestive-organ cancer in women and stomach cancer in men are increased, with no evidence of an exposure-response relationship—are difficult to compare with those of other studies. Particularly with respect to liver and biliary cancer, the probable heavy exposure to chlorinated naphthalenes cannot be ruled out as either a contributory or sole causal factor, given that chlorinated naphthalenes have been associated with substantial hepatic toxicity, including cirrhosis of the liver (a recognized precursor for liver cancer; WHO, 2001). Indeed, it seems that many, or perhaps all, of the cancer cases in this study occurred in individuals employed prior to 1952 (i.e. during the time when chlorinated naphthalenes were the only dielectric fluid used). In addition, of the nine cases of liver or biliary cancer in women, four were employed for two quarters or less, making it unlikely that exposure to chlorinated naphthalenes or PCBs was etiologically involved. As a result of this confounding, the results from the study by Mallin et al. (2003) cannot contribute meaningfully to the overall WoE and, therefore, are not used for this purpose.

The study by Ruder et al. (2006) demonstrated no statistically significant increases in mortality from liver, biliary, or gall-bladder, intestine, stomach, prostate, or brain cancer in the capacitor-worker cohort. While there was a significant increase in melanoma mortality in the lowest tertile of exposure, there was no evidence of an exposure-response trend. This finding, together with the fact that one melanoma was diagnosed pre-employment and one originated in the gall bladder, and also because there was no information on potential exposure to sunlight, suggests that there is no causal association between exposure to PCBs and increased risk of melanoma or any other cancer.

The two studies by Prince et al. (2006a, 2006b) are perhaps the most difficult to reconcile with the findings from previous studies particularly with respect to liver, biliary, and gall-bladder cancer mortality. While this grouping of cancers was significantly elevated in Prince et al.'s (2006a) total cohort, consisting of 2,572 of the most heavily exposed workers, there was no exposure-response trend, thereby undermining the likelihood of a dose-response relationship. By contrast, in the expanded cohort of 14,458 workers, there was no significant increase in the total cohort, but a significant exposure-response trend with 20-year lag. However, the liver, biliary, and gall-bladder findings are based on only 21 cases in the expanded cohort, and there was no consideration by Prince et al. (2006b) of the likelihood that some of these cases may have been metastatic from other sites. Consequently, this finding is questionable as to an association with exposure to PCBs. It is noteworthy that, when the ATSDR (2000) eliminated the metastatic liver, biliary, and gall-bladder cases from the SMR analysis, the findings were no longer significant. Because Prince et al. (2006a, 2006b) was an update of Brown (1987), which provided a detailed description of the liver, gall-bladder and biliary-tract cancer deaths (i.e. dates and length of employment, cause of death notation on death certificate and hospital or pathology report) in the cohort, it is not unreasonable to expect that similar care would have been taken to address this key issue in the updated study. While intestinal cancer was significantly increased in the Prince et al. (2006a) study in the most highly exposed female workers, there was no indication of an exposure-response relationship, thus undermining a possible causal association. Similarly, this cancer was significantly increased in women in the larger study (Prince et al., 2006b), but also with no evidence of an exposure-response trend, thereby also undermining a causal association. Because intestinal-cancer was not statistically significantly increased in Ruder et al.'s (2006) study or any previous studies of PCB-exposed workers, the WoE suggests that intestinal cancer is not associated with exposure to PCBs. Neither was prostate cancer significantly elevated in the most heavily exposed workers (Prince et al., 2006a) or in the expanded cohort (Prince et al., 2006b). The finding of a significant exposure-response trend in the later cohort is unexplained, particularly since no other study of PCB-exposed workers has reported increased mortality from prostate cancer. While the trend analysis, according to the authors, was corrected for age, information on how this was done was not provided. Consequently, despite this isolated finding, the WoE suggests that prostate cancer is not etiologically associated with exposure to PCBs.

The conclusion in 2003 was that the WoE did not support a causal association between exposure to PCBs and increased risk of cancer, and the findings from the six studies conducted subsequently do not change that conclusion. While liver, biliary, and gall-bladder cancer continues to be sporadically reported, there is uncertainty regarding

this grouping, as liver, biliary and gall-bladder cancer each has its own etiology and risk factors (DeVita et al., 1993), and as some proportion of these cases may be metastases rather than primary tumors. Of the six studies published in or after 2003, one (Mallin et al., 2004) is so confounded that the results are not useful for causal inference, another (Yassi et al., 2003) does not mention PCBs, and yet another (Ruder et al., 2006), while reporting an equivocal (i.e. no exposure–response relationship) mortality finding for melanoma, reported no significant increases in morality from any other cancer. The results of the two studies by Prince et al. (2006a, 2006b) are inconsistent. In applying the causation criteria to the findings from all studies conducted to date, several conclusions, outlined below, can be reached.

Extent to which the causation criteria are fulfilled by the available data on PCBs

Consistency of the observed association

As noted by the EPA (2005), "An inference of causality is strengthened when a pattern of elevated risks is observed across several independent studies. The reproducibility of findings constitutes one of the strongest arguments for causality." On the whole, the cohort mortality data on PCB-exposed workers demonstrate a striking lack of consistency with respect to key findings, with seemingly random associations appearing and disappearing in different studies. With the large number of studies conducted to date on highly exposed worker cohorts, including many follow-up studies of the same cohorts, it is reasonable to expect that some consistency in the reported associations would have emerged if PCBs were causally related to increased cancer risk.

Strength of the observed association

As described by the EPA (2005), "The finding of large, precise risks increases confidence that the association is not likely due to chance, bias, or other factors. A modest risk, however, does not preclude a causal association and may reflect a lower level of exposure, an agent of lower potency, or a common disease with a high background level." For liver, biliary, and gall-bladder cancer mortality, the findings from the studies by Ruder et al. (2006) and Prince et al. (2006b) do not approach statistical significance (cohort SMRs of 0.51 and 0.89, respectively), whereas the mortality finding in the first study by Prince et al. (2006a) barely does (cohort SMR = 2.11, 95%CI 1.05–3.77). The likelihood that this association was influenced by the possible inclusion of metastatic cases also weakens this finding. Mortality from intestinal cancer in the total cohort was not significantly increased in the study by Ruder et al. (2006; SMR = 1.43), was clearly increased in women in the first study by Prince et al. (2006a; SMR = 1.89), and only just significantly increased in the second study Prince et al. (2006b; SMR = 1.31, 95%CI 1.02–1.66). No other mortality studies in PCB-exposed cohorts have reported this finding. For increases in stomach-cancer incidence, which was a weakly significant finding in the study by Mallin et al. (2004; SMR = 2.25, 95%CI 1.03–4.27), which as noted above has been reported as associated with exposure to chlorinated naphthalenes, mortality from this cancer has not been reported as significantly increased in any other studies. For melanoma, other than the equivocal finding reported by Ruder et al. (2006; SMR = 3.72), no studies have reported a statistically significant finding. Prostate-cancer mortality was not significantly elevated in the total cohorts in either study by Prince et al. (2006a, 2006b; SMR = 1.14 and 1.04, respectively) or in any other studies. While multiple myeloma was significantly elevated in one of the studies by Prince et al. (2006b; SMR = 1.85) it did not approach significance in the other (Prince et al., 2006a; SMR = 2.11, 95%CI 0.84–4.34) and has not been reported in any other studies. It should also be noted that no specific chemical exposure (including PCBs) has been etiologically implicated as a risk factor for multiple myeloma. Overall, the elevated SMRs for specific cancers show a doubling at most, with most not achieving statistical significance, suggesting that occupational exposure to PCBs is not a risk factor for increased cancer mortality.

Specificity of the observed association

This criterion was originally intended to judge if one cause was associated with a single effect or disease; that is, that a finding from one study could be used to predict the outcome of other studies. As noted by the EPA (2005), "Based on our current understanding that many agents cause cancer at multiple sites, and many cancers have multiple causes, this is now considered one of the weaker guidelines for causality." However, because PCB exposure produces essentially only liver tumors in chronic bioassays (Mayes et al., 1998) with notable decreases in tumors at other sites, there is no biological basis for inferring that PCB exposure causes cancer at multiple sites in humans. Overall, the data do not support the notion that PCB exposure increases all-cancer mortality. Consequently, though a weaker guideline for causality, the seemingly random reports of different cancers in different cohorts, which typically disappear on follow-up, suggests that this criterion is not fulfilled by the available data.

Temporal relationship of the observed association

Clearly, substantial exposure to PCBs is known to have preceded the findings reported in the numerous occupational cohort studies. Indeed, the extensive follow-up of some cohorts (e.g. Bertazzi et al., 1981 and Tironi et al., 1996; Brown and Jones, 1981 and Kimbrough et al., 2003; Prince et al., 2006a and 2006b) attests to the study of temporal relationships over time. While this is "among the strongest criteria for an inference of causality", the failure to satisfy the other criteria undermines the weight that can be placed on the fulfillment of this criterion.

Biological gradient (Exposure–response relationship)

As noted by the EPA (2005), "A clear exposure-response relationship (e.g., increasing effects associated with greater

exposure) strongly suggests cause and effect, especially when such relationships are also observed for duration of exposure (e.g., increasing effects observed following longer exposure times)." As described by Golden et al. (2003), there was no exposure-response relationship for any of the randomly occurring cancers in any of the occupational cohorts reported to that time. Subsequently, as described in this review, the results of six additional mortality studies have been published. The study by Mallin et al. (2004) is not considered, as exposure was confounded by previous exposure to chlorinated naphthalenes. Ruder et al. (2006) did not find a significant exposure-response relationship for all cancers, melanoma, or brain cancer (the only cancers assessed). Prince et al. (2006a), in >,2500 of the most heavily exposed workers, did not find a significant exposure-response relationship for all cancers, liver, biliary or gall-bladder cancer, or any other type of cancer including intestine, rectum, breast, and prostate cancer. By contrast, Prince et al. (2006b) reported a significant exposure-response trend for liver, biliary or gall-bladder cancer for no lag and 10-year lag, but not for 20-year lag. With respect to the reported exposure-response trend for liver and biliary cancer, this is not supported by the most recent follow-up of the Yucheng cohort. This poisoning incident involved simultaneous ingestion-based exposure to high levels of PCBs and PCDFs (polychlorinated dibenzofurans) from contaminated cooking oil. The spectrum of toxic effects that resulted from this exposure (as well as the Yusho poisoning incident) is generally acknowledged to be predominantly due to the PCDF components of this mixture, and not to PCBs. For example, when reviewing the data for an earlier PCB toxicological profile, the ATSDR (1997) stated that "The effects from these incidents are not reviewed in this profile because CDFs appear to be the main causal agent." Additionally, the Japanese government has concluded that PCDFs, and not PCBs, as had been previously believed, were the causative agent responsible for the symptoms of the rice-oil poisoning incident, noting that "The Health, Labor and Welfare Ministry determined today that the cause of mass poisoning from cooking oil in the Kyushu region during the late 1960s to early 1970s was a type of dioxin contained in the products" (Japanese Health, Labor and Welfare Ministry, 2002). The 24-year follow-up study reported by Tsai et al. (2007) involved 1,823 Yucheng subjects. Liver and bile-duct cancer was not significantly increased (SMR = 0.7, 95%CI 0.3–1.4) and there was no indication of a morality trend based on years since first exposure (i.e. latency). These data suggest that this exposure was not a risk factor for liver cancer.

For prostate cancer mortality, while not significant in the total cohort, there was a significant trend whether lagging for 0, 10 or 20 years, although this is the only study which has reported this finding. In addition, while age was corrected for according to the authors, it is uncertain if this procedure was adequately conducted. On the basis of the experience of one of the authors of this review article (RK), the mean age at death for all workers (other than those who died from prostate cancer) in Kimbrough et al. (2003) was approximately 64 years, while the mean age of workers who died from prostate cancer was 74 years. There were no exposure-response trends across lag categories for stomach, intestine, rectum, breast, or brain cancers, melanoma, or myeloma. Taking into account all the reported findings (some of which are contradictory) on exposure-response relationships, the WoE suggests that this criterion has not been satisfied.

Biological plausibility and analogy

As noted by the EPA (2005), "An inference of causality tends to be strengthened by consistency with data from experimental studies or other sources demonstrating plausible biological mechanisms," and "Similarly, information on mode of action for a chemical... can inform decisions regarding likely causality." As stated in most of the occupational cohort morality studies on PCB-exposed workers in which the grouping of liver, biliary, and gall-bladder cancers was reported as significantly elevated, the biological plausibility of the finding is hypothetically supported for liver cancer (but not gall-bladder cancer or cancers at any other sites) by the numerous animal studies showing increased liver tumors in PCB Aroclor-dosed rats (e.g. Mayes et al., 1998). This is a correct interpretation based on the clear qualitative similarities between animals and humans—for example, both accumulate PCBs, have the same xenobiotic receptors, such as arylhydrocarbon hydroxylase receptor, constitutive androstane receptor, and pregnane X receptor, and many similar MFO enzymes. However, the analogy breaks down when quantitative differences between animals and humans are considered. The recent determination of the probable mode of action (MOA) by which PCB Aroclors 1016, 1242, 1254, and 1260 promote increased liver tumors in male and female Sprague-Dawley rats now suggests that it is biologically implausible that occupational exposure to PCBs would produce even liver cancer in humans, much less cancer at any other site (Brown et al., 2007), because of the fact that liver tumors (predominantly in female rats) were only observed after tissue accumulation of ΣPCB far greater than any known human exposure had been achieved in the Aroclor-dosed animals. Furthermore, as reviewed below in regard to breast cancer, induction of the various MFOs, such as *CYP1A1*, ultimately responsible for PCB-promoted tumors in rats simply does not occur in humans, even at occupational-exposure levels. Moreover, *in vitro* data demonstrate that human liver cells are many orders of magnitude less responsive to Aroclor 1254-induced *CYP1A1* induction than rat liver cells (Silkworth et al. 2005). The fact that *CYP1A1* induction is the first key event in the MOA for PCB-promoted tumors in rats suggests that humans would not be susceptible to PCB-induced liver tumors. In addition, as documented in Brown et al. (2007) Aroclor-dosed animals showed significant decreases in several types of extrahepatic tumors including those of the pituitary, mammary gland, adrenals, prostate, and pancreas. On the basis of knowledge of PCB levels in occupationally exposed workers, there is no evidence that body

burdens sufficient to approach the PCB levels required to produce tumors in animals have ever been achieved in humans. For example, rats fed a diet containing 100 ppm PCB would receive a daily dose of PCBs of 4–5 mg/kg body weight, which would be equivalent to a daily dose of approximately 280–350 mg for a 70 kg human. The yearly equivalent human exposure would be between 102,000 and 127,000 mg/person. However, even during occupational exposure, humans receive daily PCB doses only in μgram/kg. This further illustrates the biological implausibility that occupationally exposed humans would accumulate body burdens even remotely close to the PCB body burdens in rats that develop liver tumors. Consequently, on the basis of the recently elucidated mode of action of PCB-promoted tumors in rodents, it is quantitatively biologically implausible that PCBs would be carcinogenic in the human liver, or any other site in the human body.

There is also another important consideration pertaining to biological plausibility. Target-organ specificity is a key concept that must be addressed when assessing PCB mortality studies. Studies have shown time and again that human responses to exposures to carcinogens are consistent (i.e. of the same type or nature), although the magnitude of effect might vary among individuals or populations. There are no data suggesting that people with different levels of exposure to the same chemical will develop different types of cancers. It is well known that virtually all chemical carcinogens display striking target-organ specificity (e.g. cancer chemotherapy, aflatoxin, vinyl chloride, benzene, asbestos). This specificity is attributable to the fact that different tissues and organs have different metabolizing, detoxification, and DNA repair processes. Consequently, these organ-specific factors respond to carcinogen exposure in different ways. Carcinogen target-organ specificity, therefore, renders the striking lack of consistency for tumor types among PCB studies much more likely to be the result of random findings than of causal associations. Simply stated, there is no biologically plausible explanation for how PCBs could cause increases in the incidence of different kinds of cancer in different cohorts, or for why these chemicals would behave differently to other chemicals that have been causally associated with increases in the incidence of specific kinds of cancer. In corroboration of the above, even high-dose cancer bioassays have not shown that PCB Aroclors are capable of causing tumors in multiple target organs. In fact, as noted above, in the Mayes et al. (1998) study, PCBs were associated with increased incidence of liver tumors only, and with decreased incidence of tumors in other organs.

Breast cancer and environmental exposure to PCBs

While breast cancer was not the focus of the Golden et al. (2003) review, the findings of numerous breast-cancer studies were briefly summarized. These studies fall into several different categories. The early (generally pre-1994) studies involved few cases of breast cancer, their results were subject to considerable chance variation, and often known risk factors for breast cancer were not taken into account. In addition, the data in these studies were derived from active cases of breast cancer, in which disease-induced weight loss may have contributed to spurious elevations of PCB levels in breast tissue and serum. Moreover, PCBs and other chlorinated compounds tend to have a longer half-life in persons with greater body mass (Brown and Lawton, 2001), so that studies in which body mass was not accounted for could have produced skewed results. Critical reviews of the literature available up until about 1994 have concluded that there is no association between environmental exposure to PCBs and increased risk of breast cancer (Key and Reeves, 1994; Adami et al., 1995).

Virtually none of the numerous large studies conducted subsequently detected a statistically significant increased risk of breast cancer associated with PCB exposure. Many of these studies were prospective in design, thereby eliminating the possible confounding effects of disease-related alterations in PCB serum levels. While a few studies did report a significant association between PCBs (or individual PCB congeners, see below) and breast cancer in some population subgroup (e.g. postmenopausal women who had never lactated), the results of these studies have largely not been replicated. In a comprehensive review of environmental risk factors and breast cancer, Laden and Hunter (1998) concluded that "In summary, most of the recent large studies have not found evidence of increased breast cancer risk associated with blood levels of DDE or total PCBs. The possibility that a positive association might be limited to women with particular reproductive characteristics [e.g., women who have never breast fed, as observed by Moysich] should be examined carefully in the large ongoing studies. Nevertheless, it appears that these environmental exposures are unlikely to be responsible for rising breast cancer rates".

One of the largest studies, by Zheng et al. (2000), concluded that " ...the results do not support the hypothesis that DDE and PCBs increase the risk of breast cancer as encountered through environmental exposure". Likewise, Demers et al. (2000) concluded that " ... taken together, results from six large epidemiological studies reported during the last 2 years, including our own, provide little indication that organochlorine exposure is a risk factor for [breast cancer]". In another review, Laden et al. (2001) summarized the results of five of the largest studies, noting: "Combined evidence does not support an association of breast cancer risk with plasma/serum concentrations of PCBs or DDE. Exposure to these compounds, as measured in adult women, is unlikely to explain the high rates of breast cancer experienced in the northeastern United States".

In addition, the IPCS published the comprehensive *Global Assessment of the State-of-the-Science of Endocrine Disruptors* (2002). This assessment considered the issue of endocrine-active compounds and the strength of the evidence that exposure to weakly estrogenic substances

might be etiologically associated with breast cancer in women. The emphasis was on weakly estrogenic organochlorine compounds such as PCBs and DDT. In assessing the likelihood that breast cancer might be associated with exposure to endocrine-active compounds, the IPCS (2002) concluded that "Although numerous human epidemiological studies have been conducted to determine whether environmental EDCs may contribute to an increased risk of breast cancer, the results remain inconclusive. Overall, the current scientific evidence (from human and animal studies) does not support a direct association between exposure to environmental EDCs and increased risk of breast cancer."

Subsequent to the above-described studies, a number of additional studies have been published that address whether PCB exposure may be a potential risk factor for breast cancer. None of these studies demonstrated a significant association between serum ΣPCB levels and increased risk of breast cancer. However, several studies have reported significant associations between individual PCB congeners or specific genetic polymorphisms (e.g. *CYP1A1*) and increased risk of breast cancer. These studies are reviewed below.

Some of the more recent studies involved active cases of breast cancer. As noted above, these studies have been shown to be of questionable value compared with prospective studies. However, most of the more recent studies have been prospective in nature. These studies are briefly reviewed below. However, it is appropriate to address one key issue before turning to the studies. Most of the earlier studies sought associations between risk of breast cancer and body concentrations of total PCBs (i.e. ΣPCB). In some of the more recent studies, mainly case-control studies, the authors have sought associations between various outcomes (e.g. breast-cancer risk, estrogen-receptor status, survival) and concentrations of individual PCB congeners. Rubin et al. (2006) suggested that future research should consider the effects of individual PCB congeners, even while correctly noting that congener-specific effects can vary from estrogenic to anti-estrogenic effects. Seeking associations between breast cancer risk and individual PCB congener concentrations, rather than ΣPCB, is likely to generate results that are misleading and, frankly, useless. Suggesting that researchers use PCB congeners rather than ΣPCB as exposure metrics ignores a large body of data on receptor-mediated effects that demonstrates that competitive binding to the estrogen receptor by estrogenic (agonist) and anti-estrogenic (antagonist) substances determines the net effect. Because some PCB congeners exhibit estrogenic effects, while others exhibit anti-estrogenic effects, using ΣPCB as the exposure metric is the only way of ensuring that net effects are properly integrated and are representative of what humans are exposed to.

For example, several studies single out PCB 180 as a risk factor for breast (or other types of) cancer. However, PCB 180 has no known distinctive toxicological activity (and none is cited in studies in which this congener is suggested as having a putative role in breast-cancer etiology), other than being highly resistant to MFO-mediated metabolism. Among the more persistent PCB congeners, the activity of PCB 180 toward the Ah receptor is less than that of PCB 126 or 156, and it does not have a toxicity-equivalency-factor value. If PCB 180 happens to correlate with some response, but PCB 156 or PCB 187 (both of which are almost as persistent as PCB 180) do not, it is likely that the correlation occurred either by chance or as a consequence of differential metabolism as described below in the section on PCBS as a risk factor for breast cancer through *CYP1A1* genetic polymorphisms. It should also be noted that numerous studies (e.g. Mayes et al., 1998) demonstrate that chronic exposure to PCB Aroclors produces a decreased incidence of mammary tumors in rats, which also suggests that the effect of the mixture (i.e. ΣPCB) is the proper metric.

Pavuk et al. (2003)

Pavuk et al. (2003) conducted a case-control study involving 24 active cases of breast cancer (diagnosed between 1997 and 1999) and 88 controls. This study was designed to investigate possible associations between PCBs, DDT, DDE, and HCB exposure and risk of breast cancer in an area of high environmental exposure in eastern Slovakia. Levels of 15 individual PCB congeners, DDE, DDT, and HCB were measured in the serum of the breast-cancer patients and population controls. Known risk factors for breast cancer were considered in the analysis. The median serum levels of ΣPCB were similar in cases and controls. When PCB congeners were divided into groupings of estrogenic, anti-estrogenic, and enzyme-inducer PCB congeners, median serum concentrations of all groupings were lower in cases than in controls. However, the risk of breast cancer decreased with increasing serum levels of all congeners and groupings. This study provides no support for an association between PCBs and increase risk of breast cancer.

Hoyer et al. (2000a)

In a study designed to investigate whether organochlorine exposure (i.e. PCBs, dieldrin, DDT, DDE, and HCB) had an effect on breast cancer survival, Hoyer et al. (2000a) examined 195 breast-cancer cases in which serum concentrations of these substances had been determined in 1976–1978 and 1981–1983. Known risk factors for breast cancer were considered in the analysis. There were no differences between the first and second measurements of serum organochlorine compounds and no effect of ΣPCB on tumor characteristics or survival was observed.

Hoyer et al. (2000b)

Hoyer et al. (2000b) conducted a study on the same cohort (Hoyer et al., 2000a), which involved 155 breast cancer cases and 274 matched controls with the goal of evaluating if repeated measurements of organochlorine exposure would provide a more precise measure of breast cancer risk. Blood donated in 1976–1978 and 1981–1983 was

analyzed for ΣPCB (118, 138, 153, and 180), DDT, dieldrin, and β-HCH. Known risk factors for breast cancer were considered in the analysis. Breast-cancer risk was not significantly associated with ΣPCB or any individual congener taken over the course of two measurement periods. However, using data from the second sampling period, the concentration of one congener (PCB 118) in the second exposure quartile was significantly associated with breast cancer (OR=1.9, 95%CI 1.1–3.1). There was no attempt to explain how this congener might have played a role in breast-cancer etiology.

Woolcott et al. (2001)

In a similar case-control study, Woolcott et al. (2001) measured levels of 14 PCB congeners, DDT, DDE, HCB, chlordane, and β-HCH in breast adipose tissue from 217 cases of active breast cancer and 213 biopsy controls to investigate possible associations between organochlorine concentrations and cancer risk by estrogen-receptor and progesterone-receptor status, tumor size, and tumor grade. There were no significant associations between ΣPCB and estrogen-receptor or progesterone-receptor status, tumor size, or tumor grade. There were, however, two significant associations between the middle tertiles of exposure: PCB 156 was associated with estrogen-receptor-negative tumors and PCB 180 was associated with progesterone-receptor-negative tumors. Given the high number of comparisons made in this study, as well as the biologic implausibility of these middle tertile associations, these findings are likely to have occurred by chance given that multiple comparisons were made. Moreover, although the study accounted for known risk factors for breast cancer, it suffers from the same problem as most of the earlier studies on PCBs as potential risk factors for breast cancer—tissue samples were collected from active cases of breast cancer, resulting in uncertainty regarding whether observed associations were a cause or a result of the disease.

Rusiecki et al. (2004)

A more recent case-control study with 266 breast-cancer cases and 347 benign-breast-disease controls was conducted in India to investigate possible associations between breast cancer risk and combined estrogen-receptor/progesterone-receptor status (Rusiecki et al., 2004). Serum and breast-adipose-tissue levels of 9 PCB congeners were determined in cases and controls. There were no significant associations between joint estrogen-receptor/progesterone-receptor status and ΣPCB (i.e. 9 congeners) or any specific congeners or congener groupings (i.e. estrogenic, anti-estrogenic/dioxin-like, or phenobarbital-like). The authors concluded that "these results confirm previous findings in the literature of no positive association between environmental exposure to PCBs and risk of breast cancer".

Laden et al. (2001)

In an expanded follow-up of a previously reported study (Hunter et al., 1997), Laden et al. (2001) added an additional 143 cases of invasive postmenopausal breast cancer (a total of 381 cases). Plasma concentrations were determined for four PCB congeners (118, 138, 153, and 180) and DDE from 1 month to 4 years prior to disease diagnosis. There was no significant association between breast-cancer incidence and lipid-adjusted plasma levels of DDE, ΣPCB, or any of the individual congeners. The authors concluded that "overall, our results do not support the hypothesis that exposure to DDT and PCBs increases the risk of breast cancer".

Rubin et al. (2006)

Rubin et al. (2006) conducted a retrospective case-control study involving 63 women who developed breast cancer and 63 age-matched controls. Banked serum collected between 1981 and 1987 was available for analysis of PCBs (28 congeners), DDT, DDE, and 13 other organochlorine compounds. Most risk factors for breast cancer were accounted for in the analysis. Mean and median ΣPCB concentrations, as well as mean and median serum concentrations of individual congeners, were significantly lower in cases than in controls. The study found a significantly decreased risk for breast cancer across tertiles of ΣPCB in a univariate analysis, although the trend was no longer significant in a multivariate analysis. Overall, there was no indication that PCBs were associated with increased risk of breast cancer.

Gammon et al. (2002)

In a study of breast-cancer risk in relation to serum organochlorine levels (DDE, dieldrin, and 24 PCB congeners), Gammon et al. (2002) used blood samples from 646 cases of *in situ* or invasive breast cancer and 429 controls from a population-based case-control study on Long Island. Known risk factors for breast cancer were considered in the analysis. Potential breast-cancer associations were restricted to the sum of the concentrations of the four most prevalent congeners or "peak-4 PCBs" (i.e. 118, 138, 153, and 180), which represented approximately 50% of the 24 congeners measured. Risk of breast cancer was not significantly associated with peak 4-PCBs or any other congener groupings. There were also no significant associations between peak 4-PCBs and parity, breastfeeding, menopausal status, body mass index, tumor stage, or estrogen-receptor/progesterone-receptor status. The authors concluded that "these findings, based on the largest number of samples analyzed to date among primarily white women, do not support the hypothesis that organochlorines increase breast cancer risk..."

Negri et al. (2003)

Finally, in a recent quantitative review of the WoE concerning possible associations between environmental exposure to PCBs and risk of breast cancer, Negri et al. (2003) assessed the epidemiologic evidence on environmental exposure to PCBs and breast-cancer risk. This review was conducted with support from the Italian Association for Cancer Research and the Italian League Against Cancer. In

this review, ecological studies and studies with less than 50 cases of breast cancer were not considered. The majority (i.e. WoE) of both prospective and retrospective studies did not find any association between total PCB serum or plasma concentrations and breast-cancer risk. Furthermore, no association was found for congeners in Group I (potentially estrogenic) and Group III (biologically persistent phenobarbital-type cytochrome P450 inducers), according to the PCB-congener classification proposed by Wolff et al. (2000). Less consistent results were reported for Group II (potentially anti-estrogenic, immunotoxic, and dioxin-like) congeners. The authors concluded that "the epidemiological evidence does not support the hypothesis of an association of environmental exposure to PCBs in adulthood in the general population and risk of breast cancer". While the authors noted some uncertainties for selected subgroups of women (e.g. women who had never lactated) or individual PCB congeners, these findings were based on a very small number of cases. Importantly, Negri et al. (2003) also cited the lack of increased risk of breast cancer in female workers occupationally exposed to PCBs as strengthening a conclusion that there is no association between PCBs and increased risk of breast cancer.

Breast cancer and occupational exposure to PCBS

In virtually all the studies in which breast-cancer risk has been investigated in conjunction with environmental exposure to PCBs, there is no mention of the findings from the occupational mortality studies. These data demonstrate unequivocally that PCBs are not etiologically associated with increased risk of breast cancer. As described in Golden et al. (2003) and augmented by the additional occupational studies described in the present review, there is no evidence that occupational exposure to PCBs is associated with increased risk of breast-cancer mortality. In general, the occupational studies (now involving > 8,600 women exposed to elevated levels of PCBs) have reported a deficit in breast-cancer mortality, with the study by Prince et al. (2006a) demonstrating a statistically significant decrease in breast-cancer mortality. The results of all occupational mortality studies that reported breast-cancer mortality are summarized in Table 3. This table illustrates that occupational exposure to PCBs is not associated with increased risk of breast cancer with an overall summary SMR of ≈0.84. It should be noted that Table 3 is simply a compilation of results from individual studies, many of which are follow-ups of the same cohort, and is intended only to illustrate the consistent lack of increased mortality from breast cancer in the numerous occupational cohort mortality studies.

Finally, in a recent incidence study of breast cancer in 5752 occupationally exposed women employed at least one year in one of three capacitor manufacturing facilities, the overall breast cancer standardized incidence ratio (SIR) was 0.81 (95% CI 0.72–0.92, n = 257), and regression modeling showed little effect of employment duration or cumulative exposure (Silver et al. 2008). When broken down by race, in white women the SIR = 0.80 (95% CI 0.70–0.90, n = 244) while in women identified as non-white the SIR = 1.94 (95% CI 0.77–3.99, n = 7) was non-significantly elevated. However, in these cases there were positive, statistically significant associations with employment duration and cumulative exposure with only smoking, birth cohort, and self or proxy

Table 3. Occupational exposure levels to PCBs and breast-cancer mortality ratios.

Study	Description	Standardized mortality ratio	95% CI
Brown (1987)	Retrospective cohort mortality study of 1,318 women employed in capacitor manufacturing plants in New York (NY) and Massachusetts (MA).	0.77	0.35–1.46
Nicholson et al. (1987)	Retrospective cohort mortality study of 521 women employed in capacitor manufacturing plants in NY.	1.33	0.43–3.10
Taylor et al. (1988)	Retrospective cohort mortality study of 2,691 women employed in capacitor manufacturing plants in NY.	0.84	0.45–1.44
Sinks et al. (1992)[a]	Retrospective cohort mortality study of 846 women employed in capacitor manufacturing plants in Indiana.	0.51	0.06–1.85
Kimbrough et al. (1999)	Retrospective cohort mortality study of 2,544 women hourly workers and 469 salaried workers employed in capacitor manufacturing plants in NY.	0.82 (hourly) 1.04 (salaried)	0.53–1.21 0.34–2.44
Kimbrough et al. (2003)	Retrospective cohort mortality study of 2,544 women hourly workers and 469 salaried workers employed in capacitor manufacturing plants in NY.	0.88 (hourly) 0.87 (salaried)	0.60–1.24 0.31–1.89
Ruder et al. (2006)	Retrospective cohort mortality study of 852 women employed in capacitor manufacturing plants in Indiana.	0.83	0.4–1.6
Prince et al. (2006a)	Retrospective cohort mortality study of 1,325 highly exposed women workers employed in capacitor manufacturing plants in NY and MA.	0.59	0.33–0.98
Prince et al. (2006b)	Retrospective cohort mortality study of 7,772 women workers employed in capacitor manufacturing plants in NY and MA.	0.95	0.78–1.15
Summary standardized mortality ratio			0.84

[a]Breast-cancer mortality rate not reported in original study, obtained from Adami et al. (1995).

questionnaire completion having statistically significant explanatory power in conjunction with exposure metrics. While interesting, the exposure-related risk elevations observed among non-white workers are difficult to interpret given the small number of cases.

PCBs as a risk factor for breast cancer through *CYP1A1* genetic polymorphisms

The WoE supports the conclusion that neither environmental nor occupational exposure (both reviewed above) to PCBs is a risk factor for breast cancer. However, several recent studies have reported potential interactions between PCBs and *CYP1A1* polymorphisms that seem to be associated with increased risk. The underlying biological basis for associating PCB exposure, *CYP1A1* polymorphisms, and breast-cancer risk involves estrogen as a primary risk factor for breast cancer. Indeed, most of the known endogenous risk factors for breast cancer are in essence surrogates for internal lifetime estrogen exposure (e.g. age at menarche, parity, lactation, age at menopause). However, estrogen *per se* is not believed to be the active molecular form that causes breast cancer. Rather, estrogen metabolites produced by a number of estrogen-metabolizing P-450 CYP enzymes have been implicated. Phase 2 enzymes (e.g. glutathione S-transferase, GST, UDP-glucuronosyltransferases, UGTs, and catechol-O-methytransferase, COMT) also play key roles in the metabolism and excretion of estrogen metabolites. Oxidative metabolism of estrogens, mainly by hydroxylation, is mediated primarily by CYP1A1 (producing primarily 2OH-estradiol). The catechol estrogens that are formed are intermediates for the generation of reactive quinones and semiquinones, which have both been hypothesized to have the ability to damage DNA through redox cycling and generation of reactive oxygen species (Bolton, 2002; Jefcoate et al., 2000; Liehr 1997; 1999, 2001; Yao et al., 2003).

At least four genetic polymorphisms of *CYP1A1* have been studied for potential relationships with breast-cancer risk. Many of these are prevalent with different frequencies in Caucasian, African-American, and Asian women. The four allelic changes are termed *m1, m2, m3* and *m4* (also referred to as *CYP1A1*2A, CYP1A1*2B, CYP1A1*3,* and *CYP1A1*4,* respectively) with the 'wild-type' designated as *CYP1A1*1.* (Wormhoudt et al., 1999). The *m2* variant encodes an isoleucine (Ile) → valine (Val) substitution at exon 7 (i.e. heterozygous Ile:Val; homozygous Val:Val). While it has been reported that the allelic variants can affect the inducibility of the P-450 isoform, the evidence is mixed on this phenomenon (Landi et al., 2005; Wormhoudt et al., 1999). This is particularly true since all of the data are from *in vitro* studies and it is unknown whether there is a functional change *in vivo.* Some studies have reported that the Ile→Val substitution affected the catalytic properties of the enzyme (Kiyohara et al., 1996), while others have reported a significant effect on EROD metabolism, but no effect on the hydroxylation of benzo[a]pyrene (Zhang et al., 1996). In a study of metabolic activity toward B[*a*]P, CYP1A1*1 showed the highest total metabolism, with CYP1A1*2 at ≈50% and CYP1A1*4 at ≈70% (Schwartz et al., 2001). Other studies have reported increased inducible enzyme activity of the CYP1A1*2B variant in human lymphocytes, as measured by EROD assay, when compared with CYP1A1*. However, this has been seen only in 3-methylcholanthrene-induced lymphocytes and not in basal values of CYP1A1 activity (Cosma et al., 1993; Crofts et al., 1994). Other studies have failed to confirm a clear association between polymorphisms of CYP1A1 and increased enzyme induction as measured by EROD metabolism (Kyohara et al., 1996; Jacquet et al., 1996; Smith et al., 2001).

On the basis of *in vitro* data suggesting that polymorphic forms of *CYP1A1* are more inducible, it has been hypothesized that there could be an interactive effect between PCB body burdens and certain *P4501A1* polymorphisms to increase breast-cancer risk. Moysich et al.'s (1999) was the first study to report that, in women with serum PCB levels above the median of the distribution in the control group and with at least one variant *CYP1A1m2* allele (Ile:Val or Val:Val), there was a significantly increased risk of postmenopausal breast cancer (OR=2.93, 95%CI 1.17–7.36, 19 cases). In this study, blood was collected for PCB analysis (73 congeners) from 154 women with postmenopausal breast cancer after disease diagnosis, but prior to chemotherapy treatment and the presence of the *CYP1A1 m2* polymorphism was determined. Known risk factors for breast cancer were considered in the analysis, although it is not clear whether post-menopausal use of hormone-replacement therapy (HRT) was considered. There was no association between the *CYP1A1 m2* genotype and breast-cancer risk in women with PCB levels below the median of controls. The low and high PCB groups were 0.75–3.72 ng/g and 3.73–19.04 ng/g, respectively. Breast-cancer risk was significantly increased in women with elevated PCB body burden and a *CYP1A1* polymorphism with a history of smoking (OR=7.74, 95%CI 1.12–53.90), but not in women who had never smoked (OR=1.43, 95% CI 0.53–3.87). As in other studies, there was no increase in breast-cancer risk based on ΣPCB body burdens alone, with the suggestion that this may have been attributable to the fact that only a small fraction of the populations studied were susceptible to the effects of PCB exposure (i.e. those with a CYP1A1 polymorphism).

Layden et al. (2002) also studied breast-cancer risk with respect to potential interactions between CYP1A1 polymorphisms and PCBs. In this study, plasma PCB concentrations (21 congeners) and the CYP1A1 *m1* and *m2* polymorphisms were determined in 367 breast-cancer case-control pairs (293 postmenopausal pairs) from the Nurses Health Study. Blood was collected for PCB analysis well before disease diagnosis, thereby avoiding possible disease-related or chemotherapy-related effects on serum levels. Known risk factors for breast cancer were considered in the analysis, although it is not clear whether post-menopausal HRT was considered. There was no independent

association of either of the *CYP1A1* variants or lipid-adjusted ΣPCB with breast-cancer risk. However, based on 19 cases, there was a borderline significant interactive relative risk of post-menopausal breast cancer (RR = 2.78, 95%CI 0.99–7.82) associated with plasma ΣPCB levels in the highest tertile of the distribution (0.67–1.99 ug/g) and at least one *m2* variant allele compared with women with the wild-type allele and ΣPCB levels in the lowest tertile of exposure. There were no significant associations between post-menopausal or all breast cancer and a *CYP1A1 m1* polymorphism and plasma ΣPCB levels. As noted by the authors, because of the small number of cases (and the consequent wide CI), the finding of an increased risk of post-menopausal breast cancer in women with the *CYP1A1 m2* polymorphism and the highest PCB exposure warrants further study.

In a similar study, Zhang et al. (2004) investigated associations between serum PCB levels and *CYP1A1* polymorphisms in 374 cases of breast cancer and 406 non-cancer controls. Measurements of serum PCBs (9 congeners) and genotypes of *CYP1A1 m1, m2,* and *m4* were determined. Blood for PCB analysis was collected following a diagnosis of breast cancer and, therefore, measured levels are subject to possible disease-related effects. In addition, the extent to which chemotherapy-treatment-related effects on PCB serum levels might have influenced the results is unknown, since this potentially confounding issue was not addressed. Because variant alleles in the *CYP1A1* gene vary widely in frequency by race, the analysis was restricted to white participants, since there were few non-white participants in the total cohort. Known risk factors for breast cancer were considered in the analysis, although it is not clear whether post-menopausal HRT was considered. Lipid-adjusted serum ΣPCB levels were characterized as low (310–610 ng/g) and high (611–2600 ng/g). There were no significant independent effects on breast-cancer risk when assessed using ΣPCB. However, on the basis of analysis of the 40 cases in the total cohort, there was a significantly increased risk of breast cancer (OR = 2.1, 95%CI 1.1–3.9) in women with the *CYP1A1 m2* variant (one variant allele or homozygous alleles), which increased in post menopausal women (OR = 2.4, 95%CI 1.1–5.0). A significant interactive effect (OR = 4.3, 95%CI 1.6–12.0) in post-menopausal women (21 cases) was also observed between high serum PCB levels and the *CYP1A1 m2* variant. As noted by the authors, because of the relatively low prevalence of the variant genotypes (i.e. 6% of the *m2* genotype in the control population), it was not possible to stratify the data by potentially major confounders such as lactation and menopausal status, despite the size of this study.

In the latest study of this type, Li et al. (2004) conducted a case–control study involving 612 breast cancer cases (242 African-American and 370 white) and 599 controls to investigate possible interactions between *CYP1A1* polymorphisms and ΣPCB. In all cases and controls, plasma concentrations of PCBs were determined in addition to genotyping for *CYP1A1 m1, m2, m3,* and *m4* alleles. Blood for PCB analysis was collected following a diagnosis of breast cancer and, therefore, the measured levels are subject to possible disease-related effects. In addition, the extent to which chemotherapy treatment might have influenced PCB serum levels, and therefore study results, is unknown, since this potentially confounding factor was not considered. Because plasma ΣPCB levels were higher in African-American women than in white women, the analysis was conducted based on ΣPCB concentrations of <0.430 ng/ml and ≥ 0.430 ng/ml (lipid adjusted) for the former and ΣPCB concentrations of 0.349 ng/ml and ≥ 0.349 ng/ml for the latter groups of breast-cancer cases. Known risk factors for breast cancer were considered in the analysis, including the use of HRT or oral contraceptives.

Li et al. (2004) found no evidence of joint effects between *m1* genotypes and ΣPCB in either African-American or white women. There were also no significant interactive effects between ΣPCB and any *m2* variant in African-American or white women, any *m4* variant in white women, or any *m3* variant in African-American women. Greater than additive joint effects were reported between the *m2* genotype and ΣPCB in the total cohort with an interaction contrast ratio > 0 with $p = 0.03$. Although this statistic was not used in previous studies, which reported greater effects in post-menopausal women, the joint effects were stronger in pre-menopausal ($n = 14$) than in post-menopausal ($n = 12$) women. However, with respect to calculating additive interactions, it should be noted that, because the calculations were based on ORs, substituting ORs for risk ratios may result in misleading conclusions (Kalilani and Atashili, 2006).

In the first three of the above-described studies, the proposed mode of action for the effects reported was suggested on the basis of the hypothesis that polymorphic forms of *CYP1A1* were more inducible by PCBs (albeit with no direct *in vivo* evidence of this effect) with the increased induction leading to enhanced metabolism of estrogen to potentially toxic metabolites (which is biological plausible). However, the study by Li et al. (2004) proposes a different mode of action, suggesting instead that PCBs are metabolized by *CYP1A1* to produce free-radical-induced oxidative DNA damage in breast tissue. As support for this hypothesis, Li et al. (2004) cite a single *in vitro* study that reported that PCB dihydroxy metabolites are activated by enzymatic and non-enzymatic mechanisms (not involving *CYP1A1*) to reactive intermediates that produce oxidative DNA damage (Oakley et al., 1996). The authors did not consider the fact that while PCB-derived quinones formed DNA adducts *in vitro*, no DNA-adduct formation has been detected in PCB-dosed animals (Schilderman et al., 2000; Whysner et al., 1998). Furthermore, in an extensive analysis of the probable mode of action for PCB-induced promotion of hepatic tumors, the extent of PCB metabolism was not correlated with the development of tumors, implying that PCB metabolites did not contribute to tumor development (Brown et al., 2007).

Comment

Three of the studies summarized above suggest that there might be an interactive effect between the *CYP1A1 m2* variant and both ΣPCB serum levels and increased breast-cancer risk, predominantly in post-menopausal women. The fourth study suggested that the risk was greater for pre-menopausal women. Although all four studies, at least superficially, seem to satisfy the causation criterion of consistency and, perhaps, strength of association, there is uncertainty with respect to the dose-response criterion and, most importantly, the criterion of biological plausibility. However, because the results of these studies are based on small sample numbers and rather wide confidence intervals, the strength of association is weakened. It also should be noted that several comprehensive reviews have concluded that there is no independent association between breast-cancer risk and the *CYP1A1 m2* variant (Agundez, 2004; Masson et al., 2005; Li et al., 2005). While this does not eliminate the possibility of interactive effects with PCBs, it does illustrate the burden placed on the hypothetical role of PCBs, as these compounds are far from unique in the specific property (i.e. induction of *CYP1A1*) proposed as the key event in explaining the results of the four studies. This issue is reviewed in detail below.

Several other methodological issues also call into question the reported findings. One of the studies (Laden et al., 2002) was conducted in a cohort where blood specimens were collected prior to disease diagnosis, while three of the studies (Li et al., 2004; Moysich et al., 1999; Zhang et al., 2004) involved active cases of breast cancer with PCB levels measured in serum collected after breast cancer was diagnosed. Therefore, these three studies may very well suffer from the same problem as the early studies that used the same methodology—possible disease-induced redistribution of PCBs from breast tissue into the blood, particularly in the event that weight loss occurred. While the authors correctly note that current levels may not represent levels present when disease developed, they still fail to acknowledge the possibility of disease-related effects on current PCB levels. Related to this problem is the known effect of chemotherapy on PCB serum levels. Gammon et al. (1996) demonstrated that PCBs can be mobilized by treatment, and concluded that "the use of blood samples collected after treatment, rather than before treatment, for characterizing PCB levels may lead to misclassification of exposure". The studies by Zhang et al. (2004) and Li et al. (2004) did not address this possibility. Moysich et al. (1999) collected blood samples prior to initiation of chemotherapy, but still after disease diagnosis. The extent to which the disease and chemotherapy treatment of the disease might have altered PCB serum or plasma concentrations, thereby leading to spurious interactive relationship with *CYP1A1* polymorphisms, is unknown.

Biological plausibility

The principal explanation for the findings in the above studies is that the CYP1A1 *m2* allele is more inducible by PCBs based on data derived from a variety of *in vitro* test systems. By contrast, human data on the differential metabolism of PCB congeners in Aroclors 1242 and 1254 indicate that CYP1A1 is not induced in humans at PCB serum levels resulting from occupational, much less environmental exposures. While a PCB metabolism pattern consistent with induction of CYP1A1 is readily induced in rats (Mayes et al., 1998; Brown et al., 2007), a similar pattern is not induced in humans (Brown and Lawton, 2001). This was confirmed in a study of 48 occupationally exposed capacitor workers with mean lipid PCB levels of 195 ppm (range 37–1035 ppm), in which there was no indication of a decrease in serum levels of the specific PCB congeners that are known to be metabolized by CYP1A1 (Brown and Lawton, 2001). That is, PCB congeners 28, 74, and 118 were not reduced in concentration due to increased metabolism resulting from CYP1A1 induction. As noted by Brown and Lawton (2001), "the absence of P450 1A induction by Aroclors in the workers (or mice) could reflect either a lower responsiveness of the human (or mouse) Ah-receptor to PCBs, or the known tendency of non-coplanar PCBs to inhibit P450 1A1 induction (van der Plas et al., 1998), or both". While occupational exposure to PCBs was not sufficient to induce CYP1A1, placentas from women poisoned from the accidental ingestion of PCBs and polychlorinated dibenzofurans as a result of the Yu-Cheng incident demonstrated CYP1A1 catalytic activity increased approximately 100-fold, compared with control placenta (Lucier et al., 1987). These placentas contained 50–892 times more dioxin-equivalent chemicals than are present in background levels. By contrast, placentas from Inuit women from southern Quebec with mean blood TEQ levels approximately five times greater than those of controls showed no evidence of increased CYP1A1 activity (Pereg et al., 2002). Collectively, these data demonstrate that PCB serum levels achievable by environmental exposures are not sufficient to induce CYP1A1.

The above discussion illustrates that, while it is unlikely that CYP1A1 is induced in humans by exposure to PCBs alone, particularly from environmental exposures, sufficient exposure to TEQ can have this effect. This poses an interesting biological conundrum pertaining to the four breast cancer studies reviewed above: how could the *CYP1A1 m2* polymorphic form be induced by PCBs at environmental levels while *CYP1A1* is not induced by PCBs even at occupational-exposure levels? It is biologically implausible that the *m2* variant (i.e. either heterozygous or homozygous) would essentially change the functionality of the *CYP1A1* gene from non-inducible at occupational PCB levels to inducible by environmental PCB exposure levels. While the results of these studies seem to be in agreement, there remains considerable uncertainty whether the explanation for the reported findings is as simple as an interactive effect between the presence of a *CYP1A1 m2* variant and minimally elevated serum PCB levels, all of which are in the normal background range.

Finally, with respect to biological plausibility, the female occupational-exposure data are perhaps the most compelling. As reviewed above, these data demonstrate unequivocally that PCBs are not etiologically associated

with increased risk of breast cancer. These data, from > 8,600 women exposed to elevated levels of PCBs, take on additional importance with the publication of the studies reviewed above, suggesting an increased risk of breast cancer in women with a *CYP1A1 m2* polymorphism and environmental exposure to PCBs. While none of the occupational studies considered *CYP1A1* polymorphisms, it can only be concluded that if the mode of action by which the findings in the above four studies is hypothesized to occur (i.e. greater induction of the *CYP1A1 m2* allele leading to increased production of potentially DNA-damaging estrogen metabolites as a consequence of background exposure to PCBs), then this would have occurred to an even greater extent in occupationally exposed women. For example, approximately 12% of women carry the *m2* allele (Zhang et al., 2004) which means that ≈1000 of the women exposed in the occupational cohort studies would have been far more susceptible to PCB-induced breast cancer if the findings in the above four studies were actually due to the hypothesized events. This would have resulted in greatly increased breast-cancer mortality in this subset of women, with an overall SMR in excess of 100 instead of the observed SMR of 94, as shown in Table 4.

Given the unequivocal evidence that occupational exposure to PCBs is not associated with increased risk of breast cancer, the seemingly enigmatic covariance of slightly elevated background PCB serum levels with cancer risk in a *CYP1A- m2*-bearing subpopulation suggests an interesting biological paradox caused by two seemingly contradictory facts. The first is that the *CYP1A1 m2* variant is very similar in enzymatic activity to other *CYP1A1* types (Zhang et al., 1996; Schwartz et al., 2001), but is approximately three times more easily induced by the classical AhR agonist, 3-methylcholanthrene (Cosma et al., 1993; Crofts et al., 1994). The second is that, as described above, increased induction of CYP1A1 (or any other PCB-metabolizing CYP) could not be detected even in occupationally exposed capacitor workers with 100-fold higher levels of PCBs than those in the background-exposed study populations (Brown and Lawton, 2001). In an *in vitro* study, induction of CYP1A1 in human hepatocytes by Aroclor 1254 also required far greater PCB concentrations compared with in induction in rat or monkey hepatocytes (Silkworth et al., 2005). Therefore, even allowing for a threefold greater responsiveness of the *m2* variant, there is simply no conceivable way that PCBs at serum levels in the normal background range (i.e. ng/ml) could cause any induction of *CYP1A1 m2*. Consequently, it is necessary to explore an alternative explanation for the enigmatic correlation between increased breast-cancer risk in individuals with the *CYP1A1 m2* variant and slightly elevated background serum PCB levels.

It is well known that correlation does not necessarily equal causation. In the case at hand, it seems more likely that minimal CYP1A1 induction (not due to PCBs) is causing slightly increased PCB serum levels rather than the opposite. This is likely due to the following three inter-related factors:

Although background PCB serum levels are clearly not sufficient for CYP1A1 induction, other CYP1A1 inducers are likely to be present in the study population (e.g. smoke from cigarettes and other combustibles, cruciferous vegetables, tryptophan metabolites, certain dietary flavonoids). Thus, some induction of *CYP1A1* isozymes is occurring in the study population, particularly in individuals with the *m2* allele.

A general peculiarity of CYP induction is that agents that induce one type of CYP are often inhibitors of another (Waxman and O'Conner, 2006). For example, it has been found that CYP1A1 inducers can decrease expression of CYP2C and CYP3A isozymes in rodents (Shaban et al., 2005; Lee et al., 2006), and that similar effects occur in humans (Brown, 1989). Thus, cigarette smoke, which increases expression of CYPs *1A1* and *1A2*, decreases expression of those in the CYP3A family, CYPs *3A4, 3A5, 3A7, 3A43* (Raunio et al., 2005). Yu-Cheng patients, who ingested PCBs contaminated with the strongly CYP1A1-inducing PCDFs, exhibited not only increased clearance of mono-*ortho* PCB congeners, known to be associated with CYP1A1/2 induction (Brown et al., 2007), but also reduced clearance of the

Table 4. Observed and expected cancer mortality and standardized mortality ratios from studies of 19,825 capacitor manufacturing workers occupationally exposed to PCBs.

Type of cancer	Observed mortality	Expected mortality	Standardized mortality ratio[b]	*p* value[a]	Standardized mortality ratio[c]
All cancer	1241	1252	100	0.83	88
Liver, biliary, or gall-bladder	24	28	86	0.58	76
Intestine	121	107	113	0.35	97
Stomach	36	32	113	0.63	72
Rectum	22	21	105	0.88	81
Skin	28	19	147	0.19	110
Breast	119	127	94	0.77	-
Prostate	39	42	93	0.74	90
Lymphatic and hematopoietic	138	126	110	0.46	88
Non-Hodgkin's lymphoma	44	43	102	0.91	102

[a] *p* values are two-sided for capacitor-worker summary standardized mortality ratios

[b] Standardized mortality ratios based on Gustavsson and Hogstedt (1997), Tironi et al. (1996), Ruder et al. (2006), and Prince et al. (2006b)

[c] Standardized mortality ratios include studies in footnote b, plus observed and expected mortality data from Loomis et al. (1997).

di-*ortho* PCB congeners (Brown et al., 1989). This indicates suppression of the constitutive oxidase activity (e.g. *CYP 3A4/5*) normally involved in their metabolism.

Most of the PCBs present in human tissue residues consists of di-*ortho*-substituted and tri-*ortho*-substituted species (e.g. congeners 99, 138, 153, 170, 180, 187) that are slowly metabolized by the CYP3A, but are not metabolized by the CYP1A oxidases (Brown, 1994).

On the basis of simple pharmacokinetic considerations (Brown, 1994), individuals in a population with chronic exposure to PCBs at background levels will attain steady-state PCB levels that are inversely proportional to PCB clearance rates, which, in turn, are primarily determined by CYP-mediated metabolism. Thus, in individuals where *CYP1A1* activity has been somehow induced, which would be slightly more common in the 10–15% of the population with the *CYP1A1 m2* allele, there will be a concomitant suppression of *CYP3A* activity and consequent increase in steady state blood levels of di-*ortho* and tri-*ortho* PCB congeners. As a result, there will be an observable correlation between PCB levels (albeit in the background range) and cancer risk in the *m2* subpopulation. This occurs because low-level PCB exposure can serve as a biomarker for *CYP* induction and inhibition by other agents, even though it cannot produce observable *CYP* induction in humans.

Association between occupational exposure to PCBs and increased risk of cancer based on ATSDR methodology

As described in the introduction, the ATSDR (2000) did not rely on a WoE approach when evaluating the occupational cohort PCB mortality data, but rather used the meta-analysis-type methodology used by Nicholson and Landrigen (1994). Using this methodology, the summed observed and expected cancer mortality rates (for men and women combined) from five studies were tested for statistical significance. Importantly, this analysis only included data available up to 1994, even though the ATSDR acknowledged that additional data were available. Table 4 uses the same methodology as described by Nicholson and Landrigen (1994) and combines the data from all of the capacitor-worker occupational cohort mortality studies published to date. For the purposes of this analysis, mortality statistics for men and women are combined and, when more than one study has been conducted on the same cohort, only the most recent study is included to avoid double counting (i.e. Ruder et al., 2006 rather than Sinks et al., 1992, Prince et al., 2006b rather than Brown et al., 1987 or Kimbrough et al., 1999 or 2003, and Tironi et al., 1996 rather than Bertazzi et al., 1987). Consequently, the data from the following four studies are summarized in Table 4: Gustavsson and Hogstedt (1997), Tironi et al. (1996), Ruder et al. (2006), and Prince et al. (2006b). Data from Mallin et al (2004) are not included in Table 4, since, as discussed above, the reported effects were predominantly from exposure to chlorinated naphthalenes and not PCBs. When only observed mortality is reported, the expected mortality is calculated by dividing the observed mortality by the SMR.

In contrast to the data relied on in the ATSDR (2000) analysis, involving approximately 5386 workers, Table 4 includes mortality data on almost 20,000 PCB-exposed workers. The summary SMRs for each cancer were tested for statistical significance from a Poisson distribution as described by Nicholson and Landrigan (1994). As shown in Table 4, based on the insignificant p-values, none of the SMRs for all cancers or any specific cancer is significantly elevated. In addition, the right column of Table 4 has been added to incorporate the data from Loomis et al. (1997) into the SMR analysis. While the almost 139,000 individuals in this study were male electrical-utility workers, with probably lower exposure to PCBs than capacitor workers, there were approximately 20,000 workers with between 1,000 and 10,000 cumulative hours of exposure to PCBs. As shown in the right column of Table 4, the summary SMRs are further decreased and none are statistically significantly elevated. The above discussion demonstrates that the methodology relied upon by the ATSDR (2000), while not following any recognized guidelines, reaches the same conclusions about cancer risk in PCB-exposed cohorts as the WoE approach used in this review—that there is no evidence of a causal association between exposure to PCBs and increased risk of any type of cancer.

Overall conclusions

None of the studies published since the previous review (i.e. Golden et al., 2003) change the conclusions of that review (i.e. "The weight of evidence does not support a causal association for PCBs and human cancer"). This pertains to all cancer combined, as well as to the specific cancers that have been sporadically reported as elevated in incidence in the occupational cohort mortality studies. Special emphasis should be placed on the issue of PCBs as a risk factor for breast cancer. While the WoE supporting the conclusion that environmental exposure to PCBs is not etiologically implicated in breast-cancer risk is compelling, it is nonetheless surprising that virtually none of the breast-cancer studies mention the consistently negative findings for increased breast-cancer mortality in the occupational studies. These data, now including almost 9,000 women occupationally exposed to PCBs, show no evidence whatsoever of increased breast-cancer mortality. Similarly, virtually none of the incidence studies reviewed above, in which PCB background levels are reported to be associated with increased risk of NHL and prostate, testicular, and intestinal cancer, cite the conflicting results from the occupational cohort studies. Because the occupational studies involve PCB exposure far in excess of environmental exposures, this discrepancy should be acknowledged in future incidence studies. In addition, the possibility that serum PCB levels (all of which are in the normal population range) in such studies can be influenced by disease or

its treatment also needs to be considered. Finally, lacking any relevant or persuasive supporting evidence, there does not seem to be any biologically plausible basis for concluding that either a particular PCB congener or grouping of congeners is etiologically implicated with a particular cancer.

While the ATSDR (2000) did not follow the principles endorsed by the EPA (2005) for evaluating a body of epidemiological data, the conclusions of that document—that there is either "some evidence" or "meaningful evidence" that PCBs are carcinogenic in humans—are not supported even when the methodology employed by the ATSDR (2000) is applied to all relevant data. Notwithstanding, because of the substantial limitations of this methodology, it is suggested that this kind of procedure not be relied upon for critically evaluating epidemiological data.

All relevant data published since 2003 were evaluated in the present review with careful reliance upon and consideration of the WoE criteria established by the EPA (2005) for evaluating epidemiological data. When these criteria are followed, it can only be concluded that exposure to PCBs, whether environmental or occupational, does not increase cancer risk in humans. In another recent critical evaluation of most of the same studies considered in the present review or in previous reviews, Shields (2006) concluded as follows:

"The epidemiologic evidence fails to establish PCBs as human carcinogens. This has been an extensively studied topic in the occupational setting, and more recently in the general population. There are reported positive associations in some studies, but the literature fails to identify a consistent target organ and the animal studies do not indicate that PCBs are multiorgan carcinogens. Some cancer relationships from environmental studies are not consistent with studies of highly exposed workers."

The above conclusions are now also supported by the recent elucidation of the mode of action by which PCBs cause liver tumors in rats (Brown et al., 2007). This study, based on the Mayes et al. (1998) chronic bioassay of Aroclors 1016, 1242, 1254, and 1260, described the key events underlying the mode of action for PCB-promoted liver tumors in Sprague–Dawley rats. Most important, from the standpoint of the present review, is the fact that none of the key events in the mode of action occurred until substantial amounts of ΣPCB or TEQ had accumulated in hepatic tissues. The relevance of these findings to the human epidemiological data is that even prolonged occupational exposure to PCBs has never resulted in PCB burdens even approaching the levels required to initiate the sequence of events required for the development of tumors in rodents.

Finally, it should be noted that the few positive findings sporadically reported in the numerous studies of PCB-exposed workers (i.e. the conspicuous lack of consistency of association) can be more logically explained based on the principle embodied by Occam's razor. This is often paraphrased as 'all things being equal, the simplest solution tends to be correct'; that is, that the explanation that introduces the fewest assumptions and is dependent upon the fewest hypothetical entities is most likely to be correct. This is particularly the case now that the mode of action for PCB-promoted liver tumors in rats has been elucidated, with the strong likelihood that, because of substantial species differences, humans are unlikely to be susceptible to PCB-caused cancers. Consequently, for liver, biliary, and gall-bladder cancer, rather than assuming that this was due to PCB exposure, a less complex (and more biologically plausible) explanation for the isolated reported findings would involve the following: (a) the inappropriate grouping of these cancers to achieve statistical significance; (b) metastasis from other sites; (c) undocumented alcohol consumption; or (d) ethnic factors. For melanoma, this would include exposure to sunlight or misclassification. Other findings, such as prostate cancer, are even less likely to be due to PCB exposure.

Acknowledgements

Declaration of interest: The authors of this article were compensated by General Electric for the preparation of this review. The interpretation of the data presented, as well as the conclusions reached, is the sole responsibility of the authors. The authors alone are responsible for the content and writing of the paper.

End note

1 Although PCBs are on the list of substances the EPA intends to reevaluate as part of the Integrated Risk Information System, there is no indication that this will be done anytime soon. The most recent Integrated Risk Information System evaluation of PCB carcinogenicity is current to 1996, reviews only three studies (Bertazzi et al., 1987; Brown, 1987; Sinks, 1992) and also cites (with no review) NIOSH (1977), Gustavsson et al. (1986), and Shalat et al. (1989). Clearly, this does not qualify as a WoE assessment. Similarly, PCBs are not on the National Toxicology Program candidate list of chemicals for consideration in the 12th Annual Report on Carcinogens. With respect to international bodies, the International Agency for Research on Cancer (IARC) updated its PCB evaluation in 1987, citing a total of five studies (Brown and Jones, 1981; Brown, 1987; Bertazzi et al., 1981, 1987; Gustafasson et al., 1986). This also cannot be considered a WoE assessment and, indeed, the IARC typically does not conduct these types of assessments. The evaluation by the WHO through the International Programme on Chemical Safety (IPCS) is current as of 1993 and cites the same studies as the IARC (1987). Finally, the IPCS, in its Concise International Chemical Assessment Document series (2003), also reviewed the potential human carcinogenicity of PCBs, concluding that "Epidemiological studies suggest exposure-related increases in cancers of the digestive system, especially liver cancer, and malignant melanoma. However, the limitations of exposure information, the inconsistency of the results, and, in some cases, the presence of confounding exposures preclude a clear identification of an exposure–response relationship". While some 50 studies were considered in the IPCS evaluation, and although the conclusions seem correct, they were not based on a formal WoE process. This is true despite the existence of explicit IPCS guidelines for evaluating bodies of epidemiological data using a WoE processes (IPCS, 1999).

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Attachment J-6

TOXICOLOGICAL SCIENCES 87(2), 508–519 (2005)
doi:10.1093/toxsci/kfi261
Advance Access publication July 27, 2005

Comparison of TCDD and PCB CYP1A Induction Sensitivities in Fresh Hepatocytes from Human Donors, Sprague-Dawley Rats, and Rhesus Monkeys and HepG2 Cells

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Received July 4, 2005; accepted July 13, 2005

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related chemicals induce cytochrome P450 1A (*CYP1A*) gene expression and, at sufficient exposures, cause toxicity. Human health risks from such exposures are typically estimated from animal studies. We tested whether animal models predict human sensitivity by characterizing *CYP1A* gene expression in cultures of fresh hepatocytes from human donors, rats, and rhesus monkeys and HepG2 human hepatoma cells. We exposed the cells to three aryl hydrocarbon receptor (AhR) ligands of current environmental interest and measured 7-ethoxyresorufin-*O*-deethylase (EROD) activity and concentrations of CYP1A1 and CYP1A2 mRNA. We found that human cells are about 10–1000 times less sensitive to TCDD, 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), and Aroclor 1254 than rat and monkey cells, that relative potencies among these chemicals are different across species, and that gene expression thresholds exist for these chemicals. Newly calculated rat–human interspecies relative potency factors for PCB 126 were more than 100 times lower than the current rodent-derived value. We propose that human-derived values be used to improve the accuracy of estimates of human health risks.

Key Words: **polychlorinated biphenyls; PCB; TCDD; dioxin; dioxin equivalents; TEQ; relative potency factors; human hepatocytes; HepG2 cells; cytochrome P450 1A; CYP1A1; CYP1A2; risk assessment.**

Polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and PCBs comprise a group of highly regulated environmental contaminants of global concern. A subset of these compounds can bind to, and activate, a natural cellular receptor, the aryl hydrocarbon receptor (AhR), which is a transcription factor (Carlson and Perdew, 2002; Nebert *et al.*, 1993). This can result in the up-regulation of many genes (Boutros *et al.*, 2004; Okey *et al.*, 1994), including those encoding metabolic enzymes such as the cytochrome P450 (CYP) isozymes (Rowlands and Gustafsson, 1997). The induction of CYP1A1 correlates well with exposure in animals (Tritscher *et al.*, 1992; Vanden Heuvel *et al.*, 1994) and is used as an important basis for estimating human health risk (Birnbaum and DeVito, 1995). The World Health Organization (WHO) has established toxic equivalency factors (TEFs) for each of 29 AhR ligands, including 7 dioxins, 10 polychlorinated dibenzofurans, and 12 PCBs. Because 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the highest affinity ligand for the AhR, and by far the most potent at eliciting biologic responses, it was assigned a TEF of 1. TEFs for other chemicals are based on relative potency (REP) values relative to that of TCDD using both *in vivo* and *in vitro* animal studies, and it is assumed that these TEFs (WHO98 TEFs) are appropriate for use in human risk assessment (van den Berg *et al.*, 1998). We tested this assumption by comparing the responses of fresh hepatocytes from rats, rhesus monkeys, and human donors, and also a human-derived tumor cell line, HepG2, to TCDD, PCB 126 (3,3′,4,4′,5-pentachlorobiphenyl), and Aroclor 1254, a commercial mixture of PCBs. PCB 126 has a TEF of 0.1. Aroclor 1254 has a calculated WHO98 toxic equivalency (TEQ) of 0.000047 (Mayes *et al.*, 1998), largely contributed by PCB 126.

Studies that compared CYP1A1 induction in mammalian cells or cell lines suggest that humans are less sensitive than rats to TCDD. Some studies measured TCDD-induced 7-ethoxyresorufin-*O*-deethylase (EROD) activity in the human HepG2 cell line (Lipp *et al.*, 1992), while other studies measured EROD activity in primary human hepatocytes (Schrenk *et al.*, 1995). When the EC50s calculated in these studies were compared to those of an earlier study with rat primary hepatocytes and H4IIE cells (Schrenk *et al.*, 1991), the human cells were found to be 8–19 times less sensitive than the rat cells to TCDD. Wiebel *et al.* (1996) compared TCDD-induced CYP1A activity in human HepG2 cells and rat H4IIE

Portions of these findings were presented before the National Research Council's Committee on "EPA's Exposure and Human Health Reassessment of TCDD and Related Compounds" on March 21, 2005, at the National Academy of Sciences auditorium, Washington, D.C. and to the World Health Organization Expert Panel for the Re-evaluation of Mammalian Toxic Equivalency Factors (TEFs) of Dioxins and Dioxin-like Compounds, Public Session, June 27, 2005, WHO, Geneva, Switzerland.

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cells and found that the human cells were 20 times less sensitive. Xu *et al.* (2000) demonstrated that primary human hepatocytes were less sensitive to TCDD than primary rat hepatocytes and also reported species differences in the expression of CYP1A1 and CYP1A2 mRNA.

Few studies have measured CYP1A induction by TCDD and PCB 126 in more than one species using the same methodology. Vamvakas *et al.* (1996) tested TCDD and several PCB congeners in the HepG2 and MCF-7 human cell lines and in the rat H4IIE cell line. Zeiger *et al.* (2001) compared the induction by TCDD and several PCB congeners in primary rat hepatocytes and the rat H4IIE and human HepG2 cell lines. Recently, Peters *et al.* (2004) reported the induction of EROD activity by TCDD and PCB 126 in human MCF-7 and HepG2 cells and in rat H4IIE cells. Each of these studies showed that the human cells were less sensitive to TCDD and PCB 126 than rat-derived cells.

In addition to understanding the relative sensitivity between species to these chemicals, it is also important to determine if the REPs among these chemicals are consistent across species, especially if REPs derived from data for one species are used to extrapolate effects to other species. In fact, studies with mice (DeVito *et al.*, 2000) and human-derived cells (Zeiger *et al.*, 2001) have suggested that the REPs for several PCB congeners were inconsistent with their current WHO98 TEFs. More studies are needed to characterize how human responses to AhR ligands differ from the responses of other species while also characterizing the consistency of the relative potencies of AhR ligands across species.

Thus, to evaluate species differences in the CYP1A responses to TCDD, PCB 126, and Aroclor 1254, and to determine the REPs for PCB 126 and Aroclor 1254 in each species, we compared the responses of all cells under identical conditions. This included the use of a single lot number of each test chemical throughout the study and the preparation of only one or two stock solutions. Because immortal cell lines may not accurately reflect responses of normal human hepatocytes, we tested both fresh human hepatocytes and the HepG2 cell line. Use of donor cells also permitted an evaluation of differences among five humans. Fresh rat hepatocytes and HepG2 cells were tested to compare our methodology and results with earlier work (Lipp *et al.*, 1992; Schrenk *et al.*, 1995; Zeiger *et al.*, 2001). Hepatocytes from rhesus monkeys (*Macaca mulatta*) also were included, since both reproductive and immunological effects have been reported for this primate following exposure to PCBs (Arnold *et al.*, 1995). All four of these cell types are known to express an AhR (Roberts *et al.*, 1985, 1989, 1990). To sample the diversity of humans, we tested hepatocytes from five organ donors, two Caucasians of each gender and one African-American male.

Cells were treated over a wide concentration range of each chemical in serum-free culture medium (Hestermann *et al.*, 2000). CYP1A induction was determined after 48 h of exposure by measuring EROD activity and CYP1A mRNA. EROD activity was not obtained for one of the five donors due to experimental error. CYP1A1 and CYP1A2 mRNAs were measured in two to seven experiments for each species. Species differences were evaluated by comparing thresholds, EC50s, and maximal responses for each chemical. The potencies of PCB 126 and Aroclor 1254, relative to TCDD, were calculated for each measurement and compared across species to derive interspecies relative potency factors.

MATERIALS AND METHODS

Chemicals. TCDD (molecular weight = 322) was obtained from Accustandard (New Haven, CT; catalog no. D404N; CAS no. 1746–01–6; Lot no. 970401R-AC; 99.1% pure). The single contaminant was a pentachlorohydroxydiphenyl ether by GC/MS.

PCB 126 (molecular weight = 326.4) was obtained from Accustandard (Catalog no. C-126N; CAS no. 57465–28–8; Lot no. 081699MT-AC; 99.2% pure). The single contaminant was identified as a tetrachlorobiphenyl by GC/MS.

Aroclor 1254, lot no. 122–078 (molecular weight = 326.2) was from the same lot of material used in an earlier chronic bioassay conducted for General Electric Company (Mayes *et al.*, 1998). The calculated WHO98 TEQ (i.e., the toxic equivalency to TCDD found by summing the products of the toxic equivalency factor of each of the 12 PCB congeners with an assigned TEF multiplied by its respective measured concentration) is 47 ppm.

Hepatocyte sources. Human hepatocytes were prepared from nontransplantable human tissue acquired after informed consent for use in research by In Vitro Technologies, Inc. (IVT). An external FDA-certified Institutional Review Board approved the use of nontransplantable human tissue for ADME-Tox research at IVT. Donor 1, (IID), IVT Lot MHU-L-012303, was a 66-year-old Caucasian male who died from an intracranial hemorrhage. Donor 2, (KZO), IVT Lot FHU-L-020203, was a 42-year-old Caucasian female who died from a cerebrovascular accident. Donor 3, (WRG), IVT Lot MHU-L-052004, was a 41-year-old Caucasian male who died from an astrocytoma. Donor 4, (RFA) IVT Lot FHU-L-072004, was a 56-year-old Caucasian female who died from a cerebrovascular accident. EROD data were not collected for this donor because of experimental error but CYP1A mRNA data were obtained. Donor 5, (ZYZ) IVT Lot MHU-L-0730044, was a 46-year-old African-American male who died from anoxia. Serologies for all donors were negative for HIV, HBV, and HCV, but positive for cytomegalovirus. Urinalyses and blood chemistries for all donors were within normal limits. See www.invitrotech.com/characterizationtab.cfm for additional donor information.

Rhesus monkey hepatocytes were isolated from liver tissue from a single chemically naive young adult female for each of three trials. The tissue was purchased by IVT from approved sources that comply with all appropriate laws and guidelines.

Rat hepatocytes were isolated by IVT, from two rats (Female Crl:CD® (SD)IGS BR, Charles River Laboratories, Wilmington, MA) and pooled for each of five experiments. Rats were treated in accordance with the Animal Welfare Act.

The HepG2 human hepatoma cell line was obtained from the American Type Culture Collection, Manassas, VA.

Hepatocyte cultures. Hepatocytes were isolated according to the two-step collagenase perfusion procedure of Li *et al.* (1992). Isolated hepatocytes were counted using Trypan blue exclusion to determine yield and viability. Only hepatocyte preparations with ≥70% viability were used. Freshly isolated hepatocytes from rat, monkey, or human donors were plated, in triplicate, onto collagen-coated 24-well plates at a cell density of 3.5×10^5 cells per well in Plating Medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with bovine serum albumin, fructose, HEPES, sodium bicarbonate, L-glutamine (2.4 mM), hydrocortisone (2.38 μM), insulin (135 nM), MEM nonessential amino acids (1.2%), amikacin, penicillin (200,000 U/l), streptomycin (200 mg/l),

gentamycin, and Fungizone®). Fungizone® was excluded from HepG2 cell Plating Medium. Cultures were placed in a 37°C/5% CO_2 incubator for 2 days before use to establish the primary hepatocyte and HepG2 cell monolayers. Confluency was visually checked each day of the culture period and was generally 90–100%, except for Donor 1, for which it was 75–90%.

Chemical treatments. Each test chemical was prepared in dimethyl sulfoxide (DMSO) at 200 times (200×) the final concentration and diluted with incubation medium to the required concentrations. TCDD was soluble in DMSO at 60 μM, but not 200 μM. PCB 126 was soluble in DMSO at 2000 μM, but not 6000 μM. Aroclor 1254 was soluble in DMSO up to at least 60 mM. Two 200× stock solutions were prepared for TCDD and PCB 126. Eleven concentrations were used for each trial, in addition to the vehicle control. TCDD was used at 0.00001, 0.0001, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 10, and 100 nM. PCB 126 was used at 0.001, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 100, 1000, and 10,000 nM. Aroclor 1254 was used at 1, 3, 10, 30, 100, 300, 1000, 3000, 10,000, 30,000, and 300,000 nM.

Established primary hepatocyte and HepG2 cell cultures were treated with 500 μl of serum-free Incubation Medium (Plating Medium without serum) containing TCDD, PCB 126, or Aroclor 1254. Serum-free medium was used, since serum can significantly reduce the cellular uptake of these compounds (Hestermann *et al.*, 2000). The final concentration of DMSO in the incubations was 0.5%, consistent with previous studies with these chemicals and cells (Hestermann *et al.*, 2000; Lipp *et al.*, 1992; Zeiger *et al.*, 2001). There were no visible indications that any chemicals (or any subsets of congeners in the Aroclor 1254) had precipitated at any of the incubation concentrations, but this was not analytically confirmed. Cultures were incubated at 37°C/5% CO_2 with test chemicals for a total of 48 h. The incubation medium containing the test chemical was replaced at 24 h.

Culture viability was assessed in a replicate set of cultures for each cell type and chemical treatment by measuring the metabolic conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to the formazan product in triplicate wells (Mosmann, 1983). TCDD and PCB 126 did not affect culture viability at any concentration tested. Aroclor 1254 reduced MTT conversion in rhesus cells to approximately 50% of control levels at concentrations $\geq 10^{-5}$ M. At 3×10^{-4} M, Aroclor 1254 reduced MTT conversion to <2%, 41%, 2%, and 26% in rhesus, rat, HepG2, and 1 donor, respectively. Three donors were not affected at this concentration (data not shown).

CYP1A enzyme activity assay. EROD activity was determined using modifications of the methods of Burke *et al.* (1985) and Donato *et al.* (1993). Medium containing the test chemical was removed from the culture plates and replaced with 300 μl of Krebs-Henseleit buffer (supplemented with amikacin, calcium chloride, gentamicin, heptanoic acid, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonate), and sodium bicarbonate) containing 10 μM ethoxyresorufin and 3 mM salicylamide. Cultures were incubated at 37°C/5% CO_2 for 30 min. Incubations were terminated by removing the supernatant and adding it to 300 μl of methanol containing 2% DMSO. The amount of resorufin formed in the incubations was quantitated against a standard curve of resorufin as measured by a fluorometric assay (excitation: 530 nm; emission: 590 nm; Wallac Victor^2 plate reader, Perkin Elmer). The quantitation limit was 0.1 pmol resorufin/(min × mg protein).

Protein concentrations of the cultures were determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, ILL). Cells were lysed by adding 200 μl of 0.1 N NaOH and incubating at 37°C/5% CO_2 for 30 min. Cell lysates were scraped and harvested. Ten microliters of each cell lysate was mixed with 200 μl of BCA working reagent (reagent A:reagent B (49:1)) and incubated for 30 min at 37°C. Protein concentrations were quantified against a standard curved using absorbance at 572 nm (Wallac Victor^2 plate reader) and were generally about 0.1 mg protein/culture well.

RNA isolation. Medium containing the test chemical was removed from each well, and cultures were washed with 500 μl of phosphate buffered saline. Phosphate buffered saline was then replaced with 200 μl of TRIzol reagent (Invitrogen, Carlsbad, CA). Hepatocytes were scraped from each well with a wide-bore pipette tip, and homogenized by gently pipetting up and down. Cell homogenates were transferred to microcentrifuge tubes, and 200 μl of chloroform was added to each tube. Tubes were mixed for 15 s. Homogenates were placed on ice for 5 min and then centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous layer was carefully removed and transferred to a fresh microcentrifuge tube, and 200 μl of phenol:chloroform:isoamyl alcohol (25:24:1) was added. Tubes were mixed for 15 s. Mixtures were placed on ice for 5 min and then centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous layer was removed to a fresh microcentrifuge tube. RNA from the aqueous layer was precipitated by adding 200 μl of isopropanol and placing the tubes at −20°C overnight. Following overnight precipitation, the tubes were stored at −70°C until analysis.

RNA quantitation. The RNA samples were centrifuged at 12,000 × *g* for 15 min. Supernatants were decanted, and the RNA pellets were washed with 70% ethanol in Tris EDTA buffer. Samples were centrifuged at 12,000 × *g* for 30 min. Supernatants were decanted, and the pellets were allowed to air dry. RNA pellets were reconstituted with 50 μl of TE buffer. RNA was quantitated using a Ribogreen RNA quantitation kit (Molecular Probes, Eugene, OR). In brief, 5 μl of each RNA sample was diluted 50-fold with TE buffer. A 100-μl aliquot of the diluted RNA was mixed with 100 μl of the diluted RNA quantitation reagent and incubated for 5 min at room temperature. RNA in the samples was quantitated against a standard curve using fluorometric assay (excitation: 485 nm; emission: 535 nm; Wallac Victor^2 plate reader).

Quantitation of specific mRNA—RNA Invader® invasive cleavage assay. The RNA Invader® invasive cleavage assay is an invasive cleavage amplification assay developed by Third Wave Technologies, Inc., Madison, WI (Burczynski *et al.*, 2001; Hall *et al.*, 2000). In this method, two oligonucleotides, an upstream oligonucleotide and the probe, anneal to the target sequence. The probe contains both a target-specific and a noncomplimentary region. When the probe overlaps the upstream oligonucleotide a reporter sequence can be cleaved. The rapid turnover and production of the cleaved sequence permits a secondary reaction and the linear amplification of a fluorescent signal. This method has the ability to discriminate between two highly homologous sequences such as found with the P450s (Eis *et al.*, 2001). Total RNA isolated from each triplicate culture for each concentration and for each species was hybridized against human/rat CYP1A1 (h/rCYP1A1) or h/rCYP1A2 probes. CYP1A1 probes targeted human GeneBank Accession no. K03191 sequence (5′-CCTGATTGAGCACTGTCAGGA-3′) and rat GeneBank Accession no. NM_012540 sequence (5′-CCTCATTGAGCATTGTCTGGA-3′). CYP1A2 probes targeted human GeneBank Accession no. M55053 sequence (5′-AGGAGCACTATCAGGACTTTGACAAG-3′) and rat GeneBank Accession no. K02422 sequence (5′-AGGAACACTATCAAGACTTCAACAAG-3′). The human CYP1A1 probe was also used with rhesus hepatocytes because these two species share a consensus sequence at the targeted region. The human CYP1A2 probe could not be used for the rhesus hepatocytes because the targeted sequence is not shared, as we confirmed. CYP1A1 and CYP1A2 mRNAs are expressed as amol (10^{-18} moles) specific mRNA/ng total RNA. Human and rat GAPDH mRNAs were also measured, as reference mRNAs, and found to be consistently expressed for each chemical, all treatment concentrations, and all species (data not shown).

Each RNA sample was diluted to a maximum concentration of 10 ng/μl. If the sample concentration was below 10 ng/μl, it was used without further dilution. The amount of specific mRNA in each RNA sample was quantified using Invader® assay kits (1A1, 1A2, hGAPDH, positive control sequences, and generic reagent kits from Third Wave Technologies, Inc., Madison, WI). Specific RNA in the samples was quantified against standard curves generated using the Invader® oligo sequence test probes against positive control transcripts that included the targeted sequence in rats and humans. Fluorescence was measured as described above.

Dose-response modeling. Freshly isolated hepatocytes from the three species or the human-derived HepG2 hepatoma cell line were treated *in vitro* for 48 h. The dose-responses for EROD activity and CYP1A mRNA were modeled by combining unsummarized triplicate culture data across all experiments for each cell type at each concentration tested using the variable slope

sigmoid Hill equation (GraphPad, 2005). We defined threshold as the concentration at which the response first exceeds the model's estimated constitutive or background expression level. We estimated the threshold for each curve by determining the concentration at which a line tangent to the modeled dose-response curve at the EC05 intersects the bottom of the modeled dose-response curve, as determined by the Hill equation (Fig. 2) (GraphPad, 2005). This is an objective method to quantify the dosage at which the curve is no longer attached to its lower asymptote (i.e., the constitutive expression level). The EROD EC50 is the concentration at which the induced enzyme activity is halfway between the calculated bottom and top of each dose-response curve (GraphPad, 2005).

RESULTS

EROD Activity

EROD dose-response curves are shown in Figure 1. Species differences for each chemical are clearly evident (Fig. 1A). We conducted additional analyses of the modeled curves by examining three features of each curve: the threshold, the EC50, and the maximal response. The threshold and EC50 provide complementary information on cell sensitivity, whereas the maximal response provides information on efficacy.

For TCDD, we found the lowest thresholds in fresh rat and rhesus liver cells. Thresholds in both fresh human liver cells ($p \leq 0.05$) and HepG2 cells ($p \leq 0.05$) (Fig. 2) were about 10 times higher than in rat and rhesus liver cells. Thus, both human cell types were about 0.1 as sensitive to TCDD as were rat and rhesus cells. The EC50s show that the human cells were about 0.10 to 0.27 as sensitive to TCDD as either rat or rhesus cells (Table 1). These observations are contrary to the assumption normally used in risk assessment that humans are more sensitive than experimental animals. They also indicate that the current application of factor multipliers to compensate for uncertainties regarding species sensitivities may result in overestimating human risk by several orders of magnitude.

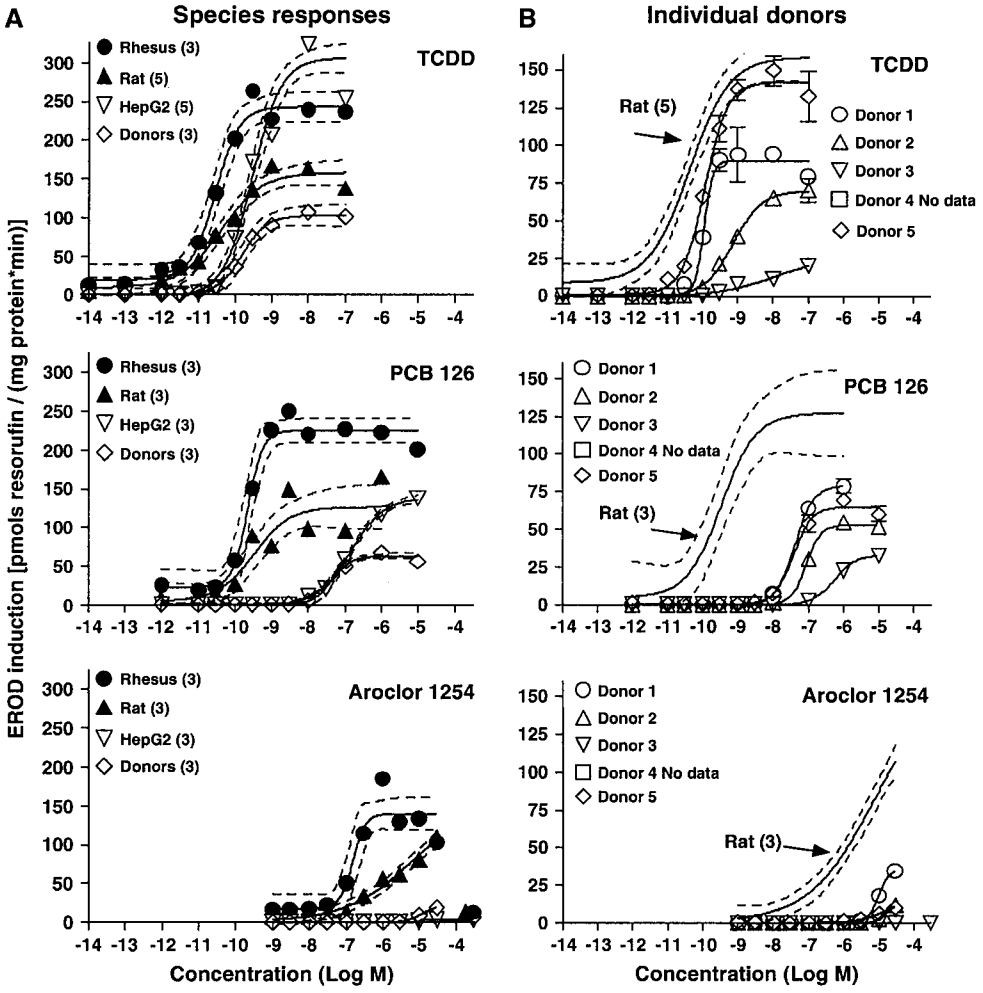

FIG. 1. Induction of CYP1A enzyme activity (EROD) in three species. (A) Dose-response showing that human cells are 10 to 100 times less sensitive than rhesus and rat cells to each chemical and that there are disproportionate changes in the potencies of these chemicals across species. Dashes represent 95% confidence limits. Parenthetical values indicate number of trials combined. (B) Responses of each donor compared to rat modeled in A showing general similarities of donors.

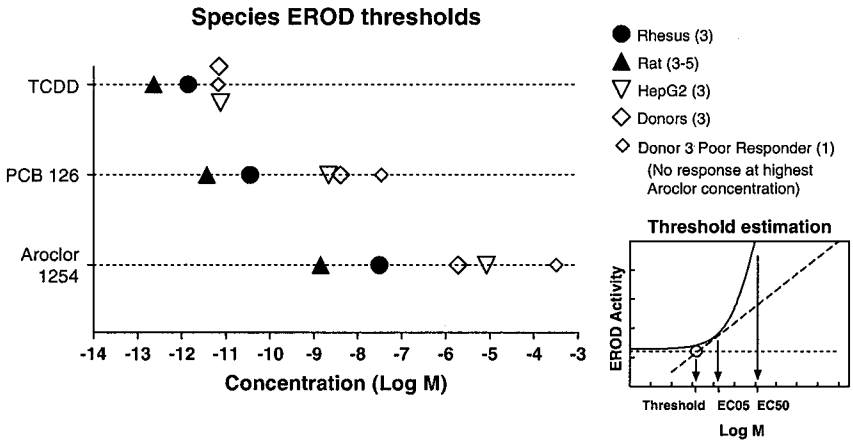

FIG. 2. Estimated thresholds for EROD induction in three species. Thresholds are estimated for TCDD, PCB 126, and Aroclor 1254 for each species by determining the concentration at which a line tangent to the fitted model curve (Fig. 1) at the EC05 response intersects the model's constitutive or background activity level (insert). Based on their statistically distinct dose-response curves, the estimated thresholds indicated that rhesus and rat cells were significantly ($p \leq 0.05$) more sensitive than human donors and the human HepG2 cell line for each chemical. Parenthetical values indicate number of combined trials. Donor 3, omitted from the modeled response in Figure 1, had a similar threshold for TCDD as the other donors and HepG2 cells but one higher for PCB 126 and one not reached for Aroclor 1254.

The maximal heights of the TCDD response curves, which measure the ability of an AhR ligand to elicit a full response, were similar for all three species (i.e., within a factor of three). This suggests that the sensitivity for EROD induction, measured by either threshold or EC50, differentiates well among species while the maximal enzyme activity level does not. This also suggests that measures of sensitivity, because they are very different among species, are more important than maximal activity levels in estimating risk across species. Because the concentrations at which thresholds, EC50s, and the maximal responses occur are similar for HepG2 and donor cells, we suggest that either type of human cells can be used to study interspecies sensitivities to chemicals, but fresh human cells have the added advantage of providing information on the extent of individual variability.

Even more pronounced differences between human cells and both rat and rhesus cells were seen when the PCB 126 EROD dose responses were compared. Rhesus cells responded in a manner more similar to rats than human cells (Fig. 1A). The thresholds and EC50s indicated that both donor and HepG2 cells were between 0.01 and 0.001 as sensitive to PCB 126 as were rat and rhesus cells (Fig. 2, Table 1). Similar species differences were observed with Aroclor 1254. Donor and HepG2 cells were $\leq$0.01 as sensitive to Aroclor 1254 as were rhesus and rat cells, based on either thresholds (Figs. 1A and 2) or estimated EC50 values (Table 1), although precise EC50s could not be calculated.

By comparing the heights of the PCB 126 response curves with those of TCDD, we see that PCB 126 is about 0.9 and 0.8 as efficacious as TCDD in rhesus and rat cells, respectively, but only 0.5 ($p \leq 0.05$) and 0.6 ($p \leq 0.05$) as efficacious as TCDD in HepG2 and donor cells, respectively. This suggests that there are species-dependent factors other than those that determine sensitivity that influence differential CYP1A1 gene expression.

In comparing the EROD response of each species to their respective TCDD response, we found that PCB 126 is about 0.1 as potent as TCDD in rat and rhesus cells. This value is consistent with the WHO98 TEF for PCB 126 of 0.1. However, PCB 126 is between 0.01 and 0.001 as potent as TCDD in donor and HepG2 cells, based on thresholds (Figs. 1A and 2) and EC50 values (Table 1).

Our data show that human cells are about 0.1 as sensitive to TCDD as rats and rhesus cells. Our data also show that human cells are 0.001 or less as sensitive to PCB 126 as rat and rhesus cells are to TCDD and less than 0.000001 as sensitive to Aroclor 1254 as rats are to TCDD (Table 1). The human EROD responses to TCDD, PCB 126, and Aroclor 1254 are compared to only the responses of rats to TCDD in Figure 3 to facilitate the direct comparison of their EROD dose-response curves and EC50s in Tables 1 and 2.

Human Diversity in EROD Response

The diversity of human responsiveness, characterized by both sensitivity and maximal response, is an important concern for risk managers responsible for protecting sensitive populations. An earlier report suggests that humans have similar sensitivities but variable maximal responses to TCDD (Schrenk *et al.*, 1995). In our study, we found similar thresholds for Donors 1, 2, 3, and 5 that were within an 11-fold concentration range (Fig. 1A) (calculation not shown). The EC50s of Donors 1, 2, 3, and 5 were within a seven-fold range, i.e., 1.1×10^{-10} M (8.9×10^{-11} M to 1.3×10^{-10} M, $r^2 = 0.95$); 7.3×10^{-10} M (5.7×10^{-10} M to 9.3×10^{-10} M, $r^2 = 0.98$); $\geq 1.2 \times 10^{-8}$

TABLE 1
EC50s for CYP1A Induction in Fresh Hepatocytes from Three Species and a Human Cell Line

Chemical	Measurement	EC50 (M) for CYP1A Induction						
		Rhesus	Rat	HepG2		Donors		
TCDD	**EROD activity**	$\mathbf{2.8 \times 10^{-11}}$	$\mathbf{4.1 \times 10^{-11}}$	$\mathbf{2.9 \times 10^{-10}}$	*a,b*	$\mathbf{1.5 \times 10^{-10}}$	*a,b*	(4)
	95% CL	1.8×10^{-11} to 4.4×10^{-11}	2.3×10^{-11} to 7.4×10^{-11}	2.2×10^{-10} to 3.9×10^{-10}		9.3×10^{-11} to 2.6×10^{-10}		
	CYP1A1 mRNA	$\mathbf{9.6 \times 10^{-12}}$	$\mathbf{2.7 \times 10^{-12}}$	$\mathbf{2.0 \times 10^{-10}}$	*a,b*	$\mathbf{1.4 \times 10^{-10}}$	*a,b*	(4)
	95% CL	1.4×10^{-12} to 6.2×10^{-11}	1.2×10^{-12} to 5.9×10^{-12}	1.2×10^{-10} to 2.7×10^{-10}		8.7×10^{-11} to 2.1×10^{-10}		
	CYP1A2 mRNA	NE	$\mathbf{3.6 \times 10^{-11}}$	NR		$\mathbf{2.0 \times 10^{-10}}$		(5)
	95% CL		4.4×10^{-12} to 2.9×10^{-10}			1.1×10^{-10} to 3.5×10^{-10}		
PCB 126	**EROD activity**	$\mathbf{2.2 \times 10^{-10}}$	$\mathbf{3.3 \times 10^{-10}}$	$\mathbf{1.5 \times 10^{-7}}$	*a,b*	$\mathbf{4.5 \times 10^{-8}}$	*a,b*	(4)
	95% CL	1.5×10^{-10} to 3.2×10^{-10}	1.1×10^{-10} to 9.9×10^{-10}	1.2×10^{-7} to 1.7×10^{-7}		3.3×10^{-8} to 6.0×10^{-8}		
	CYP1A1 mRNA	$\mathbf{3.0 \times 10^{-10}}$	$\mathbf{1.5 \times 10^{-9}}$	$\mathbf{8.0 \times 10^{-8}}$	*a,b*	$\mathbf{1.6 \times 10^{-6}}$	*a,b*	(4)
	95% CL	9.2×10^{-11} to 9.7×10^{-10}	5.4×10^{-10} to 4.3×10^{-9}	8.3×10^{-10} to 7.7×10^{-6}	*c*	1.1×10^{-8} to 2.3×10^{-4}	*c*	
	CYP1A2 mRNA	NE	$\mathbf{1.4 \times 10^{-7}}$	NR		$\mathbf{\geq 3.7 \times 10^{-7}}$		(5)
	95% CL		4.1×10^{-8} to 4.5×10^{-7}			6.4×10^{-8} to 2.2×10^{-6}		
Aroclor 1254	**EROD activity**	$\mathbf{1.5 \times 10^{-7}}$	$\mathbf{\geq 2.7 \times 10^{-6}}$	$\mathbf{> 3.0 \times 10^{-4}}$	*a,b,d*	$\mathbf{\geq 4.8 \times 10^{-5}}$	*a,b,d,e*	(3)
	95% CL	8.7×10^{-8} to 2.7×10^{-7}						
	CYP1A1 mRNA	$\mathbf{6.3 \times 10^{-8}}$	$\mathbf{7.0 \times 10^{-7}}$	NR		$\mathbf{\geq 3.9 \times 10^{-5}}$	*a,b,d,e*	(1)
	95% CL		2.8×10^{-7} to 1.8×10^{-6}			3.5×10^{-5} to 4.3×10^{-5}		
	CYP1A2 mRNA	NE	$\mathbf{1.0 \times 10^{-6}}$	NR		$\mathbf{8.6 \times 10^{-6}}$		(3)
	95% CL		LR			3.9×10^{-6} to 1.9×10^{-5}		

Note. CL, confidence limits. NE, not evaluated. NR, no dose-response detected; LR, low response. Parenthetical numbers indicate number of responding donors.
[a]Significantly different from rat at $p \leq 0.05$.
[b]Significantly different from rhesus at $p \leq 0.05$.
[c]See Fig. 4.
[d]EC50 above highest tested concentration.
[e]Estimated lowest EC50 value but cells not responsive above 3×10^{-5}M.

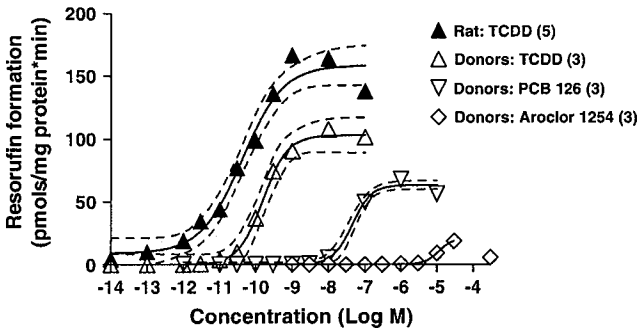

FIG. 3. Comparison of human donor hepatocyte EROD responses to the rat response to TCDD. Compared to rat cells, human cells are about 0.1 as sensitive to TCDD, ≤0.001 as sensitive to PCB 126, and <0.000001 as sensitive to Aroclor 1254.

(1.9×10^{-9} to 7.1×10^{-8}, $r^2 = 0.95$ [estimated because the maximal response could not be established with certainty]); and 1.1×10^{-10} M (8.5×10^{-11} M to 1.5×10^{-10} M, $r^2 = 0.97$), respectively. The maximal responses of Donors 1, 2, and 5 varied by less than 2-fold for each chemical (Fig. 1B). Donor 3 was a poor responder to both TCDD and PCB 126 and had no measurable response to Aroclor 1254. This donor was excluded from the aggregate model shown in Fig. 1A (thus avoiding a shift in the curve further to the right) but is shown separately in Fig. 1B. Compared to rats, all donors responded poorly to Aroclor 1254. Our findings that sensitivity differences, measured by either threshold or EC50s, span over three orders of magnitude between human and rat cells, but only vary by a factor of about ten among the human samples, suggest that species differences are a more significant source than individual differences for the uncertainty in risk estimation.

CYP1A mRNA Induction

Measurement of CYP1A1 mRNA serves as an indicator of *Ah* gene expression before any posttranslational modifications or interferences occur that could affect protein levels and/or function (Staskal *et al.*, 2005). Furthermore, mRNA levels provide a way to measure the competency of ligands with different AhR binding affinities to activate *Ah* gene expression (Chen *et al.*, 2004). Thus, mRNA levels could provide additional information on species differences not provided by EROD activity alone. Since both CYP1A1 and CYP1A2 express EROD activity (Nerurkar *et al.*, 1993), we measured CYP1A1 and CYP1A2 mRNA in a replicate set of cultures treated as described above using the RNA Invader® invasive cleavage assay (Eis *et al.*, 2001).

The induction profile of CYP1A1 mRNA for each chemical (Fig. 4A) was generally consistent with that of EROD activity for each species (Fig. 1). Interestingly, the induction of CYP1A1 mRNA by TCDD in rats, the most sensitive responder, was observed at concentrations about 10 times lower than seen for EROD activity. This has been observed by others who suggested that it was due to differences in the detection sensitivities of mRNA and enzyme activity (Vanden Heuvel *et al.*, 1994). Because this was not observed in human and rhesus monkey cells for TCDD, or for PCB 126 or Aroclor 1254 in any species, our data suggest that there are aspects of TCDD induction and of the regulation of CYP1A1 that are unique to the rat that require further investigation. Consistent with the species differences observed for EROD activity,

TABLE 2
Relative Potency Values (REPs)

Chemical	Measurement	REP	Within species (ws) and rat-human (r-h) relative potency factors (REPs)[a]			
			Rhesus	Rat	HepG2	Donors
TCDD	EROD activity	ws	1	1	1	1
		r-h			0.14	0.27
	CYP1A1 mRNA	ws	1	1	1	1
		r-h			0.01	0.02
PCB 126	EROD activity	ws	0.13	0.12	0.002	0.003
		r-h			0.0003	0.0009
	CYP1A1 mRNA	ws	0.03	0.002	0.002	0.00009
		r-h			0.00003	0.000002
Aroclor 1254	EROD activity	ws	1.8×10^{-4}	1.5×10^{-5}	$<9.8 \times 10^{-7}$	3.2×10^{-6}
		r-h			$<1.4 \times 10^{-7}$	8.6×10^{-7}
	CYP1A1 mRNA	ws	1.5×10^{-4}	3.9×10^{-6}	NR[b]	$\leq 3.5 \times 10^{-6}$
		r-h			NR[b]	$\leq 7.0 \times 10^{-8}$

[a]Within species (ws) and rat-human (r-h) interspecies REPs were calculated by dividing the EC50 for each chemical into the EC50 for TCDD in the same species or into the EC50 for TCDD in the rat, respectively.

[b]NR, no dose response.

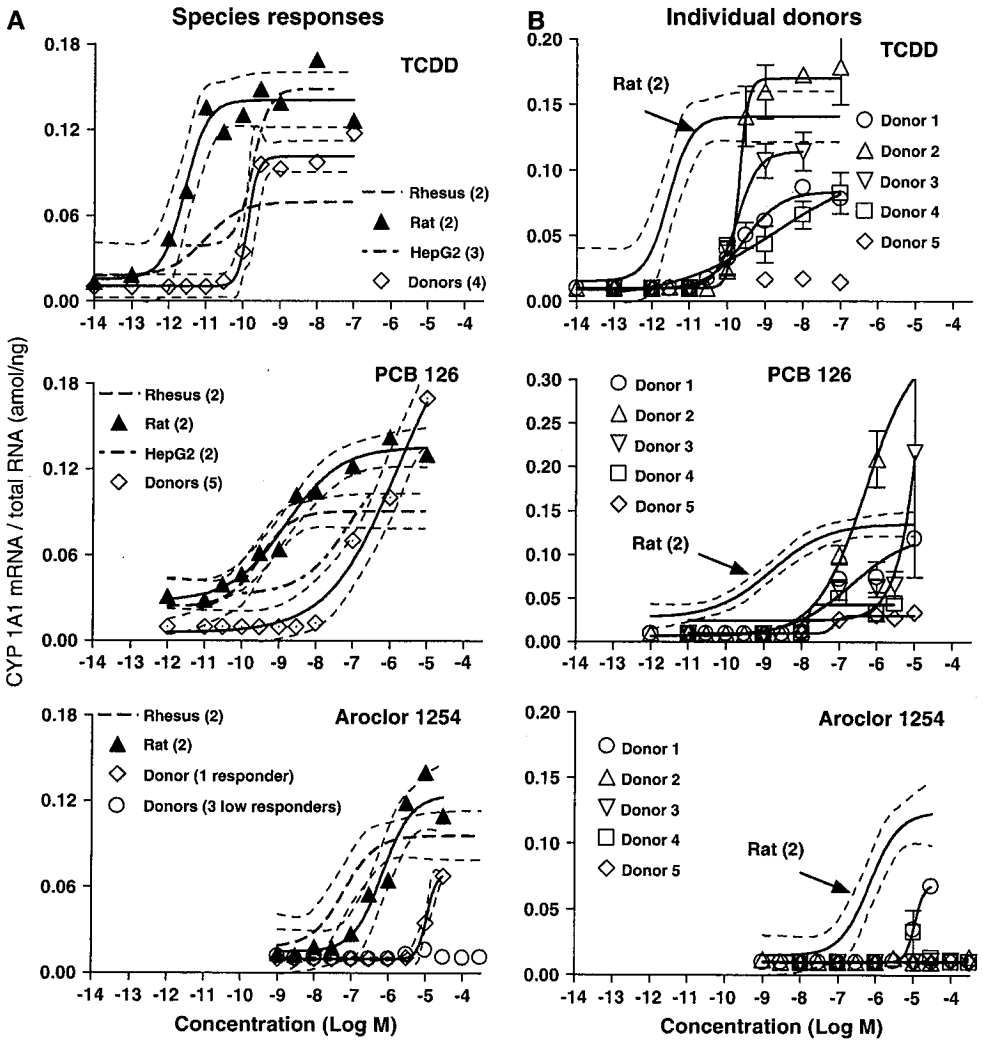

FIG. 4. Induction of CYP1A1 mRNA by TCDD, PCB 126, and Aroclor 1254. (A) Human cells (including Donor 4 but excluding Donor 5 cells, as they were nearly nonresponsive) are significantly less sensitive than rat and rhesus cells to each chemical tested. Responses were generally consistent with their respective functional assessments of CYP1A1 protein measured by EROD activity (Fig. 1). Dashes represent 95% confidence limits. Parenthetical numbers indicate number of trials combined. (B) CYP1A1 mRNA response profiles for individual donors compared to rat profile modeled in A. Cytotoxicity was observed only for Aroclor 1254 at or above $10^{-3.5}$ M.

human cells required 100 to 1000 times higher concentrations of each chemical than rat and rhesus cells to induce CYP1A1 mRNA (Fig. 4A). This suggests that such differences are due to early events in AhR binding and/or DNA activation occurring up through mRNA transcription. Our results are also consistent with a recent investigation that showed a diminished CYP1A mRNA response when the *AhR* gene in mice was replaced with human *AhR* cDNA (Moriguchi *et al.*, 2003), which the authors suggested might be due to structural differences in the AhR itself. Regardless of the explanation, the absence of such early induction events in human cells at exposure levels that elicit responses in other species suggests that animal models are overly sensitive when predicting human responses associated with *Ah* gene activation.

Individual variations in the AhR structure and function have also been described for several human cells (Cook *et al.*, 1987; Micka *et al.*, 1997). However, in our study, the CYP1A1 mRNA responses of four of the five donors were similar (Fig. 4B). These donors responded to TCDD at approximately 10^{-10} M and to PCB 126 between 10^{-8} and 10^{-7} M, and the respective responses reached amplitudes that were generally within a factor of three of one another. In contrast, the CYP1A1 mRNA responses of Donor 5 to all three chemicals were very low—but the CYP1A2 mRNA responses were robust (Fig. 5).

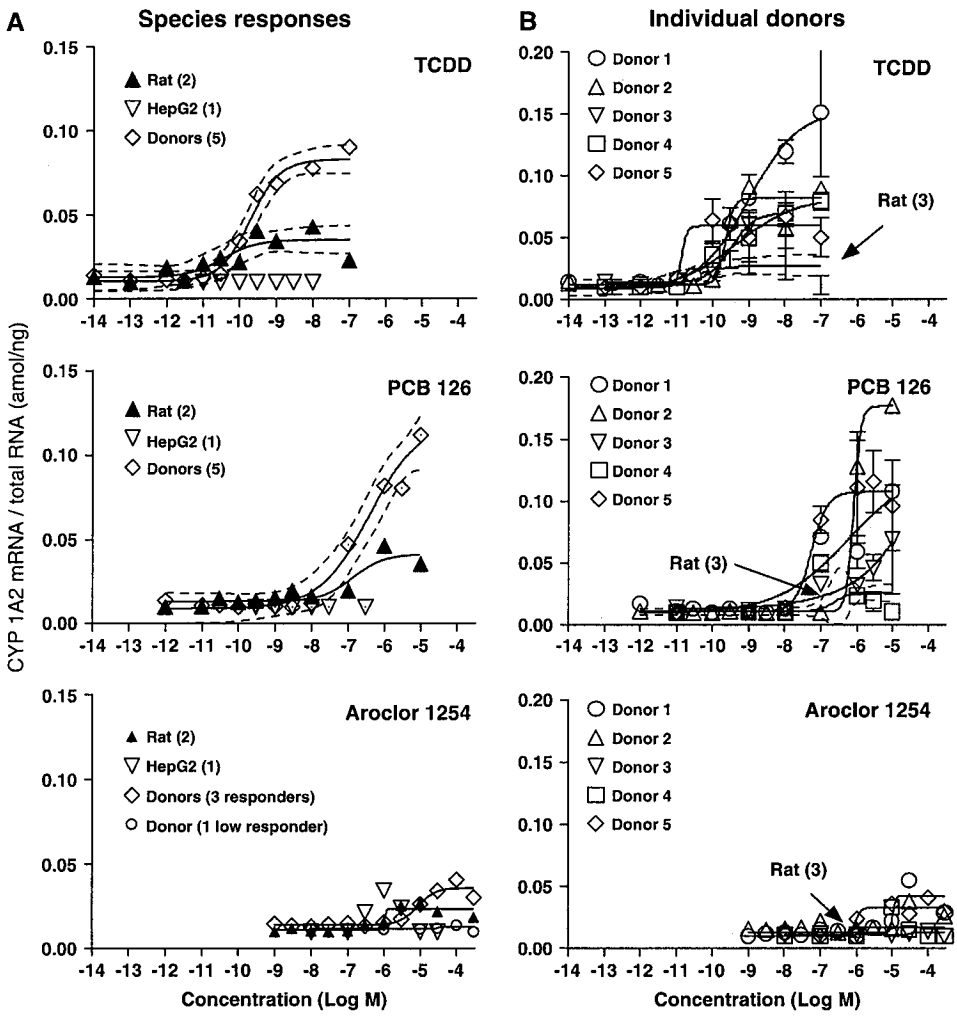

FIG. 5. Induction of CYP1A2 mRNA by TCDD, PCB 126, or Aroclor 1254. mRNA was measured using the RNA Invader® invasive cleavage assay. (A) CYP1A2 mRNA expression of donor cells was nearly equivalent to the CYP1A1 response shown in Figure 4, whereas the rat response was predominantly CYP1A1. CYP1A2 mRNA was not clearly detectable in HepG2 cells. Dashed lines indicate the 95% confidence limits. Numbers in parentheses indicate number of trials combined. (B) Responses of each donor are compared to rats modeled in A showing general similarity among donors.

Consistent with the Aroclor 1254 EROD results, only Donor 1 had a robust Aroclor 1254 CYP1A1 mRNA response, and the responses of the other four donors were barely detectable.

The regulation of both CYP1A1 and CYP1A2 mRNA induction seem to be similar in donor cells but dissimilar in rat cells (Figs. 4 and 5, Table 1). Donor cells responded to TCDD with nearly equivalent amounts of both CYP1A1 and CYP1A2 mRNA over the same concentration range. In rats, however, CYP1A2 mRNA induction was about 0.1 as sensitive as CYP1A1 mRNA induction, and the maximal CYP1A2 mRNA levels were only about 25% of CYP1A1 levels. This predominance of CYP1A2 over CYP1A1 in cultures of rat cells is consistent with earlier work (Xu *et al.*, 2000). Interestingly, both proteins have been shown to be equally induced by TCDD in a long-term rat *in vivo* study (Walker *et al.*, 1999). The CYP1A2 mRNA EC50s shown in Table 1 indicate that rat cells were about six and three times more sensitive than donor cells to CYP1A2 induction by both TCDD and PCB 126, respectively. These findings are consistent with reports that CYP1A1 and CYP1A2 may be differently regulated in rats (Drahushuk *et al.*, 1999; Santostefano *et al.*, 1997), but also indicate that there are important differences in their respective regulation between humans and rats.

CYP1A2 mRNA was barely detectable in HepG2 cells, which is consistent with earlier reports (Chung *et al.*, 1994; Li *et al.*, 1998; Vakharia *et al.*, 2001). Our finding that CYP1A1 and CYP1A2 mRNAs are strongly and similarly induced in fresh human hepatocytes demonstrates the value of using primary human cells, rather than cell lines, in studying the expression of these two AhR-associated genes.

We calculated new EROD- and CYP1A1 mRNA-based REPs for PCB 126 using the data from our study (Table 2). REPs are typically calculated by dividing the EC50 for TCDD, the reference ligand, by the EC50 for the AhR ligand of interest with both values from the same species. For PCB 126, the EROD-based REPs in rhesus monkeys and rats were 0.13 and 0.12, respectively, consistent with the WHO98 TEF of 0.1. However, the EROD-based REP for PCB 126 for human donor cells was 0.003 and only 0.002 for HepG2 cells. These values are consistent with earlier work with HepG2 cells (Zeiger *et al.*, 2001) and much lower than the WHO98 TEF of 0.1. This clearly demonstrates the inadequacy of the current TEQ approach to account for the possibility that each species may have its own unique set of REPs for different AhR ligands.

To account for the accumulating evidence not only that humans and rats may have different sensitivities to TCDD (Lipp *et al.*, 1992; Schrenk *et al.*, 1995; Vamvakas *et al.*, 1996; Wiebel *et al.*, 1996; Xu *et al.*, 2000; Zeiger *et al.*, 2001), but also that humans may have a unique set of REPs, we then calculated human REPs relative to the rat TCDD response (r-h REP) (Table 2, Fig. 6). This approach uses data from both species and is more consistent with the empirical evidence that humans and rats have different sensitivities to TCDD and other AhR ligands than using only rodent-derived data. The results show, based on EROD induction, that an appropriate r-h REP for PCB 126 is 0.0009, compared to the WHO98 TEF of 0.1. Even lower REP values were observed for CYP1A1 mRNA (Table 2).

Using the same approach for Aroclor 1254, we calculated an EROD-based r-h REP for human donor liver cells that is at least 54 times lower than predicted using the WHO98 TEFs (Table 2, Fig. 6). Comparable differences were also seen for Aroclor 1254 CYP1A1 mRNA data, with human donor cells 600 times less sensitive than predicted by the WHO98 TEF. Both assays also suggest that rhesus monkeys are more sensitive than rats to

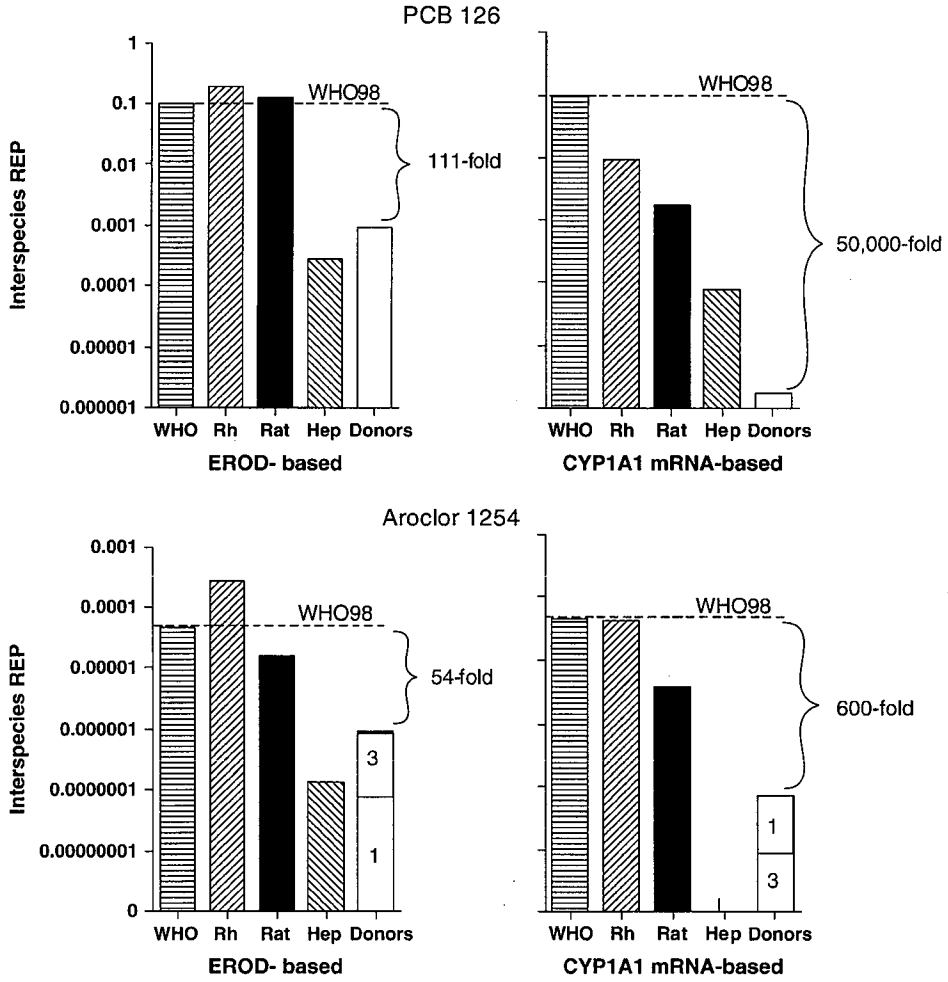

FIG. 6. The interspecies relative potency factor (REP) for each species for each chemical is the ratio of the rat TCDD EC50 to the EC50 for that species. The dashed bar labeled WHO98 represents the current WHO TEF for PCB 126 and calculated for Aroclor 1254. Number of donors responding at or below bar height REPs is shown within bars.

Aroclor 1254 and indicate that neither rhesus monkeys, although primates, nor rats are good models for humans for AhR ligand-based risk assessments for this complex mixture.

DISCUSSION

Our findings show orders of magnitude species differences in sensitivity to TCDD and PCBs and highlight the substantial uncertainties that arise when using experimental animal data to extrapolate to potential human health risks. These findings may help to explain the lack of conclusive evidence that PCBs have affected human health (Kimbrough *et al.*, 2003). To the extent that AhR-mediated events are used to predict human risk, our data demonstrate a need to compensate for the species differences in sensitivity. Interspecies relative potency factors can partially compensate for our observations that humans may be less sensitive than animals to TCDD and PCBs and that the relative potencies of these chemicals in humans may be quite different than those observed in animals. Hepatocytes from ten additional human donors have been tested for EROD responsiveness to TCDD and PCB 126. The additional results are consistent with the findings reported in this study and support our conclusions (Koganti, personal communication). Additional work with human cells is clearly needed.

ACKNOWLEDGMENT

Conflict of interest: Five of the six authors are employed by General Electric Company. This work was wholly funded by General Electric Company.

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Attachment J-7

Arch Toxicol
DOI 10.1007/s00204-008-0317-7

TOXICOKINETICS AND METABOLISM

Pharmacologic profiling of human and rat cytochrome P450 1A1 and 1A2 induction and competition

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Received: 8 January 2008 / Accepted: 6 May 2008

Abstract Strong activation of the AhR can lead to various toxic effects such as (non-genotoxic) carcinogenicity. Moreover, drug–drug interactions by non- or competitive inhibition of CYP1A1 and 1A2 may cause adverse side effects. Normally the majority of toxicity studies are performed in rats, while for the prediction of human toxicity human AhR activation and CYP1A competition should be studied. The present study focused on the deselection of strong AhR activators and/or CYP1A inducers and (non-)competitive inhibitors in the early phase of drug development, as well as on species differences between humans and rats. Induction studies were performed in the human HepG2 and rat H4IIE cell lines. A set of 119 compounds, including known AhR ligands were tested. CYP1A induction was observed for 24 compounds. In H4IIE cells, more compounds showed induction and most EC50 values were below those of HepG2 cells. Species specific CYP1A induction in H4IIE and HepG2 cells was obtained for eight and three compounds, respectively. The same compounds except four in-house NCEs were used to study differences between CYP1A1 and 1A2 competition in human and rat supersomes. Of the 115 compounds 46 showed CYP1A1 competition. Competition was human and rat specific for 12 and 10 compounds, respectively. CYP1A2 competition was observed for 37 compounds of which 14 and 3 compounds showed human and rat specific inhibition, respectively. In conclusion, for several compounds species differences between CYP1A induction and competition in human and rat were found. Therefore, parallel screening in both species might be a very useful strategy.

Keywords Aryl hydrocarbon receptor · CYP1A · Induction · Competition · HepG2 · H4IIE

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Introduction

In drug therapy, cytochrome P450 (CYP) 1A induction may lead to undesirable drug–drug interactions and toxic side effects. The regulation of CYP1A is mainly aryl hydrocarbon receptor mediated (AhR). The AhR was discovered as the receptor that binds the environmental contaminant TCDD (Bertazzi et al. 1998). The AhR is a basic helix-loop-helix protein belonging to the Per-ARNT-Sim (PAS) family (Hahn 2002) and is located in the cytoplasm in an inactive complex with Hsp90 and p23. Experiments in Hsp90 deficient yeast show that Hsp90 is needed for gene induction by the AhR (Carver et al. 1994; Whitelaw et al. 1995). Hsp90 is thought to protect AhR from degradation and to stabilize the high-affinity ligand-binding conformation of the AhR (Pongratz et al. 1992a, b). Binding of a ligand to the AhR leads to activation of the receptor and subsequently in translocation to the nucleus. In the nucleus AhR releases its partner Hsp90 and forms a heterodimer with the AhR nuclear translocator (ARNT) protein. Further activation of this heterodimer by phosphorylation and/or dephosphorylation is required for DNA binding (Chen and Tukey 1996; Long et al. 1998).

Strong activation of the AhR by TCDD results in toxic effects like a wasting syndrome, thymic involution, endocrine disorders, and very important teratogenicity and (non-genotoxic) carcinogenicity. Mortality studies involving occupational exposure to TCDD have demonstrated an

increased risk for a lot of cancers in humans (Fingerhut et al. 1991; Manz et al. 1991; Zober et al. 1990).

Important for this non-genotoxic carcinogenic effect might be the change in expression patterns of several factors that are involved in cellular growth and differentiation. Another effect contributing to carcinogenicity might be the impairment of the p53 response (Paajarvi et al. 2005; Ray and Swanson 2004).

Studies in AhR-null mice have been used to investigate the role of the AhR in mediating the toxic effects. The studies revealed that these mice were resistant to TCDD induced lesions, strongly suggesting that the toxic effects are mediated by the AhR (Fernandez-Salguero et al. 1996). Furthermore, skin tumors that appear after topical application of the AhR agonist benzo[a]pyrene (B[a]P) are not present in AhR-null mice (Shimizu et al. 2000). Besides activating the AhR, B[a]P is metabolized by CYP1A1 to a genotoxic metabolite (Jack and Brookes 1981). The metabolic activation of procarcinogens to reactive metabolites by CYP1A1 and 1A2 was also shown for other AhR agonists by using Aroclor pretreated rat liver S9-mixture for metabolic activation in the Ames test. CYP1A1 and/or 1A2 activates PAHs, nitrosamines and aryl amines into reactive metabolites that induce mutations (Hecht 1998). Induction of CYP1A enzyme activity is often used for the detection of AhR ligands as good specific CYP1A substrates are available.

Besides metabolic activation into genotoxic compounds, drug-drug interactions caused by non- or competitive-inhibition of CYP1A1 and 1A2 can also lead to adverse side effects. A lot of drugs are metabolized by CYP1A2 and therefore drug-drug interactions caused by CYP1A2 inhibition are known. CYP1A2 constitutes about 13% of the total hepatic CYP content and is one of the clinically most relevant CYP isoenzymes. Together with CYP2C9, 2C19, 2D6, and 3A4 it performs the main part of drug metabolism. Examples of drugs metabolized by CYP1A2 are the antidepressants amitriptyline HCl, clomipramine HCl, and desipramine HCl (Danie et al. 2001; Wu et al. 1998; Yoshimoto et al. 1995). The levels of CYP1A1 in the human liver are low, however, this enzyme is highly inducible in the liver and extra hepatic tissues. Induction of CYP1A1 by AhR agonists in human precision-cut liver slices and primary human hepatocytes has been shown in several studies (Drahushuk et al. 1998; Olinga et al. 2008; Pushparajah et al. 2008; Dvorak et al. 2008). Little is known about drug–drug interactions in which CYP1A1 is involved. However, some of the drug–drug interactions caused by ketoconazole might be the result of a lower CYP1A1 enzyme activity caused by inhibition or competition in the intestine (Paine et al. 1999). Furthermore synergistic embryotoxicity of AhR agonists with CYP1A1 inhibitors has been shown (Wassenberg and Di Giulio 2004). This was probably caused by a prolonged activation of the AhR.

Because of the adverse side effects it might be very useful to screen for and deselect candidate drugs that are strong AhR activators and/or strong CYP1A1 and 1A2 inhibitors/competitors in the early phase of drug development.

Induction of CYP1A enzyme activity was measured with the fluorogenic substrate 3-Cyano-7-ethoxycoumarin (CEC) and luminogenic P450-glo substrate Luciferin-CEE (Luc-CEE). The latter substrate is converted by CYP1A1 into luciferin, which in turn reacts with luciferase to produce light that is directly proportional to the activity of CYP1A1.

Because species differences have been described between the CYP1A inducing properties of AhR agonists in rats and humans (Zeiger et al. 2001), the CEC and the P450-glo 1A1 induction assays were performed in the human HepG2 and the rat H4IIE cell line.

CYP1A1 and 1A2 inhibition was measured in a 384 well high-throughput assay using CYP1A1 and 1A2 expressing supersomes and CEC as fluorogenic substrate. With these assays competition was measured between the fluorogenic substrate CEC and the tested compounds for CYP1A1 and 1A2. Whether compounds are non- or competitive CYP1A1/1A2 inhibitors cannot be determined with these assays. Moreover, in order to study species differences, competition experiments were performed by using both human and rat supersomes. A total of approximately 119 compounds were tested in the CYP1A induction and competition assays. These compounds included narcotic analgesics, hypnotics, vasodilators, specific cellular energy blockers, cellular proliferation inhibitors, ion channel blockers, estrogens, antiestrogens, androgens, progestagens, PCBs, and others.

Materials and methods

Materials

All compounds and reagents were of analytical grade. Polychlorinated biphenyls (PCBs) including 2,3',4,4'-tetrachlorobiphenyl (PCB 77), 2,3,3',4,4'-pentachlorobiphenyl (PCB 105), 2,3,4,4',5 pentachlorobiphenyl (PCB 114), 2,3',4,4',5'-pentachlorobiphenyl (PCB 118), 2,3',4,4',5'-pentachlorobiphenyl (PCB 123), 3,3',4,4',5'-pentachlorobiphenyl (PCB 126), 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156), 2,3,3',4,4',5'-hexachlorobiphenyl (PCB 157), 2,3',4,4',5,5'-hexachlorobiphenyl (PCB 167), 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169), 2,3,3',4,4',5,5'-heptachlorobiphenyl (PCB 189), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were purchased from Promochem (Wesel, Germany). All other compounds were from Sigma–Aldrich

(St. Louis, USA). Luciferin-CEE (Luc-CEE) was from Promega (Madison, USA). CEC and supersomes expressing human and rat CYP1A1 and 1A2 were from BD Biosciences (San Jose, USA). Trypsin and Dulbecco's Modified Eagles medium, Nutrient Mixture F-12 (DMEM/HAM F12 medium in a ratio of 1:1) without phenol red was from Invitrogen (Kalsruhe, Germany), defined supplemented bovine calf serum (dBCS) from Hyclone (Utah, USA) and white 96 and 384 well culture plates from Perkin-Elmer (Groningen, The Netherlands).

Cell culture

HepG2 and H4IIE cells were obtained from the American type culture collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified eagles medium and nutrient mixture F-12 mixed in a ratio of 1:1 with 10% dBCS and 1% penicillin–streptomycin (10,000 U/ml, Invitrogen). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C and medium was refreshed every 3 or 4 days with subculturing.

Preparation of compound solutions

Stock solutions of 0.1 M were dissolved in 100% dimethyl sulfoxide (DMSO). The stock solution of TCDD had a concentration of 3.16×10^{-4} M. From the stock, $\sqrt{10}$ dilutions were prepared in DMSO.

Test plate preparation induction assays

HepG2 and H4IIE cells were trypsinized, counted and seeded in 96 well plates. HepG2 and H4IIE cells were resuspended in culture medium to a final concentration of 3×10^4 and 2×10^4 cells/well, respectively. The 96 well microtiter plates were incubated for 24 h in a humidified atmosphere at 37°C under 5% CO_2. Following the incubation, 11 serial culture medium dilutions of the compounds or a control sample were added as 10 µl fractions to HepG2 and H4IIE cells leading to a final volume of 200 µl. The highest tested concentration for the compounds was 10^{-4} M. An exception was TCDD for which the highest tested concentration was 3.16×10^{-7} M. The final concentration of DMSO in the assays was 0.1%.

CEC induction assay

The induction of CYP1A activity in HepG2 and H4IIE cells was measured with the fluorogenic substrate CEC. CEC reacts with both CYP1A1 and 1A2. After incubation of cells with compounds for 24 h, cells were washed twice with PBS. Next 100 µl of 40 µM CEC dissolved in culture medium without dBCS was added. After 30 min the fluorescent signal was read on the Victor II (Perkin-Elmer) by means of excitation at 409 nm and emission measurement at 460 nm.

P450-Glo CYP1A1 induction assay

The induction of CYP1A1 activity in HepG2 and H4IIE cells was measured with the luminogenic substrate Luc-CEE. After incubation of cells with compounds for 24 h, HepG2 and H4IIE cells were washed twice with PBS and the CYP1A1 induction was assessed with Luc-CEE. To the plates 50 µl Luc-CEE (30 µM) was added. Subsequently, 50 µl luciferin detection reagent was added. Plates were shaken for 10 min and the luminescence signal was measured with a TopCountNT luminometer (Perkin-Elmer).

Cytochrome P450 1A1 and 1A2 competition assays

CYP1A1 and 1A2 competition assays were carried out by using supersomes. Compounds were tested at concentrations of 10^{-7}–10^{-4} M with $\sqrt{10}$-fold dilution steps in a 384 well plate in 0.1% DMSO. The highest concentration TCDD in the assay was 3.16×10^{-7} M. The cofactor solution was prepared in 25 mM phosphate buffer (pH 7.4) and contained 2.6 mM $NADP^+$, 6.6 mM glucose-6-phosphate, 6.6 mM $MgCl_2$ and 0.8 U/ml glucose-6-phosphate dehydrogenase. This solution was prewarmed at 37°C. Five minutes before the start of the reaction human or rat CYP1A1 or 1A2 supersomes were added to the cofactor solution leading to a concentration of 5 pmol/ml supersomes in the final reaction. To the 384 well plates, containing 10 µl compound dilution or DMSO control, 10 µl of a substrate solution containing 20 µM CEC in 325 mM phosphate buffer was added. The plates were covered with a lid and shaken for 20 min. Then plates were pre-warmed at 37°C in an incubator and 20 µl of an enzyme/cofactor solution was added leading to a final volume of 40 µl/well. Thereafter the plate was put in the Victor II reader, shaken for 20 s and pre-incubated for 2 min at 37°C. Next the fluorescence was measured ($t = 0$ min). The excitation wavelength was set at 409 nm and the emission was measured at 460 nm. Then the plate was incubated for 30 min at 37°C. Thereafter the fluorescence was measured ($t = 30$ min). The difference in fluorescence between the measurement at 0 and 30 min was used for calculation of CYP competition. The increase in fluorescence of the solvent control (0.1% DMSO) during these 30 min was set at 100% activity. For calculation of the IC50 values of competitors, the data were logit transformed. The software program Xlfit (version 4.1, ID business solutions limited) was used for the calculation of the best line fitting the experimental data. From this line the IC50 was determined. The efficacy was defined in percentage by means of the total inhibition of the fluorescence

increase at the highest tested compound concentration. This concentration was 10^{-4} M, with exception for TCDD for which the highest concentration tested was 3.16×10^{-7} M.

Statistical analysis

Each experiment was performed in duplicate in three independent experiments. Data analysis was performed by using a Student's *t* test. This indicated that an induction above control level of 20 and 50% for the CEC and P450-glo assay, respectively, was statistical significant. For the assessment of this threshold 5 independent experiments were performed with 3-methylcholanthrene, indirubin, and indigo (data not shown). Since compounds with an induction factor equal to or greater than 2-fold are defined as inducers this is based on statistically significant differences. Likewise five independent competition assays were carried out with furafylline and ketoconazole. The Student's *t* test showed that an inhibition of 20% was statistically significant. Therefore the IC50 values are based on statistically significant differences.

Results

Cytochrome P450 1A induction measured with CEC

HepG2 and H4IIE cells were exposed for 24 h to a dose-range of 119 different compounds. Thereafter the CYP1A activity was measured with CEC. Exposure to 95 of these compounds did not affect CYP1A activity (Table 1). However, 24 compounds caused an induction of the CYP1A activity in either HepG2, H4IIE, or both cell lines. These compounds are listed in Table 2 that shows also the EC50 values and induction factors (ratio of treated cells:0.1% DMSO control). Dose response curves are presented in Fig. 1.

The results clearly show that there was a difference between CYP1A induction in the human HepG2 and rat H4IIE cell line. More compounds caused CYP1A induction in H4IIE cells and most EC50 values were lower than in HepG2 cells. TCDD was as expected the most potent CYP1A inducer in both H4IIE and HepG2 cells. The EC50 value was 1.35×10^{-9} M in H4IIE cells and 36-times higher in HepG2 cells. Furthermore benzo[a]pyrene (B[a]P), β-naphthoflavone (BNF), dihydroergotamine mesylate (DHE), 2,4-dinitrophenol, ellipticin, flutamide, indigo, indirubin, 3-methylcholanthrene (3MC,) Org C, Org D, PCB 77, PCB 105, PCB 114, PCB 118, PCB 123, PCB 126, PCB 156, PCB 157, and PCB 167 induced the CYP1A activity in the H4IIE cell line. Of these 20 compounds B[a]P, BNF, DHE, indigo, indirubin, 3MC, Org C, PCB 77, PCB 114, PCB 123, PCB 126, and PCB 167 also induced the CYP1A activity in HepG2 cells. Menadione, Org A, and Org B were HepG2 specific CYP1A inducers. Moreover, Org C was more potent in HepG2 than in H4IIE cells.

Cytochrome P450 1A1 induction measured with Luciferin-CEE

Luc-CEE was used to measure the CYP1A1 induction in HepG2 and H4IIE cells. The same compounds except the four organic compounds were tested. Results were comparable with the results measured with CEC. The same compounds with exception of 2,4-dinitrophenol caused CYP1A1 induction in either HepG2, H4IIE, or both cell lines (Table 2). Although not statistically significant, 2,4-dinitrophenol showed a tendency to induction (1.8-fold) in H4IIE cells. The EC50 values of the CEC and Luc-CEE assay were almost similar. Nevertheless, the induction factors were for most compounds higher in the Luc-CEE assay.

Cytochrome P450 1A1 and 1A2 competition

CYP1A1 and 1A2 competition in human and rat supersomes was measured for 115 compound, 4 compounds were skipped from analysis i.e. Org A, B, C, and D. In Fig. 2 the dose related competition for human and rat CYP1A1 and 1A2 is demonstrated for a representative set of 8 compounds, i.e. B[a]P, ellipticine, furafylline, indigo, indirubin, ketoconazole, nitrofurantoin, and 4-NQO. The efficacies (EFF) and IC50 values are shown in Table 3. Differences were observed between CYP1A1 and 1A2 competition in human and rat supersomes. Of the 115 tested compounds, 36 compounds inhibited human CYP1A1 activity for 50% or more. Human competition for CYP1A1 was found specific for 12 compounds, being β-naphthoflavone, cisplatin, dehydroergotamine mesylate, dopamine HCl, 17β-estradiol, 4-hydroxytamoxifen, noscapine HCl, papaverine, quinidine, quinidine sulfate, reserpine and RU 58668.. The remaining 24 compounds also inhibited rat CYP1A1. Furthermore, rat specific competition for CYP1A1 was found for ten compounds.

Similar differences were observed for CYP1A2. Competition was found for 37 compounds of which 14 and 3 compounds showed specific competition for human and rat CYP1A2, respectively.

A x:y plot for the IC50 values of human vs. rat competition for both CYP1A1 and 1A2 is shown in Fig. 3. The numbers in the figure represent the compounds in Table 3. The solid line in Fig. 3 is the line of identity ($x = y$). The outlier hydralazine HCl is not shown in the CYP1A1 plot.

Table 1 Compounds that do not activate CYP1A activity in the human HepG2 and rat H4IIE cell lines

Acetylsalicylic acid	Ferrous sulfate	Orphenadrine citrate
α-Naphthoflavone	Fluorouracil	Papaverine
2-amino-3-methyl-3H-imidazo-[4,5-f]-quinoline	Furafylline	PCB 169
Aminophylline	Gentamicin A	PCB 189
Amiodarone HCl	Hexachlorobutadiene	Paracetamol
Antazoline mesylate	2,5-Hexanedione	Perhexiline
Atropine sulfate	Hydralazine HCl	Perphenazine
Bishydroxycoumarin (Dicumarol)	Hydrochlorothiazide	Phenobarbital sodium
Bromobenzene	Hydroxychloroquinone sulfate	Phentolamine mesylate
Carbon tetrachloride (CCl4)	4-Hydroxytamoxifen	Quinidine
Chlorpromazine HCl	ICI 164.384 (anti-estrogen)	Quinidine sulfate
Cis-Platin	Imipramine HCl	Raloxifen
CITCO	Indomethacin	Reserpine
Clozapine	Iodoacetate	Rifampicin
Colchicine	Iproniazid HCl	Rotenone
Cyclophosphamide	Isoprenaline HCl	RU 1881, Methyltrienolone
Cytarabine	Ketoconazole	RU 58668 (anti-estrogen)
Dacarbazine	Labetalol	Salicylamide
Dantrolene sodium	L-DOPA	Strychnine nitrate
Dehydroepiandrosterone (DHEA)	Levonorgestrel	Sulfamoxole
Dexamethasone	Medroxyprogesterone acetate	Sulphaphenazole
Diclofenac	Melphalan	Tacrine
Diethyldithio-carbamic acid	Methadone HCl	Tamoxifen
Diethylstilbestrol	Methampyrone (Dipyrone)	Tertiair-butyl-hydroperoxide
Digoxin	Methotrexate	Testosterone
2,7-Dinitrofluorene	7α-methyltestosterone	Tetracycline HCl
Dopamine HCl	17α-Methyltestosterone	Tolcapone
Doxorubicin	Naphazoline nitrate	Tularik 901317
Erythromycin	*N*-ethylmaleimide	Uramustine
Estradiol-17β	Nitrofurantoin	
Ethacrinic acid	Nitropyrene	
Ethinylestradiol-17β	4-Nitrosoquinoline-1-oxide (4-NQO)	
Ethionine	Noscapine HCl	

As can be seen in Fig. 3 there are besides the human and rat specific CYP1A1 competitors, compounds that are almost equally potent in both species (compounds just around the line of identity). However, the potency is for most compounds higher in human supersomes as compared to rat supersomes (compounds above the line of identity). The same is true for CYP1A2 as almost all competitors have a lower IC50 value in human supersomes as compared to rat supersomes.

Discussion

The present study focused on CYP1A induction, (non-)competitive inhibition and species differences between humans and rats. Strong AhR activation leads to a series of toxic effects and consequently it might be useful for the pharmaceutical industry to screen for and deselect NCEs that are strong CYP1A inducers (AhR activators) in the early phase of drug development.

Primary cultures of hepatocytes can be used for CYP1A induction studies, however, donor variation and a high number of compounds in the early developmental phase make the use of cell lines preferable. Two such cell lines might be the human and rat hepatoma cell lines HepG2 and H4IIE. Previously we demonstrated that the effects of AhR agonists in HepG2 on Cytochrome P450 enzymes are similar to the effects observed in primary human hepatocytes (Westerink and Schoonen 2007). In line with this it was reported that HepG2 cells are a better model reflecting

Table 2 Cytochrome P450 1A induction measured with CEC in human HepG2 and rat H4IIE cells

Compound	HepG2				H4IIE			
	CEC		Luc-CEE		CEC		Luc-CEE	
	EC50(M)	IF	EC50(M)	IF	EC50(M)	IF	EC50(M)	IF
B[a]P	3.73×10^{-6}	10.2	2.00×10^{-6}	2.8	2.08×10^{-8}	16.7	8.00×10^{-8}	33.2
BNF	1.22×10^{-5}	20.3	3.16×10^{-6}	9.2	2.95×10^{-8}	24.6	3.16×10^{-8}	52.9
DHE	1.21×10^{-5}	7.9	6.09×10^{-6}	11	3.35×10^{-6}	2.43	1.00×10^{-5}	6.5
Dinitrophenol	$>1.00 \times 10^{-4}$		$>1.00 \times 10^{-4}$		2.59×10^{-6}	3.79	$>1.00 \times 10^{-4}$	
Ellipticin	$>1.00 \times 10^{-4}$		$>1.00 \times 10^{-4}$		1.00×10^{-8}	4.6	1.00×10^{-8}	5.8
Flutamide	$>1.00 \times 10^{-4}$		$>1.00 \times 10^{-4}$		7.89×10^{-7}	13.1	5.00×10^{-7}	25.7
Indigo	5.79×10^{-6}	49.7	3.85×10^{-6}	116	8.52×10^{-9}	25.3	3.16×10^{-8}	63.0
Indirubin	1.73×10^{-7}	93	1.27×10^{-7}	368	4.91×10^{-8}	29	2.00×10^{-7}	68.3
Menadione	1.29×10^{-5}	16.9	1.00×10^{-5}	20.4	$>1.00 \times 10^{-4}$		$>1.00 \times 10^{-4}$	
3MC	6.48×10^{-7}	62.3	5.22×10^{-7}	222	4.45×10^{-8}	24.4	3.16×10^{-8}	69.5
Org A	2.00×10^{-6}	9.94	ND		$>1.00 \times 10^{-4}$		ND	
Org B	2.00×10^{-6}	14.0	ND		$>1.00 \times 10^{-4}$		ND	
Org C	2.00×10^{-6}	22.6	ND		3.16×10^{-5}	2.34	ND	
Org D	$>1.00 \times 10^{-4}$		ND		2.00×10^{-7}	9.50	ND	
PCB 77	8.27×10^{-6}	7.9	2.56×10^{-6}	25.5	4.35×10^{-8}	17.5	7.00×10^{-8}	25.5
PCB 105	$>1.00 \times 10^{-4}$		$>1.00 \times 10^{-4}$		$>1.00 \times 10^{-4}$	11.5	$>1.00 \times 10^{-4}$	9.6
PCB 114	$>1.00 \times 10^{-4}$	32.4	$>1.00 \times 10^{-4}$	29.8	1.30×10^{-6}	13.6	2.35×10^{-6}	15.6
PCB 118	$>1.00 \times 10^{-4}$		$>1.00 \times 10^{-4}$		$>1.00 \times 10^{-4}$	8.9	$>1.00 \times 10^{-4}$	13.6
PCB 123	$>1.00 \times 10^{-4}$	2.2	$>1.00 \times 10^{-4}$	3.5	$>1.00 \times 10^{-4}$	2.4	$>1.00 \times 10^{-4}$	3.5
PCB 126	1.65×10^{-6}	50.6	2.55×10^{-6}	65.4	1.00×10^{-9}	21.6	2.00×10^{-9}	23.6
PCB 156	$>1.00 \times 10^{-4}$		$>1.00 \times 10^{-4}$		5.37×10^{-6}	16.0	3.09×10^{-6}	24
PCB 157	$>1.00 \times 10^{-4}$		$>1.00 \times 10^{-4}$		8.15×10^{-6}	19.7	7.56×10^{-6}	40.6
PCB 167	$>1.00 \times 10^{-4}$	52.6	$>1.00 \times 10^{-4}$	45.5	3.55×10^{-8}	15.3	3.02×10^{-8}	30.5
TCDD	4.88×10^{-8}	61.8	4.00×10^{-8}	250	1.35×10^{-9}	22.3	1.25×10^{-9}	78.5

Compounds indicated in bold and italic show rat and human specific CYP1A activation, respectively. The EC50 values and induction factors (IF) are shown

B[a]P benzo[a]pyrene; *BNF* β-naphthoflavone; *DHE* dehydroergotamine mesylate; *3MC* 3-methylcholanthrene; *PCB* polychlorobiphenyl; *TCDD* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

CYP1A induction in human hepatocytes than hepatocytes from Rhesus monkeys and Sprague–Dawley rats (Silkworth et al. 2005). Others found that the H4IIE cell line is a good model to study CYP1A induction reflecting effects in primary rat hepatocytes (Zeiger et al. 2001). Thus it seems that HepG2 and H4IIE cells are good cell lines to study human and rat CYP1A induction, respectively.

For induction studies the fluorogenic substrate CEC was used. Fluorescence detection of the deethylation of CEC was reported to be 50 up to 100 times more sensitive than that of ethoxyresorufin, primarily because of the faster turnover rate of CEC (White 1988). CEC is not specific for a CYP1A isoform, it reacts with both human and rat CYP1A1 and 1A2 with a preference for CYP1A1 (Stresser et al. 2002). Besides CEC, the luminogenic substrate Luc-CEE was used for measuring CYP1A induction. The results were quite similar to results observed with CEC. Therefore Luc-CEE is a good alternative for the detection of CYP1A inducers. An advance of this substrate might be it is specificity for human and rat CYP1A1 (Promega). There is also a CYP1A2 specific luminogenic substrate (Luciferin-BE) available. However, in the present study this substrate was not used because it reacts only with human CYP1A2 and shows no reactivity for rat CYP1A2 (data not shown).

Of the 119 compounds that were tested 24 compounds were able to induce the CYP1A activity in either human HepG2, rat H4IIE, or both cell lines. Pronounced differences were observed between induction in the cells of human and rat origin. Zeiger et al. (2001) reported similar differences. In their study HepG2 and H4IIE cells were also exposed to the dioxin-like PCBs 77, 105, 114, 118, 123, 126, 156, 157, 167, 169, and 189. In H4IIE cells, they found in concordance with the present study induction after exposure to PCB 77, 105, 114, 118, 123, 126, 156, 157, and

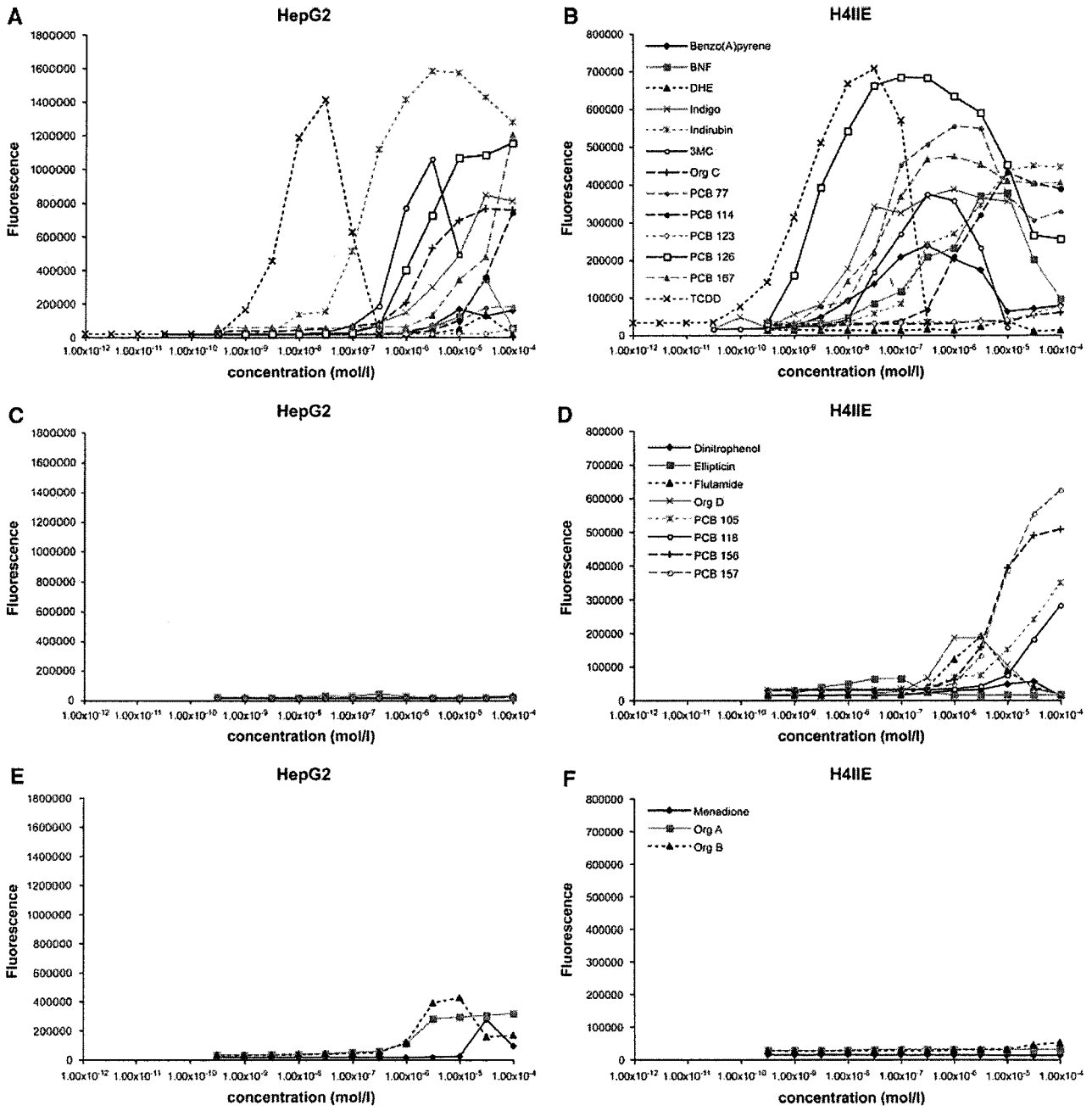

Fig. 1 Induction of CYP1A activity measured with CEC in human HepG2 and rat H4IIE cells after 24 h exposure to the compounds. Of the 119 compounds, 13 induced CYP1A activity in both HepG2 and H4IIE cells (**a**, **b**), 8 showed H4IIE specific induction (**c**, **d**), and 3 compounds showed HepG2 specific induction (**e**, **f**). Results are presented as the mean of three independent measurements. The SD is not shown as it interferes with the reading of the marker spots. However, SD is < 5% for all data points. Abbreviations: *BNF* β-naphthoflavone; *DHE* dehydroergotamine mesylate; *3MC* 3-methylcholanthrene; *TCDD* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

no induction after exposure to PCB 189. However, in contradiction we did not find induction with PCB 169 in H4IIE cells. Induction in HepG2 cells was found for less compounds at higher doses. In both studies induction was measured after exposure to PCB 77, 114, and 126. In contradiction with Zeiger et al. (2001) we found induction by PCB 123 and 167. The results suggest that the rat cell line H4IIE is more sensitive to detect CYP1A inducers than the HepG2 cell line.

The high sensitivity of rat for CYP1A inducers was also reported by Silkworth et al. (2005), who found CYP1A induction at much lower concentrations after exposure to

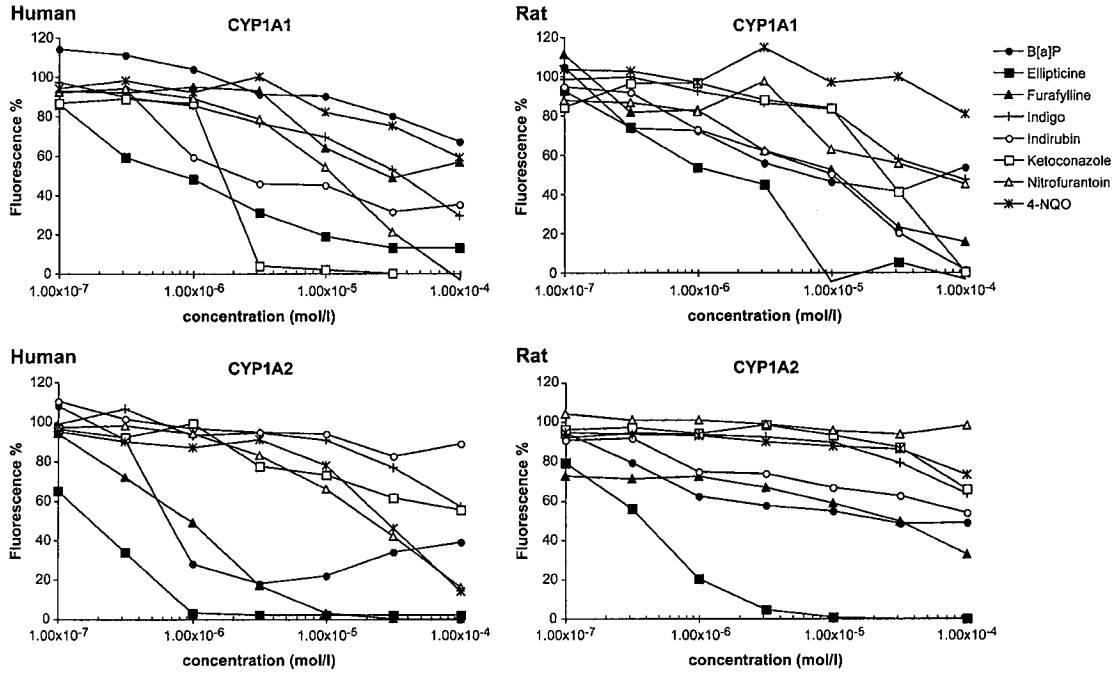

Fig. 2 CYP1A1 and 1A2 competition in supersomes from human and rat by benzo[a]pyrene (*B[a]P*), ellipticine, furafylline, indigo, indirubin, ketoconazole, nitrofurantoin, and 4-nitrosoquinolineoxide-1-oxide (*4-NQO*). The activity of the 0.1% DMSO control was set at 100%. Results are presented as the mean of three independent measurements. The SD is not shown as it interferes with the reading of the marker spots. However, SD is < 5% for all data points. Abbreviations: *B[a]P* benzo[a]pyrene; *4-NQO* 4-nitrosoquinolineoxide-1-oxide

PCB 126 and Aroclor 1254 in rat than human cells. A study by Aluru et al. (2005) also revealed species differences. While in humans α-naphthoflavone (ANF) is an AhR antagonist in rainbow trout hepatocytes it was a partial agonist.

Although mono-ortho PCBs such as PCB 114, PCB 126, and PCB 167 are considered as weak AhR agonists (Peters et al. 2006), they showed a high potency in the present study. Peters et al. (2006) showed that after purification of mono-ortho PCBs with active charcoal, mono-ortho, PCBs show only a low potency in an AhR-EGFP reporter assay in mouse Hepa1c1c7 and rat H4IIE cells. The purity of the PCBs in the present study was 99%. Therefore we do not expect large effects of impurities on CYP1A induction, however we cannot rule out that contaminations with AhR agonists of high activity had an effect on the activity.

The importance of deselecting CYP1A inducers was stressed by Org D. This rat specific CYP1A inducer caused non-genotoxic carcinogenicity in in vivo rat studies (unpublished in-house data).

We observed that Org A and B were HepG2 specific CYP1A inducers and Org C was much more potent in the HepG2 cell line. Menadione was also active in HepG2 cells and not in H4IIE cells. Apparently there are CYP1A inducers that are more potent in humans than rats. In line with this, in human, but not in mouse and in rat, the anti-ulcer drug omeprazole has been reported to induce CYP1A2 (Diaz et al. 1990; Lu and Li 2001; Roymans et al. 2005). Overall these observations make screening in cell lines from both rat and human a useful strategy.

Recently Sonneveld et al. (2007) showed that glucocorticoids enhance the induction of CYP1A1 and other AhR target genes in rat H4IIE cells but not in human cells. In the present study we did not observe an effect of dexamethasone in H4IIE cells also not when charcoal treated serum was used (data not shown).

There are also other methods available for screening CYP1A inducers and/or AhR activators. Two often used methods are the CALUX bioassay (Murk et al. 1996) and the use of 101L cells (Postlind et al. 1993). In the CALUX bioassay rat H4IIE and mouse Hepa1c1c7 cells containing a luciferase gene under control of dioxin responsive elements are used. A disadvantage of this assay is that rodent cells are used which not always reflect effects in humans. The 101L cell line is a stable cell line derived from HepG2 cells which contain a human CYP1A1 luciferase reporter. An advantage of this system might be that substrate inhibition of CYP1A1 does not play a role. However, in our assay we included a washing step after compound incubation which reduced substrate inhibition of CEC.

Ellipticine showed CYP1A induction in the H4IIE cell line. In HepG2 cells induction was just below two fold and directly after the increase in activity the signal dropped

Table 3 Compounds that show CYP1A1 and 1A2 competition in human and rat supersomes

No.	Compound	Human				Rat			
		CYP1A1 inhibition		CYP1A2 inhibition		CYP1A1 inhibition		CYP1A2 inhibition	
		EFF (%)	IC50 (M)	EFF (%)	IC50 (M)	EFF (%)	IC50 (M)	EFF (%)	IC50 (M)
1	α-Naphthoflavone	**111**	**3.47×10^{-6}**	**104**	**2.43×10^{-6}**	**101**	**1.00×10^{-5}**	**98**	**3.60E-06**
2	2-Amino-quinoline	**67**	**5.75×10^{-5}**	**95**	**2.64×10^{-6}**	**53**	**4.71×10^{-5}**	**56**	**3.11E-04**
3	B[a]P	33	$>1.00 \times 10^{-4}$	**61**	**1.50×10^{-6}**	23	$>1.00 \times 10^{-4}$	**51**	**2.30×10^{-5}**
4	BNF	**91**	**2.01×10^{-6}**	**83**	**8.07×10^{-7}**	11	$>1.00 \times 10^{-4}$	**108**	**3.80E-06**
5	Chlorpromazine HCl	**74**	**1.81×10^{-5}**	**72**	**8.56×10^{-6}**	**98**	**2.50×10^{-6}**	31	$>1.00 \times 10^{-4}$
6	Cisplatin	**72**	**3.02×10^{-5}**	**82**	**1.29×10^{-5}**	43	$>1.00 \times 10^{-4}$	**93**	**1.96×10^{-5}**
7	Clozapine	−3	$>1.00 \times 10^{-4}$	**52**	**1.00×10^{-4}**	**107**	**1.88×10^{-6}**	**47**	**1.15E-04**
8	Dantrolene sodium	**91**	**1.34×10^{-5}**	**60**	**7.74×10^{-5}**	**75**	**3.00×10^{-5}**	39	$>1.00 \times 10^{-4}$
9	DETC	37	$>1.00 \times 10^{-4}$	**71**	**5.73×10^{-5}**	12	$>1.00 \times 10^{-4}$	**87**	**3.00×10^{-5}**
10	DHE	**66**	**4.25×10^{-5}**	−6	$>1.00 \times 10^{-4}$	19	$>1.00 \times 10^{-4}$	−29	$>1.00 \times 10^{-4}$
11	2,7-Dinitrofluorene	**64**	**6.61×10^{-5}**	**86**	**3.85×10^{-7}**	**53**	**2.75×10^{-5}**	**61**	**2.62×10^{-5}**
12	DNP	**69**	**8.01×10^{-6}**	**81**	**4.20×10^{-6}**	**49**	**2.27×10^{-5}**	**51**	**5.82×10^{-5}**
13	Dopamine HCl	**84**	**3.13×10^{-5}**	8	$>1.00 \times 10^{-4}$	27	$>1.00 \times 10^{-4}$	2	$>1.00 \times 10^{-4}$
14	Doxorubicin	**73**	**3.80×10^{-5}**	**67**	**4.29×10^{-5}**	**90**	**6.16×10^{-6}**	**69**	**4.89×10^{-5}**
15	Ellipticine	**87**	**1.08×10^{-6}**	**98**	**4.71×10^{-8}**	**104**	**1.42×10^{-6}**	**100**	**3.23×10^{-7}**
16	Estradiol-17β	**73**	**2.75×10^{-5}**	30	$>1.00 \times 10^{-4}$	4	$>1.00 \times 10^{-4}$	−2	$>1.00 \times 10^{-4}$
17	Ethacrinic acid	14	$>1.00 \times 10^{-4}$	20	$>1.00 \times 10^{-4}$	**53**	**7.01×10^{-5}**	9	$>1.00 \times 10^{-4}$
18	Flutamide	30	$>1.00 \times 10^{-4}$	**71**	**8.97×10^{-6}**	−16	$>1.00 \times 10^{-4}$	9	$>1.00 \times 10^{-4}$
19	Furafylline	43	$>1.00 \times 10^{-4}$	**100**	**8.01×10^{-7}**	**77**	**3.01×10^{-5}**	**56**	**3.68×10^{-5}**
20	Hexachlorobutadiene	**83**	**1.75×10^{-6}**	5	$>1.00 \times 10^{-4}$	**59**	**1.89×10^{-5}**	5	$>1.00 \times 10^{-4}$
21	Hydralazine HCl	**69**	**5.99×10^{-5}**	**58**	**3.35×10^{-5}**	**66**	**5.80×10^{-8}**	**55**	**3.05×10^{-5}**
22	4-Hydroxytamoxifen	**81**	**3.34×10^{-5}**	−14	$>1.00 \times 10^{-4}$	6	$>1.00 \times 10^{-4}$	17	$>1.00 \times 10^{-4}$
23	Indigo	**71**	**2.83×10^{-5}**	43	$>1.00 \times 10^{-4}$	**53**	**7.00×10^{-5}**	36	$>1.00 \times 10^{-4}$
24	Indirubin	**65**	**8.90×10^{-6}**	11	$>1.00 \times 10^{-4}$	**99**	**3.53×10^{-6}**	46	$>1.00 \times 10^{-4}$
25	Isoprenaline HCl	10	$>1.00 \times 10^{-4}$	**64**	**9.43×10^{-5}**	7	$>1.00 \times 10^{-4}$	14	$>1.00 \times 10^{-4}$
26	Ketoconazole	**100**	**2.00×10^{-7}**	45	$>1.00 \times 10^{-4}$	**100**	**3.00×10^{-6}**	−14	$>1.00 \times 10^{-4}$
27	L-DOPA	18	$>1.00 \times 10^{-4}$	−62	$>1.00 \times 10^{-4}$	**56**	**1.31×10^{-4}**	**112**	**1.14×10^{-5}**
28	Melphalan	**66**	**4.68×10^{-5}**	**53**	**7.41×10^{-5}**	**63**	**6.84×10^{-5}**	**59**	**7.51×10^{-5}**
29	Menadione	**85**	**1.02×10^{-5}**	**102**	**2.43×10^{-7}**	**80**	**1.52×10^{-5}**	**93**	**2.88×10^{-7}**
30	Methotrexate	18	$>1.00 \times 10^{-4}$	**88**	**2.70×10^{-5}**	4	$>1.00 \times 10^{-4}$	28	$>1.00 \times 10^{-4}$
31	3-Methylcholanthrene	25	$>1.00 \times 10^{-4}$	**74**	**4.79×10^{-6}**	8	$>1.00 \times 10^{-4}$	**57**	**1.10×10^{-5}**
32	7α-Methyltestosterone	**64**	**5.47×10^{-5}**	6	$>1.00 \times 10^{-4}$	**116**	**1.74×10^{-7}**	10	$>1.00 \times 10^{-4}$
33	Nitrofurantoin	**103**	**1.11×10^{-5}**	**84**	**2.01×10^{-5}**	**55**	**3.48×10^{-4}**	2	$>1.00 \times 10^{-4}$
34	Nitropyrene	**83**	**1.63×10^{-6}**	**92**	**1.00×10^{-7}**	**99**	**3.58×10^{-7}**	**108**	**1.00×10^{-7}**
35	4-NQO	41	$>1.00 \times 10^{-4}$	**86**	**2.41×10^{-5}**	19	$>1.00 \times 10^{-4}$	27	$>1.00 \times 10^{-4}$
36	Noscapine HCl	**65**	**4.04×10^{-5}**	1	$>1.00 \times 10^{-4}$	12	$>1.00 \times 10^{-4}$	5	$>1.00 \times 10^{-4}$
37	Orphenadrine citrate	33	$>1.00 \times 10^{-4}$	40	$>1.00 \times 10^{-4}$	15	$>1.00 \times 10^{-4}$	**53**	**1.03×10^{-4}**
38	Papaverine	**92**	**9.15×10^{-6}**	**71**	**3.06×10^{-5}**	38	$>1.00 \times 10^{-4}$	18	$>1.00 \times 10^{-4}$
39	PCB 105	11	$>1.00 \times 10^{-4}$	**42**	**7.59×10^{-5}**	**57**	**5.18×10^{-5}**	22	$>1.00 \times 10^{-4}$
40	PCB 114	−8	$>1.00 \times 10^{-4}$	**69**	**2.21×10^{-5}**	**95**	**1.19×10^{-5}**	34	$>1.00 \times 10^{-4}$
41	PCB 118	22	$>1.00 \times 10^{-4}$	**54**	**2.54×10^{-5}**	**86**	**2.96×10^{-6}**	24	$>1.00 \times 10^{-4}$
42	PCB 123	13	$>1.00 \times 10^{-4}$	**54**	**3.60×10^{-5}**	5	$>1.00 \times 10^{-4}$	8	$>1.00 \times 10^{-4}$
43	PCB 156	11	$>1.00 \times 10^{-4}$	39	$>1.00 \times 10^{-4}$	**101**	**6.42×10^{-6}**	5	$>1.00 \times 10^{-4}$
44	PCB 167	28	$>1.00 \times 10^{-4}$	**53**	**3.22×10^{-5}**	**72**	**4.32×10^{-5}**	45	$>1.00 \times 10^{-4}$
45	Paracetamol	5	$>1.00 \times 10^{-4}$	−4	$>1.00 \times 10^{-4}$	**62**	**1.32×10^{-4}**	**57**	**5.10×10^{-5}**

Table 3 continued

No.	Compound	Human				Rat			
		CYP1A1 inhibition		CYP1A2 inhibition		CYP1A1 inhibition		CYP1A2 inhibition	
		EFF (%)	IC50 (M)	EFF (%)	IC50 (M)	EFF (%)	IC50 (M)	EFF (%)	IC50 (M)
46	Perhexiline	49	$>1.00 \times 10^{-4}$	**65**	$\mathbf{6.39 \times 10^{-5}}$	22	$>1.00 \times 10^{-4}$	16	$>1.00 \times 10^{-4}$
47	Perphenazine	**101**	$\mathbf{4.52 \times 10^{-6}}$	1	$>1.00 \times 10^{-4}$	**82**	$\mathbf{1.02 \times 10^{-5}}$	1	$>1.00 \times 10^{-4}$
48	Quinidine	**76**	$\mathbf{1.87 \times 10^{-5}}$	−2	$>1.00 \times 10^{-4}$	27	$>1.00 \times 10^{-4}$	30	$>1.00 \times 10^{-4}$
49	Quinidine sulfate	**89**	$\mathbf{1.10 \times 10^{-5}}$	24	$>1.00 \times 10^{-4}$	20	$>1.00 \times 10^{-4}$	5	$>1.00 \times 10^{-4}$
50	Raloxifen	**100**	$\mathbf{3.80 \times 10^{-6}}$	43	$>1.00 \times 10^{-4}$	**80**	$\mathbf{1.56 \times 10^{-5}}$	23	$>1.00 \times 10^{-4}$
51	Reserpine	**59**	$\mathbf{1.73 \times 10^{-5}}$	47	$>1.00 \times 10^{-4}$	23	$>1.00 \times 10^{-4}$	−5	$>1.00 \times 10^{-4}$
52	Rifampicin	**74**	$\mathbf{2.08 \times 10^{-5}}$	**90**	$\mathbf{6.22 \times 10^{-6}}$	**60**	$\mathbf{4.18 \times 10^{-5}}$	**58**	$\mathbf{4.68 \times 10^{-5}}$
53	RU 58668	**102**	$\mathbf{5.70 \times 10^{-5}}$	2	$>1.00 \times 10^{-4}$	−15	$>1.00 \times 10^{-4}$	39	$>1.00 \times 10^{-4}$
54	Tacrine	**115**	$\mathbf{6.32 \times 10^{-7}}$	**108**	$\mathbf{7.79 \times 10^{-7}}$	**131**	$\mathbf{2.01 \times 10^{-6}}$	**50**	**1.30E-04**
55	Testosterone	**64**	$\mathbf{7.47 \times 10^{-5}}$	−6	$>1.00 \times 10^{-4}$	**45**	$\mathbf{2.40 \times 10^{-4}}$	−4	$>1.00 \times 10^{-4}$
56	Tolcapone	**91**	$\mathbf{2.53 \times 10^{-5}}$	**92**	$\mathbf{9.15 \times 10^{-6}}$	**110**	$\mathbf{2.17 \times 10^{-5}}$	**92**	$\mathbf{2.11 \times 10^{-5}}$

The efficacy (EFF) and IC50 values are shown. Values are marked in bold when EFF > 50% ($P < 0.05$)

B[a]P benzo[a]pyrene; *BNF* β-naphthoflavone; *DETC* diethyldithio-carbamic acid; *DNP* 2,4-dinitrophenol; *3MC* 3-methylcholanthrene; *4-NQO* 4-nitrosoquinolineoxide-1-oxide

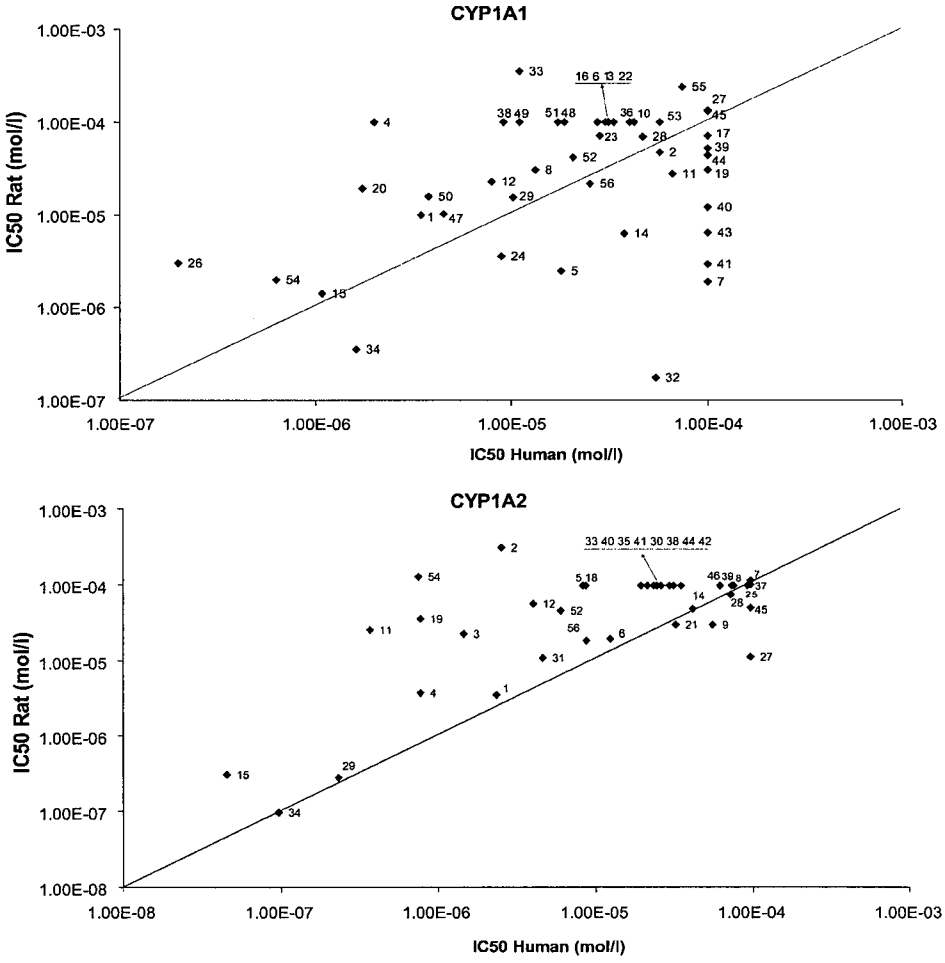

Fig. 3 Comparison of the IC50 values of human versus rat for CYP1A1 and 1A2 competition. The *numbers* represent the compounds in Table 3. The *solid line* is the line of identity ($x = y$)

sharply again. The problem with the detection of ellipticine in the HepG2 cell line was cytotoxicity. At the concentration where this compound starts to activate the AhR it shows also a strong cytotoxic effect (Schoonen et al. 2005a, b). All other compounds in the present study were tested for cytotoxicity by using the glutathione depletion and calcein-AM assay (Schoonen et al. 2005a, b). These results revealed (data not shown) that compounds not showing CYP1A induction in HepG2 cells but showing CYP1A induction in H4IIE cells were not missed in HepG2 cells because of cytotoxicity.

CYP1A induction can easily be used in the early developmental phase to detect compounds that might show dioxin-like toxicity. However, care should be taken to deselect such compounds directly as CYP1A activation does not necessarily mean dioxin-like toxicity. There are for example marketed therapeutics like omeprazole, leflunomide, flutamide, and nimodipine which are safely used but have been proven to be AhR agonists (Hu et al. 2007). Furthermore, AhR agonists like indirubin and meisoindigo have been shown to be effective in the treatment of chronic myelogenous leukemia (Xiao et al. 2002). Hu et al. (2007) also demonstrated that induction of CYP1A1 is a non-specific marker of direct AhR affinity. In the present study we measured CYP1A induction and not AhR activation. Therefore also compounds are detected that alter the CYP1A expression by pathways in addition to those mediated by the AhR. Induction of CYP1A has been reported after oxidative stress (Delescluse et al. 2001; Hazinski et al. 1995). These compounds do not have the side effects reported for some AhR activators, however, an adverse side effect of these compounds might be the effect of increased CYP1A activity on the efficacy of the compound itself or the induction might result in drug–drug interactions.

The potentially toxic effect of CYP1A inducers can be confirmed by using in vitro or in vivo transcriptional profiling. By studying the change of a broad set of genes dioxin-like toxicity might be predicted more precisely. Consequently a compound might be deselected or selected.

Thus summarized, CYP1A induction can be used as a prescreening tool to detect compounds that might show dioxin-like toxicity. However, further studies are needed to confirm this dioxin-like toxicity. When during drug development equally potent compounds without CYP1A induction are available, selection of these compounds might be preferred to avoid possible safety problems.

Besides species differences between CYP1A1 induction, species differences between the AhR receptor of human and rat could lead to differentially regulated gene expression. Recently it has been shown by Flaveny et al. (2008) that differences between the transactivation domains of the human and mouse AhR results in differential recruitment of co-activators. It is likely that this leads to a divergent regulation of target genes. Differences in the recruitment of co-activators between the human and rat AhR receptor have not been studied yet, however, similar differences as found between human and mouse might exist.

CYP1A1 and 1A2 competition assays were performed by using human and rat supersomes. The same set of compounds with exception of four in-house NCEs was tested. Like for induction species differences were observed. Of the 115 compounds 46 showed CYP1A1 competition. Competition was human and rat specific for 12 and 10 compounds, respectively. CYP1A2 competition was observed for 37 compounds of which 14 and 3 compounds showed human and rat specific inhibition, respectively. The similarity between the amino acid sequence of human and rat CYP1A1 is 79%, and of human and rat CYP1A2 73%. The difference in amino acid sequences might account for the differences in competition. Other studies also reported species differences between CYP1A metabolism in humans and rats. Shinkyo et al. (2003) studied the metabolism of TCDD and other polychlorinated dibenzo-p-dioxins in human and rat microsomes and found significant species differences. Bogaards et al. (2000) compared the CYP activities towards marker CYP substrates for human, rat, rabbit, dog, and micropig microsomes. They found that in none of the tested species metabolism was similar to CYP metabolism in man. With respect to CYP1A human metabolism was most similar to mouse, followed by rabbit, micropig, rat and dog.

In summary, we used a medium/high-throughput assay in a 96 well format for detecting CYP1A inducers in the human HepG2 and rat H4IIE cell line. Moreover, CYP1A1 and 1A2 competition assays were performed by using human and rat supersomes in a 384 well high throughput assay. The induction and competition assays revealed for several compounds a real species difference between human and rat. Therefore, parallel screening in both species might be a very useful strategy.

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