DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

I) The Ah receptor (**AhR**) mediates most of the *toxic and tumor promotional properties of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other Polycyclic Aromatic Hydrocarbons (PAH) in rodents. Humans are exposed to low levels of PAH, the long term health effect(s) of which are uncertain. To understand the significance of excessive AhR activation it is essential to understand its normal role in cellular function, which is the first long-term goal of this project.* **II)** *The toxicological significance of prolonged AhR-induced activation of CYP1A1, 1B1, and 1A2 expression may also be an important aspect PAH toxicity. Known endogenous CYP substrates play regulatory roles in directing cell growth, differentiation, proliferation or maintaining normal cellular homeostasis. Increased metabolism of such molecules by increased CYP1 expression due to AhR signaling might disrupt normal cell signaling pathways. Identification of endogenous substrates for the CYP1 family, especially those with regulatory functions, is the second long-term goal of this project.*

Two Central yet converging Hypotheses will be tested*:* ***(1)*** *There are significant levels of endogenous ligand(s) (or other endogenous regulators) for the AhR in cells, and CYP-mediated metabolism alters their regulatory effect on AhR-mediated activity*. **(2)** Endogenous substrates for CYP1B1 have regulatory roles in cellular metabolism, and one or more of the these substrates function as ligands or regulators for AhR. Both hypotheses are supported by preliminary findings from our laboratories and those of others demonstrating that constitutive AhR-mediated transcriptional activity is suppressed by CYP1 expression. Three Specific Aims will test these hypotheses: 1) Determine the mechanism by which CYP1B1 regulates AhR transcriptional activity; 2) Identify endogenous AhR ligands/regulators and characterize their mechanism of action; 3) Identify endogenous substrates for CYP1B1 that also function as endogenous ligands for the AhR via labeling with $^{18}O_2$ and ESI-MS analysis. Collectively, these studies will establish experimental systems for detecting endogenous AhR ligands and CYP substrates, and lead to identification of these compounds in mammalian cells. *Results from these aims will provide an improved mechanistic understanding of: the normal function of the AhR and CYP isozymes in cells; the role of CYPs in maintenance of normal human health and in human disease processes; as well as health risks associated with exposure to PAHs.*

PERFORMANCE SITE(S) *(organization, city, state)*

University of Whatever Health Sciences Center
Smallville, USA

The Pennsylvania State University
University Park, PA 16802

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
Scientist, John Q,	U of Whatever	Principal Investigator
Dr. Jim	UU of Whatever	Investigator
Dr, Joe	U of Whatever	Investigator
Dr. Jill	U of Whatever	Research Associate
Dr. George	U of Whatever	Post Doc
Dr. Bob	The Wonderful State University	Investigator
Jimmy Scientist	The Wonderful State University	Graduate Assistant
TBN	The Wonderful State University	Research Associate

Type the name of the principal investigator/program director at the top of each printed page and each continuation page. (For type specifications, see instructions on page 6.)

RESEARCH GRANT

TABLE OF CONTENTS

Research Plan

*Type density and size must conform to limits provided in Specific Instructions on page 6.

Appendix *(Five collated sets. No page numbering necessary for Appendix.)*

☒ Check if Appendix is included

Number of publications and manuscripts accepted or submitted for publication *(not to exceed 10)* 2

Other items (list):

1) Letter of support from Dr. Bill (University of Fun)
2) Letter of support and collaboration from Dr. Jack (University of Whatever)
3) Price Quotations for $^{18}O_2$
4) Full page reproductions of all figures contained in proposal to show greater detail
5) Supplemental figures of preliminary data shown in previous version of proposal replaced with more recent data or removed due to space limitation in current version, not essential to current application but discussed in text of proposal.

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 12/01/99 THROUGH 11/30/03

PERSONNEL *(Applicant organization only)* NAME	ROLE ON PROJECT	TYPE APPT. *(months)*	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED *(omit cents)* SALARY REQUESTED	FRINGE BENEFITS	TOTAL
John Scientist	Principal Investigator	12	25	$73,880	$19,521	$4,685	$24,206
Dr. Bill	Co-Investigator	10	5	$85,650	$4,526	$1,086	$5,612
Dr. Bob	Co-Investigator	12	10	$81,200	$8,582	$2,060	$10,642
Dr. Jill	Research Technician	12	100	$35,000	$36,991	$8,878	$45,869
Dr. George	Post Doc	12	100	$33,000	$34,877	$4,883	$39,760
	SUBTOTALS →				$104,497	$21,592	$126,089

CONSULTANT COSTS		$0
EQUIPMENT *(Itemize)* ELSD HPLC Flow detector $15,000; LN2 Cyrostat $ 1,500		$16,500
SUPPLIES *(Itemize by category)* Chromatography $4,000; Molecular Biology Supplies $5,000; Biochemicals $4,000; Mass Spec Supplies/Reagents $6,000; Cell Culture Supplies $4,000; 18-Oxygen $7,000		$30,000
TRAVEL One trip per year for each P.I. to present results/1 Trip to PI lab for collaborator PI on subcontract		$3,000
PATIENT CARE COSTS	INPATIENT	$0
	OUTPATIENT	$0
ALTERATIONS AND RENOVATIONS *(Itemize by category)*		$0
OTHER EXPENSES *(Itemize by category)* Page charges, etc. $2,200; Liquid Nitrogen $420; MS Time $3,000; Animals $1,350; Maintenance $2,500		$9470
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD	**$**	$185,059
CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	$114,187
	INDIRECT COSTS	$26,635
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)* →	**$**	$325,881

BUDGET JUSTIFICATION:

In response to review panel concerns regarding the overly ambitious nature of the original 3 year project and the recommendations for a more structured approach to the identification of AhR ligands/CYP1B1 substrates, as well as several other modifications of experimental design entailing AN increase in the number, complexity or both, of proposed experiments (such as the development of the CYP1B1 Thr=>Ala mutants and additional stable transfectant cell lines), the project duration has been increased from 3 to 4 years. Dr. XXXXX's role in the project, especially in years 3 and 4 of the project have been increased substantially, as detailed in the body of this proposal, although his overall % effort (salary request) in years 1-4 remains unchanged. In response to reviewer's concerns regarding Dr. YYYY's minimal role in the project, his participation has been changed from an unpaid consultant to 10% effort and salary support for his role in developing and conducting fractionation schemes of cellular components, and purifying and identifying the putative endogenous ligands/substrates by HPLC and MS. Also, since the initial submission of this proposal, an administrative reclassification of technical staff at UNM has significantly increased these staff salaries. Thus while this revised budget has increased over that of the initial submission, most of the increase is due to salary increases mandated by UNM or in response to reviewers requests for increased effort by Drs. YYYY and XXXX on this project, the additional year added to the project to ameliorate the somewhat ambitious nature of the original 3 year timeline, and the somewhat expanded experimental design of the project to address various reviewers concerns.

Personnel: Due in part to the challenging nature of the proposed studies, we have assembled a relatively senior and experienced team of scientists to contribute significant amounts of time toward this project. Only one graduate student will be involved (XXXX Lab) and this student has been involved with generating much of the preliminary data from the XXXX lab and is thus already experienced in most of the techniques required for this project. Hence all investigators will be able to immediately begin full contributions to the proposed experiments. Thus the investigators are already familiar with nearly all of the required techniques, most of which are currently routinely run in the various team members laboratories (in part the justification for the collaborative nature of the proposed studies), and little training or methods development in basic techniques will be required for this project. The 4% cost of living increase and different fringe rates is applied to personnel costs as per UNM HSC policy. *'Requested' salaries also include additional 'anticipated' increases for subsequent fiscal years, and actual salaries are likely to be less.*

John Scientist, Ph.D. Principal Investigator 25% effort

This project constitutes a primary research effort of the PI, and thus the PI will be involved in daily planning, design and some execution of experiments; data analysis and experimental trouble shooting. The PI will prepare manuscripts and progress reports, and be responsible for the overall execution of the research plan. The PI will supervise the research technician in his lab on a daily basis, and participate in conducting a significant number of experiments, especially those involving new methods development and troubleshooting. The PI will also coordinate the various subprojects being conducted in the ZZZZ and XXXX Laboratories, and work closely with the research scientist in the ZZZZ lab on the mass spectrometry of the ^{18}oxygen-labelled substrates.

Chris ZZZ, Ph.D. Co-investigator 5% effort

Dr. ZZZ, Professor of Chemistry is a internationally recognized expert in the field of mass spectrometry. He will direct the analysis and identification of ^{18}oxygen - labeled substrates. He will participate in the design and analysis of the MS experimental studies, and will direct and supervise the post doctoral scientist (Dr. AAAA) in his

laboratory who will conduct the actual MS analyses. Dr. ZZZZ will contribute 5% effort to this project, but requests only partial support as his summer salary.

Jerry YYYY, Ph.D. Co-investigator 10%

Dr. YYYY, Professor of Medicinal Chemistry, is an experienced analytical and synthetic chemist. He will continue in his role of providing analytical and synthetic services for this project. Specifically Dr. YYYY will assist and direct the post doctoral scientist and research technician in the fractionation of cell lysates to obtain subcelluar fractions enriched in ^{18}oxygen-labelled substrates, assist in the analytical clean up of these samples, assist and advise on the HPLC fractionations of cell lysates, assist with the 17β-estradiol metabolism assays, and conduct preliminary mass spectral and HPLC analysis of putative ^{18}O-substrate-containing fractions. Dr. YYYY's laboratory contains multiple HPLC instruments and a Shimadzu QP-5050 CI/EI GC/MS with a solid probe inlet and Pentium PC data handling and control system and a AOC-20I autosampler which he will support and make available to this project. **Dr. YYYY will contribute substantial effort to this project, by designing as well as conducting much of the HPLC fractionation and putative CYP1B1 substrate/metabolite identification studies at UNM. He will also conduct the method development for utilizing the new evaporative light scattering detector (ELSD) as a novel approach to identifying putative CYP1B1 substrates, especially those that might lack traditional chromophores that could be detected via our existing UV/VIS absorption or fluorescence flow detectors. Due to Dr. YYYY's significantly increased contributions to this project, (in part requested by the previous review panel), Dr. YYYY is now included in the budget for 10% effort and salary, instead of as an unpaid consultant. Since Dr. YYYY was previously not included in the salary request, the total budget for this project has increased concomitant with Dr. YYYY's greater commitment to the project.**

Sophie BBBB Research Scientist-Scientist Lab 100% effort

Ms. BBBB has been working in the PI's (Scientist) laboratory for the past 24 months, and has conducted many of the experiments generating the preliminary data for this project. Ms. BBBB is an experienced molecular biologist and biochemist, and is proficient in almost every technique necessary for the proposed studies. Ms. BBBB will on a daily basis conduct a significant number of the experiments described herein involved with cell culture, metabolism assays to generate ^{18}oxygen - labeled metabolites, preparation of cell fractions, and in vitro expression of CYP constructs, all in cooperation with the PI and post doc in the ZZZZ lab. Ms. BBBB will work closely with Dr. AAAA in Dr. ZZZZs lab to integrate the generation of the ^{18}oxygen-labeled metabolites and their analysis and structural identification. Ms. BBBB will also be responsible for general laboratory organization, ordering of all supplies for the Scientist, YYYY and ZZZZ labs, and general maintenance of all equipment in the Scientist lab. **Due to a University mandated reclassification of employees which has occurred since the original submission of this proposal, Ms. BBBB has been reclassified into a higher salary range than was listed on the original proposal, thus the salary requested for Ms. BBBB has increased significantly. Her experience and skills more than justify this reclassification.**

George AAAA, Ph.D. Post Doctoral Research Associate -ZZZZ Lab 100% effort

Dr. Jackson has been working in the ZZZZ lab for nearly 2 years, and is familiar with all technical and instrumental aspects of the work to be conducted on the identification of the ^{18}oxygen-labelled metabolites. Dr. Jackson will conduct all of the mass spec analyses of the ^{18}oxygen-labeled substrates, conduct much of the preliminary cell fractionations and preliminary HPLC separations, purify the various cell fractions for analysis, acquire and interpret and the mass spec results, and maintain the MS instrumentation used for this project in the

ZZZZ lab. **Dr. AAAA's current salary has been increased modestly since the original submission of this proposal.**

Equipment: (Scientist and YYYY Labs) TOTAL: $16,500

Dr. Scientist originally requested funds ($13,190) to secure the purchase of an Eagle Eye II densitometry system (Stratagene): This equipment has recently been purchased from other funding sources, and has been deleted from the current budget request. However, based upon changes in the revised proposal in accordance with recommendations of the previous review panel, we have modified and *improved* our strategies for the isolation and identification of putative AhR ligands/CYP1B1 substrates and metabolites to include HPLC approaches that include identification of essentially all organic molecules likely to be present in cell extracts. This approach will require the purchase of an extremely sensitive (low ppm or better detection capabilities) Evaporative Light Scattering (ELS) HPLC flow detector for use on existing HPLC systems and compliment and/or replace use of existing UV/VIS and fluorescence detectors in Dr. YYYY and Dr. Scientist's labs at UNM. Such a detector will especially valuable if not essential for identification of putative ligands/substrates which might lack conventional chromophores that could be detected via our existing UV/VIS absorption or fluorescence flow detectors. Thus, $15,000 is now requested for the purchase of a new ELS detector. Also, since the fist submission of this proposal, Dr. Scientist's available liquid nitrogen storage capacity has been saturated by samples from other projects, and we are currently 'borrowing' space in other investigators cryostats. $1500 is requested for the purchase of a small, additional cryostat to store the cells and other samples utilized for this project.

Dr. Scientist and Dr. YYYY will focus on the identification of CYP1B1 endogenous substrates and Dr. XXXX will focus on identification of endogenous AhR ligands. Although there will be continuous crosstalk between these two parallel yet convergent projects (Aims #2 and #3), it has become evident from our recently acquired additional Preliminary Data that it will be necessary for the labs at both UNM and Penn State to simultaneously conduct extensive HPLC-based work, hence the need for the additional specialized HPLC detector at UNM and additional HPLC system at Penn State (see Penn State Budget and justification).

Supplies: **Scientist Lab** Total: $24,000. The requested supply budget is based on recent experience in conducting the preliminary studies described herein, and is deemed appropriate for the studies described. Supplies requested include general biochemical reagents for the proposed metabolism and ligand binding/identification studies, HPLC analyses and fractionations; isotopes (including ^{18}oxygen for metabolism studies), PCR reagents, oligo nucleotide primers and internal standards, cell culture supplies and sterile plasticware, primary rat hepatocyte preparation and culture, western blotting supplies and secondary antibodies, and other molecular biology reagents. Many of the reagents for these studies are *unusually expensive,* especially the cell culture supplies, serum for other cell culture systems, PCR supplies and especially the ^{18}oxygen for metabolism/mass spectral studies. Both the Scientist and XXXX labs will need to grow and transfect a large number of cells for the proposed studies, especially to obtain sufficient cells for the ^{18}O-labeling studies. **^{18}Oxygen: Two sources of ^{18}oxygen gas for the labeling experiments are available (see quotes in Appendix). However, since the original submission of this proposal, price of ^{18} oxygen has increased 2-3-fold due to an increase in demand over supply. However, based on our preliminary experiments conducted since the original budget was prepared, we have reduced our estimate of the amount of ^{18}oxygen required for these studies by 50%, as we have developed more efficient ways to utilize the ^{18}oxygen in our experiments by decreasing dead volume in our ^{18}oxygen-incubation system. Thus the amount required for ^{18}oxygen purchases should remain essentially the same. We estimate we will require 0.5 L of the 61 atom % ^{18}oxygen per experiment, and anticipate conducting an average of one**

experiment every week. Thus purchasing ^{18}oxygen in the larger quantities from Icon Services: 50 weeks x 0.25 L x $551/L ~ $7000/yr

ZZZZ Lab: $6000 total: MS Instrumentation supplies; $1500. General labware; $1500. Reagents, chemicals and HPLC solvents; $1500. Library and copy charges; $500. Annual software upgrades for MS instrumentation and other computer supplies; $1,000.

Travel: $3000/yr for the Scientist/ZZZZ labs, to cover travel expenses for PI (Scientist, $1000) and Co-Investigator (ZZZZ, $1000) to attend 1 annual meeting each to present research results. (Scientist and ZZZZ will attend different meetings to present results.) Also, $1000 for PI to visit the XXXX laboratory for 1 week each year to discuss and evaluate results, plan and revise experimental design, and cross-train in experimental techniques.

Other Expenses: **TOTAL**: **$9470**

Maintenance contracts/annual certifications (Scientist/YYYY Lab) [costs adjusted to cover only % use each instrument on current project]: prorated share of Scientist lab and departmental maintenance contracts and certifications for those (shared) instruments to be utilized on this project (ultra and high speed centrifuges, beta counters, film processor, hoods, spectrophotometer, HPLCs, balances)	**$2500**
Liquid Nitrogen ($0.4/L X 20 L X 52 weeks)	**$420**
Page Charges, etc.: (Scientist/YYYY Lab)	
Publications charges (page charges, photography)	**$1200**
Communication (Telephone, fax,)	**$300**
FedX shipping of samples (to XXXX Lab)	**$200**
Library Charges (Interlibrary Loan - journal articles - $5.00 ea.)	**$300**
Waste Disposal	**$200**

($2200)

(Scientist/YYYY Lab - $2200): - Expenses for 3 publications/yr.; communication charges for telecommunications (annual lab and office phone line service is charged back to investigator at UNM ($258/line @ I ea., for the laboratory line; PIs office line from other sources); and FedX shipping of samples/reagents essential to project to/from other investigators in XXXX lab; Library charges - **I**nter **L**ibrary **L**oan charges for journal articles not held in University collection - 60/yr at $5.00 ea. based upon our current **ILL** usage rates). Hazardous waste disposal charges by UNM Safety Dept: (PAHs, etc): $200

Animals: A minimal number of animals have been selected for use in the proposed studies. Most of the studies will be conducted with immortalized rodent or human cell lines that have been engineered to express CYP isoforms or AhR constructs, greatly reducing the need for use of live animals. However a few studies will require either primary rodent hepatocytes in culture, or using subcellular components of cells or tissues prepared from rodent tissues or cultured cells,.

Approximately 50 male Sprague Dawley rats, 150 g, will be utilized each year as donors for primary rat hepatocyte culture experiments, and approximately 25 for preparation of liver cell microsomes. A single rat liver is sufficient to yield on average 5 x 10^8 cells, sufficient for 50 plates (treatment groups). Most of our experiments are designed to require 36 - 72 plates, i.e. 1-2 rats. Each experiment requires approximately 2 weeks to conduct, so we anticipate conducting 25 primary rat hepatocyte preps/yr. We always order 2 rats/experiment, in case of difficulties with the initial perfusion and cell harvest. Animals are typically housed for 14 days prior to and during experimental treatments, prior to hepatocyte preps or harvest of livers for microsomes. Although we anticipate the majority of the animal experiments may be conducted during the first year, during the ^{18}oxygen-metabolism assay and MS methodology development and standardization protocols, we will continue to require animals for cell and microsome preparations that will routinely be run as 'positive controls' to verify that our assays and instrumental analyses procedures are in fact working as predicted. The numbers shown for animals represent a reasonable average for the 3-year project duration.

Cost per rat (incl. Shipping/ordering costs)	$13.90
***Per diem**	$0.27
total for 75 rats x 14 days	= **$1350/yr**

***Note: per diem has increased from $0.25 to $0.27 since original submission**

Mass Spectrometer Time/Maintenance: ZZZZ Lab**:** Departmental charges to Dr. ZZZZ for use and maintenance of TSQ-7000 Mass Spectrometer. Adjusted based on anticipated annual hourly usage for on this instrument only for this project. There are no charges for the ZZZZ lab designed and constructed instrumentation, thus total Mass Spectrometer instrumentation costs will be extremely modest for the amount of work proposed. **$3000/yr**

RESOURCES AND ENVIRONMENT - (University of Whatever)

FACILITIES: Mark the facilities to be used at each performance site listed in Item 9, Face Page, and briefly indicate their capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Use "Other" to describe the facilities at any other performance sites listed in Item 9 on the Face Page and at sites for field studies. Use continuation pages if necessary. Include an explanation of any consortium/contractual arrangements with other organizations.

☒ Laboratory: John Q. Scientist Lab: One 300 sq. ft. and one 375 sq. ft of dedicated laboratory space in the Pharmacy Building at the **University of Whatever** Health Sciences Center. Access to additional shared laboratory space and instrumentation in Department and in co-investigators laboratories. Dr. John Q. Scientist's labs contain most of the necessary equipment for this project (see MAJOR EQUIPMENT). One lab contains two student/technician desk/work areas.

☐ Clinical: NOT APPLICABLE

☒ Animal: An AAALAC-accredited animal care facility, certified for rats, mice and rabbits is located within the College of Wherever building, directly adjacent to PI's laboratories. This facility includes dedicated animal care and housing, surgical preparation facilities, full-time animal care staff, and supervision by a full-time certified lab animal veterinarian.

☒ Computer: Dr. John Q. Scientist has one PII-300 IBM PC type computer and printer in his office for word processing and data analysis.. There are three 386SX-class and two 486 IBM PC class computers with dot-matrix/inkjet printers, and one P200 IBM PC computer with color inkjet and color flatbed scanner in Dr. John Q. Scientist's labs, for controlling the spectrophotometer, HPLC system, and radioisotope flow detector, and PCR thermocycler. Both Pentium Computers are connected to the INTERNET via high speed direct lines for communication with collaborators and access to on-line literature and molecular biology databases; both P5 machines are running OLIGO for primer design and DNAsis for general molecular biology data base searches, sequence manipulations, etc. We also have IMAGE QUANT for densitometric analyses, STATMOST for statistics, ORIGIN for data plotting and analysis, RASMOL, RNADRAW, PLASMID and CLONE MAP for molecular modeling and drawing.

☒ Office: Dr. John Q. Scientist has 100 sq. ft of office space adjacent to and connected to his laboratories. Dr. John Q. Scientist has access to departmental photocopy and telefax machines and limited access to shared secretarial support.

☒ Other: See following Continuation page for additional RESOURCES AND ENVIRONMENT.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Please see continuation pages.

ADDITIONAL INFORMATION: Provide any other information describing the environment for the project. Identify support services such as consultant, secretarial, machine shop, and electronics shop, and the extent to which they will be available to the project.

RESOURCES and ENVIRONMENT - University of Whatever (continued)

MAJOR EQUIPMENT: Except as noted under "REQUESTED EQUIPMENT" under BUDGET, the facilities described below will provide all the necessary space and equipment that is anticipated to be required to conduct the studies described in this proposal:

1) **Dr. John Q. Scientist's laboratory** (dedicated equipment available to project located in Dr. John Q. Scientist's laboratory spaces)

- analytical balance
- preparative balance, top loading
- liquid nitrogen cryostat 35 HC for sample storage; (2 ea)
- ultra low temperature freezer (-70°)
- freezer (-20 °)
- refrigerator and refrigerated cold box (4°) (1 ea)
- Forma digital temperature controlled water bath, ±0.2 °C (1)
- 4 L general purpose water baths ambient - 90 °C); (4 ea)
- Dry-bath block type sample heater (2)
- electrophoresis equipment & power supplies (vertical PAGE/proteins) (3 ea)
- electrophoresis equipment and power supplies (horizontal agarose/nucleotides) (4 ea)
- electrophoresis equipment and power supplies (vertical for nucleotides) (1 ea)
- isoelectric focusing apparatus and power supplies (1 ea)
- western blot equipment and power supplies (3 ea)
- mini-slot and mini-dot blotter (1 ea)
- northern blot apparatus (3 ea)
- Shimadzu 2100 dual beam UV-VIS spectrophotometer
- 30 bit color/grey scale 9600 dpi flatbed scanner/Image Quant densitometry software
- Beckman gradient HPLC system with model 166 UV-Vis variable wavelength detector; Shimadzu RF535 dual monochromator fluorescence detector, Shimadzu autoinjector, Radiomatics FLO-1 model CR radioisotope flow detector and data acquisition and analysis software
- Isocratic HPLC system\Model 153 UV-Vis detector for radioligand purifications
- Shimadzu LC-10S HPLC binary gradient system (2 pumps, controller, 1 UV-Vis variable wavelength UV detector, 1 fixed wavelength (filters) UV-Vis detector)
- Gilson fraction collector model #203
- Beckman microfuge Model E (1)
- Beckman GS-6KR refrigerated low speed centrifuge
- Stratagene Picofuge
- Laminar flow hood (4 ft) (2 ea)
- Olympus CK-2 inverted microscope

- Blue M bacterial incubator (convection)
- CO_2 water jacketed incubators (2)
- autoradiography equipment
- UV transilluminator and Polaroid camera
- surgical equipment, peristaltic perfusion pumps and small animal ventilator

RESOURCES and ENVIRONMENT - University of Whatever (continued)

- orbital shaking water bath
- orbital shakers for blotting experiments
- rocking table, dual platform
- Rot-A-Vap vacuum concentrator
- Amicon ultrafiltration concentrators (4 ea)
- column chromatography apparatus
- TLC apparatus
- homogenization equipment
- pH meters w/ standard and microtip probes
- microwave oven
- convection drying oven
- micro pipettors and liquid handling devices
- MJR PTC-200 (dual 48-well head) PCR thermocycler with dual head *in-situ* hybridization accessory
- Turner Model 20/20 luminometer w/autoinjectors
- UV crosslinker
- Hybridization oven (4 tube roller bottles)

2) **Dept. of Wherever:** (shared instrumentation adjacent to Dr. John Q. Scientist's laboratories in School of Whatever, and routinely available to Dr. John Q. Scientist).

- automated X-ray film processor & darkroom facilities
- Speed Vac Concentrator & vacuum pump
- Millipore water purification system
- Sorval RC2B preparative centrifuge and rotors
- Beckman (L6 and L7) ultracentrifuges and rotors
- Beckman Optima TLX Benchtop Ultracentrifuge and rotors
- liquid scintillation spectrometers (Wallac 1410, LKB 1212)
- Perkin Elmer LS50B spectrophotofluorometer with computer control and data acquisition; and Aminco Bowman fluorometer
- microtip sonicator
- Molecular Dynamics UV/VIS SpectraMax 340 96-well microplate reader
- BioRad GS-363 Molecular Imager
- autoclave
- Beckman DU-640 UV-Vis spectrophotometer
- Aminco-Bowman DW-2 dual beam/dual wavelength UV-Vis spectrophotometer
- Beckman Accu-6 IR
- HP 8452A Diode Array Spectrophotometer
- Pharmacia FPLC System
- Nikon inverted microscope
- Olympus BH2 RFCA fluorescence microscope

- Olympix high resolution CCD Color Camera System for Olympus Fluorescence Microscope
- Kodak Image Station CF440 - CCD camera based densitometer (direct imaging/qunatitation of ECL)
- Finnigan LCQ-DUO LC- ESI-ion trap Mass Spectrometer
- Olympus IX-70 Inverted Fluorescence Microscope

RESOURCES and ENVIRONMENT- UNM (continued)

3) **Dr. Joe's Laboratory**: Dr. Joe's laboratory is equipped with three Thermoseparations HPLC units. Two are connected to a common IN/US beta ram flow beta detector, which operates either with a solid scintilant, or as a flow cell. Two of the HPLC units are binary gradient instruments while the third is a quaternary instrument. The two instruments that are used for research samples have autosamplers while the third unit has a manual injector and is used for method development. The HPLC software is from Thermoseparations and allows us to scan peaks as they elute from the column, use a fluorescence detector, or electrochemical detector in series with the radiochemical detector. The laboratory also ncludes a Cahn C33 microbalance, a Beckman refrigerated centrifuge and rotors, a Savant speedvac, pH meters and analytical balances. The laboratory is equipped for organic synthesis with glassware, vacuum apparatus, rotary evaporator and fume hoods.

Dr. Joe also has unlimited access to Departmental equipment including a HP 5890-II GC equipped with a HP 7633 autosampler and both NP and FI detectors. This unit is controlled via a Pentium PC and data acquired and analyzed using the PC program ChromPerfect (Justice Innovations, Mountain View, CA). Also available is a Shimadzu QP-5050 CI/EI GC/MS with a solid probe inlet and pentium PC data handling and control system and an AOC-20I autosampler. High field (250 MHz and 500 MHz) FT-NMR spectrometers are located within the UNM Department of Chemistry and available to Dr. Joe for his use as needed.

4) **Dr. Jim's Laboratory:** Fully equiped 1000 ft^2. chmistry laboratory and facilities/instrumentation for Mass Spectrometry:

- TSQ-7000 triple quadrapole mass spectrometer with Electrospray and API sources
- Waters MS-500 HPLC pump system for mass spec
- Trapped ion, time-of-flight (TOF) mass spectometer (designed and constructed by Enke lab)
- Tandem time-of-flight mass spectrometer (designed and constructed by Enke lab)

5) **University Health Sciences Center:** (shared instrumentation and facilities adjacent to the School of Whaterver available on a regular basis)

- electronics shop
- machine/carpenter shop

6) **Libraries**:

- University of Whatever Library, located adjacent to Dr. John Q. Scientist's laboratory spaces in School of Wherever. Monographs and periodicals, and MEDLINE CD-ROM.; Interlibrary load services (for fees)

- Also available nearby on campus are other University Libraries, including: Chemistry and Biochemistry.

A) SPECIFIC AIMS: CV-1 cells exhibit a high level of constitutive AhR activity which is repressed by expression of CYP1B1. **Central Hypothesis:** We will test two separate but *converging* hypothesis: **(1)** There are significant levels of endogenous ligand(s) (or alternatively, endogenous regulators) for the Ah Receptor (**AhR**), in CV-1 cells that are metabolized by cytochrome P450 1B1 (**CYP**1B1), (and possibly other CYP isozymes), and that CYP-mediated metabolism alters the affinity of endogenous ligands for the AhR, or alternatively, alters the function of other endogenous AhR regulators. **(2)** Endogenous substrates for CYP1B1 exist that function as important mediators of cellular metabolism, and one or more of these substrates function as AhR ligands or regulators of AhR activity. The few known endogenous ligands for CYP isozymes do have important physiological functions in maintaining normal cellular homeostasis, growth or differentiation. Proof of *either* of these two independent yet converging hypotheses *alone* will constitute a major contribution to the understanding of the normal physiological roles of the AhR, CYP1B1 and potentially many other CYPs.

Specific Aim 1: Determine the mechanism by which CYP(1B1) regulates AhR transcriptional activity.

Aim 1a. Elucidate the potential mechanisms by which select human CYPs reduce constitutive Ah receptor transcriptional activity. Transiently co-transfect expression constructs for human CYP1A1, 1A2, 1B1, 3A4 or 2E1, mAhR, and a DRE-driven luciferase reporter, into wild-type CV-1 cells (which do not constitutively express AhR or CYP isoforms). This will accomplish three goals; **1)** The specificity of each expressed CYP capable of lowering constitutive AhR activity will yield insight as to the type of endogenous substrates that may be involved; **2)** Determe which CYP is *most* capable of metabolizing AhR endogenous ligands to develop the most efficient stable CV-1 transfectant for use in Specific Aim 1b; **3)** Prove the decrease in AhR constitutive activity is in fact due CYP *catalysis* and, not only to *expression* of a CYP enzyme that leads to indirect effects on the AhR signal transduction pathway.

Aim 1b: Determine the role and specificity of CYP *metabolism* in regulation of AhR transcriptional activity. Establish that *catalytic activity* of each expressed P-450 (Aim 1a) is obligatory for reduction of constitutive AhR transcriptional activity by creating cell lines expressing catalytically incompetent CYP isoforms.

Aim 1c. Construct a series of stable CV-1 cell lines expressing CYP1B1 that will result in low constitutive AhR activity and low CYP substrate levels. Construct a series of stable CV-1 cell lines expressing a catalytically incompetent CYP1B1 that will result in high constitutive AhR activity and high CYP substrate levels. These cell lines will be utilized for experiments to establish that CYP *activity* causes a reduction of AhR activation and as conrols for endogenous ligand experiments (Aim 2) and for endogenous substrate experiments in Aim 3. This aim has now been partially completed as described in Preliminary Results.

Specific Aim 2 :Identify endogenous Ah Receptor ligands/regulators in CV-1 cells and characterize their mechansim of action. CV-1 cells exhibit a high level of constitutive AhR activity which is repressed by expression of CYP1B1. Extracts from both "wild-type" and CYP1B1-expressing/CV-1 cells will be fractionated by HPLC. Fractions will be assayed by *cell culture AhR reporter assays* and ligand binding competition binding assays to identify molecules capable of specific binding to AhR. Fractions containing AhR ligand binding activity specific to each cell line will be further characterized. Additional complimentary studies will be conducted with cell lines derived from AhR and CYP1B1 'knockout' mice to further investigate the normal physiologic role of the AhR ligands identified.

Specific Aim 3: Identify endogenous substrates for cytochromes P450 that also function as endogenous ligands for the AhR (or as AhR regulators), via labeling with $^{18}O_2$ and ESI-MS analysis. $^{18}O_2$ Labeling Assays: Develop, calibrate and validate ESI-MS detection protocols employing ^{18}O-labeled labeling of monooxygenase substrates as a general approach for identifying unknown (endogenous) CYP substrates. Utilize this approach for the Identification of Endogenous CYP1B1 Substrates in CV-1 cells, *and* substrates that may also function as AhR ligands/regulators: Conduct *in vitro* incubations with CV-1 cells, engineered to express functional or non-functional CYPs, in the presence of $^{18}O_2$ and in the ABSENCE OF EXOGENOUSLY ADDED SUBSTRATES. CYP1B1 metabolites (and thus putative CYP substrates/AhR ligands) will be identified by ESI-mass spectrometry. Additional complimentary studies will be conducted with rodent *cell lines* derived from AhR-/- and CYP1B1-/- 'knockout' mice to further investigate the normal physiologic role of the CYP1B1 substrates identified.

B: BACKGROUND AND SIGNIFICANCE

INTRODUCTION: Cytochromes P450 (**CYP**) are a large family of enzymes catalyzing the metabolism of both endogenous and exogenous substrates in humans. Thus these enzymes are key modulators of normal cell homeostasis, growth and differentiation, and also contribute significantly to both the bioactivation and detoxification of a wide variety of human toxicants and chemical carcinogens. The improved understanding of the normal cellular functions of CYP isoforms and the molecular mechanisms governing their cell-specific expression anticipated to result from the proposed studies will have broad and significant impact on our understanding of a variety of human disease processes (including but not limited to: tumor promotion, teratogenicity, immunotoxicity, hepatic- and dermal- toxicity, alteration of cell differentiation, and proliferation, perturbations of endocrine homeostasis and steroid hormone-dependent cellular responses, and induction of gene expression), as well as mechanisms and risks of human chemical carcinogenesis. The primary CYP isozyme selected for study in this proposal, CYP1B1 has been implicated to play key roles in normal cell homeostasis, growth, differentiation, as well as the metabolism of xenobiotics, and is also reported to play a key role in at least one congenital human disease. This proposal describes a powerful and novel integration of molecular and physical approaches directed to the elucidation of the normal physiologic substrate(s) of CYP1B1 (and ultimately, *essentially any other CYP isozyme*), as well as the normal physiologic ligand(s) of the Ah Receptor (**AhR**). This will eventually allow us to elucidate the normal physiological roles for each of these two important proteins, and although such studies are beyond the scope of the current, highly focused project, such studies would obviously constitute future directions for this research group upon completion of this current project. The studies described herein are deemed to have the potential for very 'high impact' on our understanding of BOTH the normal physiologic roles of many CYP isozymes as well as normal AhR signaling events, and thus our understanding and potential treatment of a number of human disease processes. While we initially deemed the studies described herein to be somewhat 'high risk' during the original submission of this application, in view of our substantial additional preliminary data supporting our two central hypotheses, and the feasibility and validity of our experimental approaches, *we now retract that assessment and believe the proposed studies have excellent potential to be successfully completed and generate extremely interesting and useful new information.*

Cytochromes P450 (CYP): Cytochromes P-450 (**CYP**) are a large superfamily of genes, encoding hemoprotein monooxygenases catalyzing a wide array of oxidative reactions including xenobiotic metabolism and steroid biosynthesis in nearly all eukaryotic organisms. This superfamily is composed of numerous isozymes, characterized as being 'promiscuously active' with broad yet overlapping substrate specificities for the detoxification and/or bioactivation of xenobiotics {1}. Cytochromes P450 have been shown to be expressed in nearly all eukaryotic organisms and many prokaryotes. The P450 superfamily is comprised of over 750 distinct genes, divided amongst many distinct gene families and subfamilies. Humans have 37 sequenced CYP genes and 12 pseudogenes in 16 of the P450 families, and the total number of human CYP genes is currently estimated to be approximately 51.

Cytochromes P450 are widely recognized for their metabolism of foreign chemicals, usually catalyzing the insertion of atomic oxygen into substrates in a general pattern of biotransformation of small, lipophilic substrates into more polar, less biologically active metabolites. However, because of their nearly ubiquitous phylogenetic distribution and widespread evolutionary conservation, CYPs are also postulated to catalyze a variety of essential reactions with endogenous substrates. Indeed, evidence is accumulating for the role of CYP in the metabolism of a growing list of endogenous substrates, including fatty acids, steroid hormones, a number of vitamins, leukotrienes and prostaglandins. *Thus CYPs play an essential role in the maintenance of human health via synthesis of essential endogenous molecules as well as the detoxification of xenobiotics.*

Molecular Mechanisms for Regulation of CYP expression: The factors which regulate cellular levels of cytochrome P-450 (**CYP**) isozymes are complex and multi-factorial, tissue- and cell-specific differences in P-450 isozyme expression are extensive, and the mechanisms governing these processes are not yet fully understood. The mechanisms of induction for most CYP isoforms is still only partially understood at the molecular level, although the transcriptional activation of the CYP1A and 1B families via ligand activation of the aryl hydrocarbon receptor (AhR) has been widely studied and well characterized at the molecular level

{2,3,4,5}. Numerous studies have established that many CYP substrates, including naturally occurring plant secondary allelochemicals, drugs and other xenobiotics can increase (induce, or up-regulate) or decrease (down-regulate) the expression of specific CYPs, via transcriptional as well as post-transcriptional events {6,7,8,9,10,11,12,13},(Scientist and Denison, in preparation). CYP-mediated metabolism of substrates is recognized as an important initial event for regulation of expression of some CYPs {9,12,14}. The most widely studied and best understood of CYP ligand-mediated regulatory pathway is that of the *trans*-acting **Ah r**eceptor (**AhR**), which has been shown to be largely responsible for the toxic and tumor promotional activities of 2,3,7,8-tetrachlorodibenzo-p-dioxin (**TCDD**) and other polycyclic aromatic hydrocarbons (**PAH**) and is a key mediator of CYP1A transcriptional activation in most species {14}. Importantly, ligand activated AhR is also an effective *trans*-activator of a whole battery of additional genes with important physiological functions, including UDP glucuronyl transferase (**UDPGT**), quinone reductase (**QR**), epoxide hydrolase (**EH**), aldehyde dehydrogenase, plasminogen activator inhibitor 2 (**PAI-2**), Interleukin-1β, (**IL-1β**) {15}, and glutathione-S-transferase (**GST**) {9,16,17}.

The Aryl Hydrocarbon Receptor: The adverse biological effects of halogenated polycyclic aromatic hydrocarbons (HPAH) are of concern because of their widespread distribution as contaminants in the environment {18,19}. The prototypic compound that is most often used to study the toxic response to HPAH is 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD). TCDD binds with high affinity to the Ah receptor (AhR) which results in activation of this transcriptional factor to a DNA binding form. A wide range of biological effects can be attributed to ligand mediated activation of the AhR; these include thymic atrophy, a slow wasting syndrome, teratogenesis, and tumor promotion {20}. The most characterized response at the molecular level is the transcriptional activation of the CYP1A1 gene via binding of the liganded AhR heterocomplex to recognition motifs (XREs) upstream of AhR-responsive genes {21,22}. Little is yet known about the role of the AhR in development and normal cellular metabolism. Although null AhR allele mice have been developed, they exhibit no obvious phenotype. However, these mice exhibit abnormal liver growth and development, and reduced reproductive success, thus indicating a role for the AhR in development {23,24,25,26}

The AhR has been cloned, and sequence analysis has revealed a helix-loop-helix/basic region motif responsible for DNA binding and dimerization {27}. Proteins in this family of transcriptional factors usually form heterodimers prior to interacting with DNA. An AhR dimerization partner, ARNT (Ah Receptor Nuclear Translocator protein) has been cloned, and is required for formation of an AhR complex capable of enhancing gene transcription in Hepa-1 cells. Several groups have performed mapping of specific AhR functional domains including; the basic region, helix-loop-helix, PAS, ligand binding, and HSP90 binding domain {28,29,30}. Several more members of the PAS domain family have recently been cloned, including HIF-1, hSim, ARNT2, and AhR2 {31,32,33}. One interesting aspect of this emerging family of transcriptional factors is that ARNT appears to be a dimerization partner for a number of these proteins, suggesting that ligand-activated sequesteration of ARNT could alter signalling through a myriad of pathways. While much as been determined about the role of the Ah receptor in the toxic response to TCDD, little remains known about its normal biological function. In addition, the physiological role of CYPs in endogenous substrate metabolism, such as CYP1A1, CYP1A2, CYP1B1, which are regulated by the AhR, has not been determined. *Numerous investigators have speculated for many years that there is most likely at least one endogenous ligand for the Ah receptor, and that this endogenous ligand must play a key role in mediating various key parameters of cell homeostasis*.

AhR ligands: Numerous 'non classical' PAH/HAH ligands with an astounding range of structures can activate AhR (see {34} for a review). The structure/activity relationship for these 'non-classical' AhR ligands which are not consistent with the commonly accepted dogma of structure/activity relationship for PAH and HAH ligands (planar ligands of 14 x 12 x 5 Å dimensions, ca. 300 MW), is still unknown. While the molecular size and structure of the 'non-classical' AhR agonists varies considerably from that of the PAH and HAH agonists, the only common paradigm for the 'non-classical' AhR ligands is that they commonly contain at least one or more hetero atoms (N or S), as well as least one aromatic ring. Many of the known 'non-classical' AhR agonists are naturally occurring plant compounds, many of which are contained in human food plants but are safely detoxified by mammalian CYPs {35}. It has been hypothesized that no endogenous

ligands for the AhR exist, but only 'natural' ligands, derived from food source components, which serve to induce the synthesis of protective Phase I and Phase II detoxification enzymes. However, substantial evidence exists to support the existence of 'endogenous' AhR ligands as discussed below and in 'Preliminary Results'. Among the better known 'naturaly occuring' ligands for the AhR are; methylenedioxybenzenes (MDBs), {34,36,37}, (Scientist and Denison, manuscript in preparation); indoles, present in high quantities in cabbage, broccoli, cauliflower, and brussel sprouts, as well as other plants of the Brassica genus {34,38}; tryptophan (TRP), tryptophan-containing compounds, TRP-metabolites {34,39} and TRP-photoxidation products {34,40,41}, carotinoids, another group of naturally occurring plant compounds {34,42,43}; and heterocyclic amines commonly formed during cooking of meat products at high temperatures {34,44}.

Endogenous AhR ligands: Although high affinity exogenous PAH and HAH ligands are those most widely recognized as AhR agonists, evidence has been accumulating to support the hypothesis that true 'endogenous' ligands for the AhR do in fact exist and play an essential role in cell growth and differentiation and maintenance of normal cellular homeostasis. While considerable indirect evidence for the existence of such endogenous ligands exists, such a molecule has yet to be identified and characterized. *This is one of the two primary goals of the current proposal*. A number of laboratories have detected AhR complexes localized in the nucleus of cells untreated with xenobiotics, interpreting this finding as evidence for the existence of endogenous ligands {45}. More convincing evidence for the existence of endogenous AhR ligands is derived from experiments utilizing AhR 'knockout' mice which manifest a variety of physiological defects in hepatic and immune function, suggesting a tissue-specific requirement for endogenous activation of the AhR during critical windows of growth and development {23,24}. Suspension-mediated induction of CYP1A1 (an indication of AhR ligand activation) in normal human keratinocytes in culture, and of Cyp1a1 in cultures of wild-type Hepa 1c1c7 in the absence of exogenous xenobiotics has also been reported as evidence of both endogenous AhR ligands, as well as the possible metabolism of such endogenous ligands by the CYP1 family. As further evidence for endogenous AhR ligands, (and possibly CYP-mediated feedback regulation of AhR), constitutive expression of CYP1A1 in cultured cells with functional AhR but *not* in mutants with non-functional AhR, or in cells whose expression of AhR was blocked with antisense AhR molecules has been described {46,47,48}. **'**Feed-back inhibition' is a relatively common paradigm in biology whereby enzymatic metabolism of a substrate with regulatory activity controls the regulatory activity of the substrate. Metabolism converts a molecule with transcriptional activation function (or with negative regulatory functions) to a non-functional metabolite. (The alternative form of this hypothesis is that metabolism of an inactive precursor generates an active transcriptional *repressor*). *This paradigm has also been hypothesized to apply to some, if not all, of the known members of the cytochrome P450 superfamily of enzymes*.

The first evidence for such a mechanism involving interactions between the CYP1 family and (endogenous) ligands of the AhR derives from the constitutive expression of very high levels of CYP1A1 mRNA in CYP1A1 mutant cell lines unable to express functional CYP1A1 holoenzymes, and suppression of these high levels of CYP1A1 mRNA by a variety of rescue methods entailing expression of functional CYP1A1 activity {49,50}. As further evidence for CYP-mediated negative-feedback regulation of AhR activity, other investigators have reported high constitutive expression of CYP1A1 in cultured cells with non-functional mutant AhR, or in cells whose expression of AhR was blocked when treated with antisense AhR molecules {46,47,48}. Bilirubin and two other PAH-inducible products of heme catabolism, hemin and biliverdin have been shown to directly bind and activate the AhR in cultured mouse (Hepa 1c1c7) hepatoma cells {51,52,53}, also implies the existence of a regulatory feed back loop involving CYP metabolism and endogenous AhR ligands. *In vivo* treatment of rats and *in vitro* treatment of cultured hepatocytes with a mixture of the P450 inhibitor metyrapone and dexamethasone dramatically increases levels of CYP1A1 mRNA {54}, again strongly suggesting the existence of an endogenous AhR ligand that is metabolized by CYP isozymes {54} and which plays an essential role in maintaining cellular homeostasis. Further evidence for negative feedback control via the AhR and an endogenous ligand has been provided by studies with 5L rat hepatoma cells and their mutants lacking functional AhR {48}. Single hybrid analyses, comparative studies between wild-type 5L cells and mutant BP8 cells lacking AhR, BP8 cells transiently transfected and 'rescued' by AhR expression vectors, in combination with *in vitro* ligand binding assays with cytosolic extracts from these cell lines provides powerful support for the role of an endogenous AhR ligand and a negative feedback regulatory mechanism

{48}. Similarly, other laboratories have also reported evidence for endogenous ligands in lymphocytes {55,56}.

Finally, in a recent series of elegant experiments, Puga and co-workers have provided very convincing evidence for the existence of a negative feedback regulatory pathway involving an endogenous AhR ligand metabolized to an inactive form by CYP1A1 {57}. These investigators conducted a comprehensive series of experiments with CYP1A1-defective mouse hepatoma cells (c37) and AhR-deficient African green monkey CV-1 cells. Using a combination of several powerful molecular biology-based approaches, a green fluorescent protein tagged AhR, and gel mobility shift assays, the investigators demonstrated high levels of nuclear, constitutively active AhR-ARNT complexes in these cells, even in the absence of exogenously applied ligands. Constitutive expression of an AhR-driven luciferase reporter constructs was significantly increased by treatment with the CYP1A1 inhibitor ellipticine in CYP1A1-transfected CV-1 cells, or decreased as a result of expression of functional CYP1A1 in the CYP1A1-deficient CV-1 cells. This evidence strongly suggesting the existence of a CYP-metabolized endogenous AhR ligand was further supported by similar observations in wild-type hepatoma cells pretreated with CYP1A1 inhibitors. *All of the above data strongly implies the existence of endogenous AhR ligand(s) which participate in a negative regulatory feedback loop, modulated by metabolism of this active endogenous ligand by CYP1 isoforms to an inactive oxygenated metabolite. These data, along with our preliminary data, strongly support the rational for our hypothesis and the justification for conducting our proposed studies.*

Endogenous CYP substrates: Microsomal cytochrome P450s are most widely recognized for their role in the oxidative metabolism and detoxification of exogenous substrates (i.e. xenobiotics). However, increasing evidence has been accumulating that microsomal CYP isoforms may also play important roles in the metabolism of endogenous substrates as well, with the CYP-mediated metabolism of arachidonic acid and retinoic acid as two important examples {58,59,60}. In most cases, however, the endogenous substrate(s), if any, for most microsomal CYP isoforms remain as yet unidentified, leaving open the question as two whether or not all cytochrome P450s indeed metabolize important endogenous substrates that have simply yet to be identified, or whether some (or many) isoforms have evolved solely to oxidize xenobiotics {9,61,62}.

CYP1B1: Function and Molecular Mechanisms for Regulation of expression: CYP1B1 is a relatively recently identified member of the CYP1 family, simultaneously and independently characterized in mouse and human by two separate laboratories utilizing different experimental approaches {63,64,65}. Functional orthologs of CYP1B1 have been detected in adrenal, liver, mammary, skin, uterine and embryonic tissues. Importantly, CYP1B1 has been shown to contribute significantly to the bioactivation of PAH's and other procarcinogens, especially in extrahepatic tissues where it is constitutively expressed {66,67,68,69,70}. CYP1B1 has also been shown to possess substantial 17β-estradiol C-4 hydroxylase activity, and thus may play and important role in endocrine regulation and may also in part mediate the carcinogenicity and toxicity of estrogens {71,72,73}. CYP1B1 expression has been demonstrated to be regulated in part by the AhR, although other mechanisms appear to modulate both constitutive and inducible CYP1B1 expression, as well as the cell- and tissue-specificity of expression of this CYP isozyme {6,74,75,76,77,78,79,80,81,82,83, 84,85,86}. Furthermore, evidence is also accumulating that CYP1B1 may play an important role in endocrine regulatory pathways, is frequently expressed at significant levels in tumor tissue, and may also contribute in part to the altered growth potential of endocrine responsive and other human tumor types {87,88,89,90,91,92}.

Endogenous CYP1B1 substrates: CYP1B1 expressed in the trabecular meshwork of the human eye has been shown to be directly involved in the onset of primary congenital glaucoma {93}. Heritable frameshift mutations in the CYP1B1 gene resulting in non-functional truncated CYB1B1 proteins lacking the essential heme-binding cysteine residue has been demonstrated to be directly linked to and likely a cause, of this autosomal recessive disease {93,94,95}. Furthermore, it has also been shown that local ocular metabolism of arachidonate by *other* CYP isoforms may also be essential for normal function and differentiation of this tissue {96,97}, again suggesting an important role for CYPs in metabolizing endogenous substrates with important physiological functions. Additional evidence for the existence of endogenous CYP1B1 substrates is the finding that CYP1B1 efficiently and selectively hydroxylates 17β-estradiol at the C-4 position at low substrate

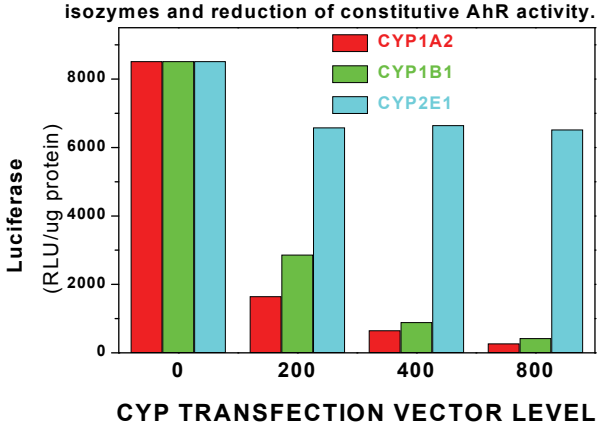
concentrations {71,72,73}. The low K_m for this reaction suggests that 17β-estradiol might be an important endogenous substrate for CYP1B1, although it does not appear to be an AhR agonist. It is very interesting to note that the expression of two other genes demonstrated to participate in the synthesis as well as degradation of the trabecular matrix in humans, interleukin-1β and plasminogen activator-2 are, like CYP1B1, are also regulated by the human AhR {98,99}, and that other genes with important endogenous function (i.e. prostaglandin synthetase) have also been shown to be regulated by the AhR {100}. These findings may also be taken as support for the hypothesis that endogenous ligands (quite possibly metabolites of CYP-mediated metabolism) exist for the human AhR and may serve to regulate the expression of important human genes with a multitude of functions.

AhR-mediated regulation of CYP1B1 has recently been demonstrated by Dr. Colin Jefcoate's research group to play a key regulatory role in adipogenesis, *and this recent report provides extremely strong corroborating evidence that CYP1B1-mediated catalysis results in the formation of oxidized products with important physiological regulatory functions* {101}. The cell lines utilized by the Jefcoate lab for these studies will be available to us as an important new alternative model system to utilize in our identification of endogenous CYP1B1 substrates with important physiologic regulatory roles. A CYP1B1 null mouse has also recently been developed, and does not exhibit an overtly distinct phenotype, even in the eye {70}. While this mouse model implies that CYP1B1 does not play an ***essential*** role in mammalian development or cellular homeostasis, it by no means ***precludes*** a role for CYP1B1 in important physiological functions. Rather, it suggests that important species differences may exist for CYP1B1 endogenous substrates, or that complex mammalian systems may be well compensated for such defects and adapt and survive via gene compensation mechanisms. Furthermore, this mouse model has only very recently been developed, and additional studies may reveal more subtle lesions or defects attributable to the CYP1B1 null genotype. However, cell lines derived from this new CYP1B1 knockout, not available when the initial version of this proposal was prepared, will now provide us with an important new model system in which to test our central hypotheses.

In summary, we believe both previous studies in other laboratories as well as our own initial studies clearly demonstrate that CYP1B1 metabolizes at least one endogenous regulator of AhR activity, and that this regulator is likely an AhR ligand. The identification of an endogenous AhR ligand (or other regulator) modulating the expression of CYP1B1 and other AhR-responsive genes would have broad impact on the understanding of how this important signaling pathway is regulated, and directly impact on questions dealing with the role of this signaling pathway in mediating normal human cellular homeostasis, growth and differentiation. Similarly, identification of endogenous substrates for CYP1B1 would provide important new information about the physiological function of this important human CYP isozyme. It is important to point out that there are no published reports utilizing a methodical approach to detect either endogenous AhR ligands or endogenous CYP1B1 substrates as outlined in this application. Thus this proposal describes a novel approach to addressing two independent yet converging questions with direct impact on questions relating to human health. Successful completion of *either* individual aim by itself via identification of endogenous AhR regulators or CYP1B1 substrates would generate important new information regarding normal function of these two important biological pathways (AhR or CYP1B1). Furthermore, identification of a regulatory feedback mechanism between CYP1B1 and AhR regulation would also provide a wealth of very important new information. And finally, it is important to stress that development of the techniques proposed utilizing $^{18}O_2$ labeling and mass spectrometry *as a general strategy to identify endogenous CYP substrates* would also provide an extremely powerful approach for the identification of putative endogenous substrates for any of the hundreds of CYPs identified to date, and thus suggest functional roles for these individual isozymes.

C: PRELIMINARY DATA:

FIGURE 1: Transient transfections of several CYP isozymes and reduction of constitutive AhR activity.



Evidence for an endogenous AhR ligand: Puga and coworkers have published data suggesting that CV-1 cells after transfection with an Ah receptor construct, have a high constitutive level of AhR/ARNT heterodimer that leads to a high level of constitutive DRE-driven reporter gene activity {57}. In addition, after co-transfection with murine CYP1A1 they observed a dose-dependent decrease in constitutive DRE-driven luciferase activity. Along with other experiments their data strongly suggest that CV-1 cells have a high level of an endogenous AhR ligand. We performed similar experiments using different hCYP constructs, in order to *focus on physiologically important human* CYPs. CV-1 cells were co-transfected with increasing amounts of various CYP mammalian expression constructs along with pGudLuc 6.1 and pCI-hAhR. After 48 h cells were harvested and luciferase activity assessed . The luciferase data was corrected for transfection efficiency and expressed on a protein basis. As shown in (**FIG. 1**), CYP1A2 and CYP1B1 at 800 ng of transfected plasmid exhibit almost total reduction in the level of constitutive AhR activation is seen. In contrast, CYP2E1 exhibited only a small decrease in constitutive activity. As a control we have tested the ability of each CYP construct to in vitro transript and translate, and each of the three constructs tested translated at essentially the same level. This data strongly suggests that CYP1B1 and CYP1A2 (like CYP1A1) are capable of metabolizing an endogenous Ah receptor ligand or regulator. This new data provides strong support for our hypothesis that an endogenous regulator of AhR activity is selectively metabolized by some CYP isozymes to an inactive oxidized form. *The effect is specific to CYP1 family* enzymes in that CYP2E1 does not cause such a decrease in AhR activity proportional to its expression level. Hence CYP1 family enzymes, which are regulated by the AhR appear to metabolize the AhR regulatory molecule, while CYP2E1, which is not under the transcriptional control of the AhR, does not metabolize the regulator. Thus this is not a non-specific general effect arising simply from expression of CYP isozymes in CV-1 cells.

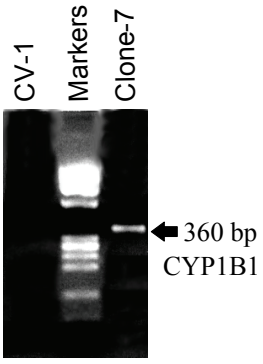
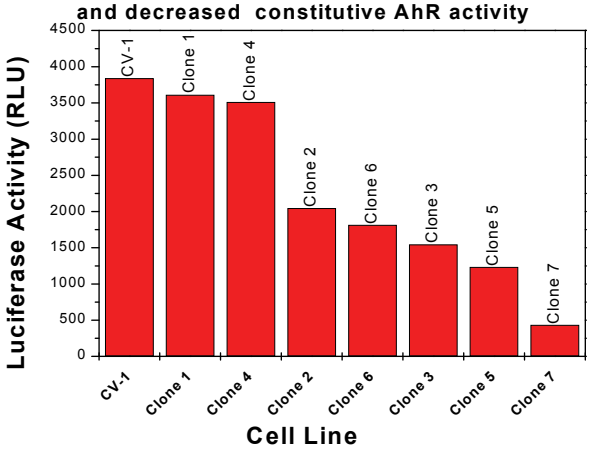


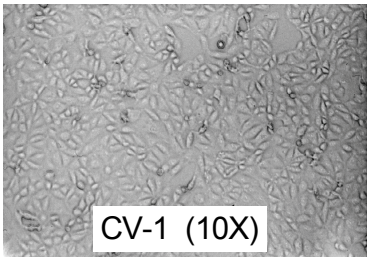
Figure 4: PCR of CYP1B1 in CV-1 and Clone - 7 cells.

FIGURE 2: Stable transfection of CYP1B1 into CV-1 and decreased constitutive AhR activity



A similar result was obtained with CV-1 cell lines engineered to **stably** express varying levels of CYP1B1. A shown in **FIG. 2,** an additional experiment was performed to determine that the concentration of hygromycin in culture medium that resulted in 100% CV-1 cell death in 14 days was 120 µg/ml. We then generated stable cell lines by transfecting CMV/CYP1B1 and pREP4/EBNA/Hygromycin at a 10/1 ratio in CV-1 cells. After two weeks of selection most of the transfected cells died and colonies were isolated and expanded. Seven clones to date have been expanded and tested for the level of constitutive AhR activation. Each CV-1 clone was transiently transfected with pGudLuc 6.1, CMV/ßGal, and pcDNA3/mAhR as above. Luciferase activity was measured and compared with nontransfected CV-1 as a control (**FIG. 2**). This data verifies our ability to efficiently generate stable clones. In fact, the use of a co-transfection protocol with a 10/1 ratio with the stable selection marker on the plasmid used at one tenth of the CYP1B1 plasmid, resulted in a high efficiency of expression in the clones isolated. In addition, this also demonstrates that these stable clones can express a

sufficient level of CYP1B1 to obtain a highly significant expression-dependent decrease in the level of constitutive AhR activity.



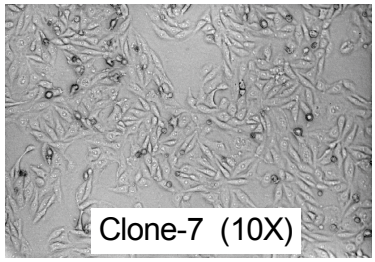
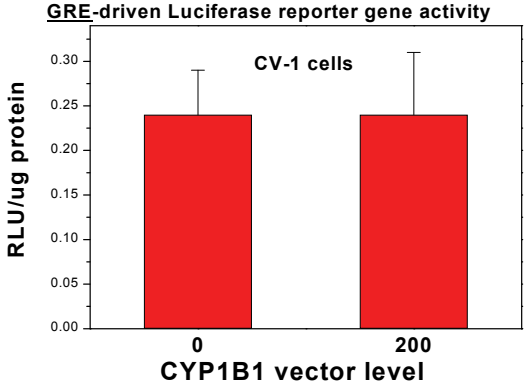


FIGURE 3 : Photomicrographs of CV-1 and Clone-7 cell lines.

FIG. 3 shows a photomicrograph of parent CV-1 cells and Clone-7, the CYP1B1 stable transfectant expressing the highest levels of CYP1B1. Both cell lines are morphologicaly similar, however the Clone-7 cell line grows at approximately one-half the rate of the parent CV-1 line. **FIG. 4** shows an RT-PCR experiment with hCYP1B1 primers, revealing no expression of hCYP1B1 in the parent CV-1 cell line and high expression in the stably transfected Clone-7 cell line as shown by the predicted PCR product at 360 bp. {90}. Preliminary *in vivo* metabolism studies revealed a dose-dependent selective toxicity of E_2 to Clone-7 cells, presumably due to bioactivation of E_2 to cytotoxic catechol estrogrens by CYP1B1, while no toxicity was observed in parent CV-1 cell line even at highest E_2 substrate concentrations tested (not shown).

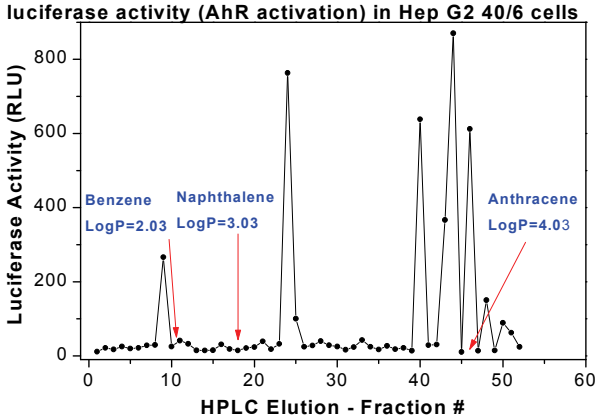
Although it is generally accepted that the pGudLuc 6.1 reporter is under the sole control of the 4 DREs and no other known or identifiable response elements {102,103}, in order to enhance our confidence that the repression of DRE-driven luciferase activity is *not to due alterations in luciferase itself or other non-specific alterations in transcription*, CV-1 cells were transfected with the Glucocorticoid Responsive Element (**GRE**)-driven reporter construct TAT3/Luc along with CMV/CYP1B1. The **GRE**-driven luciferase vector (obtained from Dr. Jeffrey Miner) contains two synthetic GRE elements immediately upstream of the SV-40 promoter. CV-1 cells have a significant level of endogenous glucocorticoid receptor and steroids are present in the medium. No difference was detected in luciferase activity upon the addition of 200 ng of pRc/CMV/CYP1B1 (**FIG. 5**). In contrast, the **DRE**-driven reporter construct is inhibited 50% by a similar level of CYP1B1 expression (**FIG. 1 & 2**). Our results thus confirm and extend previous reports {57} that expression of CYP1A1 and 1B1 leads to a depression in constitutive Ah receptor activity as measured by a DRE-driven reporter construct. (This data does not yet rule out the possibility that lowered constitutive reporter gene activity is via an endogenous ligand-independent mechanism or via some other AhR regulator).

FIGURE 5: Effect of CYP1B1 expression on 'Control' GRE-driven Luciferase reporter gene activity



To further demonstrate the existance of endogenous AhR ligands/regulators in CV-1 cells, and their stability toward our proposed isolation/purification procedures, CV-1 cell lysates were prepared, and subjected to isocratic reverse-phase HPLC fractionation. Individual HPLC fractions were collected and analyzed for AhR activation properties by the cell-culture reporter assay system described above (**FIG. 1 & 2).** Results shown in **FIG. 6** clearly demonstrate that several HPLC fractions contained AhR-activating molecules and their AhR-activating properties were preserved by this initial purification step. Since these are relatively crude HPLC fractions, we anticipate each fraction containing AhR-activating properties likely contains multiple components and will require additional purification characterization as described in Aim 3.

FIGURE 6: Effect of whole CV-1 cell extract fractions on luciferase activity (AhR activation) in Hep G2 40/6 cells



Detection of AhR Ligands. The key methods required for Specific Aim #2 are reverse-phase HPLC of crude cellular extracts and Ah receptor ligand binding competition assays. In the past we have used these methods to detect AhR ligands in indole-3-carbinol acid condensation mixtures. Indole-3-carbinol has been shown to undergo a condensation reaction to form indolo3,2βcarbazole, a high affinity AhR ligand. Briefly, indole-3-carbinol was placed in 50 mM HCl and incubated for 80 min at 37°C. The mixture was dried down, solubilized in ethanol, and warmed to increase solubility. This extract was filtered and injected onto a Ultracarb 5 ODS20 reverse phase column equilibrated in water. A gradient of 0-55% acetonitrile was applied over 5 min at 1 ml/ml followed by 10 min at 55% acetontrile, and finally 55-100% acetonitrile for 25 min. All solvents used were mixed with charcoal and filtered just prior to use. Fractions were collected and dried down under a stream of nitrogen. The OD_{254} tracing of the chromatography run is shown in **FIG. 7**. Note the large number of condensation products that are formed which provides a challenging test for the specificity of the subsequent AhR radioligand competition binding assay.

FIGURE 7: Elution profile of indole-3-carbinol condensation products

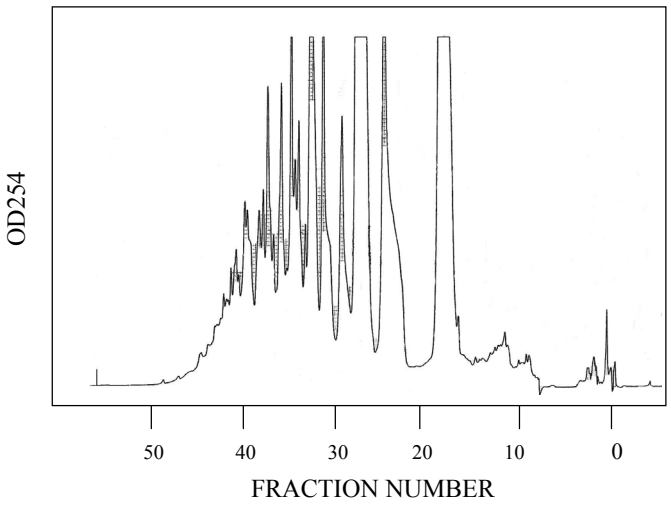
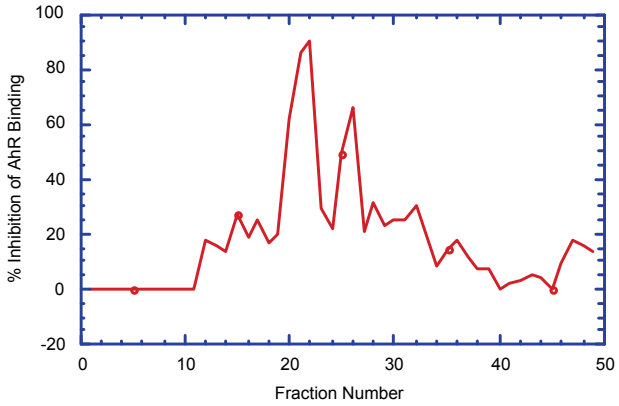


Figure 8: AhR radioligand competition binding assay of I3C condensate HPLC fractions.



Each HPLC fraction was solubilized in DMSO and an aliquot of each fraction subjected to a AhR radioligand competition binding assay (described in 'General Methods'). Because it uses a high specific activity (iodinated) reversible high affinity ligand this assay is extremely sensitive. Prior to performing these assays, a ligand binding saturation curve was generated with Hepa-1 cytosol, to optimize the amount of radioligand used in each assay. Thus ligand is added just to the point of saturation and *not* added in excess. This permits maximum sensitivity in the competition assays to be obtained. The results of the competition ligand binding assays were expressed as % inhibition of binding in each fraction and representative results are shown in **FIG. 8**, which reveals some background level of inhibition in most fractions, yet with two very predominant peaks. This preliminary result suggests that during acid condensation, at least two compounds are formed capable of binding to the AhR. In this application, the same assay conditions will be used. Two important aspects of this experiment as it relates to the proposed experimental plan should be noted. 1) Firstly, the AhR ligand binding assay is extremely sensitive and a large number of assays can be performed in a single day. 2) Secondly, there is a potential nonspecific background in the competition binding assay that we will likely encounter when screening through crude extracts, however this problem will be minimized by our use of crude extracts from a stably transfected CV-1 cell line expressing (only) CYP1B1.

Preparation of anti-human CYP1B1 antibody: A 15-mer peptide corresponding to an epitope on a putative surface loop region the human CYP1B1 protein was synthesized. This peptide, (denoted 218A) consisted of 14 amino acids (positions 332-345 of the deduced amino acid sequence; (E-K-K-A-A-G-D-S-H-G-G-G-A-R) plus an additional carboxy terminal cysteine for the conjugation reaction. The peptide was conjugated directly to KLH, and utilized to immunize a male NZW rabbit by standard protocols. Serum samples were screened for anti-CYP1B1 titer and specificity by western blotting against a human CYP1B1-maltose binding protein fusion expressed in E. coli, and human CYP1B1 expressed in COS-1 cells (not shown). Subsequently, purified anti-1B1 IgG was prepared by immunoaffinity chromatography utilizing the CYP1B1 peptide. Screening of the CYP1B1 anti-sera with a variety of cell and tissue fractions, and purified samples of human and rodent CYP1

proteins, revealed that 218A displayed highly selective affinity for human CYP1B1 {83,87,88,104}. The 218A antisera has also been tested by several other laboratories and verified to be highly selective for human CYP1B1. ECL-visualized western blots of the 218A antisera are shown in **FIG. 9** for microsomes prepared from control (untreated) or TCDD-treated MCF-7 cells, and microsomes prepared from Chinese hamster V-79 cells engineered to constitutively express human CYP1B1.

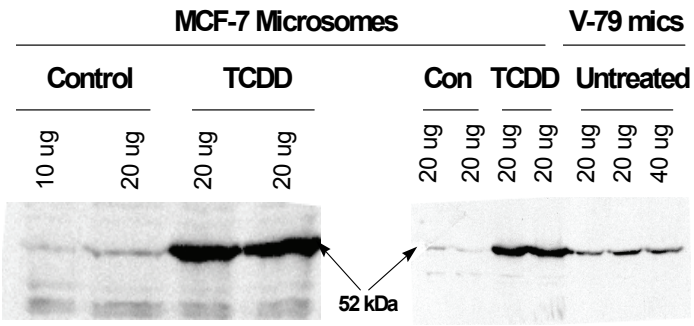


FIGURE 9: ECL Western Blots with anti-human CYP1B1

^{18}Oxygen incorporation into CYP substrates and detection by ESI-MS:: Preliminary studies were conducted to demonstrate both the absolute sensitivity of this analytical method as well as our ability to spray and ionize a model CYP1B1 endogenous substrate 17β-estradiol (**E_2**), and its metabolites (2-hydroxy and 4-hydroxy 17β-estradiol). E_2 was selected as the model compound for these preliminary studies since it has been demonstrated to be an endogenous substrate of CYP1B1, and undergo typical high-affinity but low-turnover CYP-catalyzed monooxygenation reactions {71,72, 105,106}. Thus, although E_2 does NOT appear to be the endogenous substrate and ligand of interest to us, it IS an endogenous substrate for hCYP1B1 {71,72} and does serve as the best available model compound to test and validate our proposed methodology. Using synthetic 4-OH-β-estradiol obtained from Sigma as a standard, we have demonstrated our ability to detect less than 1.1 x 10^{-14} moles by negative ESI mass spectroscopy in order to demonstrate the extreme sensitivity of detection possible with this methodology. The 4-OH-β-estradiol standard was prepared at 3.5 x 10^{-8} M, and analyzed on the Finnigan TSQ 7000 instrument in Dr. ZZZZ's lab; ESI voltage 3.5 kV, flow rate of 1 uL/min with a 20 second scan time (data not shown)

A number of preliminary experiments have been conducted to verify our ability to detect CYP-catalyzed ^{18}O incorporation into low turnover substrates. Preliminary *in vitro* metabolism studies were first conducted to: a) verify our ability to remove essentially all ambiant $^{16}O_2$ from our reaction mixtures, and; b) that running *in vitro* metabolism assays under 95% O_2 was not toxic or inhibitory toward assays conduted with microsomes or cell lysates (data not shown). **FIG. 10** shows a negative ESI MS of a of a crude methylene chloride extract of a 20 min. microsomal metabolism assay utilizing 100 uM E_2 as model substrate, conducted in a sealed system in which $^{16}O_2$ was removed by evacuation and sparging with argon and then replaced by sparging with 95% $^{16}O_2$). The mass spectrum readily shows formation of the 2-^{16}OH- and 4-^{16}OH-estradiol with a detection limit of ca. 150 picomoles. This demonstrates the feasibility of high sensitivity detection of oxygenated metabolites <u>even in very crude mixtures/extracts with no sample clean-up or fractionation</u>.

Figure 10: Negative ESI-MS of $^{16}O_2$-estradiol metabolism.

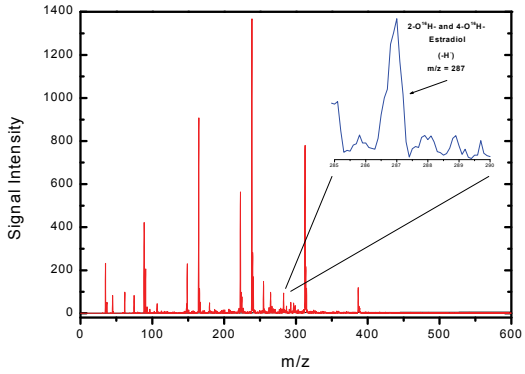
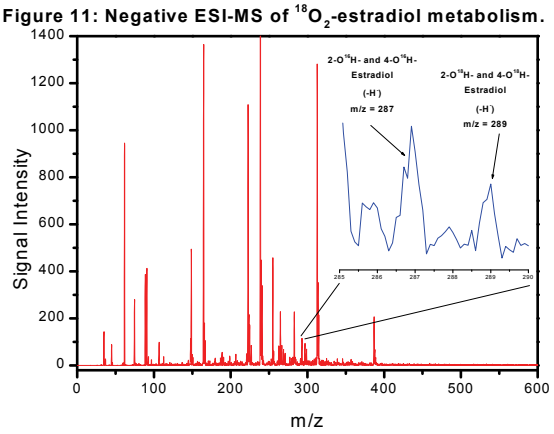
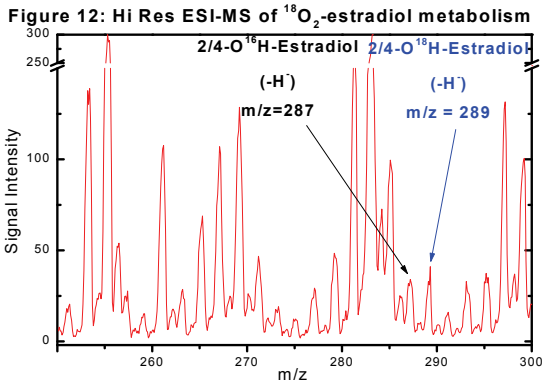


FIG. 11 shows the negative ESI MS of a of an identical experiment as shown in **FIG. 10** EXCEPT that all $^{16}O_2$ was removed by evacuation and sparging with argon and then replaced by sparging with 61% $^{18}O_2/^{16}O_2$. In comparison to FIG 7., the spectrum readily shows formation of BOTH the 2-^{16}OH- and 4-^{16}OH-estradiol **<u>AND</u>** 2-^{18}OH- and 4-^{18}OH-estradiol with a detection limit of ca. 150 picomoles. This demonstrates the feasibility of high sensitivity detection of ^{18}O-labeled metabolites <u>even in crude mixtures</u>. (Incorporation of

Figure 11: Negative ESI-MS of $^{18}O_2$-estradiol metabolism.



the ^{18}O-label appears to be ca. 30-40% of total, compared to the theoretical maximum of 61%.) **FIG. 12** shows a higher resolution negative ESI MS of the sample shown in **FIG. 11**, clearly revealing incorporation of the ^{18}O-label at 50-60% of total, in close agreement with the theoretical maximum of 61%. Further enhancements in sensitivity may be obtained if necessary by utilizing 100% $^{18}O_2$ which is also commercially available.



One additional preliminary experiment was conducted to further demonstrate our ability to detect the presence of unique molecules (i.e. ^{18}O-labeled metabolites) in complex mixtures by subtractive or 'difference' ESI MS. This powerful technique will be utilized with experiments employing duplicate incubations in the presence of $^{16}O_2$ compared to incubations in the presence of $^{18}O_2$, or incubations plus/minus catalyticaly functional CYP1B1, etc. in the studies proposed below in Aim 3 to detect formation of endogenous CYP1B1 substrates. Subtractive or 'difference' ESI-MS is a very powerful approach for subtracting out backgrounds in complex mixtures and greatly assisting in the initial identification and detection of unique components. **FIG. 13** shows a 'difference' ESI-MS spectrum between ethyl acetate extracts of media collected from CV-1 cells after 24 hrs incubation and a similar sample to which 50 uM E_2 had been added. The difference spectrum is highly simplified, and readily identifies the presence of E_2 even in such a complex mixture. While additional peaks do still appear in the 'difference' spectrum, much of the background can be subtracted out via this approach and greatly assist in identifying unique components of complex mixtures. (NOTE: Difference spectrum shown does not align perfectly with normal specta in figure because instrument does not permit printing the spectra on identical scales). **Thus our preliminary data, in corroboration with prior reports in the literature, provides convincing evidence for the existence of endogenous AhR ligands or regulators, and that at least some of these ligands are metabolized by CYP1B1 to less potent activators of AhR signalling.**

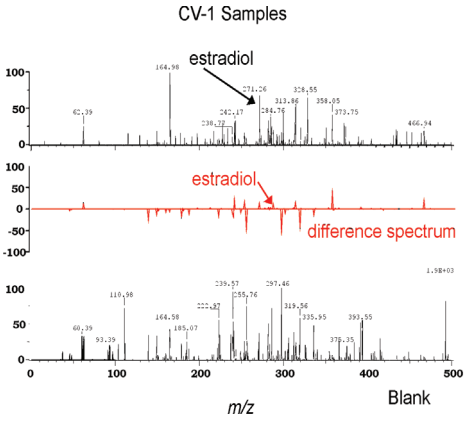
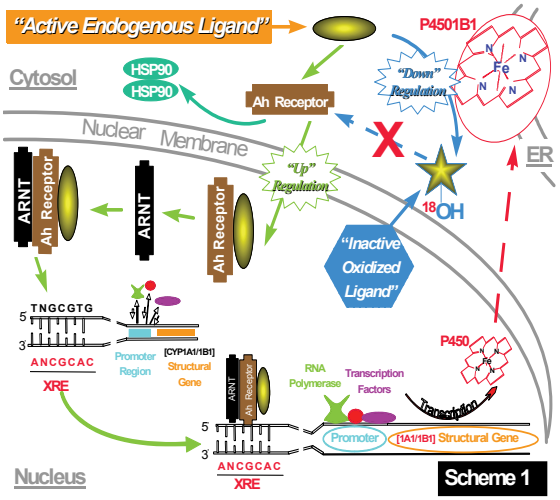


FIGURE 13: Negative ESI-MS difference spectra between extracts of CV-1 cells incubated with and without estradiol.

D: RESEARCH PLAN : Experimental Design and Methods:

Our preliminary data clearly demonstrates that CV-1 cells exhibit a high level of constitutive AhR activity which is repressed by expression of CYP1B1. Thus we will address the following **Central Hypotheses (Scheme 1):** Endogenous ligands exist for many CYP isoforms, and these ligands most likely have important physiological functions in maintaining normal cellular homeostasis, growth or differentiation. Specifically, we will test two separate but converging hypothesis: (1) There are significant levels of endogenous ligand(s) for the Ah receptor, or alternatively, endogenous regulators of the AhR, in CV-1 cells that are metabolized by cytochrome P450 1B1 (CYP1B1), and possibly other cytochrome P450 isozymes, and that CYP-mediated metabolism alters (increases or decreases) the affinity of endogenous ligands for the AhR or

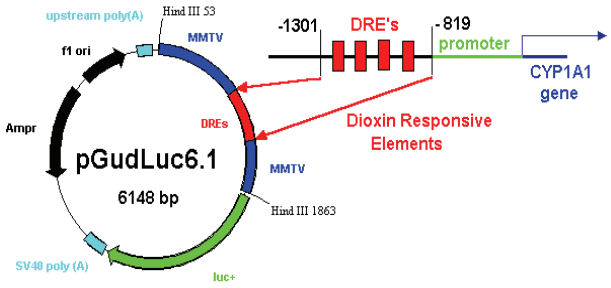


alternatively, alters the function of other endogenous AhR regulators. (2) There exist endogenous substrates for CYP1B1 that function as important mediators in cellular metabolism, and one or more of the these substrates function as AhR ligands or regulators of AhR activity. Proof of either of these two converging hypotheses will make a major contribution to the understanding of the normal physiological roles of the AhR, CYP1B1 and potentially many other CYPs.

Specific Aim 1: Determine the mechanism by which CYP(1B1) regulates AhR transcriptional activity.

Aim 1a. Elucidate the potential mechanisms by which select human cytochrome P-450s (**CYP**) reduce constitutive Ah receptor transcriptional activity.

Rationale: In the preliminary data section we have shown that cytochrome CYP1B1 is capable of lowering the constitutive level of AhR-mediated transcriptional activity using a DRE-driven reporter construct in CV-1 cells (**FIG. 1 & 2**). This effect was specific in that CYP1B1 is not able to influence glucocorticoid receptor activity as assessed with a GRE-driven reporter construct in CV-1 cells (**FIG. 5**). The first set of studies will assess the level of metabolic specificity of various CYP enzyme's ability to lower constitutive AhR activity. This will accomplish three goals; **1)** the specificity of each expressed CYP enzyme that is capable of lowering constitutive AhR activity will yield possible insight as to the type of endogenous substrates that may be involved; **2)** Determination of which CYP is *most* capable of metabolizing AhR endogenous ligands will allow the development of the most efficient stable transfectant in CV-1 proposed in Specific Aim 1b; **3)** to determine that the decrease in AhR constitutive activity is in fact due to metabolism and not just to expression of a CYP enzyme that leads to indirect effects on the AhR signal transduction pathway. The CYPs that have been selected for these expression studies are CYP1A1, CYP1A2, CYP1B1, CYP3A4, and CYP2E1. Each CYP selected is an important enzyme in drug and xenobiotic metabolism. CYP2E1 was chosen to serve as a control, because it metabolizes substrates that are quite small (e.g., acetone, ethanol) and thus CYP2E1 is deemed unlikely to metabolize an endogenous AhR ligand. Considering that CYP1A2, CYP1A1, and CYP1B1 are directly regulated by the AhR, it is possible that each enzyme is capable of metabolizing an endogenous AhR ligand thus composing an endogenous substrate feedback mechanism for clearing excessive levels of certain compounds. This would be consistent with the feedback mechanism hypothesis that has been discussed in the background and significance section.



Experimental approach: Each CYP expression construct will be transfected into CV-1 cells using a Lipofectamine protocol routinely used in the XXXX laboratory. This procedure results in approximately 40% transfection efficiency in CV-1 cells. To assess the ability of a given CYP to alter constitutive AhR activity, 60 mm^2 dishes of CV-1 cells will be transfected with a CYP construct, pcDNA3/mAhR, pGudLuc 6.1 (This reporter construct responds only to the liganded AhR and contains no other known or identifiable response elements {102}, and CMV-β-gal constructs. After transfection the cells will be cultured for a total of 40 h and harvested. Cells will be directly lysed in lysis buffer prior to performing luciferase, β-galactosidase, and protein assays. Cells harvested for detection of CYP enzyme expression will be treated with trypsin/EDTA, washed with PBS and homogenized for isolation of homogenate and microsomes. We have obtained all but one of the expression constructs necessary to perform these studies. hCYP1A1 cDNA in a bacteria expression construct has been obtained from Dr. Ron Hines and will be subcloned into pCI vector. hCYP1A2 mammalian expression vector has been obtained from Dr. Robert Tukey. pRc/CMV/CYP1B1 has been obtained from Dr. William Greenlee. A CYP3A4 expression construct has been obtained from Dr. Frank Gonzalez, and hCYP2E1 cDNA in a bacterial expression vector has been provided by Dr. Judy Raucy. All three have been already subcloned into the pCI vector.
Control experiments will be performed on transfected cells to ensure that each CYP is expressed *and* exhibits the appropriate catalytic activity. CYP1A1 and CYP1A2 activity will be assessed with the EROD and MROD

assay, respectively {107,108,109}. CYP1B1 catalytic activity will be assessed by EROD activity assay. While there is no single highly selective specific assay for CYP1B1 activity, the EROD assay is a sensitive measure of CYP1B1 activity. CYP1B1 catalyzes EROD activity at 1/10 the rate of CYP1A1 {109,110}. As an alternate approach to verify CYP 1A1, 1A2 and 1B1 activity, metabolism studies can be performed utilizing DMBA (7,12-dimethylbenzanthracene) as the model substrate. This assay is routinely conducted in the Scientist lab {37,65,104}. Each CYP catalyzes a distinct pattern of regioselective DMBA metabolism, distinct from other CYP isoforms metabolizing this substrate {65,66,67,111,112,113}. However*, since only single CYP isoforms will be expressed in each cell line*, sensitivity rather than selectivity will be the major concern in these *in vitro* metabolism assays. CYP3A4 will be assayed using a nifedipine oxidation assay {114}. CYP2E1 will be assayed by the N-nitrosodimethylamine demethylase assay (115}. Expression of each CYP will be verified and quantified by western blots of microsomal preparations from transfected cells, and by competitive RT-PCR. The XXXX lab has a monoclonal antibody that works well against human cytochrome CYP1A1 obtained from Dr. John Stegeman. The Scientist lab has prepared a highly specific rabbit polyclonal antibody to CYP1B1 that is routinely used in both the principal and co-investigator's laboratories. The Scientist lab has also generated a polyclonal antibody against rCYP1A1 that cross-reacts with hCYP1A1/2. In addition, antibodies against hCYP1A2 are commercially available. Polyclonal antibodies against CYP2E1 have been obtained from Dr. Judy Raucy, and a commercial hCYP3A antibody has been purchased from Oxford {116}. Quantitative competitive RT-PCR measurements of CYP mRNA in cultured cells is routinely conducted in the Scientist lab. The PCR primers and internal standards for most of these CYP isoforms are already available in the Scientist lab, or will be commercially synthesized.

Expected Results: The expected result is that CYP1A2, CYP1A1, and CYP1B1 will all be able to decrease AhR constitutive activity, although, the efficiency of each CYP enzymes to metabolize an endogenous ligand may vary. CYP2E1 will have no effect on AhR constitutive activity (as recently demonstrated in **FIG. 1**). Whether CYP3A4 will be able to metabolize the endogenous AhR ligand(s) is difficult to predict, although based on our preliminary data presented herein, we anticipate that it will not. We expect that each CYP will exhibit considerable catalytic activity in CV-1 cells because: a) previous studies by others have demonstrated that transfected COS-1 cells were suitable for carcinogen metabolism studies, and CV-1 cells are the parent cell line for COS-1 which have been cells transformed with T-antigen. {117,118,119}; and b) that CV-1 cells are apparently capable of supporting the activity of transfected CYP1A1 {57}.

Aim 1b: Determine the role and specificity of CYP *metabolism* in regulation of AhR transcriptional activity.

Rational: Another key aspect of these studies that we wanted to establish is that *catalytic activity* of each expressed P-450 is necessary for reduction of constitutive AhR transcriptional activity. This will be achieved by generating catalytically non-functional CYP1B1 (and the other CYPs to be studied) mutants and stably transfecting CV-1 cells with these mutants (analogous to preliminary studies), and then repeating the above studies with the CV-1 cell lines expressing the catalytically incompetent CYP isoforms. As an *alternative* approach, we may also selectively inhibit CYP catalytic activity with chemical inhibitors.

Approach: Catalytically non-functional CYP1B1 will be generated by site-directed mutagenesis of the highly conserved and invariant active-site diooxygen-binding Threonine in the I-Helix. Mutation of this Threonine (Threonine 334 in CYP1B1 to Alanine 334) will generate a catalytically non-functional CYP1B1 that is properly folded and with normal expression, and not suffer from the rapid turnover observed for catalytically inactive, non-heme binding mutants of the invariant Cysteine of the heme-binding pocket {120,121}, Dr. Bill Peterson, personal communication). Such Thr => Ala mutants typically have 100-1000-fold lower catalytic activity toward normal substrates, although substrate binding *may* induce slightly higher rates of uncoupled hydrogen peroxide generation than by the native enzyme. Site directed CYP1B1 mutants will be generated by a procedure currently employed in the Scientist and Omdahl labs for generation of vitamin D 1-hydroxylase mutants with the QuikChange Site Directed Mutagenesis Kit from Stratagene. Briefly, this entails the use of two-inverse complementary primers that contain the mutation and about 15 bp of wild type sequence on either side. The kit uses Pfu Turbo DNA polymerase which is a high fidelity thermal-stable enzyme that gives blunt ends for convenient blunt-end ligation. Thus while one can routinely achieve greater than 80% mutation and

transformation efficiency with nearly negligible error rates, mutagenesis will be confirmed by sequencing. This approach will be utilized to generate similar Thr => Ala mutants of the other CYP isozymes as needed.

As an alternative approach, various chemical inhibitors of CYP catalytic activity may be used to confirm the results obtained with the site-directed catalytically incompentant CYPs. These inhibitors have been widely used *in vivo* and *in vitro* and are generally non-toxic to cells at the doses required to inhibit CYP activity, although they have not yet been tested with CV-1 cells for toxicity. We would first verify that doses required to inhibit CYP isozymes were not cytotoxic if we find need to apply this alternate approach. A number of such mechanism-based CYP inhibitors are available: (1-aminobenzotriazole (ABT), 2 mM {122,123}; 1-ethynyl-pyrene (1EP), 1-(1-proprynyl)-pyrene (1PP) and 2-ethynyl-pyrene (2EP) have been shown to be selective inhibitors of CYP1A1 (1PP) and CYP1B1 (1EP, 2EP) {124}. 2EP has been shown to be a superior inhibitor for human 1B1, while 1EP is superior for mouse 1B1 (C. Jefcoate, W. Alworth personal communication). 3,4,5,3',4',5'-hexachlorobiphenyl (HCB) has recently been shown to be a potent and selective inhibitor of CYP1B1 {125,126}, C. Jefcoate, personal communication). *If required as an alternative approach*, these inhibitors will be purchased from comercial sources or synthesized and purified by Dr. YYYY via published methods {124,127}.

To clearly demonstrate that the decrease in constitutive luciferase activity is due to decreased AhR activity and not alterations in luciferase mRNA expression, or enzymatic activity by constitutive CYP expression, several control experiments will be performed. A CMV/pGL3 luciferase vector, differing from pGudLuc 6.1 *only* in the enhancer region will be transfected with or without hCYP1B1 into CV-1 cells. If CYP1B1 expression does not influence luciferase activity we expect to observe no differences. An additional experiment will examine the influence of CYP1B1 expression on a Glucocorticoid Response Element (GRE)-driven reporter construct. This experiment will be performed in a similar fashion, with and without CYP inhibitors, as shown in the **FIG 5**.

Expected Results: CV-1 cells transfected with catalytically non-functional CYP1B1 mutants will not display reduced levels of endogenous AhR activity compared to wild-type CV-1 cells as will CV-1 cells transfected with functional CYP1B1.

Specific Aim 1c. Establish stable CV-1 cell lines expressing CYP1B1 that will result in low constitutive AhR activity and low CYP1B1 substrate levels.

Rationale: This cell line will be utilized for experiments to establish that CYP *activity* does lead to a reduction in the ability of the AhR to be activated and form heterodimers. This cell line will also be used as a control cell line in endogenous ligand experiments in Specific Aim 2 and as a control cell line in endogenous substrate experiments in Specific Aim 3. This Aim has already been partially completed since the initial submission of this proposal (See Preliminary Data section), and the preliminary results obtained with these cell lines serves to strongly support our Central Hypothesis.

Approach: The pRc/CMV/CYP1B1 construct expressing the neomycin resistance factor was transfected into CV-1 cells using a Calcium Phosphate Transfection kit (Promega). After transfection, cells were split into a number of 100 mm culture dishes, G-418 was added at 400 ug/ml for 10-14 days and the resulting colonies isolated. Each colony was expanded and cultured in 24-well plates. We screened 50-100 clones to obtain each positive clone {102}. Screening of stable transfectants for stable expression of CYP1B1 was performed by transient transfection of clones in 24-well plates using a Lipofectamine method. Cells were transfected with pGudLuc 6.1, CMV/βgal, and mAhR/pcDNA3 and harvested after 36 h (see General Methods section). Luciferase and β-galactosidase activity was determined, and several clones expressing CYP1B1 showing a significant decrease in constitutive activity were isolated, as well as several negative clones as control cell lines. These clones are being assessed for CYP1B1 expression by isolation of microsomes and detection of CYP1B1 apoprotein via Western blotting and mRNA by quantitative RT-PCR as employed in the Scientist lab (**FIG. 3 & 4**).

After initial characterization, each clone will be transfected with mAhR/pcDNA3 and the total cytosolic level of AhR vs. the amount of nuclear AhR/ARNT heterodimer will be assessed. This will be accomplished using methods that the XXXX lab has previously published to assess heterodimer levels in HeLa cells {128}. Briefly, cells will be mechanically homogenized and the nuclei isolated by centrifugation at 1,000 g for 20 min. The supernatant will be spun at 100,000 g for 60 min. to obtain the cytosolic fraction. Nuclei will be washed three times with MENG, a low salt buffer, followed by a single wash with MENG + 50 mM NaCl. The nuclei will then be incubated for 1 h in MENG + 500 mM NaCl. This high salt extract will be used to assess AhR levels and in EMSA (gel shift) analyses. The key point here is to compare AhR levels in cytosolic vs. nuclear fractions from both CYP1B1 expressing clones and clones that do not express CYP1B1. Additional experiments with cell lines expressing catalytically incompetent CYP1B1 (Aim 1b), which should lead to an increase in AhR activation due to accumulation of endogenous AhR ligand(s), will confirm the role of CYP1B1 catalysis as a modulator of AhR activation.

Expected Results: The expected result is that constitutive CYP1B1 expression will cause a significant decrease in AhR/ARNT levels in the nucleus and decreased constitutive DRE-binding activity. This would firmly support the transient transfection experiments by directly demonstrating that CYP1B1 metabolism leads to a decrease in AhR activation, which would be expected if an endogenous AhR ligand is being metabolized.

Specific Aim 2. **Identify endogenous AhR ligands or regulators of AhR activity in CV-1 cells and characterize their mechanism of action.**

Rationale: One of the most important questions regarding the role of the AhR in normal cellular biology is whether there are endogenous ligands or other regulators for the AhR. As discussed in the Background and Significance section, there are a number of recent reports in the literature that support the conclusion that there are endogenous AhR ligands. In addition, the preliminary data in this application supports the assertion that there are AhR ligand(s) in CV-1 cells. We have devised a method based upon using HepG2 cells stably transfected with a DRE-driven reporter construct as a reporter assay for identifying such ligands/regulators that will overcome the primary problem of screening crude cellular extracts (high backgrounds), and *an alternative* AhR competition binding assay. Both assays can be conducted utilizing cell fractions prepared via HPLC separations. The technical problem encountered with this second approach is that considerable background competition is observed in many fractions due to the non-specific binding of a large amount of hydrophobic compounds, such as a phospholipid, in the competition assay (**FIG. 8**). In order to achieve a high level of *specificity* in detecting an AhR ligand in such assays, a control HPLC fraction will be performed using crude extracts from the CYP1B1 stable CV-1 cell line to account for the background of nonspecific competition in the AhR ligand competition assay. In this application, the stable cell line expressing CYP1B1, generated in Specific Aim 1b, will be used to control for the absence of an AhR ligand of interest. Extracts from both "wild-type" and CYP1B1/CV-1 cells will be applied in separate HPLC runs and ligand binding competition binding assays performed. ONLY the inhibition peaks specific to the "wild-type" extracts will be further characterized. This system will allow us to methodically isolate and characterize endogenous CYP1B1 substrate(s) that is/are also an AhR ligand. Putative ligands will also be tested for their metabolism by CYP1B1 (Aim 3).

Approach CV-1 and hCYP1B1/CV-1 stable transfectant cell lines will be homogenized and spun at 15,000 x g to remove intact cells, nuclei, and mitochondria. Assuming that the compound(s) of interest are likely metabolized in either the cytoplasmic or microsomal compartments, these cellular fractions will be the logical initial source for the putative AhR ligand. The post-mitochondrial fractions will be applied directly to a Varian Bond Elut C-18 column after acidification. Compounds on the column can than be eluted with methanol and applied directly to an HPLC column. An alternate method to prepare samples for HPLC is to apply solvent extracted compounds from the post mitochondrial fraction directly to a Varian Bond Elut C-18 column. In considering the appropriate solvent extraction techniques and HPLC chromatography conditions it is important to keep in mind that the nonpolar/polar character of endogenous ligands is unknown. Nevertheless, it can be expected that the compound will be of intermediate hydrophobicity and more polar than TCDD (see Aim 3 for discussion of strategies to direct isolation of putative AhR ligands/CYP1B1 substrates (**FIG. 6**)). The

extracted compound will be solubilized in anhydrous methanol, and subjected to chromatography on a Beckman Ultrasphere ODS (250 x 4.6 mm column) and eluted with 70% methanol. An alternate HPLC chromatography system is the use of reverse-phase columns with an isocratic solvent system such as hexane/2-propanol/acetic acid (95:5:0.1) or acetonitrile/2 propanol/trifluoroacetic acid (4:54:0.1). Fractions will be collected and dried down under a stream of nitrogen and re-solubilized in five µl DMSO.

Each solubilized fraction will be added to 24-well cell culture plates containing a HepG2 cell line with a DRE-driven luciferase reporter vector stably intergrated. We have been able to detect as little as 50 pmol of TCDD in this assay system, so it is extremely sensitive. This system will not suffer from the background problems encountered with the competitive ligand competition binding assay, *which is our alternate screening approach.* This assay is rapid and hundreds of fractions can be tested each day. Results using this assay system are shown in the preliminary data section (**FIG. 6**). HPLC fractions containing AhR activating activity will also be subjected to an AhR competition binding assay described in the General Methods. This assay is *extremely* sensitive using 2-^{125I}iodo-7,8-dibromodibenzo-p-dioxin (2,176 Ci/mmol) as a radioligand. Dr. XXXX's and Dr. Poland's laboratories are the only laboratories that we are aware of that have synthesized this radioligand. A large number of AhR competition binding assays can be run in a single day. After a peak is identified from CV-1 extracts that is not present or significantly reduced in extracts from the CYP1B1/CV-1 stable cell line it will be further purified for Mass Spec analysis. Details of further purification of putative endogenous AhR ligands/CYP1B1 substrates are described in Aim 3 The chromatography conditions of the initial fractionation will be optimized and the scale of the isolation and column size increased. Either reverse-phase or normal phase chromatography systems will be developed to further purify the compound of interest to homogeneity for Mass Spectrometry analysis.

Alternative Approach: A powerful new alternative approach has recently become available to us via the development of a CYP1B1 null mouse model and cell lines derived from this animal and its wild-type progenitor. We will be able to utilize these cell lines in analogous experiments to those described above, however with the major advantage that we may be more likely to identify true CYP1B1 substrates with physiologically important roles from such cell lines which normally express CYP1B1, rather than our comparatively artificial system with CV-1 cells and transfected CYP1B1, even though our preliminary results strongly indicate the existance of such CYP1B1 substrates in the CV-1 lines.

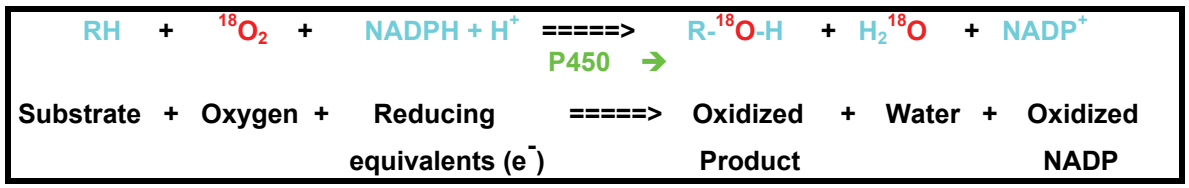
Expected results: One aspect of this series of experiments that we do not know is whether there will be more than one compound that will be discovered through these studies, an our preliminary studies suggest that this may be a possibility (**FIG. 6**). However, even if only one compound is identified, this may be highly significant. *Using the CV-1 parent line as a 'background control' and CYP1B1 stable CV-1 transfectant should allow us to compare chromatograms and 'subtract out' false positives, and thus efficiently identify the AhR ligand(s) of interest.*

Specific Aim 3: Identification of Endogenous Substrates for CYP1B1: Substrates that serve as endogenous ligands for the AhR.

Rationale: We postulate that endogenous substrates exist for some if not all CYP isoforms. Of particular interest is that subset of substrates for CYP isoforms that serve as regulatory molecules controlling expression of CYP isoforms (or other proteins), in particular those molecules which may serve as endogenous substrates for CYP1B1 **AND** as ligands for the AhR. While this aim is narrowly focused on the identification of endogenous substrates for CYP1B1, and specifically those serving as AhR ligands, *it is extremely important to recognize that the approaches developed under this Aim will be broadly applicable to the identification of most if not all, endogenous substrates of the CYP superfamily. Extension of this methodology to the identification of endogenous substrates for CYP isoforms other than CYP1B1 is beyond the scope of the current proposal, but clearly feasible and a logical extension of the proposed studies for future work.* Although independent by design, the studies in this Aim will be highly integrated and convergent those described in Aim 2. In addition to identifying endogenous CYP1B1 substrates the studies in Aim 3 will provide a powerful alternative approach to identifying putative AhR ligands, while simultaneously verifying their status as CYP1B1 substrates. Candidate

ligands identified in Aim 2 will be tested for metabolism by CYP1B1 by the approaches described in Aim 3, and CYP1B1 substrates identified in Aim 3 will be tested for AhR binding and activation as described in Aim 2. Thus, while there will be considerable interaction and 'convergence' between Aims 2 and 3, each will be able to proceed completely independently.

Approach: Identification of ^{18}O-labeled CYP1B1 metabolites as putative AhR ligands: We postulate that it will be possible to identify at least some endogenous AhR ligands by virtue of their properties as CYP metabolites. The cytochrome P450 system functions as a monooxygenase, cleaving diatomic molecular oxygen and inserting one atom of oxygen into the substrate (hence the name monooxygenase) and the other atom of oxygen from the diatomic oxygen into water via the simplified general reaction scheme:



This reaction scheme thus offers a unique advantage in that all CYP metabolites will contain one atom of oxygen derived from molecular, diatomic oxygen (not water). Thus, reactions conducted in the presence of $^{18}O_2$ will thus result in the incorporation of one atom of ^{18}O into the substrate, which may serve as a ***label*** for subsequent analysis and identification by mass spectrometry as described below. Incorporation and detection of this stable isotope into CYP metabolites will serve as a powerful strategy for identification of endogenous CYP substrates. As further enhancement to this strategy, we will utilize only 61% $^{18}O_2/^{16}O_2$ for our incubations. This will allow us to scan for $^{18}O/^{16}O$ doublets of 60:40 ratio in the mass spectrums acquired, further facilitating our ability to identify and discriminate ^{18}O-labeled molecules, since only CYP-monooxygenated metabolites should incorporate the expected ratio of the two isotopes as additional confirmation of the origin of the labeled metabolites (**FIG. 10, 11, 12**). Subtractive difference mass spectrometry will also be employed to further assist in the detection of endogenous substrates (**FIG. 13**)

While the monooxygenation reaction described above is characteristic of most CYP-mediated reactions, a potential problem may arise from the occurrence of much less common CYP-mediated reactions resulting in formation of unstable oxidized products/metabolites that undergo rearrangement via de-alkylation. In these cases, the atomic oxygen inserted by CYP may be eliminated from the parent molecule during significant non-enzymatic rearrangement reactions, rather than being stably inserted. There are also a number of even less common reactions mediated by CYP isoforms, including dehydrogenation reactions, (including oxidations of saturated carbon-carbon bonds) oxidations of alcohols to ketones, oxidation of aldehydes to carboxylic acids, and even oxidation of certain nitrogen compounds to more unsaturated metabolites, that also potentially may generate metabolites lacking incorporation of the $^{18}O_2$ label into the parent substrate. A few such reactions are even known for endogenous substrates (i.e. aromatase, progesterone 17α-hydroxylase/17,20 lyase and lanosterol 14α-demethylation). However, we have developed methods to identify the occurrence of such reactions and general strategies to cope with this potential, although unlikely, possibility (described below). Fortuitously, most of these elimination products (i.e. dealkylations) will be identifiable via our ^{18}O-labeling strategy and could thus serve in the identification of the parent endogenous substrate molecule. A second potential problem could arise from the existence of non-P450-mediated monooxygenation reactions, resulting in insertion of a single ^{18}O-label into substrates from molecular oxygen (for example reactions catalyzed by flavin monooxygenases or via co-oxidations by prostaglandin synthetase). However, such reactions will be identified and excluded by <u>two</u> approaches: 1) Comparison of reactions conducted with: CV-1 cells (expressing no CYP), CV-1 cells expressing transfected mutant, non-functional CYP), and CV-1 cells expressing transfected functional CYP isozymes, to allow us to identify and eliminate non-P450-mediated monooxygenations; or <u>*alternatively*</u> 2) their *insensitivity* to inhibition by carbon monoxide and their *sensitivity* to inhibition by relatively specific inhibitors of these reactions (i.e. methimazole, alpha-naphthylthiourea for FMO, and indomethacin or other NSAIDs or COX inhibitors for PGS).

Approach: We will conduct *in vitro* incubations with cells and/or cell lysates prepared from CV-1 cells, CV-1 cells transfected to express functional CYP1B1, and CV-1 cells transfected to express non-functional CYP1B1 in the presence of a defined ratio of $^{18}O_2/^{16}O_2$ (61%). *Alternatively*, similar studies with newly available cell lines derived from CYP1B1 null mice and their wild type progenitors may also be conducted. After incubations are complete, the incubation mixtures will be group-fractionated to isolate various classes of chemicals (to minimize the number of compounds subjected to LC/MS analyses at any one time). LC/MS analysis will then identify molecules containing single atoms of ^{18}O /^{16}O in the 61% ratio present in the incubation system. Candidate endogenous substrates will be further tested to verify they do in fact serve as endogenous substrates for CYP isozymes and also be tested for their ability to serve as AhR ligands (Aims 1 and 2).

The utility of LC/MS as an ***initial*** screening technique for identification of ^{18}O-labeled metabolites of cytochrome-P450 mediated reactions is moderately limited by the availability/cost of MS instrument time and the expense of $^{18}O_2$. To reduce the initial requirement for LC/MS instrument time and isotope costs, we will utilize an evaporative light scattering detector (ELSD) to direct HPLC fractionation procedures for use with the initial group-fractionated samples isolated from *in vivo* and *in vitro* incubations with $^{18}O_2$, The utilization of a light scattering detector will provide preliminary information concerning the extent of separations of molecules contained within extracts from non-labeled incubations, and perhaps even more importantly, the ability to detect putative ligands/substrates that might lack conventional chromophores and thus have only little or no absorbtivity in the UV, thus limiting our ability to detect them with conventional UV-Vis HPLC flow detectors. We will also sample the *in vitro* incubations to evaluate the possibility that a volatile ^{18}O-containing material is being produced in the incubations, as might be the case for CYP-mediated dealkylations. Since all reactions will be in gas-tight vessels, we will sample the headspace above the reactions at the conclusion of the incubations. A head space sample will be removed from the incubation with a gas tight syringe and the head space gas will be injected into a GC/MS (Dr. YYYY's lab) using a capillary column and a temperature program which will separate formaldehyde and acetaldehyde (likely products from O- or N-dealkylation reactions) and then ramp to 250^{o} C to insure that other ^{18}O-containing organic compounds are not present.

Aim 3a: $^{18}O_2$ Metabolism Assays: The ^{18}O-labeling studies will be conducted in two stages. First, to ensure successful identification of low abundance endogenous substrates, optimize sensitivity, minimize non-specific 'background' ^{18}O-labeling, and thus minimize false-positive identifications of putative substrates, it will be *critically important to thoroughly characterize and optimize* both the ^{18}O-labeling reactions and the ^{18}O-mass spec instrumental analyses. Secondly, we will need to develop reliable 'positive' and 'negative' controls for the ^{18}O-labeling experiments, and to serve as quantitative standards for our studies. We anticipate that it will require considerable effort during the first year of this project to accomplish this essential goal. We will utilize the metabolism of 7,12-dimethylbenzanthracene (DMBA) and 17β–estradiol as model substrates to validate and calibrate our ^{18}O-labeling assays, extraction, clean-up, and mass spectral analysis protocols, and to serve as 'positive' controls for our subsequent attempts to identify unknown endogenous CYP1B1 substrates. Both reactions are catalyzed by CYP1B1 and quantifiable by non-^{18}O-labeling HPLC assays. One substrate is a widely studied xenobiotic substrate of CYP1B1, the other a proven endogenous substrate of physiological importance. The PI has extensive experience with the DMBA and E_2 HPLC metabolism assays. The DMBA assay offers the advantage that CYP1B1 oxidizes DMBA at multiple positions, and thus will serve as a rigorous test of our ability to separate and identify ^{18}O-labeled molecules.

- Initial studies will utilize the CV-1 cell lines we have already created. We will optimize substrate concentrations by conducting dose-response studies for each of the two reactions; 5-25 uM DMBA {66,67,110,129} and 0.1 to 10 uM 17β -estradiol from {73,89}. *Analogous to the studies in Aim 2, this will also provide us with an essential approach to determine the 'background' of non-CYP-mediated oxygenations, by comparing reactions from control, wild-type and CYP1B1-transfected CV-1 cells*. Time courses for each reaction will also be conducted, with incubations from 1-30 minutes, to determine both the linearity and maximize conditions for ^{18}O incorporation. Based upon results obtained above, similar studies as described above will then be conducted with microsomes prepared from rat livers and primary rat hepatocytes grown in culture, to validate our ability to discriminate CYP-mediated incorporation of ^{18}O into

specific substrates against what we would anticipate to be a greater background of non-specific oxidative reactions.

AIM 3b: Identification of endogenous CYP1B1 substrates in CV-1 cells. After the $^{18}O_2$/MS procedures are optimized (Aim 3a), actual experiments to identify *endogenous* CYP1B1 substrates will be conducted.

- *In vitro* incubations in the presence of $^{16}O_2$ or $^{18}O_2$, utilizing CV-1 cells, CYP1B1 expressing CV-1 cells, and CV-1 cells expressing the catalytically incompetent CYP1B1 mutants will be conducted as described above, EXCEPT IN THE ABSENCE OF EXOGENOUSLY ADDED SUBSTRATES. Following incubations, cell lysates will be fractionated as described and putative CYP1B1 substrates (and AhR ligands) will be identified by ESI-MS as described. Putative CYP1B1 substrates identified by this procedure will be purchased, purified or synthesized and tested for AhR binding and activation as described in Aims 1 and 2. Similarly, putative endogenous AhR ligands identified under Aim 2 will be added to incubations with CV-1 or CYP1B1-expressing CV-1 cells to determine whether or not they are CYP1B1 substrates.

Initial fractionation scheme for cell lysates to identify CYP endogenous substrates: At the conclusion of *in vitro* incubations with cells, cell lysates, or microsomes plus various cell fractions in the presence of $^{18}O_2$, as described above, the reaction systems will be lysed (if necessary), then extracted and group fractionated to partially purify putative ^{18}O-labeled CYP metabolites into three initial pools for subsequent instrumental analysis and identification as described below. As an *Alternative Approach* for the purification/fractionation of potentially hydrophobic CYP1B1 substrates/AhR ligands, we may also utilize Immobilized Artificial Membrane chromatography {65,130,131}. These HPLC columns have been shown to be uniquely effective in separating a variety of compounds with varying physiochemical properties, and especially for hydrophobic molecules. All reactions and extractions will be carried out in acid washed and siliconized glassware to avoid false positives and to prevent the absorption of the ligands on the glass surface. Pesticide quality solvents will be employed in the extractions and all water utilized will be HPLC quality. In addition we will perform a blank extraction of water to insure that our extraction procedures are not introducing organic molecules that might be identified as substrates. Crude cell-fraction extractions (discussed below) will subsequently be subjected to reverse phase HPLC to further purify and characterize endogenous CYP1B1 substrates and putative AhR regulators.

Since we do not have *a priori* knowledge of the exact structure of the endogenous CYP1B1 substrates and AhR ligands/regulators we are seeking to characterize, we will begin with a broad-based fractionation scheme to be directed by our biological assays of AhR activity and CYP1B1-mediated metabolism. Our Preliminary Data does in fact provide some physico-chemical information regarding the general properties of the molecules of interest. Our initial reverse phase HPLC separations of candidate compounds is based on the partition of the analyte between the liquid phase and the solid C_{18} phase of the column, whereby non-polar compounds have much longer retention times than more polar compounds. In general, a compounds retention time is directly related to the log P for the compound. Thus the relative retention times for the elution of benzene (logP 2.03, Ghose-Crippen calculation from Spartan/Wavefunction Inc.); naphthalene (logP 3.03 Ghose-Crippen calculation); and Anthracene (logP 4.03 Ghose-Crippen calculation) from a C_{18} column eluting with a mixture of acetonitrile and water are predicted by the relative logP values of the respective compounds, as shown in **FIG. 6**. Although one can not make exact predictions of the retention times of compounds it does follow that the order of elution is related to the lipid solubility (log P) of the compound(s) in question. Thus the elution of the biologically active peaks of interest from our initial HPLC fractionations occur within the range of LogP values for benzene, naphthalene and anthracene (**FIG. 6**), suggesting that the logP values for at least some of our target compounds fall within this range. These retention times are consistent with our prediction that the molecules in question are very non-polar in character, and we can utilize this information to *in part* further direct our purification and identification of the unknown endogenous CYP1B1 substrates and AhR regulators. Several complex structure-activity models have been developed to analyze and predict AhR-ligand binding interactions and CYP-substrate interactions {132,133,134,135, 136,137}. While Dr. YYYY's laboratory possesses two powerful Silicon Graphics computer workstations and molecular modeling software, and while we might at later stages of the project apply these models to evaluate putative AhR ligands and CYP1B1 substrates as we characterize their structure(s), we believe the simpler approach based upon LogP will provide

an adequate, (although not complete) strategy to initially direct purification and identification of our target molecules and characterization of their interaction with the AhR and CYPs {138,139,140}. Since the precise molecular properties/structure of the substrates and ligands we are searching for are unknown, we will also utilize a new, Evaporative Light Scattering Detector (ELSD), in *combination* with traditional UV and fluorescence detectors to detect components in our HPLC elution fractions that may not possess traditional chromophores and might other wise be 'missed' during fractionation. This detector is based upon relatively new technology and is capable of detecting virtually any non-volatile, small MW organic molecule.

Although our Preliminary Results (**FIG. 6**) indicate that although at least some molecules with AhR activating properties may be isolated from cell fractions via simple organic solvent extractions, we will utilize the following general strategy to further direct our isolation and purification of endogenous AhR ligands and CYP1B1 substrates:

1) Extraction procedure for **Endogenous CYP Substrate/^{18}O-metabolite Identification.**

a. cells, cell lysates or microsomal incubations will be treated with $HClO_4$
b. centrifuge to remove ppt protein
c. remove the aqueous phase
d. wash ppt with water
e. centrifuge to remove protein
f. combine the water wash with the aqueous phase from step c.
g. split sample into two parts 1:2:

i) The larger sample will be extracted with ethyl acetate 3 x 5 ml with each extraction being combined and then evaporated to dryness under a stream of nitrogen. The remaining material will then be diluted in MeOH 200 ul and subjected to HPLC/MS and GC/MS analysis. This fraction will contain neutral and acidic organic compounds. The aqueous material will be made basic with K_2PO_4 and then extracted with 3 x 5 ml portions of ethyl acetate. The combined ethyl acetate extracts will be dried under nitrogen and diluted with 200 ul of MeOH and subjected to HPLC/MS and GC/MS analysis. This fraction will contain basic organic compounds.

ii) The smaller sample will be placed in a speedvac and evaporated to dryness. The material will then placed in 200 ul of MeOH for HPLC/MS and GC/MS analysis.

2) Extraction procedure for isolation of small peptides:

a. cells, cell lysates or microsomal incubations will be dispersed (and solubilized if necessary), and then passed through a C_{18} Sep-Pac column. The column will be eluted with three volumes of water and then eluted with 5 volumes of methanol. The methanol will be collected and evaporated under vacuum.

b. The residue from methanol evaporation will be reconstituted in methanol/water and subjected to HPLC/MS analysis using the standard protocol for the determination of ^{18}O incorporation into molecules. Preliminary GC/MS analysis will not be attempted on this fraction.

3) Procedure for isolation of volatile metabolites: Since all reactions will be in gas-tight vessels, we will sample the headspace above the reactions at the conclusion of the incubations. The procedure for the evaluation of volatile ^{18}O-containing organic molecules in the headspace will utilize GC/MS techniques. A head space sample will be removed from the incubation with a gas tight syringe and the head space gas will be injected into a GC/MS using a capillary column and a temperature program which will separate formaldehyde and acetaldehyde (likely products from O- or N-dealkylation reactions) and then ramp to 250^{o} C to insure that other ^{18}O-containing organic compounds are not present. In addition we will utilize this procedure to check the ratio of ^{18}O /^{16}O at the completion of the incubations. Samples of the extracts described below will be subjected to both GC/MS and LC/MS analysis to look for the presence of ^{18}O-containing molecules.

Electrospray ionization (ESI) mass spectrometry to identify ^{18}O-containing organic molecules (and putative AhR ligands) arising from CYP metabolism of endogenous substrates: The coupling of electrospray ionization (ESI) to mass spectrometers {141,142,143,144} has placed mass spectrometry on the forefront of biological analysis. The success of ESI is primarily due to the technique's ability to ionize usually nonvolatile analytes such as biomolecules and to place multiple charges on large molecules that have multiple charge sites. Multiple charging effectively reduces the mass-to-charge (*m/z*) ratio of large biomolecules making analysis of very large molecular weight compounds possible on instruments that have limited *m/z* ranges. Furthermore, the ease of coupling liquid chromatography to ESI {145} and the extremely high sensitivity (700 zeptomoles) achieved with ESI {146} are additional important advantages of this ionization method. For example, in the ZZZZ laboratory, one femtomole of heroine and morphine can be detected using conventional constant infusion ESI coupled to a Finnigan TSQ 7000 triple quad mass spectrometer (data not shown). Another very significant advantage of ESI is the mild conditions to which the analytes are exposed. Samples are prepared in the appropriate liquid solvent at 4 ^{o}C, and then held at room temperature only for a brief period prior to instrumental (MS) analysis. Thus, as long as the analytes of interest are stable for brief periods of time at room temperature in the chosen solvent that will be sprayed, the analytes of interest will be stable throughout the mass spectral analysis. Analytes are **NOT** exposed to harsh conditions or extremes of temperature, and thus even labile molecules are readily identified by ESI. These are among the reasons which make electrospray ionization mass spectrometry (ESI-MS) a powerful method for the identification of the endogenous substrates for cytochrome P450 monooxygenases in cells.

In order to further increase our ability to employ mass spectral detection of endogenous CYP substrates, cells or cell fractions will be incubated in the presence of 61% $^{18}O_2$. Once ^{18}O has been incorporated into substrate, we will then be able to search for peaks separated by a *m/z* of two or four (incorporation of one or two ^{18}O). Only monooxidase substrates involved in oxidative hydroxylation will exhibit this pattern, and *ONLY* monooxidase substrates will display a pattern where the mass change is two. Creation of this pattern in the mass spectrum inherently increases our power of detection. However, if "noise" from other molecules is too prevalent and interferes with this pattern, other approaches will be applied to identify the endogenous substrates. For example, we will run reactions with and without the $^{18}O_2$ or with cells expressing functional or non-functional CYP1B1, and then perform a "spectral subtraction." In other words, the mass spectrum from the reaction containing no $^{18}O_2$ (other than normally present in nature) would be treated as background. Once this spectrum has been "subtracted" from the spectrum that contains products from the $^{18}O_2$ reaction (the resulting spectrum is appropriately called the difference spectrum), product ions and fragments that have incorporated $^{18}O_2$ will be more readily identifiable in the difference spectrum (**FIG. 13**).

Sensitivity in ESI is extremely high. In conventional ESI (metallic spray needle with an inner diameter of 50-500 mm) samples as dilute as 10^{-10} M can be detected. These samples are usually infused out a 50-250 mL syringe at a flowrate of 5-10 mL/min. It takes approximately 30 seconds to scan enough times to gather a mass spectrum at these low concentrations. Furthermore, with micro-ESI (glass spray needle with an inner diameter from 10-50 mm; {146,147} *sensitivity is usually 10-100 times greater*. In this technique samples are infused out of a 1-250 mL syringe at a flowrate from 100-5000 nL/min. Once again, even with extremely dilute samples, 30 seconds is usually sufficient to gather a mass spectrum. Micro-ESI has become an experimental staple in the ZZZZ laboratory. An alternative approach, if sensitivity continues to be a problem, on-line HPLC will also be performed. In this technique the chromatographic separation is performed in series with the ESI. The column may be before the needle, or the ESI needle itself may be packed and serve as a column {148}. On-line HPLC will further enhance the sensitivity of the technique by effectively pre-concentrating the sample *and potentially removing many of the peaks that could interfere with the signal of the desired analyte*. In LC/MS, mass spectrum are usually gathered for a shorter length of time (1-10 seconds) in order to increase chromatographic resolution. Any loss in sensitivity due a decrease in the number of scans is usually more than compensated for by the pre-concentration and sample cleanup provided by LC. This combination of *high selectivity* and *high sensitivity* is anticipated to allow us to readily identify endogenous CYP substrates.

If sensitivity is an issue, on-line chromatography will also be performed to separate and pre-concentrate components in the mixture. Furthermore, if questions arise concerning the source of a fragment ion, the tandem mass spectrometry (MS/MS) capabilities of the TSQ 7000 will be utilized. In tandem mass spectrometry, the

ion of interest is isolated in the first quadrupole. It then undergoes collisions in the second quadrupole at high pressure (this is called the collision cell). The fragment ions produced by these collisions are then analyzed in the third quadrupole. Thus with ESI, the analytes are not subjected to harsh conditions until ionization has already occurred, and parent ions are not lost or damaged. For example, if a fragment that could arise from multiple reaction products incorporates the ^{18}O, each product may be isolated and fragmented. *Thus, it will be possible to identify the source (or parent ion) of the fragment*. Additionally, each fragment ion that appears in the original mass spectrum may also be isolated and fragmented as well. In this way structural information will be inferred from the tandem mass spectrometric experiments. Also, confirmation of the ^{18}O inclusion will be obtained by comparing it with the ^{16}O fragmentation pattern. Finally, data on the site of the inclusion will be obtained by determining which fragment or fragments contain the ^{18}O. Although a similar mass spectral analysis has been performed (solely to confirm oxygen insertion into the T-2 toxin formed by *Fusarium sporotrichiodes* grown in the presence of $H_2{}^{18}O$ and $^{18}O_2$ molecular oxygen {149}, to our knowledge *we will be the first group to apply this much more powerful technique to the identification of unknown endogenous CYP substrates.*

Structural identification of ^{18}oxygen-labeled CYP metabolites and putative AhR ligands: (Endogenous) substrates which have been oxidized by CYP will be detected by LC/MS experiments, utilizing the appearance of a doublet in the molecular ion (2 mass unit difference) as a marker for monooxygenated metabolites in reactions conducted under an atmosphere of 61% $^{18}O_2/^{16}O_2$, as described in Preliminary Results. The TARGET molecular ions of interest, i.e. those converted to a molecular ion doublet in the presence of $^{18}O_2$ will require additional analysis in order to elucidate the molecular structure of the metabolite and thus the parent substrate (or endogenous ligand.) We will subject the parent ions of the putative endogenous CYP1B1 substrates or AhR ligands, identified as described above, to MS/MS analysis to generate fragments of the parent ion to facilitate the identification of the ligand. Such identification of the structure of compounds based on the molecular ion and fragmentation pattern is the basis of mass spectral identification of unknown compounds. This powerful bioanalytical technique will be our primary approach for the structural identification of endogenous substrates and ligands. However, a number *of alternative and complementary approaches* are also available to us if the identification of the substrates/ligands structure by MS/MS proves equivocal. In this case, as several examples of alternative approaches, we would:

A) Utilize chemical modification of the unknown compounds to provide additional structural information. In short, experiments of this type involve the LC/MS of the parent mixture followed by chemical modification and LC/MS to determine which of the doublet ($^{18}O/^{16}O$) containing peaks have diminished in intensity and which have appeared. Peaks subjected to chemical modification will be further evaluated by MS/MS techniques designed to fragment the parent ion and provide structural identification.

B) Utilize model compounds of similar molecular weight and functional groups as the unknown molecule to produce representative fragments in the MS/MS experiments. Since preliminary experiments suggest that the ligands are very lipid soluble we would concentrate our efforts on molecules consistent with known biosynthetic pathways leading to non-polar compounds. As one example, the isoprene unit associated with steroid biosynthesis might serve as an attractive model compound. Such utilization of model compounds would be similar to producing a 'library' of fragmentation patterns to aid in the MS/MS identification of the putative CYP1B1 substrates and AhR ligands.

C) Utilize 300 or 500 MHz ^1H NMR (available to us at UNM Department of Chemistry NMR Facility) for structure identification. (We are confident that purification by HPLC will produce compounds with sufficient purity for such studies). The consistency of the molecular doublet ($^{18}O/^{16}O$) will be utilized to insure that the compound has not decomposed during purification as well as to verify that we are purifying the putative metabolite(s).

D) Utilize ^{13}C NMR techniques, although these may be limited by the comparatively low concentrations of putative CYP1B1 substrates/metabolites and AhR ligands initially available to us, and thus the utilization of ^1H spectra for structural identifications is deemed more likely to be applicable to these studies.

Alternative Approaches: We recognize that CV-1 cells may not contain ALL potential endogenous AhR ligands and especially those potentially metabolized by CYP1B1, or those which may be the most physiologically relevant. Thus, a similar menu of experiments as described above will be conducted utilizing other cell systems, primarily those derived from the CYP1B1 'knockout' mouse and now available to us via the collaboration with Dr. Colin Jefcoate. Utilization of these cell models will enhance our likelihood of

identifying AhR ligands and/or CYP1B1 substrates with important physiological functions. Ultimately it would be highly desirable to develop an *in vitro* ocular cell model system and employ the ^{18}O-labeling strategy to identify endogenous CYP1B1 substrates contributing to the development of glaucoma {93,94,95}

Expected Results: In view of our preliminary results demonstrating our ability to detect ^{18}O-incorporation into at least one low turnover endogenous substrate of CYP1B1 (i.e. E_2), we anticipate we will be able to use this approach to identify at least one (additional) endogenous CYP1B1 substrate in the CV-1 cell models. Since we have now demonstrated inverse proportionality between endogenous AhR activity and CYP1B1 in CV-1 cells (**FIG. 1 & 2**), and E_2 is *not* an AhR ligand or activator of our DRE-driven reporter construct, it follows that it is highly likely that at least one of the endogenous CYP1B1 substrates identified via ^{18}O-labeling will be an AhR ligand or other regulator of AhR-mediated gene regulation. The converging strategy of integrating Aims 2 and 3 will further ensure we discover at least on such molecule. Finally, we anticipate that application of this approach will identify at least one endogenous CYP1B1 substrate in the cell lines derived from the CYP1B1 null mouse and its wild-type progenitor, and that such a molecule(s) is even more likely to have an important physiological role in mammalian cells and/or function as an AhR ligand/regulator.

General Methods:

Transient and stable transfections: We will be using two methods for transfections, 1) calcium phosphate for stable transfection using a Promega kit, or 2) transient transfections will be performed with a relatively efficient Lipofectamine reagent (Life Technologies) method, optimized in our laboratories to yield up to 40% transfection efficiency in COS-1 and CV-1 cells.

Relative AhR levels: Protein samples will be separated by tricine SDS-PAGE. After SDS-PAGE on an 8.0% polyacrylamide tricine gel, the proteins will be electrophoretically transferred to PVDF membrane (Immobilon P). The electrophoretic transfer will be performed at 8 V for 6 h in a Genie electroblot unit (Idea Scientific Co., Minneapolis, MN). Transfer buffer will be composed of 20 mM Tris, 185 mM glycine, and 20% methanol (v/v). Following protein transfer the membrane will be blocked with 3% bovine serum albumin in 10 mM Na phosphate, 150 mM NaCl, pH 7.4 (PBS), containing 0.5% Tween 20 for 30 minutes at room temperature. The blots will be rinsed once in wash buffer, consisting of 0.1% bovine serum albumin in PBS containing 0.5% Tween. The AhR (Rpt 1 and Rpt 9) and Arnt (2B10) mAbs that will be used in these experiments were generated and are already routinely used in the co-investigator's laboratory. The blots will then be incubated with either mAb Rpt 1 (0.5 ug/ml) or mAb 2B10 (0.1X hybridoma culture supernatant) for one hour at room temperature, followed by five 5 minute rinses with wash buffer. The blots will then be incubated with biotinylated goat anti-mouse IgG (Fc specific), followed by ^{125}I-streptavidin in wash buffer for 1 hour each, followed by three 5 minute rinses with wash buffer after each incubation. In some experiments, Rpt 1 will be visualized with ^{125}I goat anti-mouse IgG (Fc fragment specific). The blots will be dried and visualized by autoradiography. The individual radioactive bands containing either AhR or Arnt will be excised and counted in a gamma counter. Standard curves will be routinely plotted to establish the linear antibody response over a range of cytosolic protein concentrations. A standard curve for the quantitation of AhR and Arnt, using both mAb Rpt 1 and 2B10, will be generated.

Nuclear AhR/ARNT heterodimer levels: Washed cells will be homogenized in MENG-Mo (25 mM Mops, 2 mM EDTA, 0.02% NaN_3, 10% glycerol, and 20 mM sodium molybdate, pH 7.5) with 25 strokes in a Dura-Grind dounce tissue grinder at 4^{o}C. Extent of cell disruption will be routinely monitored microscopically to ensure complete cellular disruption. The nuclear pellet will be isolated by centrifuging the cell homogenate at 1,000 x g for 15 minutes. The supernatant will be centrifuged at 100,000 x g for 30 minutes to obtain the cytosolic fraction. The nuclear pellet will be resuspended and washed 3X with MENG-Mo and once with MENG containing 50 mM NaCl. High salt nuclear extract will be prepared by suspending the nuclear pellet in MENG-Mo + 500 mM NaCl for one hour, 4^{o}C, followed by centrifugation at 100,000 x g for 1 hour.

Gel Retardation Assays. Gel retardation assays will be performed with high salt nuclear extracts isolated from untreated and TCDD treated cells. Nuclear extract (about 7.5 ug of protein) will be added to a mixture of 25 mM Hepes, pH 7.5, 10% (v/v) glycerol, 100 mM KCl, 540 ng of poly (dIdC), 5 mM DTT, 4 mM $MgCl_2$, 4 mM

spermidine, 2.5% CHAPS, and 1 ug of sonicated E. coli DNA (final concentrations in 25 ul assay volume). The mixture will be incubated at room temperature for 15 min followed by addition of 0.5-0.75 ng of ^{32}P-labeled oligonucleotide (DRE) and further incubated for an additional 15 min at room temperature. Wild type DRE, and mutant M1 and M2 DREs {150} have been kindly provided by Dr. Denison (University of California, Davis CA). The purified oligonucleotides will be radiolabeled with ^{32}P-ATP as described by Denison et. al. {150}. Upon addition of 2.5 ul of 0.25% xylene cyanol in 20% (w/v) ficoll to the reaction mixture, samples will be loaded onto a 4% nondenaturing polyacrylamide gel and the products separated by electrophoresis. The gels will be dried and subjected to autoradiography. The specificity of the binding will be assessed by incorporating 100-fold molar excess of either unlabeled DRE or mutant DREs in the assay system.

HepG2 cell culture reporter assay for AhR activity: Our primary approach to assay for putative endogenous ligands is the use of a stable HepG2 cell line HG40/6 which expresses pGudLuc 6.1 vector. In studies published elsewhere we have demonstrated that clone HG40/6 luciferase activity is highly induced by TCDD {102}. These cells will be utilized to assess the presence of ligands in crude extracts from CV-1 and other cells. Cells are homogenized in buffer and run through a Varian Bond Elut C-18 prep column. The column is extensively washed with water, and hydrophobic compounds eluted with methanol. The eluted fraction is dried down, suspended in methanol, and injected onto a Beckman Ultrasphere ODS (250 x 4.6 mm column) and then eluted with 70% Methanol at 1 ml/min. One ml fractions are collected and dried down, re-solubilized in five µl DMSO. Each solubilized fraction is subsequently added to 24-well cell culture plates containing a HepG2 cell line with a DRE driven Luciferase reporter vector stably integrated. We have been able to detect as little as 50 pmol of TCDD in this assay system, thus it is extremely sensitive. This system will not suffer from the background problems encountered with the ligand competition binding assay (described below), which is our alternate screening approach. As can be seen in **FIG. 6** the background is very low (especially compared to the competition binding assay; FIG. 8) and at least six peaks of significant AhR activity were detected. We are in the process of running extracts from clone 7 as one of our controls.

AhR Competition Binding Assay. An alternative approach to assess the ability of endogenous compounds in HPLC fraction extracts to bind to the Ah receptor, a competition binding assay will be used. All solvents to be used in extractions are incubated with 1% charcoal for 15 minutes, followed by centrifugation and Millipore filtration just before use. Each solvent extract is dried down and re-solubilized in dimethyl sulfoxide (DMSO) at one-quarter the volume of initial extract. A series of different concentrations of each extract and 480,000 cpm of 2-125Iiodo-7,8-dibromodibenzo-p-dioxin (2,176 Ci/mmol) will be added to a set of 12 x 75-mm glass tubes. This is the concentration of radioligand that just reaches saturation and thus most appropriate for competition assays. The total volume of DMSO in each tube is always adjusted to 6 ul. The radioligand will be synthesized in the XXXX lab {151}. Mouse hepatoma cell line 1c1c7 cytosol (500 ul, 150 ug/ml) will be added to each tube, and the tubes incubated for 30 minutes at room temperature. The concentration of radioligand used saturates the Ah receptor binding sites in the cytosol added to each assay. Excess radioligand is removed by the addition of 50 ul of 3% charcoal-0.3% dextran and incubation for five minutes at room temperature. The amount of charcoal used was optimized to yield the highest levels of specific binding with acceptable nonspecific binding. Assay tubes are centrifuged at 3,000 g for 10 minutes at 40C, and 300 ul of the supernatant from each tube will be counted in a gamma counter. The level of nonspecific binding is determined in separate competition assays by blocking all specific binding to the AhR by co-incubating the radioligand with a 200-fold excess of 2,3,7,8-tetrachlorodibenzofuran. The ratio of specific to nonspecific binding is routinely 6:1.

***In Vitro* metabolism assays in the presence of $^{18}O_2$:** Metabolism of DMBA and 17β-estradiol are by standard protocols routinely utilized in the Scientist lab {37,65,66,67,73,89,104,110 ,129}. Since the metabolism of DMBA by CYP1B1 and many other hCYP isozymes is well characterized and extremely sensitive, this assay will serve as an important 'control' assay to validate and calibrate the ESI-MS procedures, and serve as a positive control to verify and quantitate expression of functional CYP1B1 in our cell models. Additional assays utilizing 100 uM 17β-estradiol as substrate; {71,105,152} will also be utilized as a positive control to verify and quantitate expression of functional CYP1B1 in our cell models. Similar assays IN THE ABSENCE of exogenous substrates as detailed in Aim 3 will be conducted to identify endogenous CYP substrates. *In vitro* metabolism assays will be conducted with

intact cells suspended in growth media, cell lysates, and microsomes prepared from cells, or subcellular fractions plus cell lysate fractions (**FIGS. 10-12**). Incubations are conducted in reaction vials sealed with rubber stoppers and parafilm. Gas exchange is effected via 20 ga needles inserted through the stoppers. Reaction mixtures are flushed with argon gas then evacuated 3 times in succession, then flushed and saturated with 61% $^{18}O_2$, prior to initiation of the reaction via addition of substrate by syringe needle through the stopper. Similar experiments may be conducted using undisturbed adherent cells in tissue culture flasks by replacing the cap with a rubber stopper and proceeding as described above. Although we do not anticipate this being a problem, we will monitor for potential oxygen toxicity in incubations with intact cells, and substitute a mixture of nitrogen, carbon dioxide and $^{18}O_2$ for pure $^{18}O_2/^{16}O_2$, if necessary.

Animals, animal treatments, microsome preparations, and cell isolations: Male Sprague Dawley will be utilized for some control experiments in this project. Rats will be treated *in vivo* with chemical inducers at various concentrations via i.p. injection, in 0.5 ml saline or corn oil vehicles as appropriate. Tissue microsomes are prepared by differential centrifugation and stored in liquid nitrogen until use. Hepatocytes will be isolated via a modification of a standard collegenase perfusion protocol routinely utilized in Dr.Scientist's lab {13}. Microsomes are prepared by differential centrifugation of tissue homogenates or cell lysates by standard protocols as previously described {13,36,65,104}. Total protein is quantitated by the BCA reaction (Pierce) with BSA as standard. CYP holoenzyme levels will be quantitated from the reduced carbon monoxide difference spectrum {36,104,65}.

Cell culture: CV-1, V-79 and MCF-7: MCF-7 and V-79 cells expressing CYP1B1 will be cultured by standard protocols in the Scientist lab {104,153}. CV-1 cells and stably transfected clones are cultured as previously described {57,154} in both the Scientist and XXXX labs.

Research Time Table:

Project/Specific Aim	Year 1	Year 2	Year 3	Year 4
1	<----------	----------	---------->	
2	<----------	----------	----------	---------->
3	<----------	----------	----------	---------->

Each project will be performed primarily by the personnel listed below:
PROJECT/AIM:

1- Gary XXXX, Technician (XXXX Lab), Chris CCCC (XXXX lab)
2- John Scientist, Sophie BBBB, Gary XXXX, Technician (XXXX Lab), Chris CCCC (XXXX lab)
3- John Scientist, Sophie BBBB, George AAAA, Jerry YYYY, Chris ZZZZ

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