Estrogenic effects of perfluorooctanoic acid (PFOA), a persistent contaminant found in common consumer products

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Statement of proposed activity

My research is focused on the toxic effects of environmental chemicals that are suspected endocrine disrupters, and specifically on the subcategory that affects mammalian estrogen-based functions. I employ *in vitro* systems (cultures) of cells related to estrogen synthesis (JEG-3 cells) and estrogenic response (MCF7 cells). A recently emerged chemical of concern is perfluorooctanoic acid (PFOA) that is suspected to cause disruption of reproductive system development during gestation and early life stages in animals. Practically nothing is known about PFOA's mechanism of action that could explain the emerging abnormalities observed in very recent animal studies. PFOA is the molecule of interest for the proposed research activity.

Significance of project

PFOA has emerged as a chemical of serious public health and ecological concern for the following reasons:

a) It is a highly stable and therefore **environmentally highly persistent** chemical because it is highly fluorinated and consequently resistant to degradation inside the body or in the environment. This property is similar to that of polychlorinated chemicals (e.g. PCBs) because of the presence of the halogens, fluoride (F) and chlorine (Cl), in these structures.

b) It is a component of a large number of consumer products, including detergents, Teflon coatings, stain resistant fabrics for clothing or furnishings, and a solvent in multiple applications that lead to its release in the environment and contamination of groundwater systems. These uses and applications lead to **multiple paths of human exposures**.

c) National (CDC) and international (Europe, Japan) biomonitoring studies of human populations reveal a wide range of **blood levels in humans** with toddlers and young children at higher levels than adults. It is also detected in **breast milk of nursing mothers** with serious implications for infant exposures.

d) Toxicology studies are very limited and most of the key studies are very recent (2008-09). In addition to the **paucity of toxicological information**, the studies addressing endocrine effects are even fewer and indicative of impacts deserving deeper exploration.

e) Some of the endocrine disrupting effects observed in animals (impaired mammary gland development in female offspring) are **similar to still unexplained trends in human pre-adolescents**.

f) I have significantly extended the work of a **student-initiated risk assessment project** on PFOA that was produced as part of the Environmental Health program in 2008-09 and which culminated in a **recently presented conference poster/platform presentation** (attached) that attracted attention from several scientists from both industry and government agencies.

Research Activities

I have developed a functional laboratory employing an estrogen producing (JEG-3) and an estrogen responsive (MCF-7) cell lines and I can assess two major mechanisms: estrogen synthesis and receptor activation. PFOA very likely affects one or both of these pathways that determine timely estrogenic activity in the developing organism. These *in vitro* systems offer an efficient assessment approach of the potential estrogenic or antiestrogenic activity of this chemical.

The goals of the proposed work are a) to assess the ability of PFOA to activate the estrogen receptor (ER) and the subsequent expression of estrogen-regulated genes, using the ER-transactivation assay based on a reporter gene transcription system in the estrogen-responsive cell line, MCF-7 and b) to assess the ability of PFOA to alter the synthesis and secretion of estradiol, using a simple immunoassay (ELISA) in the estrogen-producing cell line, JEG-3.

a) Estrogen receptors alpha and beta (ERα and ERβ) are activated upon binding of estrogen and translocate to the nucleus where they mediate estrogenic effects via regulation of gene expression. An *in vitro* model will be employed to examine the ability of PFOA to activate ERα, the receptor predominantly expressed in MCF-7 cells (already owned). Expression of the reporter gene coding for Chloramphenicol

Acetyl Transferase (CAT) under the control of an Estrogen Responsive Element sequence (ERE) allows the quantitative assessment of ERα activation. Cells transfected with the ERE-CAT plasmid (already owned: obtained as a gift from Dr K. Korach, NIEHS, NC) will be treated with a series of PFOA (already owned) concentrations in duplicates for 24 or 48 hours, in growth media, MEM (already available, more may be needed) supplemented with estrogen-stripped charcoal-dextran-treated (CD) serum (some already available, more is needed). CAT gene expression will then be evaluated with semi-quantitative (due to lack of quantitative, "real-time" PCR instrumentation on campus) reverse transcription – polymerase chain reaction (RT-PCR), using commercial reagents (needed) and established protocols. Alternatively, if this semi-quantitative approach is not sensitive enough to detect differences in expression (assessed with a positive control, 17b-estradiol), a radiometric approach that quantitatively assesses CAT's enzymatic activity can be adopted. This uses a radiolabeled substrate (^{14}C-acetyl CoA) (needed) and thin layer chromatography (TLC) (materials already owned) that is then processed for imaging and quantification of labeled acetyl chloramphenicol with a scintillation counter (already available). The latter is a very sensitive assay that has been used widely for this purpose. My lab is licensed for the use of radioactivity.

b) Estrogen synthesis follows a timely and tightly regulated series of enzymatic reactions involving several enzymes and intermediate products. The final product, 17-β estradiol, is secreted from cells into their growth media. I plan to employ a simple immunoassay (ELISA) to measure 17-β estradiol in the media, of estrogen-producing JEG-3 cells (already owned). Cells will be treated with a series of PFOA concentrations in duplicates for 24 or 48 hours in CD-serum growth media, DMEM (some available, more is needed). The media will then be analyzed for the amount of secreted estradiol in PFOA-treated samples compared to control samples. Specifically, ELISA involves the competitive binding of a specific (primary) antibody (some already available, more is needed) to the secreted estradiol in the samples, followed by the binding of an HRP-conjugated secondary antibody (some already available, more is needed) onto the primary antibody. HRP catalyzes a color-producing reaction that is measured spectrophotometrically at 490nm. Because the analyte of interest (estradiol) is soluble, a competitive ELISA will need to be employed, using estradiol (antigen)-coated ELISA plates (needed). The intensity of the color is inversely proportional to the estradiol present in the sample, which is quantified using a calibration curve made of known purified estradiol (already available) concentrations.

Professional Development

The proposed laboratory-based research activity is critical for my continuous professional development as a member of the scientific community within and outside the college. This would greatly enhance my contribution in my field, following up on my recent presentation at the annual conference of the Society for Risk Analysis (December 2009, Baltimore, MD) and providing urgently needed toxicological data on PFOA's estrogenic potential. It will also enhance my position as a faculty mentor in the classroom and in research projects to support student learning, provide an excellent opportunity for student engagement in lab research and work toward a peer review publication and professional conference presentations including students.

In addition, the results of this study will be very useful as a demonstration of my research capabilities in supporting external funding proposals. Along with the improved overall lab set up from last year's purchase of key equipment, the demonstration of productivity in the form of results will allow me to make a stronger case for obtaining external funding.

Dates and Length of Request: The proposed work will take place during winter, spring and summer of 2010. I am requesting support for the purchase of essential consumables and reagents as described in the budget below. Sufficient minimum funding is estimated approximately at $4,680.

Current Curriculum Vita: Attached.

Other Sources of Funding: I am not currently funded by any grant.

Budget:

Item	Description	Source, Cat#	List Price	Number of items	Total price
Primary antibody	Mouse monoclonal antibody against human estradiol, 200ug	Thermo Scientific MA1-83370	$215	2	$430
Secondary antibody	Goat anti-mouse, pre-diluted, 2mL, stabilized HRP-conjugated	Thermo Scientific 32430	$95.00	2	$190
MEM	Growth media for MCF-7 cells (pck 10x1L in powder)	Sigma	$32.90	1	$32.90
DMEM	Growth media for JEG-3 cells (pck 10x1L in powder)	Sigma	$32.90	1	$32.90
CD-serum	Charcoal-dextran stripped serum, 500 mL	VWR, 101301-472	$281 + dry ice	1	$350
DNA polymerase	Main enzyme for PCR, 500units	Promega, A3800	$123	5	$615
ImPropm-II Rev. transcr/ase	Main enzyme for RT-PCR, 100 reactions	Promega, A3802	$117	5	$585
Pen/strep	Growth media supplement, 100ml	Sigma, 15070-063	$16.56	2	$33.12
Insulin	Growth media supplement, 5ml	Sigma, I0516	$38.4	2	$76.80
Qiagen Maxi kit	Plasmid purification, 25 columns	QIAGEN 12162	$199	1	$199
ELISA plates	96-well high binding (pck100)	Fisher, 07-200-721	$373.18	1	$373.18
ELISA plates	96-well round, mixing (pck100)	Fisher, 07-200-99	$122	1	$122
^{14}C-acetylCoA	CAT substrate, 10uCi	Perkin Elmer NEC313L010UC	$704	1	$704
Multi-channel pipetter	8-Channel, 5-50uL, Fisherbrand	Fisher, 21-377-827	$435.08	1	$435.08
Multi-channel pipetter	8-Channel, 50-300uL, Fisherbrand	Fisher, 21-377-829	$435.08	1	$435.08
Pipetter basins	Nonsterile basins (pck100)	Fisher, 13-681-100	$62.37	1	$62.37
Total					**$4,676**

Antibodies: Essential reagents for ELISA assays.
Media: DMEM is different than MEM; cells have different optimum growth media requirements
CD serum: It is critical that, during the time of cell treatment with the chemical, animal hormones are removed from the fetal bovine serum used as a supplement in growth media. This CD-serum is specifically formulated to ensure absence of hormones, external to the experimental treatment conditions.
RT-PCR reagents: Additional enzymes for the RT-PCR reactions are needed, DNA polymerase and Reverse transcriptase, because the RT-PCR reagents available are not enough for the project.
^{14}C-acetyl CoA: this is needed as an alternative, if the RT-PCR approach is not sensitive enough.
Media supplements: insulin and penicillin/streptomycin are indispensable supplements for cell growth.
Qiagen Kit: the most efficient, effective and clean method to purify the CAT plasmid and avoid use of unsafe organic solvents such as phenol and chloroform.
96-well ELISA plates: Specifically formulated to bind the competing antigen with high affinity, and non-binding for reagent mixing/incubation (two steps in competitive ELISA assays of soluble molecules.
Multichannel pipetters and basins: for low and higher volume ranges; essential for even, time-sensitive and reproducible pipetting in ELISA 96-well plates; basins hold the buffer for 8 pipette tips.
Plate reader (not requested here): Normally the assay requires an automated plate reader but this instrument is not currently available on campus. Unless this is purchased for multi-faculty and program support in the near future, the alternative, manual (laborious and time-consuming!), single-sample measurement of color production will have to be used in a conventional single-cuvette spectrophotometer.

Respectfully,
Maria Bastaki