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thesis entitled

In Vitro Studies of the Biochemical Toxicity of Perfluorooctane Sulfonic Acid and its possible Interaction with 2,3,7,8-Tetrachlorodibenzo-p-dioxin

presented by

Wen Yue Hu

has been accepted towards fulfillment
of the requirements for

M.S. degree in Zoology and
Institute of Environmental
Toxicology

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Date 4/24/00

IN VITRO STUDIES OF THE BIOCHEMICAL TOXICITY OF PERFLUOROOCTANE SULFONIC ACID AND ITS POSSIBLE INTERACTION WITH 2,3,7,8 – TETRACHLORODIBENZO-*P*-DIOXIN

By

Wen Yue Hu

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Zoology
Institute of Environmental Toxicology

2000

ABSTRACT

IN VITRO STUDIES OF THE BIOCHEMICAL TOXICITY OF PERFLUOROOCTANE SULFONIC ACID AND ITS POSSIBLE INTERACTION WITH 2,3,7,8 – TETRACHLORODIBENZO-*P*-DIOXIN

By

Wen Yue Hu

In the current study, three aspects of the biochemical toxicity of perfluorooctane sulfonic acid (PFOS) were investigated using *in vitro* cell culture systems. The effects of PFOS on aryl hydrocarbon receptor (AhR) mediated cytochrome P4501A1 (CYP1A1) activity were tested using *in vitro* cell bioassays. The results showed that PFOS had neither adverse effect on cell viability nor direct effect on CYP1A1 activity within the dose range tested. However when cells were dosed with PFOS and TCDD in combination, interactive effects on both CYP1A1 induction and AhR activation were observed at environmentally relevant concentrations, in which PFOS increased the effects of TCDD by 30-40 %. It was further tested with time course experiment and transcription inhibition experiment that this interactive effect possibly occurred at transcriptional level. In addition, the effects of PFOS on gap junctional intercellular communication (GJIC) were tested. PFOS inhibited GJIC in a dose-dependent manner, and the inhibitory effect occurred in a very short time period. It was proven that this inhibitory effect was neither species-specific nor tissue-specific. Finally aromatase assay was conducted, results from which indicated that PFOS at a concentration of 50 mg/L could induce aromatase activity *in vitro* by 1.5 fold for 24 hrs exposure, and by 1.7 fold for 48 hrs exposure for PFOS concentration at 10 mg/L and 50 mg/L.

To my dear parents Hua Hu and Xiao Ru Liu,
for their never stopped love and encouragement.

ACKNOWLEDGMENTS

I sincerely thank my academic advisors Dr. John Giesy and Dr. Paul Jones for their continuous support and tremendous help. I really appreciate the cooperative tradition in the aquatic toxicology laboratory at Michigan State University, only with the friendly supports from all the personnel in this laboratory, could I make through all the difficulties during the process of doing this project and writing my thesis. I am honored to be able to have Dr. Don Hall, Dr. Will Kopachik and Dr. Robert Roth on my graduate committee, and to receive invaluable advises and comments from them. Special thanks were owed to Dr. James Trosko and Dr. Brat Upham at Michigan State University for their kindly providing experimental instrument and instructions that made part of this thesis possible. I am thankful to Dr. Wim Decoen at Antwerp University, Belgium for his advises on cell bioassays; Dr. Thomas Sanderson and Marjoke from Utrecht University at Netherlands for their help on NCI-H295R cell culture and aromatase assay. Debts are owed to Dr. Heather Eisthen for her support during the first year and a half in my graduate studies, and especially for her understanding on my switching program.

Thanks are owed to 3M company for their funding of this project, and the aquatic toxicology laboratory at Michigan State University for providing me the graduate assistantship.

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LIST OF SYMBOLS AND ABBREVIATIONS

AhR	--- aryl hydrocarbon receptor
ATCC	--- American Type Culture Collection
CDK	--- Carvan dolphin kidney cell line, isolated from a prematurely born female-bottle-nose dolphin
CYP19	--- cytochrome P450 19 (aromatase)
CYP1A1	--- cytochrome P4501A1
DMEM	--- Dulbecco's Modified Eagle Medium
DREs	--- dioxin-responsive elements
EC50	--- concentration of 50% effectiveness
EROD	--- ethoxyresorufin-*o*-deethylase
FBS	--- fetal bovine serum
GJIC	--- gap junctional intercellular communication
H4IIE-luc	--- rat hepatoma cell line, which was stably transfected with firefly luciferase reporter gene
MEM	--- Minimum Essential Medium Eagle
NADPH	--- β-nicotinamide adenine dinucleotide phosphate (reduced form)
NCI-H295R	--- human adrenocortical carcinoma cells
PBS	--- phosphate buffer saline
PFDA	--- perfluorodecanoic acid
PFFAs	--- perfluorinated fatty acids
PFOA	--- perfluorooctanoic acid
PFOS	--- perfluorooctane sulfonic acid
PFOSA	--- perfluorooctanoic sulfonamide
PKC	--- protein kinase C
PLHC-1	--- hepatocellular carcinoma cells derived from desert topminnow (*Poeciliopsis lucida*).
PPAR	--- peroxisome proliferator activated receptor
PPRE	--- peroxisome proliferator responsive element
TCDD	--- 2,3,7,8 – tetrachlorodibenzo-*p*-dioxin
WB-F344	--- rat liver epithelial cells

INTRODUCTION

Perfluorinated fatty acids (PFFAs) are commonly used in industrial materials such as wetting agents, lubricants, corrosion inhibitors, stain resistants for leather, paper and clothing, as well as in foam fire extinguishers (Sohlenius, *et al.* 1994) due to their low surface tension, stable physical and chemical properties. PFFAs also possess unique biological characteristics that make them suitable for red blood cell substitutes and hepatic drugs (Ravis, *et al.*, 1991). Because of their growing list of applications and increasing potential for exposure to humans and wildlife, toxicologists are now assessing the potential toxicity of PFFAs at environmentally relevant concentrations.

Based on the fact that PFFAs are chemically stabilized by strong covalent bonds between carbons and fluorines, they were historically considered to be metabolically inert and non-toxic (Sargent *et al.* 1970). However, only recently, has it been found that they are biologically active and can induce effects on peroxisomal proliferation, lipid metabolizing enzyme activity, xenobiotics metabolizing enzyme activity, and other important biochemical processes in exposed organisms (Obourn, *et al.*, 1997; Sohlenius, *et al.*, 1994). The major target organ of PFFAs is the liver, but this does not exclude other possible target organs such as the pancreas, testis and kidney (Olson, *et al.*, 1983).

Acute Toxicity

The most well studied compounds in the PFFA family are perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA). The acute toxicities of these two compounds were

evaluated in male Fisher rats, and the LD50/30 days for PFOA was found to be 189 mg/kg-body weight and 41 mg/kg-body weight for PFDA (Olson and Andersen, 1983). Rats treated with a lethal dose of PFOA exhibited incipient death within the first five days; however, those exposed to PFDA showed a delayed lethality after two weeks (Olson and Andersen, 1983). This difference is probably due to their different rate of accumulation and elimination in male rats.

Tissue Distribution, Metabolism and Excretion

PFFAs are completely ionized in hydrophilic environments, and their basic hydrocarbon backbone is similar to fatty acids, except that all of their carbon atoms are covalently bound with fluorine atoms, which is also responsible for their chemical and metabolically inertness. When Wistar rats were treated with a single intraperitoneal dose (20 mg/kg-body weight) of PFDA, approximately 15% of the administered PFFAs were found in the serum, with more than 99% bound to the serum proteins. In the liver, 5% of PFFAs were found to be either in the free anionic form or bound to lipid portion (Ylinen and Aurivla, 1990). Most of the PFFAs administered via the diet were unaffected by metabolic enzymes. Elimination of PFFAs was primarily through urinary excretion, with little bilary and fecal excretion, and the rate of elimination was sex-related (Ylinen, *et al.*, 1989; Hanhijarvi, *et al.* 1987). The renal elimination rate of PFOA in female Wistar rats was ten-fold greater than in male rats. It was suggested that estradiol played a considerable role in controlling PFOA excretion (Ylinen, *et al.*, 1989).

Induction of Oxidative Stress and Lipid Metabolizing Enzyme Activities

Although the mechanism by which PFFAs elicit their toxic effects is unknown, the one consistent conclusion drawn by most researchers is that they acted as peroxisome proliferators. Peroxisome proliferators include a number of structurally diverse compounds. Regardless of their dissimilarities in structure, these compounds all have one thing in common: they all induce the proliferation of peroxisomes, and result in an increase in both the number of peroxisomes and their corresponding enzyme activities (Kawashima, *et al.* 1989). PFFAs can interfere with lipid metabolism by increasing peroxisomal fatty acid β-oxidation, and induce several hepatic enzyme activities (Sohlenius and Reinfeldt, 1996). Both *in vivo* and *in vitro* exposures to PFFAs result in increased activities of peroxisomal Acyl-CoA oxidase, which is known to catalyze the first and rate-limiting step in fatty acid oxidation (Sohlenius, *et al.*, 1994). Fatty acid oxidation is also a process that can produce hydrogen peroxide, a oxidative radical, which can cause oxidative stress and result in DNA damage (Sohlenius, *et al.*, 1994). The peroxisome proliferator activated receptor (PPAR), a member of steroid hormone receptor family, can be activated by peroxisome proliferators and bind to the peroxisome proliferator responsive element (PPRE). Previous studies identified several PPREs which located upstream from the structural gene for Acyl-CoA oxidase (Sohlenius and Reinfeldt, 1996; Braissant, *et al.*, 1996). A good correlation had been observed between PPAR activation and peroxisome proliferation potency (Green, 1992).

PFFAs have been shown to be involved in regulating tissue fatty acid composition and content. PFFAs can reduce cholesterol and triacylglycerol level in serum, increase liver

triacylglycerol concentration, and reduce hepatic lipid output (Haughom and Spydevold, 1992). It has also been found that treatment with PFFAs can inhibit Acyl-CoA synthetase activity and result in an increase in the level of free fatty acids (Reo, *et al.*, 1996). Free fatty acids are known to be able to activate protein kinase C (PKC), which leads to a signaling cascade that is important for normal cell function, cell proliferation and gene expression.

Effect on Hepatic Microsomal Cytochrome P450 Enzyme Activity

Cytochrome P450 enzymes (CYP) are a group of primary oxidative enzymes involved in phase I metabolism, a process that detoxifies xenobiotics by making them more polar so that they can be conjugated and excreted easily. Microsomal cytochrome P450 enzymes were induced in rats treated with PFFAs (Permadi, *et al.*, 1992). This induction was sex-related and organ-specific, based on the fact that male rats were more sensitive than female rats, and liver was the major target organ compared to the kidney. For example, administration of PFOA to male rats induced CYP4A1 enzyme activity by 6.8 fold in liver and 2.1 fold in kidney (Diaz, *et al.*, 1994). The CYP4A sub-family is a group of nine enzymes that are specific for fatty acid ω-hydroxylation. Other CYP enzymes may be induced depending on the administration pathways and duration of exposures.

Effect on Leydig Cell Function

PFFAs can affect Leydig cell function and produce Leydig cell adenomas (Liu, Hahn, and Hurtt, 1996). So far most information available is for the effects of ammonium perfluorooctanoate (C8) on Leydig cells of adult male rats. Three levels of effects have

been observed: 1) overall depression of Leydig cell function *in vitro* (Cook, *et al.*, 1992); 2) decreased testosterone release and increased estradiol level *in vivo* (Biegel, *et al.*, 1995); 3) elevation of aromatase (CYP 19) activity by 16 fold *in vivo* (Liu, *et al.*, 1996).

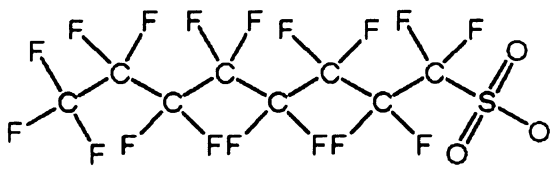
Non-genotoxic Tumor Promoter

Treatment with PFFAs has been associated with the induction of hepatic necrosis, hepatocyte carcinomas, Leydig cell adenomas, and pancreatic tumors (Obourn, *et al.*, 1997). It has been postulated that the increase in oxidative stress and alteration in protein kinase C level are responsible for the possible carcinogenic property of PFFAs (Reo, *et al.*, 1996). Recently the alternative hypothesis has been suggested that these effects may be non-genotoxic and caused by the disruption of hormone regulation (Cook, *et al.*, 1992) or blocking of intercellular communication (Upham, *et al.*, 1998).

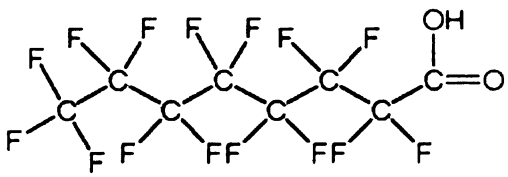
Perfluorooctane sulfonic acid (PFOS) appears to be the end metabolite of a number of perfluorinated compounds used extensively in commercial applications. The amount of PFOS produced is much greater than any other PFFAs (3M internal report). However so far most studies have been conducted on PFOA instead of PFOS. Whether PFOS can cause similar adverse biological effects as PFOA and other PFFAs is still under investigation, and its possible mechanism of action remains to be elucidated.

In this study three aspects of biochemical toxicity of PFOS were investigated using *in vitro* cell culture systems. The effect of PFOS on aryl hydrocarbon receptor (AhR) mediated cytochrome P4501A1 activity and its possible interaction with 2,3,7,8-

tetrachlorodibenzo-p-dioxin (TCDD) were tested using EROD and Luciferase bioassays. In addition, the effects of PFOS on gap junctional intercellular communication were tested using the scrape/loading dye technique. Finally, *in vitro* aromatase assays were conducted on human adrenocorticol cells in order to determine the possible effects of PFOS on steroid hormone metabolism. Together these studies investigate possible modes of action of PFOS based on known toxicological end-points.



PFOS



PFOA

CELL BIOASSAY

BACKGROUND

The acute toxicity of PFFAs has been tested using Fisher rat *in vivo* (Olson and Andersen, 1983). In most cases, rats treated with PFOA ate less and exhibit a significant weight loss compared to the control groups, this phenomenon is called "wasting syndrome". This is usually accompanied simultaneously with an increase in relative liver weight. These effects are remarkably similar to those caused by dietary exposure to TCDD. The dioxin-like effects of PFOA indicate that PFOS, as an end metabolite of PFOA, may have either direct or indirect effects on certain gene products whose synthesis or activity is disrupted by TCDD. Since these two toxicants could occur together in the environment, and are known to have the potential to elicit the same toxic response, their effects should be considered together.

It is known that TCDD elicits most of its toxic effects through the aryl hydrocarbon receptor (AhR) and that the AhR-mediated induction of CYP 1A1 is probably one of the most sensitive methods to detect exposure to dioxin-like compounds (Okey, *et al.*, 1994). Therefore, it is necessary to measure both the direct and the indirect effects of PFOS on CYP 1A1 activity and to investigate possible interactions between PFOS and TCDD. Furthermore, since the AhR-mediated induction of CYP 1A1 by TCDD is a relatively well-defined pathway (Roman, *et al.*, 1998; Yoshiaki, *et al.* 1992)), study of the interaction of these two compounds could shed light on the receptor-mediated mechanism

of PFOS. These two cell lines were chosen as representatives for mammalian and non-mammalian species.

MATERIALS AND METHODS

Chemicals

Perfluorooctane sulfonic acid (PFOS) was obtained from 3M company (St. Paul, MN) as a mixture. Based on NMR analysis the mixture consisted of 68% straight chain PFOS and 17% branched chain PFOS. 2,3,7,8–tetrachlorodibenzo-*p*-dioxin (TCDD) was synthesized at the Aquatic Toxicology laboratory at Michigan State University. The purity was determined by high resolution gas chromatography and high resolution mass spectrometry (HRGC/HRMS) to be >99.9%. α-Amanitin was purchased from Molecular Probe (A 6920).

Cell Culture

H4IIE-luc cells are rat hepatoma cells that were stablely transfected with firefly luciferase reporter gene under direct control of dioxin-responsive elements (DREs) (Sanderson, *et al.* 1998). Due to this unique feature, the H4IIE-luc cell line can be used for both luciferase assay and ethoxyresorufin-*O*-deethylase (EROD) assay. PLHC-1 cells are derived from a hepatocellular carcinoma of desert topminnow (*Poeciliopsis lucida*). Previous studies have indicated the presence of AhR and inducible cytochrome P450 1A1 activity (Hahn, 1993).

H4IIE-luc and PLHC-1 cells were cultured in 100 mm disposable tissue culture dishes (Corning, 25020). All cells were grown under sterile conditions (pH=7.4) in a humidified 5/95% CO_2/air incubator (Forma Scientific, Model 8173). H4IIE-luc cells were cultured at 37 C°, and the PLHC-1 cells were grown at 30 C°. H4IIE-luc cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma D-2902), supplemented with 10% fetal bovine serum (FBS, Hyclone). PLHC-1 cells were cultured in Minimum Essential Medium Eagle (MEM, Sigma M3024) supplemented with 292 mg/L L-glutamine (Life Technologies), and 10% FBS (Hyclone). All cells were passaged when cells became confluent, and new cultures were started from frozen stocks after 30 passages.

Bioassay Procedure

Day 1 Plating cells

When cells reached 80-100% confluence, they were trypsinized from tissue culture dish using 1x trypsin–EDTA (Sigma), and resuspended in cell culture media. The concentration of stock cell solution was estimated using a hemocytometer. H4IIE-luc cells were diluted to a concentration of approximately 7.5 x 10^5 cells / ml, and PLHC-1 cells were diluted to a concentration of approximately 1.25 x 10^6 cells / ml. Cells were seeded into the 60 interior wells of the 96 well flat bottom micro-plate (view plates, Packard 600-5181) using Eppendorf repeater pipette (Brinkmann Instruments, NY). 250 µl of cell solution was seeded into each well. The 36 exterior wells were filled with 250 µl culture medium to avoid marginal effects. Cells were incubated for 24 hrs before dosing to allow for cell attachment.

Day 2 Dosing cells

TCDD was dissolved in isooctane, and the solvent for PFOS was methanol. Each standard consisted of six concentrations prepared by 5 fold and 10 fold serial dilutions for TCDD and PFOS, respectively. The concentration range for TCDD was determined based on previous studies, which showed the whole range of dose-response; where as PFOS concentration centered at the environmental average concentration 0.1mg/L:

TCDD: 1 μg/L, 0.2 μg/L, 0.04 μg/L, 0.008 μg/L, 0.0016 μg/L, 0.00032 μg/L;

PFOS: 10 mg/L, 1 mg/L, 0.1 mg/L, 0.01 mg/L, 0.001 mg/L, 0.0001 mg/L.

Before dosing, cells were briefly inspected under microscope, checking for contamination and even cell distribution. Control wells and treatment wells were dosed with 2.5 μl of appropriate solvent and chemicals. Blank wells received no dose (see the spread sheet below for example). Each treatment was tested in triplicate. All exposures except for those in the time course experiment were 72 hrs.

	TCDD alone 1ug/L			TCDD 1ug/L + PFOS 0.001mg/L			TCDD 1ug/L + PFOS 0.1mg/L			TCDD 1ug/L + PFOS 10mg/L	
	0.2ug/L			0.2ug/L+ 0.001mg/L			0.2ug/L + 0.1mg/L			0.2ug/L + 10mg/L	
	0.04ug/L			0.04ug/L + 0.001mg/L			0.04ug/L + 0.1mg/L			0.04ug/L + 10mg/L	
	0.008ug/L			0.008ug/L + 0.001mg/L			0.008ug/L + 0.1mg/L			0.008ug/L + 10mg/L	
	0.0016ug/L			0.0016ug/L + 0.001mg/L			0.0016ug/L + 0.1mg/L			0.0016ug/L + 10mg/L	
	0.00032ug/L			0.00032ug/L + 0.001mg/L			0.00032ug/L + 0.1mg/L			0.00032ug/L + 10mg/L	

Day 5 Cell Viability Assay

Cytotoxicity was measured using the live/dead viability kit (Molecular Probes L-3224). The kit comprises two probes: calcein AM and ethidium homodimer. Calcein AM is a fluorogenic esterase substrate that when hydrolyzed produces green fluorescence. Thus, green fluorescence is an indicator of living cells that have esterase activity as well as intact cell membranes. Ethidium homodimer is a red fluorescent nucleic acid stain that is only able to pass through the broken membranes of dead cells. Therefore by measuring the ratio of these two fluorescent emissions at two different wavelength, an estimation of the ratio of live to dead cells can be obtained.

Preparation of the viability assay reagent was done by diluting the appropriate amounts of calcein and ethidium with the appropriate volume of media without FBS. Plates were removed from the incubator and media was aspirated, then cells were rinsed twice with phosphate buffer saline (PBS). 50 µl of PBS with calcium and magnesium and 50 µl of viability assay reagent were added to cell containing wells using 8-channel pipette. Plates were incubated at 30 C° for 10 min and then scanned in the Cytofluor 2300 Fluorescence Measurement System (Millipore, Bedford, MA) at 500 nm and 600 nm wavelength for calcein and ethidium respectively.

Day 5 EROD Assay

The ethoxyresorufin-*O*-deethylase (EROD) assay is a useful tool for identification of dioxin-like compounds which can induce P450 1A1 (CYP 1A1) activity. EROD assay

with H4IIE-luc and PLHC-1 cells were performed following a modified version of the EROD assay procedure (Sanderson and Giesy 1998).

On the day of assay, exposed cells were briefly inspected under microscope, checking for degree of confluence, homogeneity among wells, and any sign of contamination and cytotoxicity. Cell culture medium was aspirated by vacuum aspirator, and cells were rinsed three times with PBS. 30 µl of distilled water was added to each cell-containing well, and cells were lyzed by freezing and thawing. Cells were treated with 70 µl of Hepes-dicoumarol buffer (Sigma, M1390) and 50 µl of 20 µM 7-ethoxyresorufin (Molecular Probes, Eugene, OR), and incubated at 30 C° for 20 min to ensure temperature uniformity. Reactions were initiated by adding 50 µl of 1.25 mM NADPH (Sigma N-6505) in Hepes, and plates were incubated exactly for 1 hr. at 30 C°. Reactions were stopped by adding 50 µl of 1.08mM fluorescamine (Sigma, F-9015) in acetonitrile, and plates were incubated for another 15 min. Fluorescamine is intrinsically non-fluorescent, but reacts with amine groups on protein to yield a fluorogenic derivative; thus it was used to determine the protein concentration of cell lysate (Udenfriend, *et al.*, 1972). Resorufin was measured using a Cytofluor 2300 Fluorescence Measurement System (Millipore) at λ_{ex} =530 nm and λ_{em} =590 nm, and fluorescamine was measured at λ_{ex} = 400 nm and λ_{em} = 460 nm. P4501A1 induction was expressed as relative EROD activity, which was calculated as resorufin illumination divided by protein concentration.

Day 5 Luciferase Assay

Luciferase assay is an *in vitro* technique using a genetically modified system to identify Ah receptor-active compounds. H4IIE-luc cells were stablely transfected with an AhR controlled luciferase reporter gene, and these cells express firefly luciferase in response to Ah receptor agonists.

On the day of assay, exposed cells were briefly inspected visually for signs of contamination and abnormal cell growth. Luciferase Reporter Gene Assay Kit (Packard, 6016916) were reconstituted freshly right before performing the assay. One bottle of lyophilized substrate was dissolved with 10ml buffer, and agitated gently until a homogeneous solution was formed. Cell culture media was aspirated, and cells were rinsed three times with PBS. The bottom of the view plates was sealed with self-adhesive Topseal-A (Packard, 6005185). 75 μl of PBS including calcium and magnesium was added to 60 interior wells using 8-channel pipette. Under subdued light condition, 75 μl per well of reconstituted substrate solution was added and agitated, and the plates were incubated for 10 min at 30 C°. Luminescence was measured on a plate-reading luminometer (Microlite ML3000, Dynatech).

Cell Bioassay Data Analysis

All cell bioassay data were collected electronically and converted into spreadsheet for analysis. Dose response curves were drawn using microsoft EXCEL 98, and statistical tests were conducted using SYSTAT 8.0.

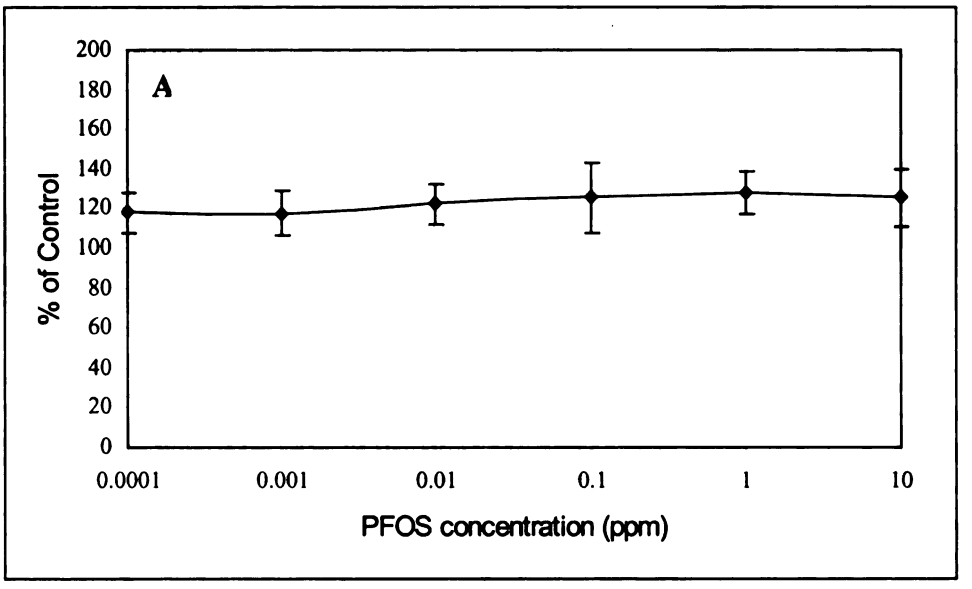
RESULTS

Cell Viability Assay

H4IIE-luc cells exposed to PFOS and TCDD showed no sign of cytotoxicity or abnormal cell growth within the concentration range tested (Fig. 1A and 1B). Live/dead ratios of cells treated with PFOS alone up to 10 mg/L were not significantly different from that of the control (Fig. 1A). When cells were treated with PFOS and TCDD in combination, the live/dead ratios for treated cells were not significantly less than that of the control (Fig. 1B). In fact, at relatively high dose of TCDD and PFOS, the live/dead ratios of treated cells were greater than that of the control, even though the difference was not significant. This could be explained by excessive cell growth observed with visual assessment which was possibly due to the inhibition of cell-cell communication (see GJIC assay results) and the block of contact inhibition.

Direct Effect of PFOS on EROD activity and luciferase activity

Two cell lines H4IIE-luc and PLHC-1 were used to assess any direct effects of PFOS on EROD activity and luciferase activity. Cells were exposed to PFOS or TCDD for the purpose of comparison. PFOS alone did not induce Cytochrome P450 1A1 (CYP1A1) activity compared to that of the control (Fig. 2A). EROD activity of H4IIE-luc cells dosed with PFOS was similar to that of the control, whereas TCDD induced EROD activity in a dose-dependent manner, with the greatest induction being 17 fold. In order to check whether this effect is species-specific, the same experiment was conducted using fish PLHC-1 cells (Fig. 2B). Results were very similar to those observed for H4IIE-luc



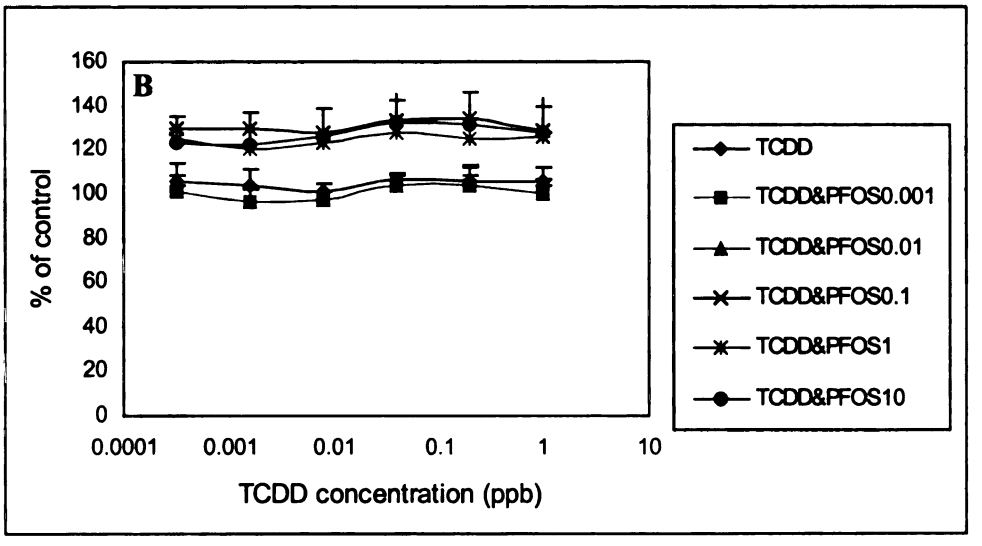


Fig. 1 Effects of PFOS on H4IIE-luc cell viability in the absence or presence of TCDD. A) cells were dosed with PFOS only; B) cells were dosed with different concentrations of PFOS and TCDD in combination. PFOS concentration in mg/L, TCDD concentration in μg/L. Cell viability was measured as live/dead ratio, and expressed as % of control, which was cells dosed with solvent (methanol). Error bars represent standard deviation of three measurements.

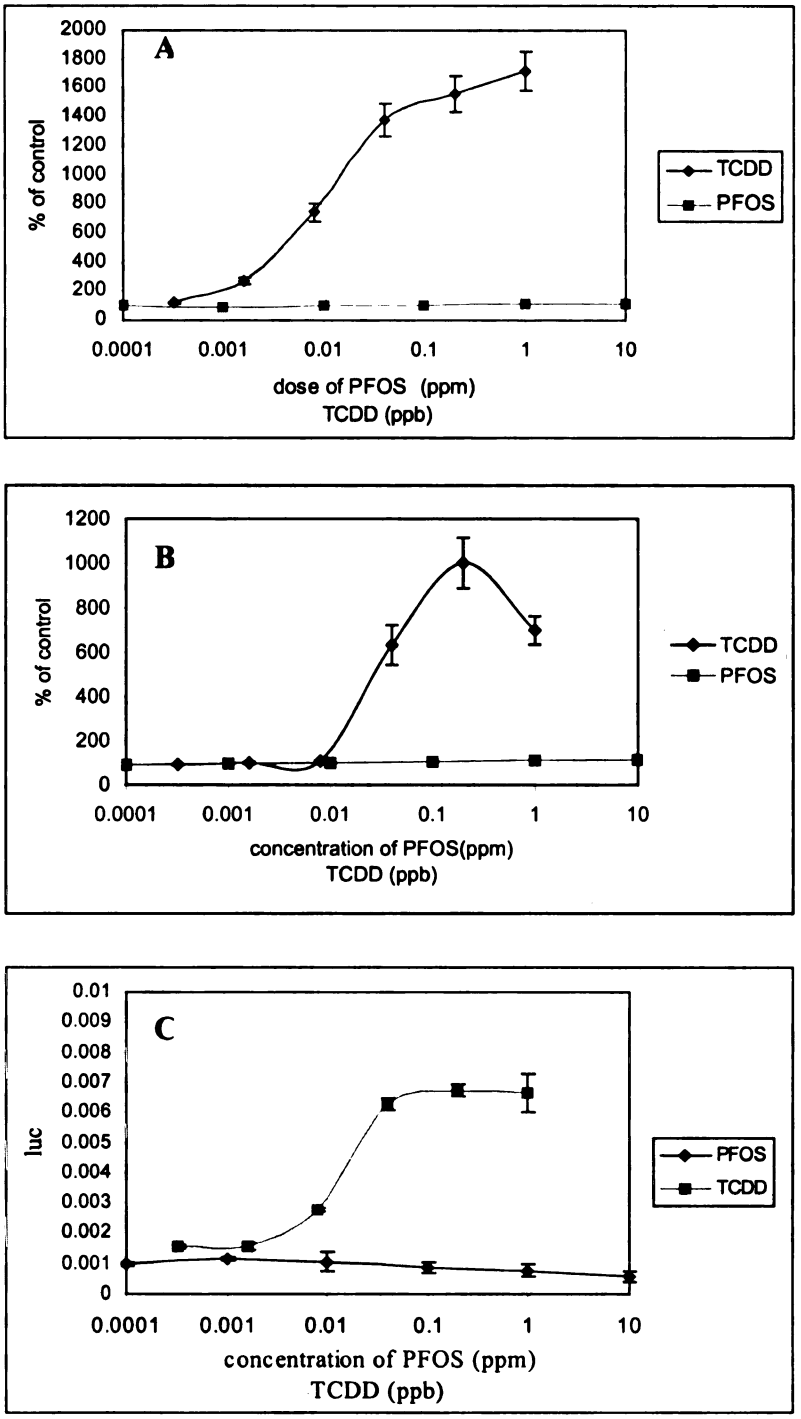


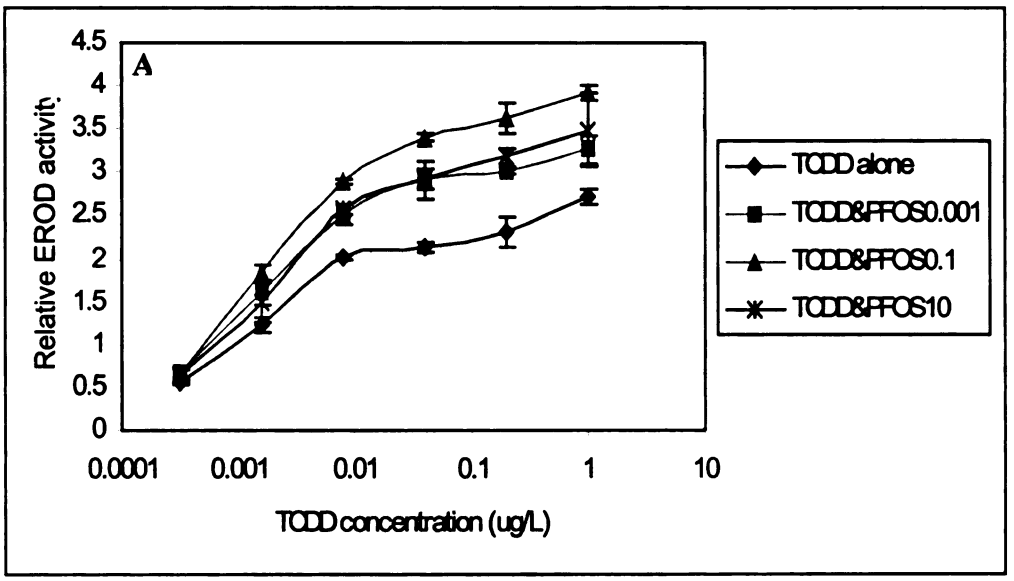
Fig. 2 Direct effects of PFOS on H4IIE-luc cell and PLH C-1 cell EROD activity, and on H4IIE-luc cell luciferase activity compared with the effects of TCDD. A) EROD activity of H4IIE-luc cells dosed with PFOS or TCDD; B) EROD activity of PLH C-1 cells dosed with PFOS or TCDD; C) luciferase activity of H4IIE-luc cells dosed with PFOS or TCDD. EROD activity was expressed as % of control, luciferase activity was expressed as luciferase light production. Control cells were dosed with 0.1% (v/v) solvent (methanol) only. Error bars represent standard deviation of three measurements.

cells: PFOS exhibited no detectable effect on CYP1A1 induction. Luciferase assay was conducted on H4IIE-luc cells dosed with PFOS or TCDD. PFOS when dosed alone did not induce AhR-mediated luciferase activity relative to that of the control. In contrast, TCDD induced luciferase activity in a dose-dependent manner (Fig. 2C).

Interactive Effects of PFOS and TCDD in EROD Assay

In order to assess the possible interaction between TCDD and PFOS, cells were exposed to these two chemicals in combination. Cells were dosed with TCDD standard alone, and TCDD in combination with 0.001, 0.1 or 10 mg/L of PFOS (Fig. 3A). Co-exposure of cells to PFOS and TCDD increased the CYP1A1 activity induced by TCDD. Compared to the TCDD standard dose-response curve, the addition of PFOS increased both the slope of the curve and the magnitude of maximum response, with the medium concentration of PFOS (0.1 mg/L) having the most significant interaction with TCDD.

In order to confirm the interactive relationship between these two chemicals, more PFOS concentrations were tested. Cells were exposed to PFOS with concentrations ranging from 10mg/L to 0.0001mg/L in a serial dilution of 10 fold, in combination with the TCDD standard concentration. A similar interactive relationship between PFOS and TCDD was observed (Fig. 3B). To permit visual assessment, the same data was plotted as a 3-D graph and General Linear Model (GLM) pairwise comparison was conducted (Fig. 4). Significant interactive effects were observed at 0.2 μg/L TCDD plus 0.1 mg/L PFOS ($p<0.05$), 1 μg/L TCDD plus 0.01 mg/L PFOS ($p<0.05$), and 1 μg/L TCDD plus 0.1mg/L



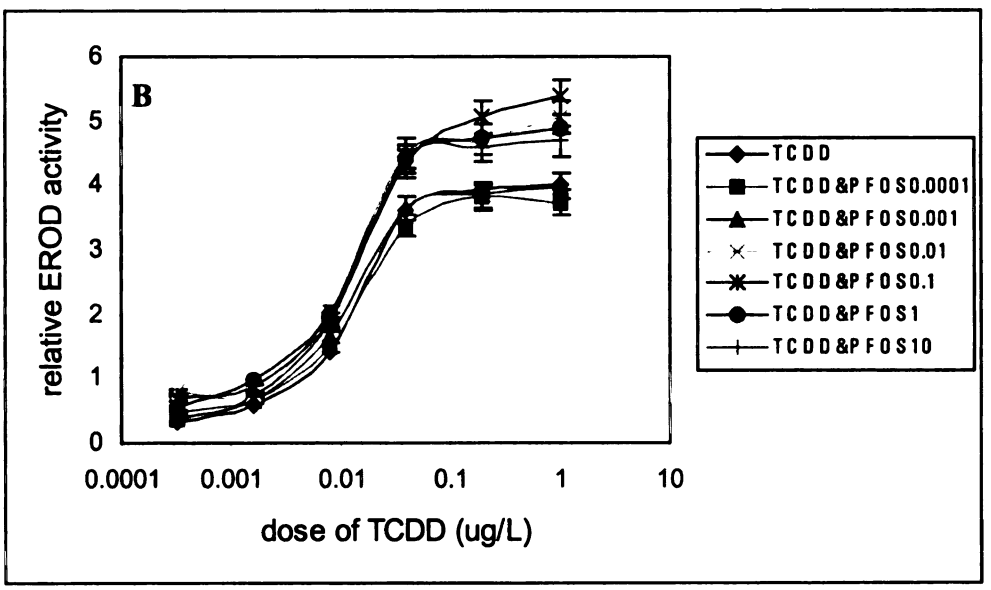


Fig. 3 Interactive effects of PFOS and TCDD on H4IIE-luc cell EROD activity. A) H4IIE-luc cells were exposed to PFOS at concentrations of 0.001 mg/L, 0.1 mg/L and 10 mg/L in the presence of TCDD; B) H4IIE-luc cells were exposed to PFOS at wider concentration range, from 10 to 0.0001 mg/L with 10 fold dilution, in the presence of TCDD. EROD activity was expressed as relative EROD which equals to resorufin fluorescence divided by protein concentration. Error bars represent standard deviation of three measurements.

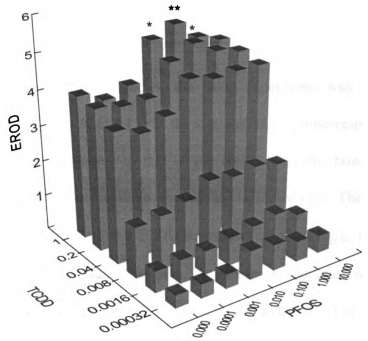


Fig. 4 3-D plot of interactive effects of PFOS and TCDD on H4IIE-luc cell EROD activity (with the same data as in Fig. 3B. X-axis represents PFOS concentration in mg/L; Y-axis represents TCDD concentration in μg/L; Z-axis represents relative EROD activity which is resorufin fluorescence divided by protein concentration. General linear model pairwise comparison was conducted (* $P<0.05$, ** $P<0.01$).

PFOS ($p<0.01$). In the last combination, the addition of PFOS increased the effect of TCDD by 40%.

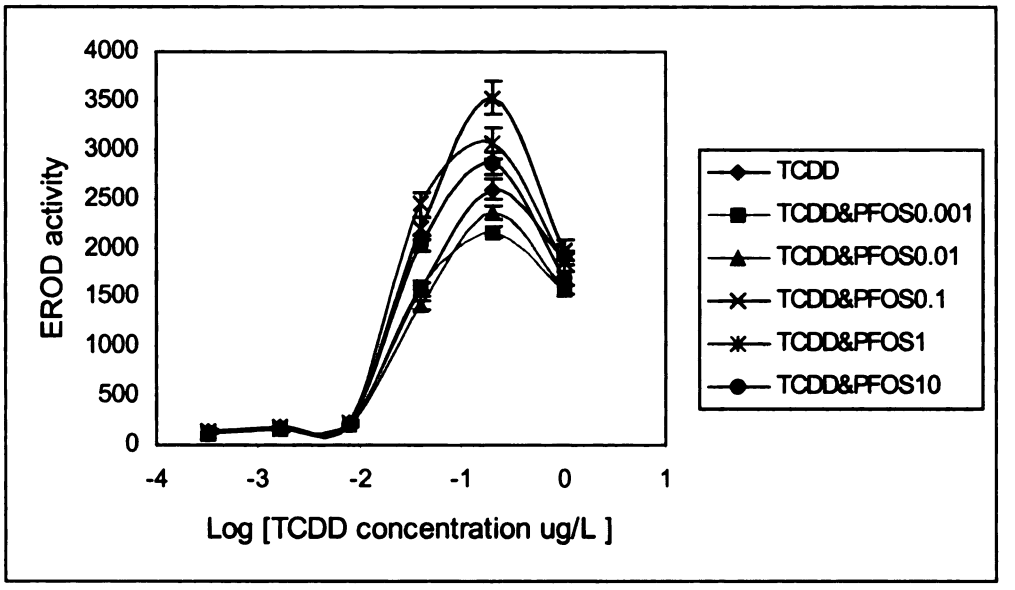
To compare responses between species, the same experiment was conducted using PLHC-1 cells (Fig. 5A). For PLHC-1 cells the standard TCDD dose-response curve had a slightly different shape compared to that of the H4IIE-luc cells, however the general trend of interactive effects was similar to that of the H4IIE-luc cells. The most significant interactive effects were observed at a TCDD concentration of 0.2 μg/L, therefore, this set of data was plotted in a histogram (Fig. 5B). The maximum induction was observed at a PFOS concentration of 0.1 mg/L ($p<0.01$), which increased the effect of TCDD by 40%.

Interactive Effects of PFOS and TCDD on Luciferase Expression

The luciferase assay was conducted using H4IIE-luc cells dosed with TCDD and PFOS in combination, at the same concentrations used as in the interactive EROD assay (Fig. 6A). The same data was plotted as a 3-D histogram for better visual assessment (Fig. 6B), and general linear model (GLM) pairwise comparison was conducted. Exposure to 1 μg/L TCDD plus 0.1 mg/L PFOS ($p<0.05$), and 0.2 μg/L TCDD plus 0.1 mg/L PFOS ($p<0.05$), significantly increased induction over TCDD alone, with the maximum of increase by 40%.

Time-course Experiment

To determine whether the interactive effects on EROD induction was due to interaction between PFOS and CYP 1A1 enzyme or EROD reaction substrate, a time course



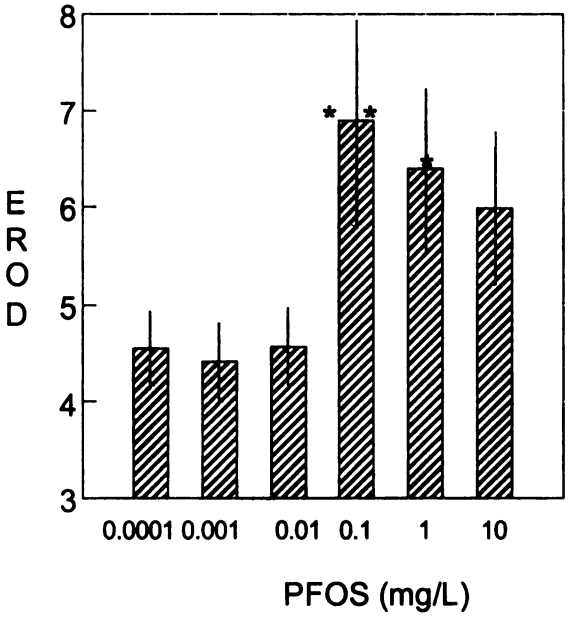
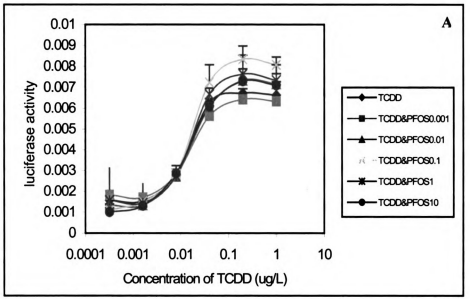


Fig. 5 Interactive effects of PFOS and TCDD on PLH C-1 cell EROD activity. A) PLH C-1 cells were dosed with different concentrations of PFOS (mg/L) and TCDD (μg/L) in combination; B)a set of data in A where TCDD concentration equals to 0.2μg/L were plotted in a histogram, general linear model pairwise comparisons were conducted (* $p<0.05$; ** $p<0.01$).



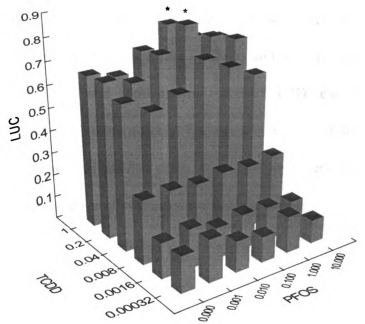


Fig. 6 Interactive effects of PFOS and TCDD on H4IIE-luc cell luciferase activity. A) H4IIE-luc cells were dosed with different concentrations of PFOS and TCDD (μg/L) in combination; B) 3-D graph with same data from A, X-axis represents PFOS concentration in mg/L, Y-axis represents TCDD concentration in μg/L, Z-axis represents luciferase light production. General linear model pairwise comparisons were conducted (* $p<0.05$, ** $p<0.01$).

experiment was conducted. Instead of dosing cells with both TCDD and PFOS on day 2, which is 72 hrs before performing the assay, PLHC-1 cells were dosed with TCDD standard only. On the same day of the assay, cells were then dosed with PFOS at 5 min, 20 min, and 60 min before running the EROD assay (Fig. 7B, 7C, 7D). A PFOS concentration of 0.1 mg/L was used in this experiment, because based on previous results, this concentration of PFOS caused the greatest interaction between TCDD and PFOS on both EROD and luciferase induction. In all three assays, there was no significant difference between cells dosed with TCDD alone and TCDD in the presence of PFOS added at different time intervals before the assay. In contrast, when cells were exposed to TCDD and PFOS at 72 hrs before assay, a significant interaction was observed (Fig. 7A). Thus, it can be concluded that the significant interactive effects occurred only in the long-term exposure (72hrs), and it was not due to the direct interaction between PFOS and P450 enzyme or the EROD reaction substrate, which should have happened much more quickly. Species-specificity was studied by conducting the same experiment with H4IIE-luc cells. The result was very similar to that of the PLHC-1 cells, with interactive effects observed only in long-term exposure (Fig. 8).

Transcription Inhibition Experiment

α-Amanitin is an inhibitor of eukaryotic polymerase II (pol II) enzyme, which is responsible for mRNA transcription. This experiment was conducted to determine whether PFOS could still elicit its interactive effects with TCDD after pol II transcription was inhibited. Cells were seeded into the 96 well plate on day 1 as described in materials

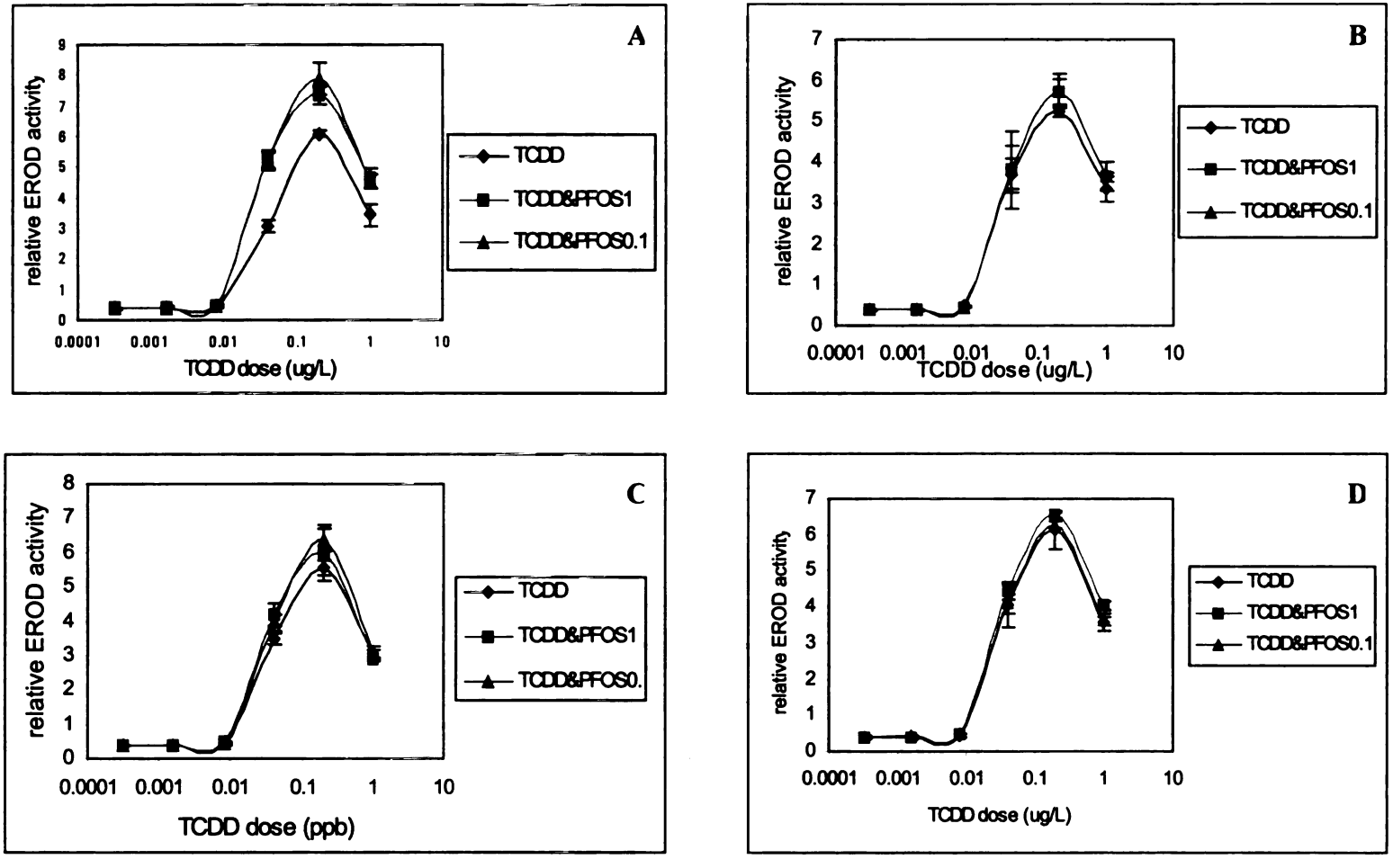


Fig. 7 Time Course experiment of interactive effects of PFOS and TCDD on PLH C-1 cell EROD activity.
A) PLH C-1 cells were dosed with TCDD and PFOS on day 2 (72 hrs before run the assay); B) cells were dosed with TCDD only on day 2, and with PFOS on day 5 (5 min before running the assay); C) cells were dosed with TCDD only on day 2, and with PFOS on day 5 (20 min before running the assay); D) cells were dosed with TCDD only on day 2, and with PFOS on day 5 (1 hr before running the assay). Error bars represent standard deviation of three measurements. (PFOS at concentration of 0.1 mg/L).

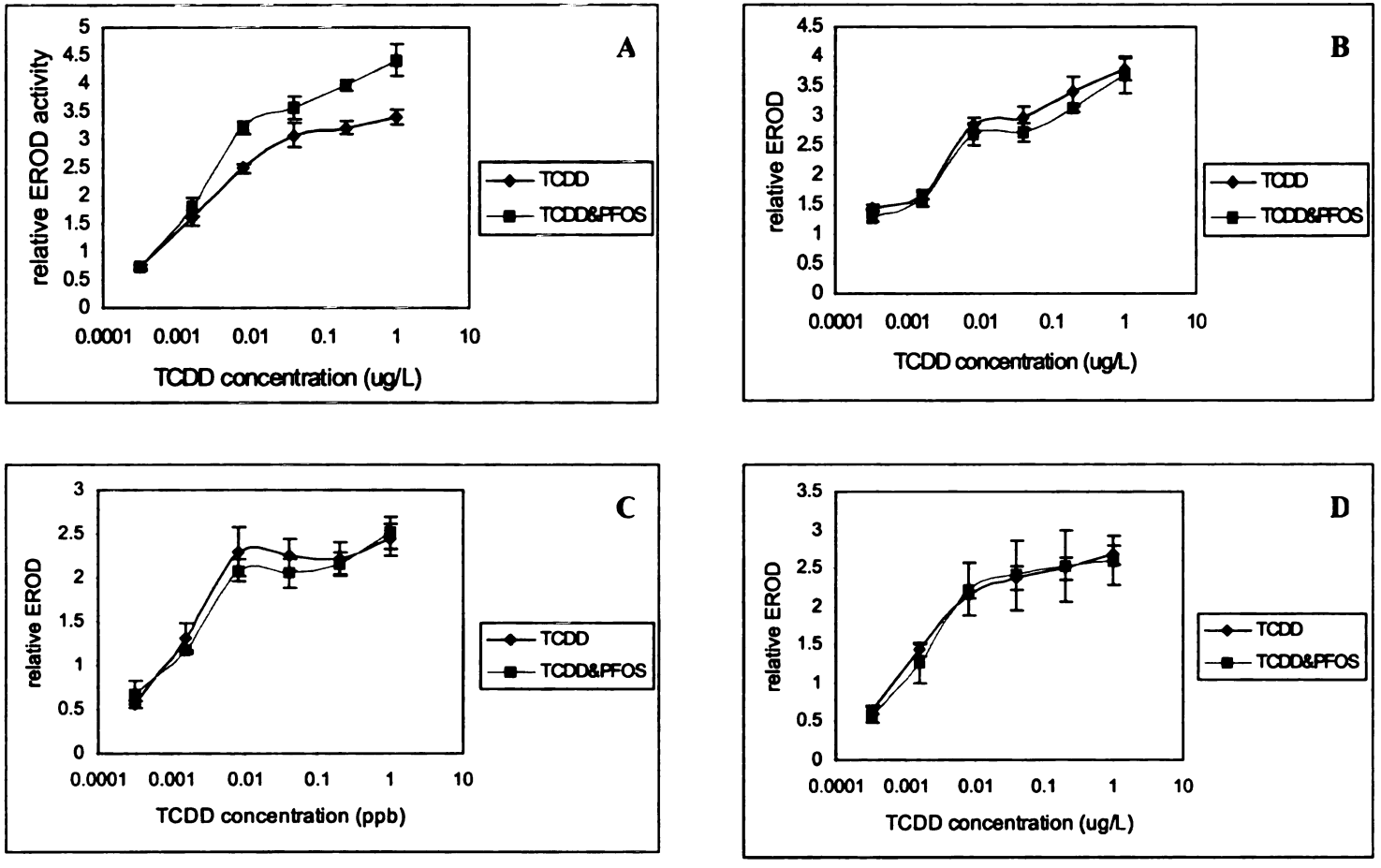


Fig. 8 Time Course experiment of interactive effects of PFOS and TCDD on H4IIE-luc cell EROD activity.
A) cells were dosed with TCDD and PFOS on day 2 (72 hrs before running the assay); B) cells were dosed with TCDD only on day 2, and with PFOS on day 5 (5 min before running the assay); C) cells were dosed with TCDD only on day 2, and with PFOS on day 5 (10 min before running the assay); D) cells were dosed with TCDD only on day 2, and with PFOS on day 5 (30 min before runing the assay). Error bars represent standard deviation of three measurements. (PFOS at concentration of 0.1mg/L).

and methods. On day 2 cells were dosed with TCDD, and incubated at 37 C° for 36 hrs to allow for TCDD to induce luciferase activity. On day 3 cells were dosed with either amanitin or amanitin and PFOS in combination. The amanitin concentrations used were 10 μM and 100 μM, and the PFOS concentration used was 0.1 mg/L based on previous studies. Luciferase assay was conducted on day 5 as previously described.

Amanitin at a concentration of 100 μM inhibited luciferase gene transcription efficiently, whereas a concentration of 10 μM had no effect (Fig. 9A and 9B). Both concentrations showed no sign of cytotoxicity based on visual observation and cell viability assay results. Cells dosed with 10 μM amanitin plus TCDD were observed in the same magnitude of luciferase induction as those dosed with TCDD only, and cells dosed with TCDD, amanitin plus PFOS had a significantly greater luciferase induction (Fig. 9A). In contrast, cells dosed with 100 μM amanitin plus TCDD showed a much lesser luciferase induction relative to cells dosed with TCDD only (Fig. 9B). Cells dosed with TCDD, amanitin plus PFOS were observed in the same magnitude of luciferase induction as those dosed with TCDD and amanitin (Fig. 9B). When cells were dosed with TCDD only, TCDD and PFOS in combination (no amanitin added), PFOS increased the TCDD induced luciferase activity by 40 % (Fig. 9C), which was of the same magnitude of interaction as that observed in Fig. 9A and was consistent with results from the interactive luciferase assay (Fig. 6). This result suggested that interactive effects occurred when there was no transcription inhibitor added or the concentration of the inhibitor was not high enough to inhibit transcription. The addition of PFOS did not have any effect on

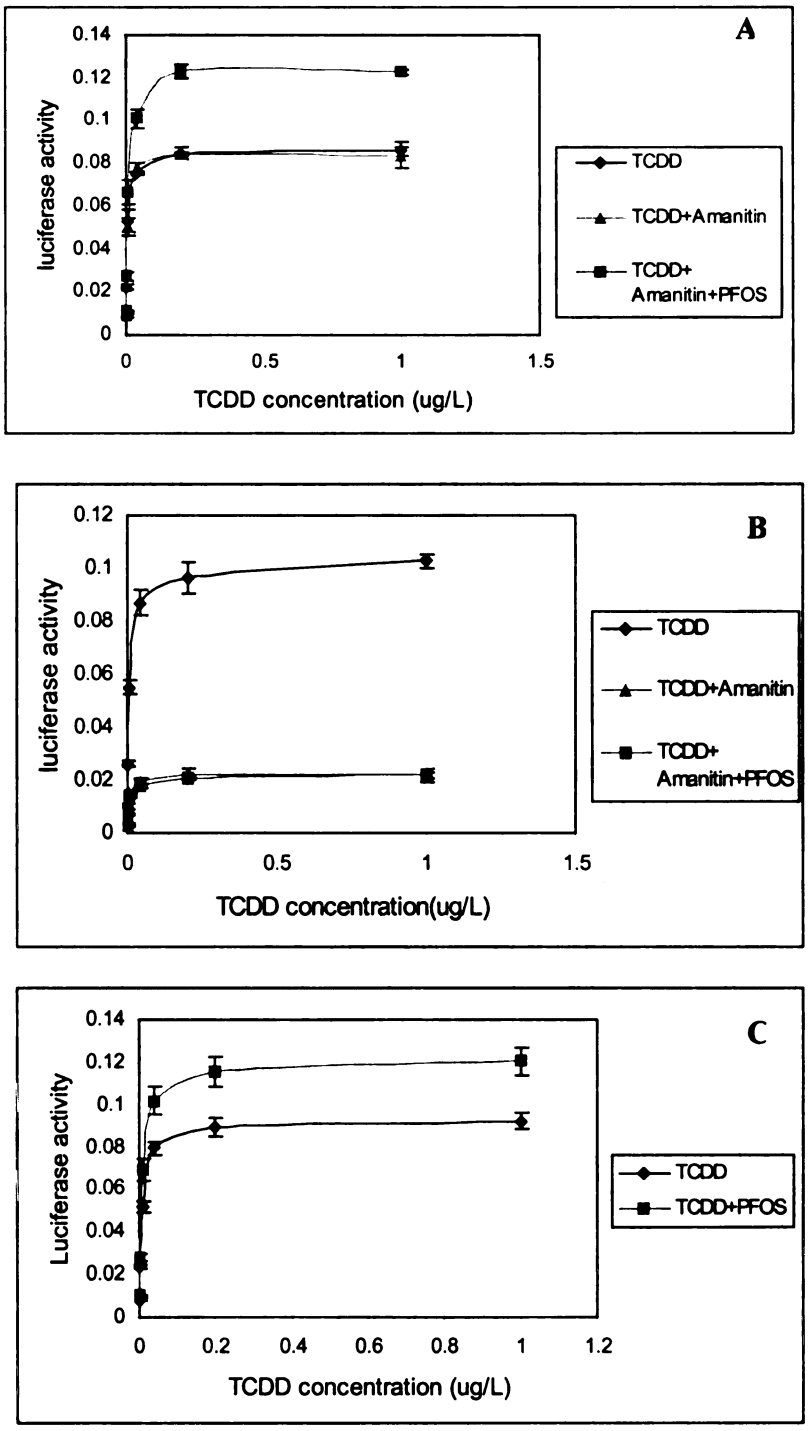


Fig. 9 Transcription inhibition assay on interactive effects of PFOS and TCDD on H4IIE-luc cell luciferase activity. A) cells were dosed with TCDD, TCDD in the presence of 10μM amanitin and TCDD in the presence of 10μM amanitin plus 0.1mg/L PFOS; B) cells were dosed with TCDD, TCDD in the presence of 100 μM amanitin and TCDD in the presence of 100 μM amanitin plus 0.1mg/L PFOS; C) cells were dosed with TCDD and TCDD in the presence of 0.1mg/L PFOS, no inhibitor was added.

TCDD induced luciferase activity when transcription of luciferase gene in these cells had been inhibited.

DISCUSSION

The mechanisms of EROD assay and Luciferase expression are diagramed in Fig. 10A and 10B, respectively. Induction of EROD activity is an endogenous response of cells to dioxin-like compounds. When TCDD or other dioxin-like compounds are introduced into the cell, they bind to aryl hydrocarbon receptor (AhR), and form a receptor-ligand complex facilitated by heat shock protein (Hsp70). This complex translocates to the nucleus and interacts with specific sequences on the DNA, termed dioxin-responsive elements (DREs), which is an enhancer of the CYP1A1 gene. This binding can up-regulate the transcription of CYP1A1 gene, increase the amount of CYP1A1 mRNA, and subsequently increase the amount of CYP1A1 protein. CYP1A1 is involved mainly in oxidative metabolism of exogenous chemicals, and one of its characteristic activities *in vitro* is to catalyze the reaction from ethoxyresorufin to resorufin. Resorufin is a fluorogenic compound. Therefore the induction of CYP 1A1 can be quantitated by fluorometric measurement of EROD activity (Sanderson, *et al.* 1998; Roman, *et al.*, 1998).

In contrast to the EROD assay, luciferase assay is a genetically engineered system to identify AhR agonists. H4IIE-luc cells were stably transfected with firefly luciferase reporter gene, which is under direct control of the DRE. Binding of the agonist to the

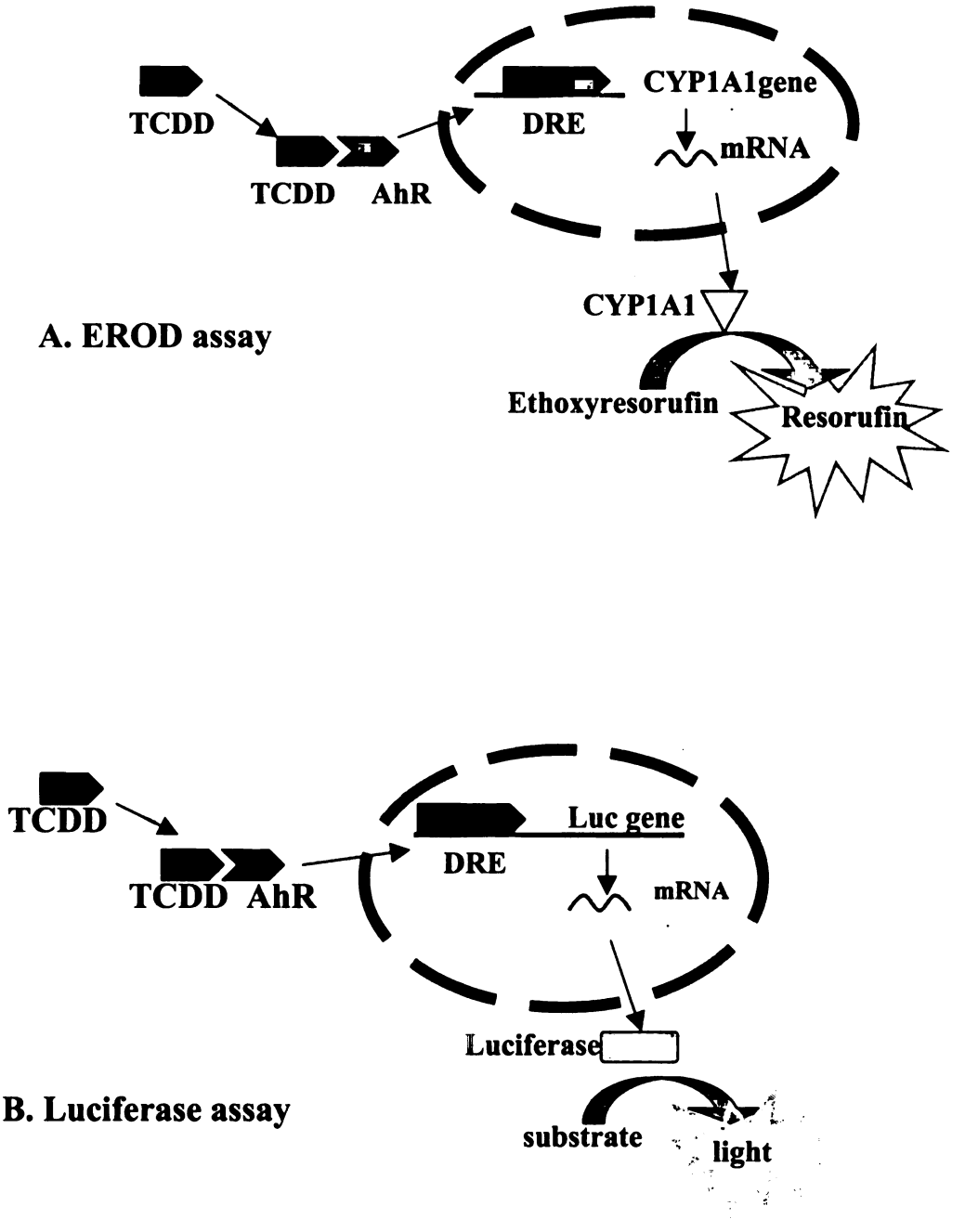


Fig. 10 Diagram showing the mechanisms of EROD assay and Luciferase assay.

receptor results in an activated receptor-ligand complex that translocates to the nucleus. There it interacts with the *cis*-acting regulatory sequences DREs, which are localized 5' upstream of the luciferase reporter gene, and increases the production of luciferase enzyme. This enzyme can cleave the luciferin substrate to produce light. Hence light production is an indicator of AhR binding affinity (Sanderson, *et al.*, 1996).

Because of the differences in the mechanism of these two *in vitro* bioassays, their results have different implications. First, the induction of EROD activity has biological significance. Good correlation exists between the AhR binding affinity and the EROD induction potency *in vitro* and toxic potency *in vivo*. Whereas luciferase induction is only an *in vitro* transcription monitoring system, and it is not associated directly with any biological effects. Secondly, an alteration in EROD activity dose not necessarily mean that it is an AhR mediated effect. Since EROD activity is an endogenous response, it is regulated at transcriptional, post transcriptional and -translational levels. Compared to the EROD assay, the expression of exogenous luciferase reporter gene may be less affected by other factors. Thus, it is a more direct indicator of AhR mediated response, and it has a greater sensitivity as well (Sanderson, *et al.*, 1996).

Based on above discussion, the results of *in vitro* bioassays tell us that PFOS by itself has no direct effect on cytochrome P450 isoenzyme activity. Even though PFOS can cause wasting syndrome similar to that caused by TCDD, it probably elicits its effect through a different, non AhR mediated pathway. However PFOS can induce interactive effects with TCDD not only in the EROD assay, but also in the luciferase assay, this implies a more

complicated transcription receptor interaction rather than direct AhR binding. As shown in previous studies peroxisome proliferator activated receptor (PPAR) is involved in mediating most of the effects of peroxisome proliferators. PPAR has been qualified as a member of the nuclear transcription receptor family. It was once called orphan receptor because its exact mode of action was unknown. The tentative hypothesis here is that PPAR may have interacted with AhR itself or AhR-associated factors, such as ARNT. However so far there is no evidence on the relationship between PPAR and AhR. Further studies would need to be conducted to reveal the mechanism of the interactive effects.

To eliminate the possibility that PFOS simply interacts with EROD and luciferase assay substrates, and to confirm the finding that PFOS interacts with TCDD at a transcriptional level, two additional experiments were conducted. The time course experiment was based on the theory that alterations at the transcriptional level did not occur instantaneously, but required certain amount of time to elicit effects, whereas interactions directly with the substrates would occur rapidly (5~10 min). Since interactive effects were only observed in long-term exposure (72 hrs), it can be concluded that the observed effects were not due to direct interaction with the substrates. The transcription inhibitor experiment was designed to test effects occurring at the transcriptional level using transcription inhibitor amanitin. The idea was that if PFOS interacted with TCDD at stages other than the transcriptional level, the interactive effects between PFOS and TCDD should still persist after the transcription of luciferase gene had been inhibited. The result in Fig. 9 suggests the opposite, which is that the interaction does occur at the transcriptional level since the interactive effects between PFOS and TCDD disappeared after the transcription of

luciferase gene was inhibited by amanitin. Another possible explanation of the effects observed is that after cells were exposed to fairly great amount of amanitin for 36 hrs, they were not in a physically healthy status, even with the live/dead ratio stays the same. Therefore, no interaction could be observed no matter which stage it happened. If this is true, then the transcription inhibition assay might not be a good way to determine the effect at transcription in this study.

The purpose of this study was to investigate potential mechanisms of action of PFOS, that could be used to provide general information for environmental risk assessments. Thus, the environmental relevance of the data becomes an important issue. To illustrate the environmental relevance, a dose range box was developed (Fig. 11). The two dashed lines indicate the environmental extreme value for these two chemicals. Here 2mg/L and 500 μg/L were considered the maximum concentration measured in wildlife for PFOS and TCDD respectively. Thus, the lower left area of the box indicates the concentration range of environmental concern. Based on chemical analysis data obtained in ATL this is also the concentration range within which these two chemicals can be measured together out in the field. The interactive dose range based on cell bioassay results is 0.01mg/L to 0.1mg/L for PFOS and 200 μg/L to 1000 μg/L for TCDD, shown as a cross. This dose range falls into the lower left area of the environmental ranges.

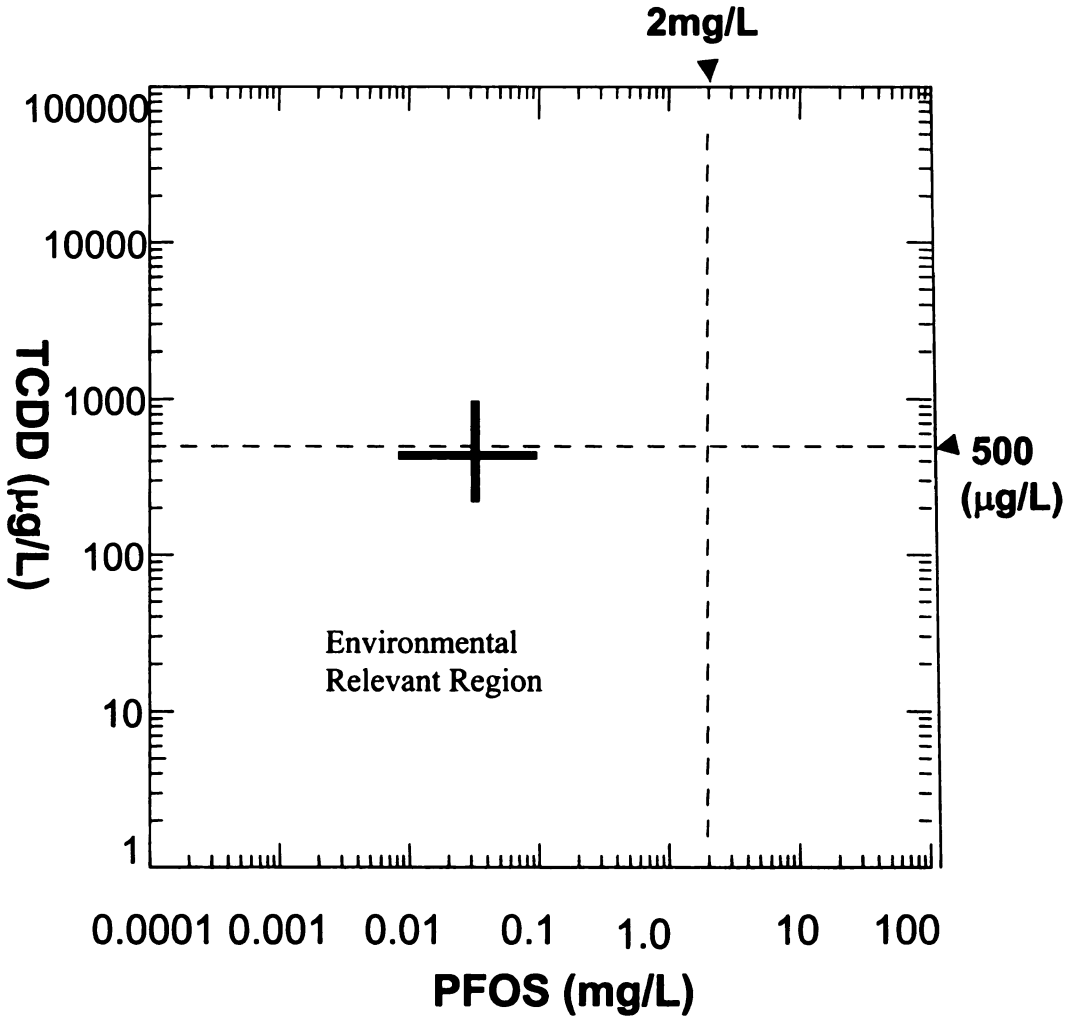


Fig. 11 Dose Range Box showing the environmental relevance of data. X-axis shows PFOS concentration, and Y-axis shows TCDD concentration. The two dash lines indicate the maximum concentrations of these two chemicals found in wildlife, 2 mg/L and 500 µg/L for PFOS and TCDD respectively. The lower left area of the box indicates the concentration range of environmental concern. The interactive dose range based on cell bioassay results is 0.01 mg/L to 0.1 mg/L for PFOS and 200 µg/L to 1000 µg/L for TCDD, shown as a cross.

GAP JUNCTIONAL INTERCELLULAR COMMUNICATION ASSAY

BACKGROUND

Gap junctional intercellular communication (GJIC) is a major pathway for cells to communicate with each other, thus, it plays a crucial role in the maintenance of normal cell growth and function. The down-regulation of GJIC has been linked to tumor promoting properties of many non-genotoxic carcinogens. For a wide range of chemicals, the correlation between tumor promotion and GJIC inhibition is greater than 80% (Trosko and Ruch, 1998). Previous studies have shown that some perfluorinated fatty acids can inhibit GJIC in a dose-dependent fashion. This inhibition occurs within a short time period (<1hr), and is rapidly reversed by removing the inhibitors (Upham, 1998). In order to compare the effects of PFOS on GJIC to those of other PFFAs, and to determine the species specificity of the effects, GJIC assay was conducted using WB F-344 rat liver cells and CDK dolphin kidney cells. The dolphin cell line was used here in the effort to develop a marine mammalian model for testing the effect of PFOS, because PFOS was also detected in a fairly great amount in marine mammal samples (3M internal data).

MATERIALS AND METHODS

Chemicals

Perfluorooctane sulfonic acid (PFOS) was obtained from 3M company (St. Paul, MN) as a mixture. Based on the NMR analysis, the mixture consisted of 68% of straight chain

PFOS and 17% of branched chain PFOS. Perfluorooctanoic sulfonamide (PFOSA) was obtained from Sigma.

Cell Culture

WB-F344 cells are rat liver epithelial cells obtained from Drs. J.W. Brisham and M.S. Tsao of University of North Carolina. This cell line has been well characterized for its expression of gap junctional proteins (Tsao, *et al.*, 1984). Carvan dolphin kidney (CDK) cell line is an epithelial cell line isolated from a prematurely born female-bottle-nose dolphin. Same as WB-F344 cells CDK cells are also non-tumorigenic primary cells.

WB-F344 and CDK cells were cultured in 75cm flask (Corning 430720). All cells were grown under sterile conditions (pH=7.4) at 37 C° in a humidified 5/95% CO_2 /air incubator (Forma Scientific, Model 8173). WB-F344 cells were cultured in Dulbecco's Modified Eagle Medium (Formula 78-5470-EF, Gibco), supplemented with 5% FBS (Gibco). CDK cells were cultured in Dulbecco's Modified Eagle Medium and Ham's F12 (Sigma D-2906), supplemented with 10% FBS (Gibco), and other nutrients (see Appendix A for detailed formula).

GJIC Assay

GJIC was measured by the use of the scrape loading dye transfer technique. Two mammalian cell lines (WB-F344 and CDK) were used to compare species specificity, and two chemicals (PFOS and PFOSA) were tested to compare structure specificity.

Cell Plating

After reaching 80-100% confluence, cells were trypsinized with 1x trypsin-EDTA (Gibco-BRL 15400-054)) and cell solution was collected. Cell number was determined using a hemocytometer. WB-F344 cells were diluted to a concentration of approximately 1 x 10^6 cells / ml, and CDK cells were diluted to a concentration of approximately 1 x 10^5 cells / ml. 2 ml of the cell solution was then transferred to 35 mm tissue culture plates, and cells were incubated for 24 hrs before dosing to allow for cell attachment.

GJIC Measuring

PFOS (MW 499) and PFOSA (MW stock solutions were prepared in acetonitrile, in a 2 fold dilution series from 20 g/L to 0.3125 g/L. 20 µl of PFOS and PFOSA stock solution or solvent was added to each cell culture plate containing 2 ml cell culture medium. Following the chemical exposures, cells were rinsed three times with PBS and then approximately 1 ml of 0.05% lucifer yellow dye was added to each plate. The size of the dye molecule is large enough to keep them from entering the intact cell membrane, but is small enough that they can go through gap junction between cells. A surgical steel blade was used to make three scrapes through the monolayer of cells. So the dye can be up-taken by the broken cells, and then transferred to adjacent cells through gap junction. After three minutes incubation at room temperature, the dye was discarded, and the cells were rinsed three times with PBS, and then fixed with 0.5 ml of 4% formalin. Dye migration was observed and photographed at 200X using a Nikon epifluorescence phase contrast microscope illuminated with an Osram HBO 200W lamp and equipped with a COHU video camera. The average distance of dye migration from the scrape indicates

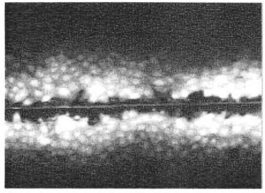
the ability of cells to communicate with each other through gap junction, and it was calculated using the Nucleotech Gel Expert program. Each treatment was tested in triplicate. The average was calculated, and plotted using Excel. Differences among clones and between compounds were determined by 2-way ANOVA, followed by Turkey's multiple range test.

In the dose response experiment, cells were treated with PFOS at concentrations ranging from 200 mg/L to 3.125 mg/L with a 2 fold dilution series, but with the same exposure time (30 min). In the time course experiment, cells were treated with the same dosage of PFOS (50 mg/L), but with different duration of exposure: 2min, 5 min, 10min, 30min, 1 hr and 24 hrs. Dosage ranges and exposure times were determined based on previous studies.

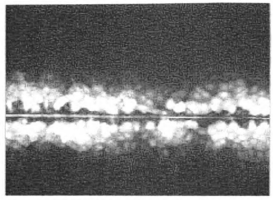
RESULT

Dose-response Experiment

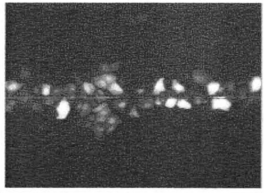
Fig. 12 showed the pictures of WB F-344 cells in GJIC dose-response experiment taken under phase-contrast fluorescent microscope. As described in methods and materials, the distance from the front of the dye to the scrape is directly proportional to the level of cell-cell communication. The dye migrated to the greatest distance in cells treated with solvent. PFOS inhibited dye migration in a dose-dependent manner (Fig. 12 and Fig. 13). From the dose response curve, the EC50 for GJIC inhibition was determined to be approximately 20 mg/L PFOS for 30min exposure. The maximum inhibition of GJIC was



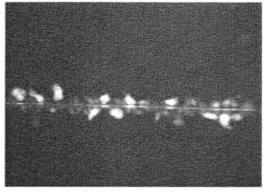
WB cell Blank Control



WB cell exposed to 6.25 mg/L PFOS



WB cell exposed to 25 mg/L PFOS



WB cell exposed to 100 mg/L PFOS

Fig. 12 Image of WB-F344 cells dosed with different concentration of PFOS(exposure time of 30 min).

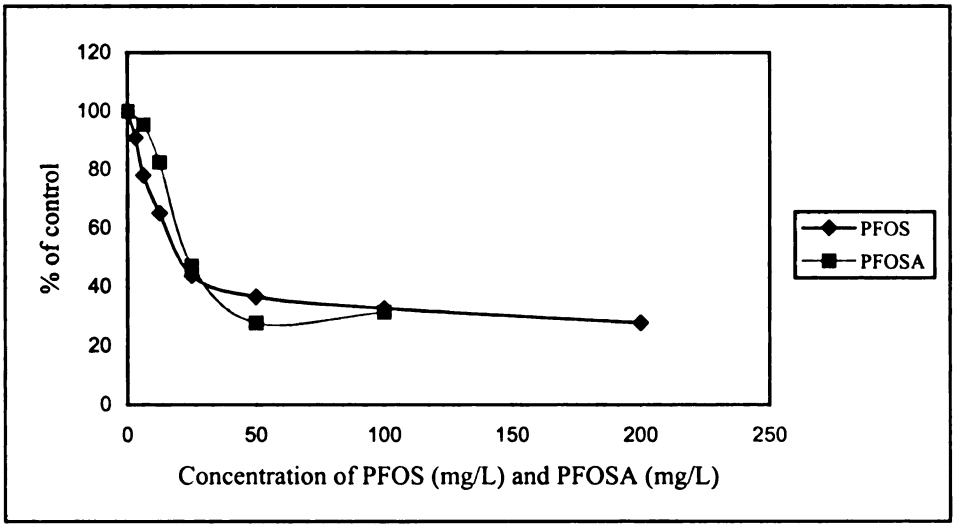


Fig. 13 Dose Response Effects of PFOS and PFOSA on WB cells Gap Junctional Intercellular Communication (with exposure time of 30 min).

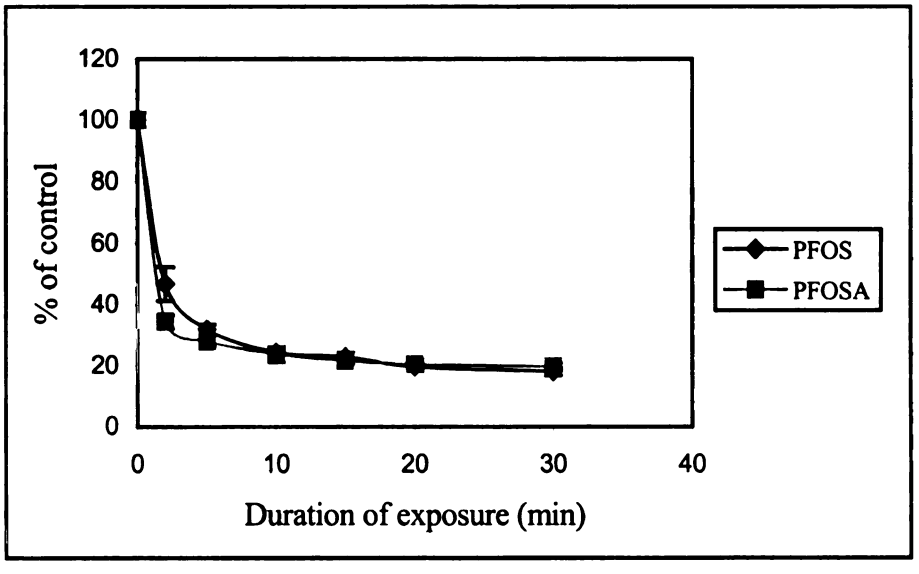


Fig. 14 Effects of PFOS on CDK cells Gap Junctional Intercellular Communication (with exposure time of 30 min).

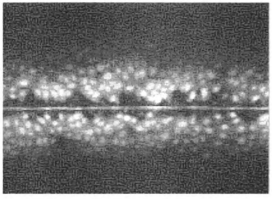
caused by PFOS concentration of 50 mg/L or greater. No sign of cytotoxicity was observed within the concentration range tested. To determine the structure specificity of the chemicals, PFOSA (an insecticide) was tested at the same dose range as PFOS. A similar dose-dependent inhibition of GJIC was observed. The EC50 value for PFOSA was approximately 24 mg/L, with the maximum inhibition occurred at 100 mg/L PFOSA. To determine the species specificity of this inhibitive effect, another cell line, CDK cells were tested with the same treatment (Fig. 14). A very similar dose-response curve was obtained, with an EC 50 value of approximately 14 mg/L PFOS. The maximum effect was observed at 50 mg/L PFOS or greater.

Time course experiment

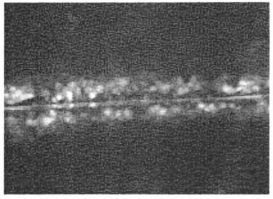
The phase-contrast images of WB F-344 cells exposed to 50 mg/L PFOS for 2 min, 5 min, and 10 min were shown in Fig. 15. GJIC was inhibited in a short time period by the exposure to 50 mg/L PFOS. A 50% inhibition was observed after WB F-344 cells were exposed to PFOS for only 2 min, and the maximum inhibition of 90% occurred within 5 to 10 min (Fig. 16). These results were similar to those observed for PFOSA. When WB cells were exposed for 1hr and 24 hrs, no further inhibition of GJIC was observed.

DISCUSSION

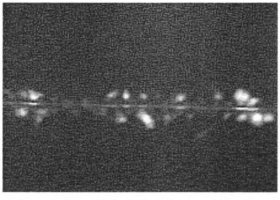
The mechanism of the GJIC inhibition by fluorinated compounds is poorly understood, however, inhibition of GJIC does depend on the fluorinated carbon tail. Previous studies have shown that perfluorinated fatty acids (PFFAs), such as perfluorooctanoic acid



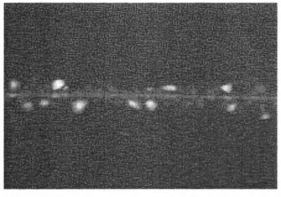
WB cells control



WB cells exposed to PFOS for 2 min



WB cells exposed to PFOS for 5 min



WB cells exposed to PFOS for 10 min

Fig. 15 Phase Contrast Image of WB-F344 cells exposed to PFOS (50 mg/L) for different time period.

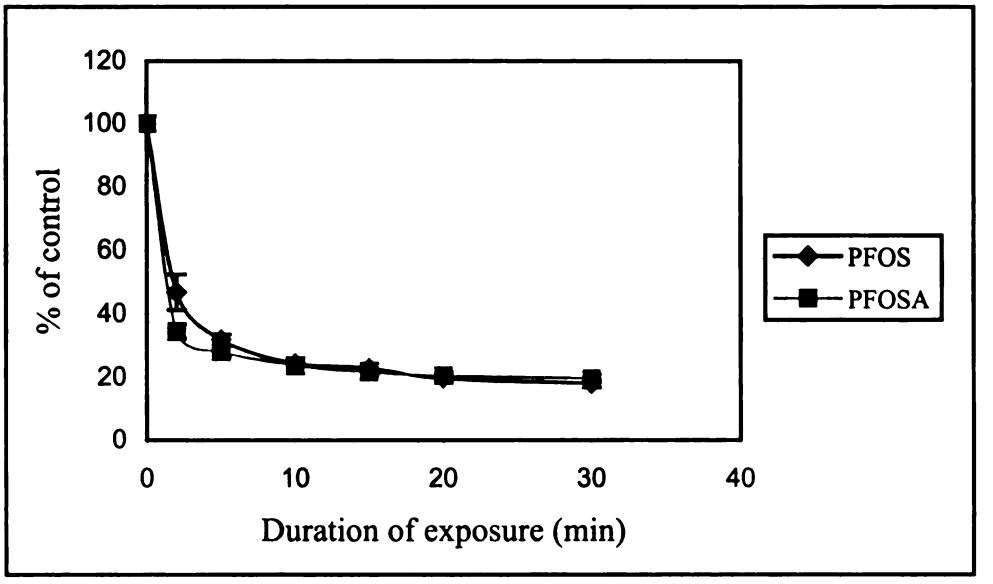


Fig. 16 Time Course of the Inhibitory Effects of PFOS and PFOSA on WB cells Gap Junctional Intercellular Communication (with PFOS / PFOSA concentration at 50 mg/L).

(PFOA) and perfluorodecanoic acid (PFDA), can inhibit GJIC in a dose-dependent manner, whereas the non-fluorinated fatty acids do not have such effects (Ketcham and Klaunig, 1996). The inhibitory potency of PFFAs depends on the length of its carbon chain. PFFAs with carbon chain length less than 5 or more than 16 did not inhibit GJIC. In contrast, PFFAs with carbon chain lengths of 7, 8, 9 and 10 can completely inhibit GJIC at a concentration of 50 mg/L (Upham *et al.*, 1998).

Results from the current study are consistent with previous published result. PFOS which has an 8 carbon chain effectively inhibits GJIC, with EC50 value of 18 mg/L. PFOSA has the same carbon chain length as PFOS but with a modified functional group. PFOSA inhibits GJIC with a potency similar to that of PFOS. This indicates that the critical feature that determines the GJIC inhibition efficiency is the length of the carbon chain, but not the functional groups. This result suggests a receptor-mediated mechanism, that is only ligand of certain structure can be recognized by the receptor, and elicits its effect on GJIC consequently. Peroxisome proliferator activated receptor (PPAR) may be a potential mediator of this process, however, to date there is no direct evidence of such a relationship between GJIC and PPAR.

To date most of the studies of GJIC inhibition have been conducted using the well-developed rat liver cell model. In this study dolphin kidney cells CDK was used to test species specificity. The result showed that the inhibitory effect of PFFAs on GJIC is neither species- nor tissue-specific.

The results of the time course experiment indicate that inhibition of GJIC occurred within a very short period of time, which is not sufficient enough for effects at transcriptional level to occur. This suggests a possible mode of action, that is the post-translational modification of gap junctional protein was involved in this effect.

Even though there is a good correlation between tumor promotion and GJIC inhibition, so far no direct evidence has been provided on the carcinogenesis of PFOS. Long term in vivo exposure studies need to be conducted to provide further information on PFOS in order to reach a definite conclusion.

AROMATASE ASSAY

BACKGROUND

In recent years there has been growing concern about the endocrine disruptive potential of environmental contaminants and commercial products. Chemical disturbances of endocrine functions can be caused by either direct interaction with steroid hormone receptors, particularly the estrogen receptor, or by interference with enzymes that are involved in steroid synthesis and breakdown (Kavlock, *et al.*, 1996).

In the adrenocortical cortex of mammals, cholesterol is transformed into 17β-estradiol in several steps by several enzymes. An estrogenic or antiestrogenic effect may occur due to interference with one or more enzymes involved in this pathway (Drenth, *et al.*, 1998; Sanderson and Van den Berg, 1998). The aromatase enzyme complex consists of the microsomal CYP19 enzyme and the flavoprotein NADPH-reductase. It is this enzyme that catalyzes the last step in the cholesterol to estradiol pathway, which converts testosterone to estradiol (Simpson, *et al.*, 1994).

Ammonium perfluorooctanoate (C8) has been reported to induce hepatic aromatase activity by up to 16 fold in dietary exposed Fisher rats (Cook, *et al.*, 1992; Liu, *et al.*, 1996). Therefore, the effect of PFOS on aromatase activity *in vitro* was investigated using human adrenocortical cells in order to compare the effect of PFOS with that of

ammonium perfluorooctanoate, and to assess the effect of PFOS on hormonal regulation *in vitro*.

MATERIALS AND METHODS

Chemicals

Perfluorooctane sulfonic acid (PFOS) was obtained from 3M company (St. Paul, MN) as a mixture. Based on the NMR analysis, the mixture consisted of 68% of straight chain PFOS and 17% of branch chain PFOS. [1β-^{3}H(N)]-Androst-4-ene-3,17-dione (28.5Ci/mmol) was obtained from New England Nuclear company (NEN-926).

Cell Culture

NCI-H295R is a human adrenocortical carcinoma cell line, which was obtained from the American Type Culture Collection (ATCC # CRL-2128). The NCI-H295R cells have the physiological characteristics of undifferentiated human fetal adrenal cells. They have the ability to synthesize steroid hormones and to express steroidogenic cytochrome P450 enzymes including aromatase (CYP19) activity (Rainey, *et al.*, 1993).

NCI-H295R cells were cultured in 75 cm flask (Corning 430720) under sterile conditions (pH=7.4) at 37C° in a humidified 5/95% CO_2 /air incubator (Forma Scientific, Model 8173). NCI-H295R cells were cultured in DMEM/Ham's F12 medium (Gibco), supplemented with other nutrients (see Appendix A1). Medium was changed twice a week. When cells reached confluence and were ready for passaging, medium was removed, cells were washed twice with 10 ml PBS, and trypsinized with 1x trypsin

/EDTA (Sigma). Cells were then incubated at 37C° for 5 min, and 10 ml medium was added. The cell suspension was split into two new flasks each containing 12ml medium.

Aromatase Assay

Plating

NCI-H295R cells were grown until reached 90% confluent. Medium was aspirated and cells were washed twice with PBS. 2 ml of 1 x trypsin/EDTA was added, the flask was incubated at 37 C° for 5 min, then cells were suspended in 10 ml medium. Cell number was determined using a hemocytometer, and cell concentration was adjusted to 2~5 x 10^5 cells per ml. 1 ml of diluted cell suspension was added in each well of the 24 well flat bottom view plates (Corning, 25820). Cells were incubated at 37 C° for 24 hrs to allow cell attachment.

Dosing

Medium was changed the day after plating, and cells were dosed at 0.2% (v/v) of PFOS stock solution dissolved in Methanol. The final concentrations of PFOS were 0.01 mg/L, 0.1 mg/L, 1 mg/L, 10 mg/L, and 50 mg/L. Methanol was used as solvent control. Each concentration was tested in triplicate. After dosing cells were incubated at 37 C° for 24 hrs or 48 hrs exposure.

Assay Procedure

The aromatase assay was conducted following the modified procedure of Lephart and Simpson (1991). The method measures the production of [^{3}H-] H_2O, which is formed as a result of the aromatization of the substrate [1β-^{3}H]-androstenedione.

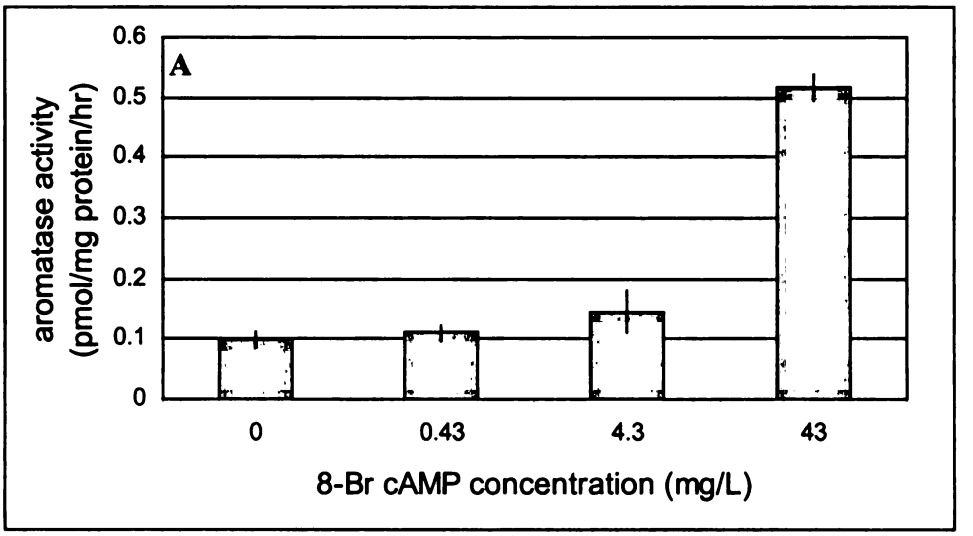
Medium was aspirated from the 24 well plates, and cells were washed twice with PBS. A working solution of 54 nM [^{3}H]-androstenedione in serum-free medium was prepared. A volume of 0.25 ml of working solution was added to each well and plates were incubated at 30 C° for 1.5 hrs. 50 µl of working solution was added, in duplicate, directly to a scintillation vial, as a check of the total amount of radioactivity in the working solution. 200 µl of the rest working solution were added in duplicate to enpendorf tubes, these were used as background check, and they would go through the same extraction procedure as other treatment samples. After incubation, plates were immediately placed on ice. Exactly 200 µl of medium were withdrawn from each well, and transferred to eppendorf tubes containing 500µl chloroform. Each tube was vortexed for 15 sec, and centrifuged at 11,000x g for 2 min. 100 µl of the supernatant was carefully transferred to an eppendorf tube containing 100µl dextran-coated charcoal solution. This mixture was vortexed for 15 sec, and allowed to stand for 5 min. Then it was centrifuged at 11,000x g for 15 min. 125 µl of the supernatant was transferred to a scintillation vial, to which 4 ml of scintillation cocktail were added, and the tritium isotope activity was measured in a scintillation counter.

Fluorescamine Protein Assay

To correct for heterogeneity in plating and in differential cell growth, a fluorescamine protein assay was carried out on the lysed cells. Fluorescamine is intrinsically nonfluorescent but reacts in milliseconds with primary amine groups on proteins to yield a fluorescent derivative. It is widely used to determine protein concentrations of aqueous solutions (Udenfriend, *et al.*, 1972). After completing the aromatase assay, the plates were washed twice with PBS. 200 µl of distilled water was added to each well, and cells were lysed by freezing and thawing. 50 µl of 1.08 mM fluorescamine (Sigma, F-9015) in acetonitrile was added and mixed by agitation. After 10 min incubation at room temperature, fluorescence were measured using a Cytofluor 2300 Fluorescence Measurement System (Millipore) at λ_{ex} = 400 nm and λ_{em} = 460 nm. The aromatase activity was expressed as picomole reaction per hour per milligram protein (see Appendix B for aromatase activity calculation).

RESULT

Aromatase was expressed constitutively by NCI-H295R cells, which responded in a predictable manner to known aromatase inducers. Aromatase activity was induced by 8-Br cAMP in a dose-dependent manner, with the maximum of 5.2 fold induction occurred at 43 mg/L 8-Br cAMP (Fig. 17A). In contrast, aromatase activity was only slightly induced by PFOS (Fig. 17B and Fig. 17C). When cells were treated with PFOS for 24 hrs, only a concentration at 50 mg/L showed a significant effect ($p<0.05$) compared to the solvent control, with the aromatase activity increased by a factor of 1.5 (Fig. 17B). The



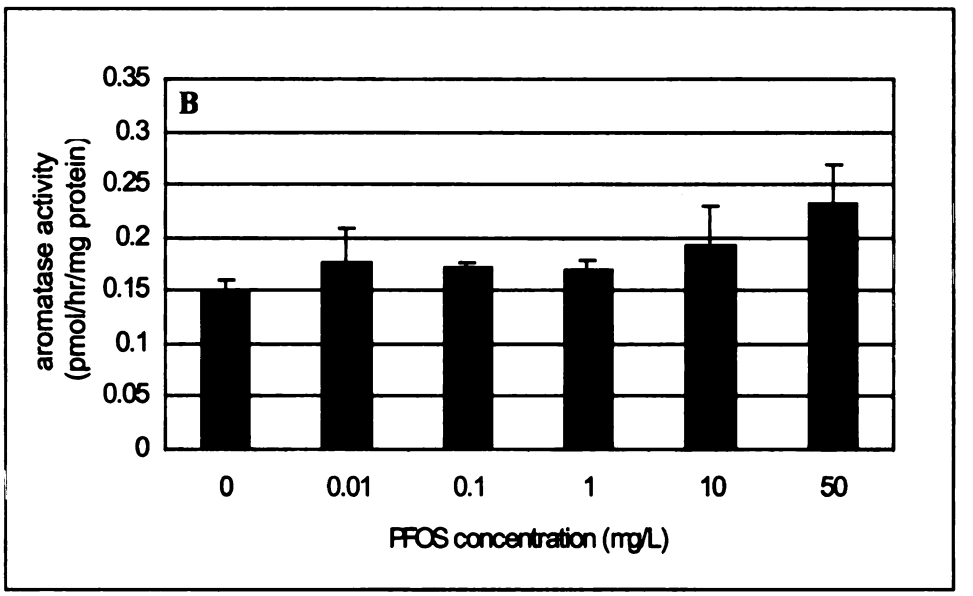
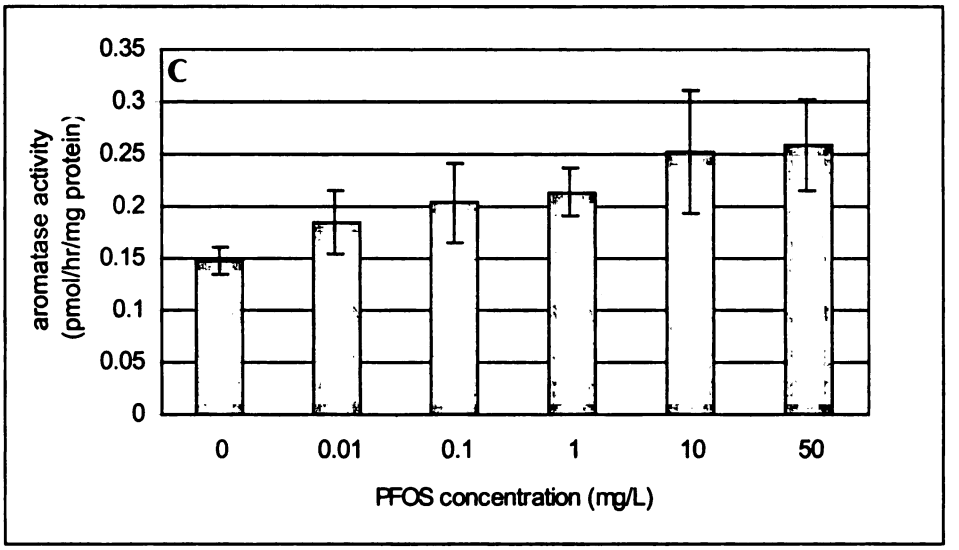


Fig. 17 Effect of PFOS on NCI-H295R cell aromatase activity. A) cells dosed with 8-Br cAMP as positive control; B) cells dosed with PFOS for 24 hrs. Aromatase activity was expressed as picomole tritiated water formed per mg protein per hr. Control wells were incubated with 0.02% (v/v) solvent (methanol). Error bars represent the standard deviation of three measurements.



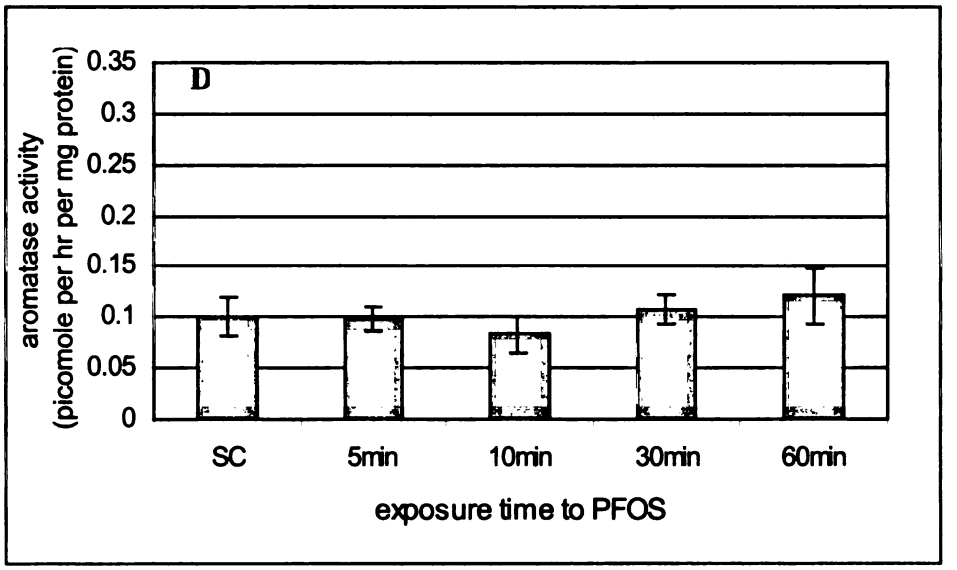


Fig. 17 Effect of PFOS on NCI-H295R cell aromatase activity. C)cells dosed with PFOS for 48 hrs. D) cells dosed with 50mg/L PFOS at different time interval before running the assay. Aromatase activity was expressed as picomolar substrate aromatized per mg protein per hr. Control wells were incubated with 0.02% (v/v) solvent (methanol). Error bars represent the standard deviation of three measurements.

magnitude of effect of PFOS on aromatase activity was proportional to the duration of exposure. The aromatase increased in a dose-dependent manner for cells treated with PFOS for 48 hrs (Fig. 17C). PFOS at concentrations of 10 and 50 mg/L significantly increased aromatase activity ($p<0.05$) by a factor of 1.7.

To test the hypothesis that PFOS affected aromatase activity by changing membrane properties of cells and allowing a greater influx of substrate, a time course experiment was conducted. NCI-H295R cells were dosed with 50 mg/L of PFOS at 5min, 10min, 30min or 60min before performing the assay (Fig. 17D). During all these short termexposure to PFOS (< 1 hr), no significant effects on aromatase activity was observed. This indicates that the initial hypothesis was false, that is the effects of PFOS on NCI-H295R cell aromatase activity is was not simply due to change of membrane properties and increase of substrate influx.

DISCUSSION

The NCI-H295R cell line was established from a human adrenocortical carcinoma. Multiple pathways of steroidogenesis are expressed by NCI-H295R cells, including formation of corticosteroids, mineralocorticoids, androgens, and estrogens (Gazdar, *et al.*, 1990). All of the major adrenocortical enzyme systems are present in NCI-H295R cells, including desmolase (P450scc), 11 β-hydroxylase (P450c11β), 21 α-hydroxylase (P450c21), 17 α-hydroxylase (P450c17), lyase and aromatase (P450c19) (Gazdar, *et al.*, 1990). The cytochrome P450 steroid hydroxylase activity can be controlled at two levels: at the level of substrate mobilization, and at the level of gene transcription (Parker and

Schimmer, 1995). Some trophic hormones such as ACTH, FSH and LH regulate steroidogenesis by mobilizing substrates across mitochondrial membranes to their corresponding steroidogenic cytochrome P450 enzymes. This process is dependent on the cAMP and cAMP related protein kinase A signaling cascade (Parker and Schimmer, 1995; Pon, *et al.*, 1986). The genes encoding for cytochrome P450 steroid enzymes are regulated in a cell selective way by some nuclear receptors and growth factors. One of the candidates is a DNA binding protein termed steroidogenic factor 1 (SF-1). SF-1 can bind to SF-1 responsive elements that reside close to the sequences for many of the steroid hydroxylases (Parissenti, *et al.*, 1993). The activation of SF-1 and its subsequent binding to SF-1 responsive elements requires the phosphorylation of SF-1by a cAMP-dependent protein kinase (Pon, *et al.*, 1986; Parissenti, *et al.*, 1993). This is supported by the fact that most of the steroidogenic P450 enzymes can be up-regulated in a dose-dependent fashion by 8-Br cAMP, which is an activator of the protein kinase-A pathway.

The specific mechanism under which PFOS may elicit its effect on the aromatase (CYP19) activity is still under investigation. It has been found that treatment with PFOA and PFDA can affect the level of protein kinase C, which is an important signaling pathway that may interfere with steroidogenesis (Reo, *et al.* 1996). Another feasible possibility is that the peroxisome proliferator activated receptor (PPAR) is involved, and it interacts with the nuclear receptors and growth factors that regulate aromatase gene expression.

Aromatase (CYP19) enzyme is responsible for the formation of estrogens from their androgen precursors. In most vertebrate species that have been examined, aromatase expression occurs primarily in the gonads and in the brain (Simpso, *et al.*, 1994). In some species estrogen biosynthesis in the brain has been implicated in sex determination during development and sex related behavior such as mating (Jeyasuria, *et al.*, 1994; Antonopoulou, *et al.*, 1995). Therefore regulating aromatase activity can have tremendous effect on estrogen biosynthesis and estrogen related physical responses.

CONCLUSION

The results from cell bioassays, GJIC assay and aromatase assay can be summarized as following:

- PFOS exhibits no cytotoxicity within the concentration range tested;
- PFOS by itself has no significant effects on cytochrome P4501A1 isoenzyme activity;
- TCDD when dosed in the presence of PFOS, elicited a greater magnitude of P4501A1 induction and Aryl Hydrocarbon activation;
- The interactive effects of PFOS and TCDD occurred at the level of transcription;
- PFOS inhibited Gap Junctional Intercellular Communication in a dose-dependent manner with a EC50 value of 20 mg/L PFOS;
- The inhibition of GJIC by PFOS occurred within a short period of time;
- This inhibitory effect is neither species- nor tissue-specific;
- PFOS slightly induced aromatase activity in NCI-H295R cells, significant induction only occurred at a relatively great dose of PFOS (50 mg/L);
- Induction increased with prolonged duration of exposure from 24 hr to 48 hr;
- The concentration range at which significant effects were observed on AhR and CYP1A1 activity were marginally environmentally relevant, the concentration of PFOS required to affect GJIC and aromatase activity were greater than environmentally relevant concentration (Fig. 18).

Results from the current study provide useful information on three aspects of the biochemical toxicity of PFOS. To estimate the environmental risk of a compound to

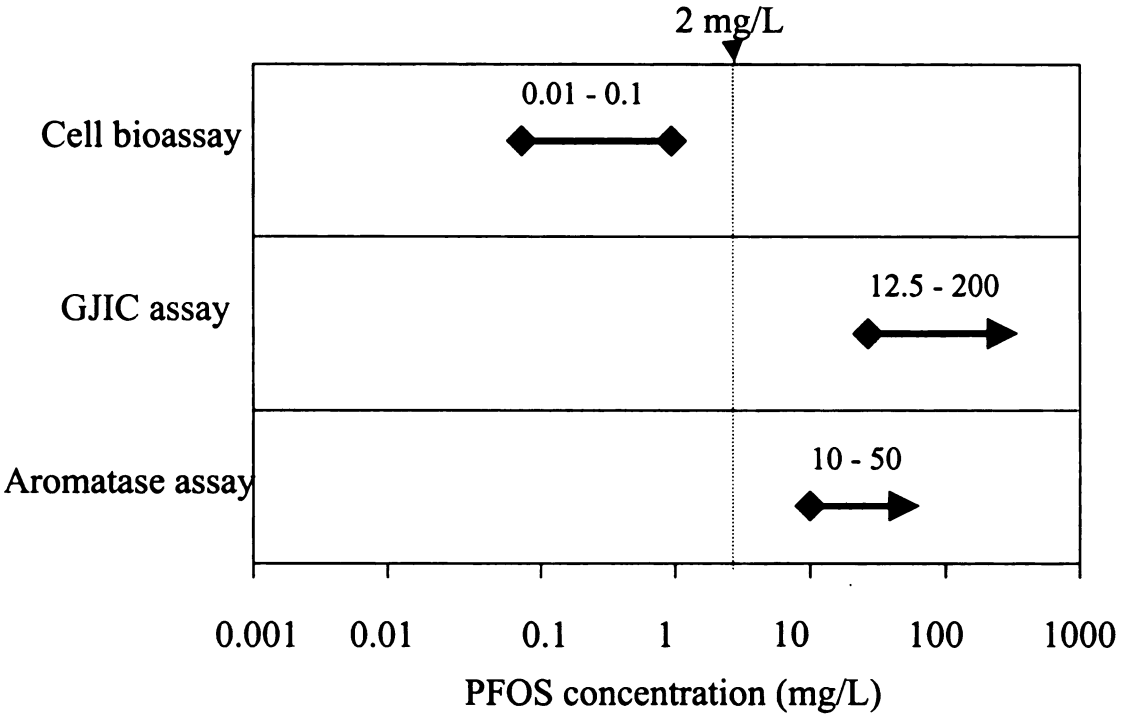


Fig. 15 Diagram shows the environmental relevance of the data.
The PFOS concentration that caused interactive effects with TCDD in cell bioassays ranges from 0.01 mg/L to 0.1 mg/L; the PFOS concentration that caused significant inhibitory effects on GJIC ranges from 12.5 mg/L to 200 mg/L; the PFOS concentration that caused significant effects on aromatase activity ranges from 10 mg/L to 50 mg/L. (dashed line indicates the maximum concentration of PFOS found in wildlife); ◆—◆ indicates range of concentration determined at both end; ◆—▶ indicates range of concentration determined at only one end.

human and wildlife, we have to take into account of both hazard and exposure. In cell bioassays the concentration at which significant interaction between PFOS and TCDD was observed falls into the range of environmental relevance. Furthermore, this interaction only resulted in an increase of CYP1A1 induction by 30-40%. This means that when human and wildlife are exposed to moderate concentrations of PFOS and a fairly great concentrations of TCDD at the same time, it is possible that the effect of TCDD may be increased by 30-40%. The concentrations of PFOS that significantly inhibited GJIC are approximately 10 fold greater than the maximum concentration of PFOS detected in wildlife. Similarly the effective concentration of PFOS on aromatase activity also exceed the environmentally relevant concentration range of PFOS. Furthermore, the aromatase activity was only induced by a factor of 1.5-1.7 fold. All the concentrations I mentioned in this study were the concentration of PFOS in cell culture medium, eventually how much of them end up getting into the cell still remain undetermined. Based on above discussion, it is unlikely that PFOS is currently causing significant effects on any of the biochemical pathways investigated on wildlife. Of course efforts should be made to assure that concentrations at which PFOS may elicit significant adverse effects are not reached in the future.

Another issue of concern is whether it is feasible to extrapolate these *in vitro* results *to in vivo* situations. This has been a point of discussion among environmental toxicologists. Predicting *in vivo* toxicity from *in vitro* effects must be done with caution. Toxicokinetics, levels of organization and functional integrations *in vivo* complicate the extropolation. *In vitro* studies are efficient tools to study the mechanisms of effects. Once

the mechanisms of effects are known, greater generality in extrapolating to *in vivo* systems can be made. Also it is only by knowing the mechanism of action that effective biomarkers and monitoring programs can be established.

The mechanism of action of PFOS is still under investigation and needs to be further clarified. No definite conclusion can be drawn upon the limited information currently available. A more in depth study is currently being conducted in the aquatic toxicology laboratory at Michigan State University. Hopefully we will be able to elucidate the mechanism of action so that accurate risk assessments of the potential effects of PFOS in a wider range of organisms can be valid.

APPENDICES

Appendix A

Table A1. Culture Medium for NCI-H295R Cells.

Ingradient	Abbreviation	Sources and catalog #	Amount
Dulbecco's Modified Eagle Medium / Ham's F12	DMEM	GIBCO-BRL (12400-016)	7.8g dissolved in 440ml diH_2O
Insulin, Transferrin, Selenium	ITS-G	GIBCO-BRL (41400-037)	100x, 5ml
Bovine	BSA	SIGMA (A-9647)	1.25mg/ml, 5ml
1,000U penicillin/streptomycin		GIBCO-BRL (15140-114)	0.5ml
10% Dextran Charcoal Coated Fetal Bovine Serum	DCC-FBS	GIBCO-BRL (16000-044)	50ml

Table A2. Culture Medium for CDK Cells.

Ingradient	Abbreviation	Sources and catalog #	Amount
Dulbecco's Modified Eagle Medium / Ham's F12 medium	DMEM/F12	SIGMA (D-2906)	7.8g dissolve in 850ml water
10% Fatal Bovine Serum	FBS	GIBCO-BRL (16000-036)	5ml
10mM NaCl		EM Science (SX0420-1)	10mM
50mg/L Gentamicin		GIBCO-BRL (15710-064)	5ml
3x MEM amino acid including L-glutamin		SIGMA (M6725)	15ml
3x MEM non-essential amino acid		SIGMA (M-7145)	15ml
3x MEM vitamins		SIGMA (M-6895	15ml

APPENDIX B

AROMATASE CALCULATION

The aromatase activity was expressed as picomole ^{3}H-Androst-4-ene-3,17-dione catalyzed per milligram protein, this was calculated based on following equation:

Aromatase activity (pmol/mg) = (cpm/ 35.19%)*4/1.5/73964.5/ protein content per well

Cpm --- count per minute (background substracted)

35.19% --- measuring and labeling efficiency

4 --- dilution

1.5 --- incubation time (hr)

73964.5 --- factor used to convert dpm to picomole

Protein content measurement

In order to convert fluorescence data from fluorescamine assay to protein content, a BSA protein standard curve was produced.

Table B1 Fluorescamine protein assay data for BSA standard.

protein(mg)	fluorescence
0.1	563
0.05	352
0.025	216
0.0125	144
0.00625	104
0.003125	85

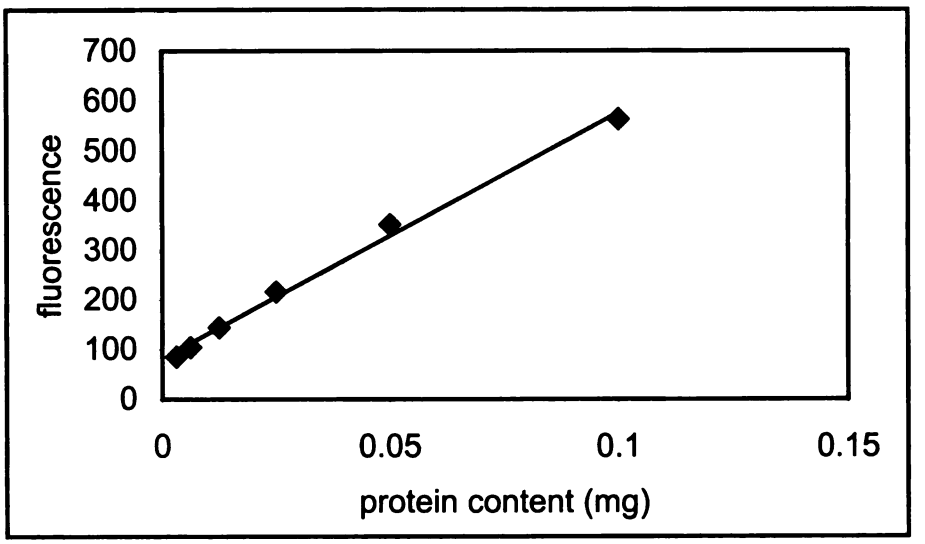


Fig. B1 BSA protein standard curve.

slope 5683.441
intercept 70.08333

fluorescence=5683.4*protein + 70
protein content per well (mg) = (fluorescence - 70) / 5683.4

REFERENCES

REFERENCES

Antonopoulou, E., Mayer, I., Berglund, I., and Borg, B. (1995). Effects of aromatase inhibitors on sexual maturation in Atlantic salmon, *Salmo salar*, male parr. *Fish Physiology and Biochemistry*. 14, 15-24.

Biegel, L. B., Liu, R. C. M., Hurtt, M. E., and Cook, J. C. (1995). Effects of ammonium perfluorooctanoate on Leydig cell function: *in vitro*, *in vivo*, and *ex vivo* studies. *Toxicol. Appl. Pharmacol.* 134, 18-25.

Bojes, H. K., and Thurman, R. G. (1994). Peroxisomal proliferators inhibit Acyl-CoA synthetase and stimulate protein kinase C *in vivo*. *Toxicol. Appl. Pharmacol.* 126, 233-239.

Cook, J. C., Murray, S. M., Frame, S. R., and Hurtt, M. E. (1992). Induction of Leydig cell adonomas by ammonium perfluorooctanoate: a possible endocrine-related mechanism. *Toxicol. Appl. Pharmacol.* 113, 209-217.

Diaz, M. J., Chinje, E., Kentish, P., Jarnot, B., George, M., and Gibson, G. (1994). Induction of cytochrome P450 4A by the peroxisome proliferator perfluoro-n-octanoic acid. *Toxicology*. 86, 109-122.

Drenth, H.-J., Bouwman, C. A., Seinen, W. and Van den Berg, M. (1998). Effects of some persistent halogenated environmental contaminants on aromatase (CYP19) activity in the human choriocarcinoma cell line JEG-3. *Toxicol. Appl. Pharmacol.* 148, 50-55.

Gazdar, A. F., Oie, H. K., Shackleton, C. H., Chen, T. R., Triche, T. J., and Myers, C. E. (1990). Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. *Cancer Research*. 50, 5488-5496.

Green, S. (1992). Commentary: receptor-mediated mechanisms of peroxisome proliferators. *Biochemical Pharmacology*. 43, 393-401.

Hahn, M. E., Lamb, T. M., Schultz, M. E., Smolowitz, R. M. and Stegeman, J. J. (1993). Cytochrome P4501A induction and inhibition by 3,3',4,4'-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma cell line (PLHC-1). *Aquatic Toxicology*. 26, 185-208.

Hanhijarvi, H., Ylinen, M., Kojo, A., and Kosma, V. M. (1987). Elimination and toxicity of perfluorooctanoic acid during subchronic administration in the Wistar rat. *Pharmacol. Toxicol.* 61, 66-68.

Haughom, B., and Spydevold, O. (1992). The mechanism underlying the hypolipemic effect of perfluorooctanoic acid (PFOA), perfluorooctane sulphonic acid (PFOSA) and clofibric acid. *Biochimica et Biophysica Acta*. 1128, 65-72.

Jeyasuria, P., Roosenburg, W. M., and Place, A. R. (1994). Role of P450 aromatase in sex determination of the diamondback terrapin, *Malaclemys terrapin. J. Experi. Zoo*. 270, 95-111.

Kavlock, R. J., Daston, G. P., DeRosa, C., Fenner-Crisp, P., Gray, L. E., Kaattari, S. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the USEPA-sponsored workshop. *Environ. Health Perspect*. 104 (*suppl* 4), 715-740.

Kawashima, Y.,Suzuki, S., Kozuka, H., Sato, M., and Suzuki, Y. (1994). Effects of prolonged administration of perfluorooctanoic acid on hepatic activities of enzymes which detoxify peroxide and xenobiotics in the rat. *Toxicology*. 93, 85-97.

Kawashima, Y., Uy-yu, N., and Kozuka, H. (1989). Sex-related difference in the inductions by perfluoro-n-octanoic acid of peroxisomal β-oxidation, microsomal 1-acylglycerophosphocholine acyltransferase and cytosolic long-chain acyl-CoA hydrolase in rat liver. *Biochem. J*. 261, 595-600.

Ketcham, C. A., and Klaunig, J. E. (1996). Effect of protein kinase C inhibitors on hepatic gap junctional intercellular communication blockage by peroxisome proliferators. *Fundament. Appl. Toxicol*. 30(*suppl.*), 1054-1062.

Lephart, E. D. and Simpson, E. R. (1991). Assay of aromatase activity. *Methods Enzymol*. 206, 477-483.

Liu, R. C. M., Hahn, C., and Hurtt, M. E. (1996). The direct effect of hepatic peroxisome proliferators on rat Leydig cell function *in vitro*. *Toxicol. Appl. Pharmacol*. 30, 102-108.

Liu, R. C. M., Hurtt, M. E., Cook, J. C., and Biegel, L. B. (1996). Effect of the peroxisome proliferator, ammonium perfluorooctanoate (C8), on hepatic aromatase activity in adult male Crl:CD BR (CD) rats. *Fundam. Appl. Toxicol*. 30, 220-228.

Obourn, J. D., Frame, S. R., Bell, R. H., Longnecker, D. S., Elliott, G. S., and Cook, J. Cl. (1997). Mechanisms for the pancreatic oncogenic effects of the peroxisome proliferator wyeth-14, 643. *Toxicol. Appl. Pharmacol*. 145, 425-436.

Okey, A. B., Riddick, D. S., and Harper, P. A. (1994). The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. *Toxicology Letters*. 70, 1-22.

Olson, C. T., and Andersen, M. E. (1983). The acute toxicity of perfluorooctanoic and perfluorodecanoic acids in male rats and effects on tissue fatty acids. *Toxicol. Appl. Pharmacol.* 70, 362-372.

Parissenti, A. M., Parker, K. L., and Schimmer, B. P. (1993). Identification of promoter elements in the mouse 21-hydroxylase (CYP21) gene that require a functional cyclic adenosine 3',5'-monophosphate-dependent protein kinase. *Mol. Endocrinol.* 7, 283-290.

Parker, K. L., and Schimmer, B. P. (1995). Transcriptional regulation of the genes encoding the cytochrome P450 steroid hydroxylases. *Vitamins and Hormones.* 51, 339-370.

Permadi, H., Lundgren, B., Anderson, K., and Depierre, J. W. (1992). Effects of perfluoro fatty acids on xenobiotic-metabolizing enzymes, enzymes which detoxify reactive forms of oxygen and lipid peroxidation in mouse liver. *Biochemical Pharmacology.* 44, 1183-1191.

Pon, L. A., Hartigan, J. A., and Orme-Johnson, N. R. (1986). Acute ACTH regulation of adrenal corticosteroid biosynthesis. Rapid accumulation of a phosphoprotein. *J. Biol. Chem.* 261, 13309-13316.

Rainey, W. E., Bird, I. M., Sawetawan, C., Hanley, N. A., et al. (1993). Regulation of human adrenal carcinoma cell (NCI-H295) production of C19 steroids. *J. Clin. Endocrinol. Metab.* 77, 732-737.

Ravis, W. R., Hoke, J. F., and Parsons, D. L. (1991). Perfluorochemical erythrocyte substitutes: disposition and effects on drug distribution and elimination. *Drug Metabolism Reviews.* 23, 375-411.

Reo, N. V., Narayanan, L., Kling, K. B., and Adinehzadeh, M. (1996). Perfluorodecanoic acid, a peroxisome proliferator, activates phospholipase C, inhibits CTP: phosphocholine cytidylyltransferase, and elevates diacylglycerol in rat liver. *Toxicology Letters.* 86, 1-11.

Roman, B. L., Pollenz, R. S., and Peterson, R. E. (1998). Responsiveness of the adult male rat reproductive tract to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure: Ah receptor and ARNT expression, CYP1A1 induction, and Ah Receptor down-regulation. *Toxicol. Appl. Pharmacol.* 150, 228-239.

Sanderson, J. T. et al. (1996). Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-*o*-deethylase induction in H4IIE cells: implicaiton for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* 137, 316-325.

Sanderson, J. T., Kennedy, S. W., and Giesy, J. P. (1998). *In vitro* induction of ethoxyresorufin-*o*-deethylase and porphyrins by halogenated aromatic hydrocarbons in avian primary hepatocytes. *Environ. Toxicol. Chem.* 17, 2006-2018.

Sanderson, J. T. and Van den Berg, M. (1998). An *in vitro* system for the detection of compounds that can interfere with the expression of steroidogenic cytochrome P450 (CYP) enzymes. *Organohalogen Compounds*. 37, 77-80.

Sargent, J. and Seffl, R. (1970). Properties of perfluorinated liquids. *Fed. Proc.* 29, 1699-1703.

Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., et al. (1994). Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Rev.* 15, 342-355.

Sohlenius, A. K., et. Al. (1994). Effects of perfluorooctanoic acid, a potent peroxisome proliferator in rat, on morris hepatoma 7800C1 cells, a rat cell line. *Biochimica et Biophysica Acta*. 1213, 63-74.

Sohlenius, A. K., Reinfeldt, M., Backstrom, K., Bergstrand, A., and Depierre, J. W. (1996). Hepatic peroxisome proliferation in vitamin A deficient mice without a simultaneous increase in peroxisomal Acyl-CoA oxidase activity. *Biochemical Pharmacology*. 51, 821-827.

Trosko, J. E., and Ruch, R.J. (1998). Cell-cell communication in carcinogenesis. *Frontiers Biosci.* 3, 208-236.

Tsao, M. S., Smith, J. D., Nelson, K. G. and Grisham, J. W. (1984). Adiploid epithelial cell line from normal adult rat liver with phenotypic properties of oval cells. *Exp. Cell Res.* 154, 38-52.

Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W. and Weigele, M. (1972). Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science*. 178, 871-872.

Upham, B. L., Deocampo, N. D., Wurl, B., and Trosko, J. E. (1998). Inhibition of gap junctional intercellular communication by perfluorinated fatty acids is dependent on the chain length of the fluorinated tail. *Int. J. Cancer*. 78, 491-495.

Witzmann, F. A., Parker, D. N., Jarnot, B. M. (1994). Induction of enory-CoA hydratase by LD50 exposure to perfluorocarboxylic acids detected by two-dimensional electrophoresis. *Toxicology Letters*. 71, 271-277.

Ylinen, M., and Auriola, S. (1990). Tissue distribution and elimination of perfluorodecanoic acid in the rat after single intraperotoneal administration. *Pharmacol. Toxicol.* 66, 45-48.

Ylinen, M., Hanhijarvi, H., Jaakonaho, J., and Peura, P. (1989). Stimulation by oestradiol of the urinary excretion of perfluooctanoic acid in the male rat. *Pharmacol. Toxicol.* 65, 274-277.

Yoshiaki, F. K., Imataka, H., Sogawa, K., Yasumoto, K. I. And Kikuchi, Y. (1992). Regulation of CYP1A1 expression. *The FASEB J.* 6, 706-710.