Biochemical and Molecular Mechanistic Studies of N-Alkyl Perfluorosulfonamides

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LONG-RANGE GOAL: ESTABLISH A SCIENTIFICALLY-BASED METHOD TO MONITOR FOR POTENTIAL HEALTH RISKS ASSOCIATED WITH WORKER OR CONSUMER EXPOSURE TO PERFLUOROALKYL ACIDS AND THEIR DERIVATIVES.

SHORT-TERM GOAL (3-5 years):

1. DEFINE RELIABLE BIOMARKERS FOR ASSESSING BOTH EXPOSURE AND BIOLOGICAL REACTIVITY.

2. DEFINE THE MOST APPROPRIATE EXPERIMENTAL SYSTEM (IN TERMS OF BEST REPRESENTING THE HUMAN SITUATION) FOR ESTABLISHING RELATIONSHIPS BETWEEN EXPOSURE DOSE AND BIOLOGICAL EFFECTS.

There exists considerable experimental evidence demonstrating that perfluoroalkyl acids and their derivatives have the potential of being toxic to humans, which raises serious concern for the safe production, use and disposal of these chemicals in the work place, by the consumer, and in the environment. Many of the current and pending regulatory decisions for these products are based on experimental evidence gathered from rats, for which there is strong evidence for the induction of peroxisome proliferation, metabolic wasting and carcinogenesis [1,2,3]. However, the evidence for comparable effects in other mammalian species, including primates, is less convincing which suggests profound and important differences among species. Therefore, although rats may be the most convenient and extensively studied species, the evidence may not

yield accurate indications of potential adverse human health effects. A more prudent means of managing potential human health risks associated with the production and use of these important products requires careful selection of a representative, yet convenient surrogate species test organism. The fact that chemical residues of perfluoroalkyl acids have been detected in sera of production workers [4] adds urgency to the need to understand potential health risks, to establish guidelines to safeguard human health, and to estimate margins of safety for exposed populations.

In order to address the surrogate species issue, it is first necessary to establish one or more reliable indicators of toxic tissue damage upon which the species comparisons can be made. The proposed investigation is designed to provide an understanding of the underlying mechanisms of toxic tissue injury and to identify reliable biomarkers of tissue damage that can be used for risk-management purposes.

IMMEDIATE OBJECTIVES (24 months):

1. Define the "dominant" mechanism by which perfluoroalkyl acids and their derivatives manifest the well documented "metabolic wasting" effect observed in experimental animals *in vivo*.

2. Based on an understanding of the biological mechanism of action, identify potential biomarkers that can be used to provide reliable and quantitative estimates of exposures and the biological consequences associated with such exposures.

3. Using the biomarkers described for Aim 2, determine whether differences exist among species in their sensitivity to perfluoroalkyl acids and their derivatives and in the cellular mechanism by which the toxicity is manifested.

NOTE:

- The research plan is being submitted with the understanding that publication of the results will be subject to a 30 day first-right-of-review by the sponsor.
- The sponsor will provide all required quantities of test chemicals.
- Selected tissue samples from the *in vivo* exposures will be archived for subsequent histological examination and trace residue and metabolite analyses, both to be performed by the sponsor.
- The sponsor will be briefed on the progress of the funded project on a quarterly basis.
- In addition to the 18 month contract, an unrestricted gift of $100,000 is requested to support more empirical exploratory research on related topics.

INTRODUCTION

Perfluoroalkyl acids and their derivatives represent a large and important class of synthetic chemicals, with production amounting to several thousands of pounds each year. These compounds constitute a broad market including surfactant and foaming applications as well as potent pesticidal activity. Prudent regulation of the production, distribution, storage, application and disposal of these products necessitates a thorough understanding of the probability and nature of any potential adverse health effects. This proposal is directed at improving the confidence and judiciousness with which such risk management decisions are made.

Although each member of this class possesses distinct structural and physical chemical properties, they share very similar biological activities when administered at high doses to experimental subjects. From a structure-activity basis, this suggests that the N-alkyl substituent, along with the length and branching of the carbon chain, determine the rates of absorption and the biological distribution (pharmacokinetics) of the compound but that the biological reactivity (toxicity) resides in the sulfonic acid or sulfonamide that is common to all members of the class [5]. A fully comprehensive characterization of the risks associated with this class of chemicals would require rigorous investigation of the biological reactivity of each member compound. Such an analysis would be extremely costly, both in terms of resources and time. However, Dr. S. C. Gordon has proposed a tentative metabolic pathway for many of the N-alkylated perfluorooctane sulfonamides which consists of successive N-dealkylations leading to perfluorooctane sulfonic acid as the final common metabolite (Fig. 1). In view of the converging metabolic pathway, it may be possible to gain an accurate indication of potential health risks by examining only selected metabolites that are believed to be primarily responsible for the biological activity of the entire class. Thus, in the interest of efficiency, this initial investigation is limited to testing N-ethyl perfluorooctane sulfamido ethanol, N-ethyl perfluorooctane sulfamido acetate, the sulfonamide of perfluorooctane, and perfluorooctane sulfonic acid. The structures of all 4 proposed test compounds are bracketed in figure 1. Perfluorooctanoic acid will serve as a positive control for all exposures. A rigorous understanding of the biological reactivities of these metabolites will provide the most efficient means of gaining a better understanding the potential health risks associated with the entire class of N-substituted perfluorooctane sulfonamides.

Acute and chronic toxicity of N-alkyl perfluorooctane sulfonamides -

Both the sulfonic acid and the N-ethyl sulfonamide of perfluorooctane are essentially non-toxic on an acute basis. Only at very high oral doses (2-5 g/kg) are vague signs of gastrointestinal discomfort and weight loss observed. The primary concern with acute exposure to these compounds is their persistence in the body, the half-lives being between 1 and 11 days depending on the dosing schedule and the tissue examined [6,7]. Many of these chemicals bioconcentrate in the liver. Perfluorooctane N-ethyl sulfonamide is rapidly de-ethylated *in vivo* and it is the des-ethyl sulfonamide that accumulates in liver. This biopersistence raises serious concerns about the potential cumulative and long-term toxicity associated with continuous exposures to low concentrations of these chemical agents.

Both perfluorooctane N-ethyl sulfonamide and sulfonic acid elicit profound subchronic toxicities regardless of the route of administration. Ninety-day feeding studies in rats caused deaths at doses of 100-150 ppm. Although high sub-lethal doses cause assorted neurological, musculoskeletal, dermatologic and hemopoetic abnormalities, the most consistent symptoms observed at the lowest doses are anorexia, weight loss, emaciation, and elevation of liver enzymes in the plasma [8-12]. This apparent hepatotoxicity is observed in rats, rabbits, dogs and monkeys and is most prevalent in males, possibly due to the more rapid renal elimination of the metabolite in females. Regardless, there are no substantive histopathology or gross morphological changes that accompany the hepatotoxicity observed at low doses of either the N-ethyl sulfonamide or the sulfonic acid of perfluorooctane [9,12]. This, by itself, is strong evidence suggesting that the observed toxicity is more a manifestation of metabolic or functional deterioration, rather than structural damage.

Proposed mechanisms of toxicity -

Many of the signs and symptoms associated with intoxication by perfluoroalkyl acids and their derivatives, such as the anorexia and weight loss, resemble that of a metabolic disorder. In deed perfluorooctane sulfonamide, like many other weak acids, has been demonstrated to uncouple mitochondrial respiration *in vitro* [1,2]. It is presumed, but has yet to be confirmed, that this leads to mitochondrial depolarization and the depletion of ATP in cell cultures as well as *in vivo*. Whether perfluorooctane sulfonic acid also uncouples mitochondrial oxidative phosphorylation has yet to be determined. It is, however, well established that the sulfonic acid is a potent peroxisome proliferator *in vivo* in rats, which is evident as an hypertrophic hepatomegaly accompanied by proliferation of both mitochondrial and microsomal membranes

[3]. Associated with this is the stimulation of a number mitochondrial enzyme activities, including Mn-superoxide dismutase and fatty acyl CoA-oxidase. The reported interference with spermatogenesis and inhibition of sperm motility by N-ethyl perfluorosulfonamide is consistent with an effect on inhibiting mitochondrial bioenergetics [10].

The accumulation of triacylglycerols and free cholesterol in liver implies interference with fatty acid or lipid metabolism as a primary mode of expression of the toxicity [13,14]. In deed, the effects of perfluorooctane sulfonic acid on fatty acid and cholesterol synthesis mimics those of the HMG-CoA reductase inhibitors, which are some of the most effective agents in treating hypercholesterolemias clinically. It may be more than coincidence that many of these lipid-lowering agents, such as the fibric acids and the phthalic acid plastisizers, are classical peroxisome proliferators. Although circumstantial, this lends strong support for an important effect of the perfluorocompounds on lipid metabolism. Haughom and Spydevold [13] suggest that the hypolipemic effect of perfluorooctane sulfonic acid results from its inhibition of mitochondrial carnitine acyl-CoA transferase activity, which is required for the transport of long chain fatty acids for beta-oxidation within the mitochondrial matrix.

Although not much is known regarding the biological action of perfluoroalkyl acids and their derivatives, there is considerable information available for other peroxisome proliferators. As a class, these agents are known to interfere with mitochondrial respiration (although the precise mechanism is not known), to inhibit fatty acyl-CoA synthesis, acylcarnitine translocase and mitochondrial fatty acid beta-oxidation, and cholesterol synthesis. Peroxisome proliferators also stimulate the expression of several immediate early response genes (such as c-fos and c-jun), CYP4, cyclin-dependent kinases, proliferating nuclear antigen, and the peroxisome proliferator-activated receptor, which is a ligand-activated nuclear transcription factor that up-regulates the expression of genes that transcribe various lipid metabolizing enzymes [15-17]. It is the expression of many of these early response genes that has been implicated in the non-genotoxic carcinogenic activity of peroxisome proliferators. Whether these same genes are activated by the perfluoroalkyl acids and their derivatives has yet to be determined. Regardless, the similarity in biological response with classic peroxisome proliferators provides ample opportunities to unravel many of the unresolved questions concerning their mechanism of action.

It is curious, and perhaps unfortunate from a regulatory stand-point, that the vast majority of knowledge gained for perfluoroalkyl acids and their derivatives as well as for the classic peroxisome proliferators is derived from the laboratory rat. The concern lies in the fact that, although strongly predicted by rat data, there is little evidence for the proliferation of liver peroxisomes in humans exposed to these compounds. In fact, there are a number of non-primate species, such as guinea pigs and dogs, that don't respond to conventional peroxisome proliferators [18-21]. This then raises doubts regarding the validity of using rat data to establish

regulatory policy for these agents. Of particular concern is the debate over whether the peroxisome proliferators, including the N-alkyl perfluorosulfonamides, are non-genotoxic carcinogens [22]. The outcome will have profound effects on the regulation of these products, particularly in the context of the additives or residues in foodstuffs (e.g., N-ethyl perfluorooctane sulfamido ethanol). Resolution of this apparent species difference warrants immediate and aggressive attention.

RESEARCH PLAN

- **SPECIFIC AIMS**

1. Determine whether perfluoroalkyl acids and their derivatives interfere with mitochondrial electron transport and/or fatty acid metabolism by isolated rat hepatic mitochondria.

2. Identify molecular and peroxisomal biomarkers of N-ethyl perfluorooctane sulfamido ethanol exposure *in vivo* in rats.

3. Compare and contrast the response of hepatocyte cell cultures isolated from rats, guinea pigs and primates exposed *ex vivo* to perfluorooctane sulfonic acid and its N-alkyl derivatives and to compare the molecular and peroxisomal biomarkers and the histopathology in rats and guinea pigs in response to *in vivo* exposure to N-ethyl perfluorooctane sulfamido ethanol.

- **EXPERIMENTAL APPROACH**

1. DETERMINE WHETHER N-ETHYL PERFLUOROOCTANE SULFAMIDO ACETATE, PERFLUOROOCTANE SULFONAMIDE, AND/OR PERFLUORO-OCTANE SULFONIC ACID INTERFERES WITH CELLULAR ENERGY METABOLISM *IN VITRO*.

Mitochondrial bioenergetics -

N-Ethyl perfluorooctane sulfonamide uncouples oxidative phosphorylation in kidney mitochondria *in vitro* [1,2]. The authors attribute this activity to the des-ethyl metabolite. We propose to expand on this observation to determine whether the perfluorooctane sulfonic acid and its sulfonamide derivatives uncouple mitochondrial respiration in the liver and to conduct a full scale characterization of the effects of these agents on mitochondrial bioenergetics *in vitro*. The uncoupling protonophoric activity will be assessed by the chemical-induced depolarization of mitochondrial membrane potential, stimulation of cyanide-insensitive state 4 respiration and acidification of the mitochondrial matrix. All experiments will be performed with freshly isolated rat liver mitochondria. Mitochondrial membrane potential will be measured using a TPP^+-selective electrode. Oxygen consumption will be analyzed polarographically in a closed-chamber system employing an oxygen-selective Clarke-type electrode. Protonophoric acidification of the mitochondrial matrix will be assessed with an high-sensitive pH electrode.

Fatty acid metabolism -

We have preliminary evidence demonstrating that weak acids (both substituted and unsubstituted acrylates, phthalic acids, valproic acid, salicylic acid, etc.) inhibit the mitochondrial carnitine acyltransferase I required for fatty acid oxidation. Depending on chain length, this inhibition of translocase activity results from the depletion of either the cytosolic or mitochondrial coenzyme A due to the formation of thioesters of CoA with the corresponding acid. We expect that many perfluoroalkyl acids and their derivatives are also capable of forming thioesters of coenzyme A, thereby inhibiting fatty acid oxidation. It is quite possible that this interference with fatty acid metabolism may be responsible for the well-characterized peroxisome proliferating effect reported for many of the perfluoroalkanes.

Accordingly, we propose to study the effect of perfluorooctane sulfonic acid and its sulfonamide derivatives on palmitic acid oxidation by intact rat liver mitochondria. We will attempt to distinguish whether any deficit is due to inhibition of acyl-CoA synthase or carnitine acyl-CoA translocase by varying the substrate for beta-oxidation between palmitic acid, palmitoyl CoA, and palmitoyl carnitine. We will also determine whether the perfluorocompounds deplete mitochondrial coenzyme A, suggesting the formation of a thioester. Furthermore, we can identify a specific effect on fatty acid oxidation by demonstrating an inhibition of mitochondrial respiration using palmitoyl carnitine as substrate, but not when either glutamate or succinate are substrates. This isolates the deficit to the acyl-CoA dehydrogenase flavoprotein, independent of effects on any other portion of the mitochondrial electron transport chain.

2. IDENTIFY POTENTIAL BIOMARKERS OF TOXIC TISSUE DAMAGE ASSOCIATED WITH *IN VIVO* EXPOSURE TO N-ETHYL PERFLUOROOCTANE SULFAMIDO ETHANOL

Identification of potential biomarkers of toxic tissue damage is based on a thorough understanding of the mechanisms of action of these agents as explored in Aim 1 of this proposal. Therefore, it isn't possible at this time to describe the precise details regarding which biomarkers to use to monitor *in vivo* exposure and toxic tissue damage. The ultimate experimental plan for identifying specific biomarkers is contingent on the outcome of the experiments listed in Aim 1. The results will provide a sound basis for discussing the most probable markers and for designing future experiments to address dose-response relationships in an attempt to define margins of safety in exposed populations.

Biomarkers of mitochondrial uncoupling and liberation of oxygen free radicals -

Although the expression of oxidative stress *in vitro* is very well characterized, attempts to identify a quantitative biomarker of tissue damage *in vivo* have met with limited success. One of the most promising markers that most consistently yields correlations to the extent of oxidative stress is the accumulation of 8-hydroxydeoxyguanosine (8OHdG) adducts to mitochondrial DNA (mtDNA), which can be measured in circulating peripheral lymphocytes. There is a large and growing body of evidence that demonstrates a progressive accumulation of 8OHdG adducts to

mtDNA with age [20-24], giving rise to the widely held theory of "mitochondrial aging" [23]. Furthermore, the rate of "mitochondrial aging" can be influenced by environmental exposures. We've recently demonstrated an organ-specific and preferential oxidation of cardiac mtDNA following acute exposures to adriamycin *in vivo*. Similar organotropic oxidations of mtDNA have been reported for many other genotoxic carcinogens. In fact, Takagi et al [24] report a 2-fold increase 8OHdG adducts of nuclear DNA (nDNA) in livers, but not kidneys, from rats treated acutely with perfluorooctanoic acid. Based on our experience, we expect that the 8OHdG adducts of mtDNA would have been 2-10 times higher had the mitochondria been examined.

For this aim, we will investigate whether exposure of rats *in vivo* to N-ethyl perfluorooctane sulfamido ethanol causes the preferential oxidation of mtDNA in liver, more so than in other organs which exhibit minimal organopathy. Rats will be injected intraperitoneally with varying doses of the perfluorocompound and killed at different times thereafter. The tissues will be harvested and the nDNA and mtDNA extracted from the corresponding cell fractions. 8OHdG adducts will be quantified by HPLC/EC and the results expressed as the ratio of mtDNA/nDNA adducts. We expect to observe a dose-dependent accumulation of 8OHdG adducts to mtDNA. Once we establish an effective single acute dose, we plan to repeat the dosing experiments on a sub-chronic basis, where the rats will receive multiple low daily doses. Subsets of animals will be killed at various dosing intervals and the total amount of DNA adducts expressed as a function of the "cumulative" dose. To complete the dosimetry experiments, we will characterize the persistence of the DNA adducts. Rats will be exposed to a cumulative dose of perfluorocompound that is known to cause a modest degree of DNA oxidation. They will then be allowed varying periods of exposure-free recovery prior to sacrifice and measuring DNA adducts. Being that mitochondria lack the proficient DNA repair enzymes found in the nucleus, we expect that the mtDNA adducts will persist for some time after dosing. Such a phenomenon will be of great advantage in providing a lasting measure of past and repeated exposure histories.

We also will screen for the induction of several early response genes that are known to be induced in response to mitogenic and/or oxidative stress, including the classic peroxisome proliferators [15-17]. These include GST Ya, metallothionein IIA, c-fos, NFκB, GADD153 and GADD45, p53, HSP70, and GRP78, all of which are available commercially (Xenometrix). Differential induction of these genes will reveal important insight into discrete modes of action, specifically distinguishing between oxidative stress, genotoxicity, and induction of cell proliferation.

types will be compared in terms of their sensitivity to chemical-induced depolarization of mitochondrial membrane potential, depletion of ATP, increased fatty acyl oxidase activity, induction of the early response genes and the peroxisome proliferator-activated receptor, and cell death. The comparisons between species will be both qualitative and quantitative.

The final objective will be to compare (again both qualitatively and quantitatively) the response of guinea pigs and rats exposed *in vivo* to N-ethyl perfluorooctane sulfamido ethanol exposure. The objective will be to gauge the appropriateness of the respective species as a representative surrogate for estimating potential human health risks. Using the biomarkers identified in Aim 2, we will compare and contrast the effects of *in vivo* exposure to varying doses on the accumulation of 8OHdG adducts to mtDNA, stimulation of early response genes, stimulation of fatty acyl CoA oxidase activity, and induction of the peroxisome proliferator-activated receptor gene. Dosing regimens will resemble those described for Aim 2 and the calculated effective doses compared between species. To complement this, we will also examine the tissues histologically for evidence of peroxisome proliferation in one or both species. It is these experiments that will determine the suitability of the rat as a representative surrogate species for regulatory purposes.

SUMMARY -

The ultimate goal of the proposed investigation is to provide a scientifically sound basis for managing the potential risks associated with human exposures to perfluoroalkyl acids and their derivatives. The existing evidence suggests that regulation on the basis of peroxisome proliferation and non-genotoxic carcinogenicity may be overly restrictive in that these responses are unique to rats and mice. There is little evidence to support this biological reactivity in guinea pigs, dogs, or primates, including humans. The proposed investigation is designed to provide a scientific basis to support or refute such a claim. The individual aims will walk the investigation through the succession of steps needed to identify valid biomarkers to make the appropriate surrogate species comparisons. As such, the investigation has the potential of having a profound impact on the regulation of these important products, not only in designing effective monitoring programs to insure worker safety, but also for setting guidelines to safeguard consumer health. The urgency for such information is highlighted by the fact that, 1) perfluoroalkyl acids and their derivatives are known to have the potential to be toxic, and 2) residues have been detected in the sera of production workers. The pressing question is: What is the margin of safety for these individuals? The proposed investigation will provide information critical to answering this question.

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ATTACHMENT A

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Biochemical and Molecular Mechanistic Studies of N-alkyl Perfluorosulfonamides

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The initial phase of this investigation revealed that all of the perfluorochemicals (PFC's) examined were capable of interfering with the bioenergetic properties of freshly isolated intact rat liver mitochondria *in vitro*. However, the precise mechanism by which each PFC interacted with the mitochondrial membranes differed, the activities ranging from a specific protonophoric uncoupling of oxidative phosphorylation to a full-blown detergent-like disorganization of the membranes. Regardless of the specific molecular mechanism, the results indicate that sufficiently high concentrations of any of the PFC's have the potential of inhibiting oxidative phosphorylation and ATP synthesis. It is presumed that at the cell or tissue level, the resulting deficit of ATP will be manifested as an interference with the regulation and integration of the various energy-dependent intermediary metabolic pathways, cell signaling, and cell cycle control points, including the balance between ATP-dependent apoptosis and cell proliferation.

At this point, all that has been established is that the PFC's have the potential to interfere with mitochondrial bioenergetics and presumably cell number and function, provided the concentration is sufficiently high. Whether such concentrations are achieved in realistic exposure scenarios has not yet been established. Accordingly, two pressing questions that remain are: 1) Is the inhibition of mitochondrial bioenergetics *in vitro* by the various PFC's manifested as a bioenergetic deficit and interference with metabolic

regulation at higher levels of biological organization, such as in cell cultures, intact tissues, or whole organisms? and 2) Are the nominally effective concentrations within the range to warrant concern for human exposures? Inherent in this latter question is the suitability of isolated rat liver mitochondria to predict adverse human health outcomes *in vivo*.

We propose to extend the investigation to address these questions by assessing the response of primary cultures of isolated hepatocytes to selected PFC's *in vitro* (Gray et al., 1983). The specific design is to assess the effects of exposing cells to individual PFC's on various indicators of cell bioenergetics, including changes in oxygen consumption, mitochondrial membrane potential, ATP phosphorylation potential, and cell viability. In addition, we intend to incorporate into these cell studies measures designed to assess the effects of PFC's on the expression of genes believed to be responsible for mediating the effects of related chemicals on peroxisome proliferation, lipid metabolism and cell proliferation. The entire suite of genes to be analyzed is believed to be under the control of the "peroxisome proliferator response elements" (PPRE) and include peroxisomal fatty acyl CoA oxidase, cytosolic fatty acid binding protein (FABP), microsomal CYP_{IVA}, mitochondrial HMG-CoA synthase, activating adipocyte protein (aP2), and apoproteins AI and CIII (Latruffe and Vamecq, 1997; Gonzalez, 1997). All of these proteins have been implicated to be responsible for the effects of peroxisome proliferators on altered lipid metabolism *in vivo* and may prove to be valuable markers of the biological response to PFC exposures in the corresponding species.

Finally, since there are strong arguments questioning the validity of data from rats and mice to predict the proliferation of peroxisomes and promotion of tumorigenesis in humans, we propose to include in the study design the opportunity to compare and contrast the metabolic and genetic response of rat and human hepatocyte cell cultures to PFC exposure *in vitro*. There is substantial evidence that exposures of non-human primates to related compounds does not induce the proliferation of peroxisomes, hepatomegaly, and tumorigenesis as is characteristic of rats and mice.

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Epidemiological evidence concurs with the suggestion that rats and mice are poor surrogates for the human response. Based on published experimental evidence, our original suggestion was that guinea pigs are more representative of the human response to exposure to peroxisome proliferators. We intend to test this by including primary cultures of guinea pig hepatocytes in the cell exposure experiments. The intent is to compare and contrast the response of rat, guinea pig and human hepatocytes to exposures to the individual PFC's. Comparisons will be quantitative and based on dose-related changes in bioenergetic and genetic indicators of a metabolic response to PFC exposure.

EXPERIMENTAL DESIGN

Isolated rat, guinea pig, and human hepatocytes will be purchased from a common vendor in order to minimize the possibility of artifactual differences among the different cell types due to cell isolation procedures. The cells will be suspended in Hepes-based minimum essential media at 37°C and distributed among wells at a density of approximately 10^6 cells/ml. Cells in suspension will be exposed to the individual PFC's for up to 24 hr. The concentration of each PFC will be varied in order to establish an effective range of concentrations for each end-point (bioenergetic or gene expression) for each PFC. This range of effective concentrations will serve as the basis for quantitative comparisons amongst the individual PFC'S and for assessing differences in sensitivities of the 3 species-specific cell types. The duration of exposure will be dictated by the kinetics of the response and its relationship to indications for gross changes in cell viability.

The bioenergetic parameters to be assessed during the course of the cell exposures include oxygen consumption, mitochondrial membrane potential, ATP phosphorylation potential, and cell viability. Whole cell oxygen consumption will be monitored in a manner similar to that employed for the

isolated mitochondria. Cells will be suspended in a stirred, closed chamber into which is inserted a Clark-type oxygen electrode metered to a continuous strip-chart recorder. The cell suspending medium will be supplemented with glucose and insulin to provide the requisite metabolic fuels. Once an equilibrium is established, sequential additions of the selected PFC will be made and any change in the rate of oxygen consumption recorded. In order to confirm and normalize any effect on mitochondrial respiration, all reactions will be terminated by first adding antimycin A followed by FCCP. Oxygen consumption in the presence of antimycin A will serve to reflect non-mitochondrial respiration, as one might expect if the agent in question undergoes cytochrome P450-dependent oxygenation or redox cycling, for example. The rate of oxygen consumption in the presence of the uncoupler, FCCP, will provide a measure of any direct effect of the individual PFC on the electron transport chain.

The effect of the individual PFC's on mitochondrial membrane potential of the cells in culture will be monitored continuously on the basis of the distribution of the cationic fluorometric dye, rhodamine 123 (Rh123) as we have published previously (Palmeira et al., 1996). Cells will be pre-loaded with Rh123, followed by washing, and then allocated to individual exposure wells. Again, increasing concentrations of the respective PFC will be added sequentially and the fluorescence recorded. The reactions will be terminated by adding FCCP to achieve a full depolarization of membrane potential, followed by digitonin to assure complete dissolution of cell membranes and release of non-specific latent fluorescence.

Cell viability will be recorded in parallel with mitochondrial membrane potential using the fluorometric indicator propidium iodide (PI) according to standard procedures in our laboratory (Palmeira et al., 1996). Sequential additions of the individual PFC's and termination of the reaction with FCCP and digitonin will be performed just as described for Rh123 fluorescence. Unfortunately, PI apparently quenches some of the Rh123 fluorescence, prohibiting the concurrent measurement of both membrane

potential and cell viability in the same cells. Instead, the experiments will be conducted in parallel using the cells from the same culture sample for both measures.

The concentrations of the various adenine nucleotides will be assessed by HPLC (Jones, 1981). Isolated hepatocytes from each of the 3 species will be exposed in culture to varying concentrations of the individual PFC'S. The exposures will be stopped by alkalinizing the reaction medium with KOH to stabilize the adenine nucleotides in the respective phosphorylation state. Samples from each exposure well will be centrifuged to remove debris, neutralized and injected onto a Waters Radial Pak C18 column. Individual nucleotides are eluted at 1 ml/min in 100 mM potassium phosphate (pH 6) followed by a gradient to 5% methanol. Individual nucleotides will be detected at 260 nm and quantified by integrating the peak area as compared to known standards.

Activation of PPAR-responsive genes will be assessed by differential display using standard molecular biology techniques (Wang and Morais, 1997). The method consists of exposing all 3 species of hepatocytes to the corresponding PFC. At pre-determined times, the reactions will be stopped in liquid nitrogen and analyzed for the enhanced expression of a select group of genes encoding for peroxisomal and mitochondrial proteins. This entails isolation of total RNA followed by gel separation and Northern hybridization for selected mRNA's. Probes have been prepared by PCR for the two "house-keeping" genes β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which are supposedly un-inducible and thus valid measures for gene copy number (cell number). The PFC-induced activation of all other genes will be normalized to the copy number for these two genes in order to quantitatively gauge the degree of stimulation of gene expression on the basis of total genomic DNA.

Exposure-related proliferation of peroxisomes will be estimated on the basis of the stimulated expression of the following genes: peroxisomal fatty

acyl CoA oxidase, cytosolic fatty acid binding protein (FABP), microsomal CYP_{IVA}, mitochondrial HMG-CoA synthase, activating adipocyte protein (aP2), and apoproteins AI and CIII (Latruffe and Vamecq, 1997; Gonzalez, 1997). Mitochondrial proliferation will be assessed on the basis of the enhanced expression of citrate synthase and succinate dehydrogenase (SDH). Amplification of the mitochondrial genome (mtDNA per mitochondrion as opposed to an increase in mitochondrial number per cell) will be assessed by an increase in gene copy number for mitochondrial transcript of cytochrome b (CYTb) and ND1 relative to SDH (a nuclear encoded gene) using gene dosing experiments (Boultwood et al., 1996). ^{32}P-labeled probes have been generated by PCR for all 3 genes. Increased expression of CYTb and ND1 relative to SDH has been used as an indicator of an increase in mtDNA copy number in cardiac tissue following acute intoxication of rats with adriamycin (unpublished observation).

The stimulation of both peroxisomal and mitochondrial proliferation is ascribed to altered transcriptional control. To verify this, we will substantiate our results of the gene dosing and differential display experiments by measuring the corresponding enzyme activities following each of the various cell treatment paradigms. The exposure incubations will be stopped and the cells recovered by centrifugation. Fatty acyl CoA oxidase activity will be estimated from the cyanide insensitive oxidation of palmitoyl CoA according to the spectrophotometric technique of Lazarow (1981). Succinate dehydrogenase (SDH), citrate synthase and cytochrome oxidase(COX) are measured by the corresponding spectrophotometric methods (Pennington, 1961; Trounce et al., 1996; Lash and Jones, 1993).

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SUMMARY

The proposed investigation is designed to take our current findings regarding the effects of PFC's on the bioenergetic properties of isolated mitochondrial membranes to the next level of biological integration. The objective is two-fold: to determine whether the effects of PFC's on isolated mitochondria is manifested as bioenergetic dysfunction at the level of whole cell and to compare and contrast the response of hepatocytes from different species to PFC exposure in culture. The metabolic and genetic studies will yield valuable understanding into the mechanism(s) of biological activity as well as reveal potentially important biomarkers for assessing PFC exposure *in vivo*. The comparisons among species will further test the question regarding the suitability of the rat as an experimental model for predicting adverse health outcomes in humans exposed to peroxisome proliferators and whether the guinea pig may prove to be a more suitable surrogate. These data are essential to extrapolating dose-response relationships from experimental evidence to predict no effect levels and margins of safety for potential human exposures, especially in light of our preliminary evidence which indicates that isolated hepatic mitochondria from guinea pigs is 3-6 times more resistant to PFC-induced interference with bioenergetics than are rat liver mitochondria (unpublished observations).

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Effect of N-Alkyl Perfluorooctylsulfonamides on Mitochondrial Bioenergetics In Vitro

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- Executive Summary -

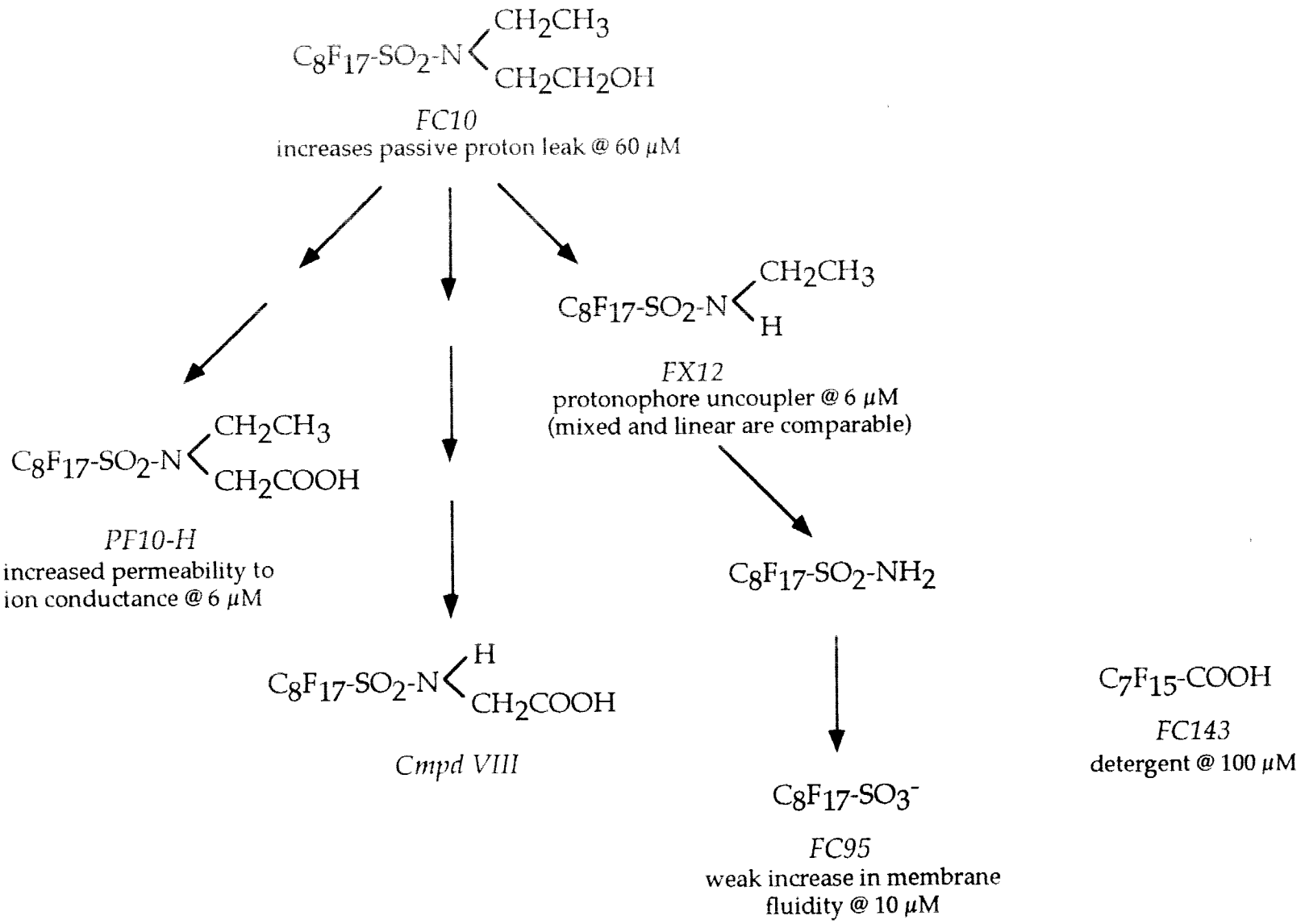
The following report summarizes research conducted the past 7 months concerning the effects of 6 different perfluorooctanonyl compounds (PFC's; supplied by The 3M Company) on the bioenergetic characteristics of isolated rat liver mitochondria *in vitro*. For purposes of confidentiality, the trade names and chemical structures were not disclosed to those conducting the research and analyzing the results. Consequently, the identities of the individual compounds are codified throughout the report. They are:

PF10;	FC10
PF10H;	the carboxylic acid of FC10
PF12L;	linear FX12
PF12M;	mixed FX12
PF95;	FC95
PF143;	FC143

[Results for the sulfonamide and the N-acetic acid of the sulfonamide are not summarized in this report.]

All six PFC's examined to date exhibited some effect on mitochondrial bioenergetics. FC10 increased the passive proton leak, resulting in a stimulation of state 4 (non-phosphorylating) respiration without uncoupling oxidative phosphorylation. The acid of FC10 increased membrane permeability to ion conductance, resulting in membrane depolarization and release of cytochrome c to inhibit respiration. Both linear and mixed FX12 were potent protonophore uncouplers of oxidative phosphorylation. Both FC95 and FC143 exhibited a general detergent-like effect on mitochondrial membranes as reflected by a decrease in respiratory control without uncoupling oxidative phosphorylation.

In conclusion, although all PFC's interfered with mitochondrial bioenergetics, they differed in both potency and mechanism. Regardless, the ultimate effect is the same; inhibition of ATP synthesis. At the cellular level this may well manifest itself as failure of assorted energy-dependent metabolic or cell signaling pathways leading to disruption of metabolic regulation and cell cycle control. Whether and by what mechanism this may relate to the alleged peroxisome proliferating activity and tumorigenic activity of these compounds remains to be defined.



(~ 4.5 ppm)

log P Anion - correlates to ox. Phosphorylation Uncoupling.