

Mechanistic

- 1) Reports from University of Minnesota Duluth Research (Kendall Wallace):
 - a) Kendall B. Wallace, Biochemical and Molecular Mechanistic Studies of N-Alkyl Perfluorosulfonamides, Research Proposal, April 8, 1997, and Updated Proposal May 7, 1998
 - b) Kendall B. Wallace and Anatoli Starkov, The Effect of Perfluorinated Arylalkylsulfonamides on Bioenergetics of Rat Liver Mitochondria, Feb. 4, 1998
 - c) Report on Covance Studies, assessment of mitochondrial bioenergetics, undated
 - d) Summary of the Effects of PFC's [Perfluorinated Compounds] on Mitochondrial Bioenergetics In Vitro, undated
 - e) Report, Effects of Selected Perfluoro-compounds on Mitochondrial Beta-Oxidation, Dec. 20, 1999
 - f) Report, Effect of Acute FC Administration on Catalase and acylCoA Oxidase Expression, January 27, 2000
- 2) Nabbefeld, et al., Displacement of a Fluorescently Labeled Fatty Acid Analogue from Fatty Acid Carrier Proteins by Wyeth - 14,643, Ammonium Perfluorooctanoate, Potassium Perfluorooctane Sulfonate and Other Known Peroxisome Proliferators, Abstract, Society of Toxicology, 1998 Annual Meeting

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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

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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.

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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.

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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. Haugthom 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

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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.

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• **EXPERIMENTAL APPROACH**

1. DETERMINE WHETHER N-ETHYL PERFLUOROOCTANE SULFAMIDO ACETATE, PERFLUOROOCTANE SULFONAMIDE, AND/OR PERFLUOROOCTANE 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.

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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

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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.

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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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