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Inhalation Toxicity Study of a Haloalkene Degradant of Sevoflurane, Compound A (PIFE), in Sprague-Dawley Rats

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Background: **Under certain circumstances in the clinical setting, contact of the anesthetic sevoflurane with a CO_2 absorbant (*e.g.*, soda lime, Baralyme) leads to the formation of a degradant designated as pentafluoroisopropenyl fluoromethyl ether (PIFE; Compound A). Previous studies have shown that the kidney is the primary target organ for toxicity in the rat. This study was designed to determine the impact of PIFE on rat renal histology correlated with functional changes. The findings are discussed in terms of probable mechanism of action and relevance to humans.**

Methods: **Male and female Sprague-Dawley rats were exposed to 0, 30, 61, 114, or 202 ppm PIFE for a single 3-h period *via* nose-only inhalation. Rats were observed daily for behavioral changes or external physical signs of toxicity (*i.e.*, lacrimation, dyspnea, piloerection, *etc.*) and body weights were recorded at 6, 4, and 1 day preexposure and 1, 3, 7, and 13 days postexposure. Animals were evaluated for hematologic, clinical chemistry and/or urinalysis changes immediately postexposure and/or at 1, 4, and 14 days postexposure. Rats were killed, subjected to a macroscopic postmortem examination, and evaluated for histopathologic changes in all major tissues and organs at 1, 4, and 14 days postexposure.**

Results: **Labored breathing was observed in 3 of the 20 and 2 of the 20 rats in the 114 ppm and 202 ppm groups, respectively, during the 3-h exposure period. No significant reductions in body weight gain were noted during the 2-week study period. Clinical chemistry evaluations revealed increases in blood urea nitrogen and creatinine 1 day postexposure in males and females exposed to 202 ppm PIFE. Changes in urinary glucose, protein and N-acetyl-β-glucoaminidase/creatinine were evident one day postexposure in males and females exposed to 202 ppm and in males exposed to 114 ppm PIFE. Most values were within normal ranges by 4 or 14 days postexposure. No drug-related alterations in hematolologic parameters were noted. Evidence of olfactory epithelial degeneration and desquamation in the nasal turbinates was noted at 4 days postexposure in male and female rats exposed to 202 ppm PIFE. Concentration-dependent renal tubular necrosis and tubular cell hyperplasia, in the corticomedullary border, were observed in males and females exposed to 114 and 202 ppm PIFE. The severity of tubular necrosis in both males and females was considered minimal to slight at the 114 ppm exposure concentration and slight to moderate at the 202 ppm exposure. Both the numbers of affected animals and severity were reduced over time. The most marked changes in serum and urine chemistry were associated with the animals described as having moderate renal necrosis. Male rats appeared more susceptible to nephropathy than female rats. There were no other PIFE-related histopathologic findings.**

Conclusions: **The renal histopathologic findings in this study are consistent with those reported in previous acute studies in rats after PIFE administration. Functional changes in the kidney, as evidenced by serum chemistry and urinalyses, were observed at exposure concentrations that induced morphologic alterations. (Key words: Anesthetics: sevoflurane. Degradants: Compound A; PIFE. Haloalkenes: nephrotoxicity.)**

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SEVOFLURANE is a highly fluorinated derivative of methyl isopropyl ether and has been marketed in Japan as an inhalational anesthetic since early 1990. In the clinical setting, contact of sevoflurane with CO_2 absorbants (*e.g.*, soda lime, Baralyme, Allied Healthcare Products, Inc., St. Louis, MO) under certain circum-

stances leads to the formation of low concentrations of a degradant designated as pentafluoroisopropenyl fluoromethyl ether (PIFE; Compound A). The interaction of halogenated anesthetics with CO_2 absorbants is not unique to sevoflurane and, as shown with halothane, most likely is the result of extraction of the acidic proton by a strong base (KOH and/or NaOH) followed by loss of halide to form an alkene.[1]

In one previous study, the LC_{50} of PIFE in Wistar rats after a 3-h exposure in a closed whole-body inhalation system was calculated to be in the range of 350 to 490 ppm for males and 340 to 460 ppm for females.[2] Tubular necrosis was observed in animals dying 4 days after exposure (2/12 at 340 ppm), accompanied by an increase in urinary ketone bodies and glucose in their urine. No changes in renal morphology were noted in the rats surviving the 2-week observation period. In another series of acute whole-body inhalation studies, Gonsowski *et al.*[3,4] calculated a 3-h LC_{50} of 331 and found 50 ppm as the threshold for induction of morphologic change in the kidneys of male Wistar rats. However, only minimal changes were observed at this concentration, no female rats were studied, and no testing was done to evaluate possible functional changes.

The purpose of this study was to evaluate the potential toxicity of PIFE in male and female rats after acute nose-only inhalation exposure and to determine the impact of PIFE on rat renal histology correlated with functional changes. This study was conducted in accordance with the United States Food and Drug Administration Principles of Good Laboratory Practices set forth in 21 CFR Part 58 and with the requirements and recommendations of the Animal Welfare Act regulations: 9 CFR Parts 1, 2, and 3/ Animal Welfare Standards: 9 CFR Part 3.

Materials and Methods

Test Material

Pentafluoroisopropenyl fluoromethyl ether was obtained from Central Glass (Ube City, Japan; lot number 930906; 99.84% pure with 500 ppm butylated hydroxytoluene as a preservative). Their synthetic process used a similar pathway to the mechanism responsible for the generation of PIFE in closed anesthetic conditions (*i.e.*, a strong base such as potassium hydroxide or sodium hydroxide extracts the acidic proton followed by loss of fluoride to generate PIFE).

Test Animals

Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) aged approximately 6 weeks (weighing between 181 and 248 g for males and 129 and 177 g for females) at initiation of exposure were used in this study because of the availability of historical control data on hematology, clinical chemistry, urinalysis, and pathologic parameters for this species and strain, as well as their acceptance in pharmaceutical safety evaluation. The rats were acclimated to environmental room conditions for 2 weeks before the start of the study. Animals were housed individually in hanging stainless steel wire mesh cages. Room temperature and humidity were maintained at 21 ± 1°C and 50 ± 9%, respectively, with a 12-h daily light:dark cycle. Food (Purina Certified Rodent Diet #5002, PMI Feeds, Inc., St. Louis, MO) was available *ad libitum* except during the 3-h exposure period and when rats were fasted overnight (approximately 5 PM–8 AM) before blood sampling. Rats were also allowed water *ad libitum,* except during the 3-h exposure period and the 4-h urine collection period. Animals were randomly assigned to treatment groups and individually identified by ear tag.

Exposure Apparatus

The nose-only exposure apparatus (ADG Instruments, Ltd., Condicote, England) used in the current study is a system validated for consistent vapor generation and environmental conditions[5] and used previously on multiple GLP (good laboratory practices) studies. This system allowed for greater control of variables (*e.g.*, vapor distribution, temperature, and CO_2 levels without the use of a CO_2 absorber) than those systems used in previous studies of PIFE.[2–4]

On the day of exposure, rats were placed head first in polycarbonate tubes that were arranged around a central exposure cylinder with their snouts projecting into the main cylinder. Rats were exposed for a single 3-h period to target concentrations of 0, 25, 50, 100, and 200 ppm PIFE. The chamber had a total volume of 40 l and was operated at a calibrated airflow of 10 l/min. The nose-only exposure chambers, in turn, were contained within a 10 m^3 stainless steel and glass chamber. Recordings of chamber temperature, relative humidity, airflow rate, and static pressure were made every 30 min during exposure. Vapors were generated by bubbling nitrogen gas through the PIFE in a glass impinger. The resultant PIFE-laden nitrogen gas was mixed with the carrier gas (40% oxygen/60% nitrogen)

and delivered to a side portal of the nose-only exposure chamber. The delivery rates of PIFE were adjusted by varying the flow rates of nitrogen gas through the impingers. The initial flow rates ranged from 3 to 16.5 standard cm^3/min. The animals remained in the chamber for at least 30 min after exposure to exhaust the chamber with 40% oxygen/60% nitrogen gas delivered at the same flow rate used during exposure.

Concentrations of PIFE within the chamber were measured using a Gow Mac model 3396A gas chromatograph (Bridgewater, NJ) equipped with a flame ionization detector. Chamber atmosphere was drawn into a gas sampling valve inside the gas chromatograph via a vacuum pump and injected directly onto the analytic column for analysis. Pentafluoroisopropenyl fluoromethyl ether concentrations within the chamber were determined three times during exposure (at 15–20 min, at 75–80 min, and 135–140 min into the 3-h exposure period). Chamber distribution analyses were performed during trial exposures before the initiation of the study. The lowest limit of quantitation for this method was 11.6 ppm PIFE.

Study Design

On the day of exposure, all animals were observed for gross behavioral changes and external physical signs of toxicity (*i.e.*, lacrimation, dyspnea, piloerection, *etc.*) immediately before exposure, at approximately 15-min intervals during the first hour of exposure and hourly for the remainder of the exposure period, upon removal from the exposure apparatus, and at 1 and 2 h postexposure. Detailed behavioral/physical observations were recorded once daily thereafter. Body weights were recorded at 6, 4, and 1 day preexposure and 1, 3, 7, and 13 days postexposure, and at termination.

Groups of five males and five females were evaluated for hematology, clinical chemistry, and urinalysis parameters immediately postexposure and/or at 1, 4, or 14 days postexposure as outlined in table 1. Animals were fasted overnight (~5 pm to 4 am) before urine and blood collection, except those animals scheduled for blood collection only immediately postexposure. Urine samples were limited to collection during a 4-h period (approximately 4 AM–8 AM) to avoid confounding water deprivation factors. After urine collection, blood was obtained (at between approximately 8 AM–9 AM) from the lightly anesthetized (60% CO_2/40%O_2) animals by venipuncture of the orbital plexus (retrobulbar) for hematology and clinical chemistry.

At each time point (1, 4, and 14 days postexposure) after blood collection the rats were killed by exsanguination under CO_2/O_2 anesthesia (at between approximately 9 AM–10 AM), subjected to a macroscopic postmortem examination and evaluated for histopathologic changes in all major tissues and organs, including the kidney, liver, brain, heart, lung, and nasal turbinates. Although tissues were evaluated on a nonblinded basis, all histopathologic findings were confirmed by a second independent pathologist. Animals used for blood collection immediately postexposure did not undergo necropsy. Tissues for microscopic examination were fixed in 10% neutral buffered formalin, decalcified in 10% formic acid (nasal turbinates only), embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Most histopathologic findings were graded as to severity according to a visual estimation of the percentage of tissue section involved as follows: minimal (up to 2%); slight (2–10%); moderate (10–30%); moderately severe (30–70%); and severe (over 70%).

Data Analysis

Statistical comparisons were made between the control and the four exposure groups at each time period and also between the same exposure group over time. Statistical evaluation of equality of means was made by an appropriate one-way analysis of variance, followed by testing for equal variance (Bartlett's test). If the variances were equal, parametric analysis using one-way analysis of variance with the F distribution to assess significance was conducted. If significant differences among the means were indicated, Dunnett's test was used to determine which means were significantly different from control. If the variances were unequal, nonparametric analysis using the Kruskal-Wallis test was conducted, followed by a summed rank test (Dunn) to determine which means were significantly different from control. A statistical test for trend in the doses was also performed, either with the parametric standard regression technique or nonparametric Jonckheere's test for monotonic trend. Bartlett's test was conducted at the 1% two-sided risk level. All other statistical tests were conducted at the 5% and 1%, two-sided risk levels.

Results

Exposure Concentrations

Measured exposure concentrations were 0, 30 ± 16, 61 ± 16, 114 ± 15, and 202 ± 61 ppm (mean ± SD)

Table 1. Study Design

Exposure (ppm)	No. of Rats		Clinical Pathology*				Pathology			
	M	F	A†	B	C	D	A	B	C	D
Control	20	20	5	5	5	5		5	5	5
30	15	15	5	5	5			5	5	
61	15	15	5	5	5			5	5	
114	20	20	5	5	5	5		5	5	5
202	20	20	5	5	5	5		5	5	5

A = immediately postexposure; B = 1 day postexposure; C = 4 days postexposure; D = 14 days postexposure.

* Includes hematology, clinical chemistry, and urinalysis.

† Hematology and clinical chemistry only.

in the 0, 25, 50, 100, and 200 ppm target groups, respectively. The mean temperature and humidity within the exposure chambers were maintained at 22 ± 1°C and 30 ± 10%, respectively, during the 3-h exposure period.

General Observations

All animals survived to scheduled termination. During the 3-h exposure period 3 of the 40 and 2 of the 40 rats in the 114 and 202 ppm exposure groups demonstrated labored breathing. In the early postexposure hours (1–2 h postexposure) after removal from the chamber, exposed rats, including control animals, displayed chromodacryorrhea, nasal discharge, anogenital staining, and matted coat. During the 14-day postexposure observation period, there were no PIFE-related gross behavioral changes or external physical signs of toxicity. No significant changes in body weights or body weight gains were observed in comparison to control values at any exposure concentration during the course of the study (data not shown).

Clinical Pathology

Hematology. No PIFE-related alterations in hematologic or coagulation parameters (hemoglobin, hematocrit, erythrocyte, and leukocyte counts, reticulocytes, platelets, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, prothrombin time, activated partial thromboplastin time), were noted in either male or female exposed rats (data not shown).

Clinical Chemistry (tables 2 and 3). Increases in mean blood urea nitrogen (BUN) and creatinine levels were observed in male ($p \leq 0.01$) and female rats exposed to the highest concentration (202 ppm) of PIFE in comparison to control values at 1 day postexposure. All 202 ppm exposed male rats killed at 1 day postexposure were observed to have moderate renal necrosis (see later). The BUN values range from 13.5 mg/dl to 23.4 mg/dl in these 202 ppm exposed male rats compared to 9.0 mg/dl to 13.6 mg/dl in control males. The creatinine values ranged from 0.6 to 0.9 mg/dl in 202 ppm exposed male rats to 0.4 to 0.5 mg/dl in control males. For the five 202 ppm exposed females killed at 1 day postexposure, one was described as having minimal renal necrosis, one slight necrosis, and the remaining three moderate renal necrosis. No increase in BUN or creatinine level was noted in the female with minimal necrosis (11.4 and 0.5 mg/dl, respectively). A small increase in BUN and creatinine level was noted in the female described as having slight renal necrosis (17.7 and 0.6 *vs.* mean control values of 11.2 and 0.4 mg/dl, respectively). However, the three 202 ppm exposed females described as having moderate renal necrosis showed more marked increases in BUN (range in 202 ppm exposed: 0.7 to 1.6 mg/dl *vs* control range of 0.4 to 0.5 mg/dl). A small increase in mean BUN was also noted in the 202 ppm exposed females ($p \leq 0.05$) immediately postexposure with values ranging from 14.4 to 28.4 mg/dl compared to a control range of 13.6 to 17.0 mg/dl. These increases in BUN and creatinine levels appeared to be transient and all rats sampled at 4 and 14 days postexposure showed values within the control range, with the exception of one male exposed to 202 ppm displaying a slightly elevated (0.8 mg/dl) creatinine level and one female exposed to 202 ppm with a slightly elevated BUN level (25.8 mg/dl) at 14 days postexposure.

All other measured clinical chemistry parameters, including serum potassium, chloride, calcium, phosphorus, sodium, albumin, globulin, and bilirubin, as well as serum enzyme activities of aspartate amino-

Table 2. Serum Chemistry and Urinalysis Values in Male Rats

Concentration	Study Day	Control	30 ppm	61 ppm	114 ppm	202 ppm
Serum blood urea nitrogen (mg/dl)	A	12.2 ± 0.7 (5)	14.9 ± 2.6 (5)	12.5 ± 1.1 (5)	14.2 ± 2.7 (5)	14.5 ± 1.1 (5)
	B	12.3 ± 2.1 (5)	11.7 ± 1.5 (5)	10.4 ± 2.2 (5)	9.8 ± 1.6 (5)	18.6 ± 4.8 (5)*
	C	12.6 ± 5.1 (5)	11.4 ± 1.4 (5)	11.3 ± 1.1 (5)	12.8 ± 1.2 (5)	11.5 ± 1.8 (5)
	D	14.7 ± 1.6 (5)	ND	ND	14.1 ± 0.9 (5)	14.6 ± 2.7 (5)
Serum creatinine (mg/dl)	A	0.5 ± 0.1 (5)	0.4 ± 0.0 (5)	0.5 ± 0.1 (5)	0.4 ± 0.1 (5)	0.4 ± 0.0 (5)
	B	0.4 ± 0.0 (5)	0.4 ± 0.1 (5)	0.4 ± 0.0 (5)	0.4 ± 0.0 (5)	0.7 ± 0.1 (5)*
	C	0.5 ± 0.1 (5)	0.4 ± 0.1 (5)	0.4 ± 0.1 (5)	0.4 ± 0.0 (5)	0.4 ± 0.1 (5)
	D	0.6 ± 0.1 (5)	ND	ND	0.5 ± 0.1 (5)	0.5 ± 0.2 (5)
Serum glucose (mg/dl)	A	166 ± 17 (5)	170 ± 31 (5)	154 ± 7 (5)	162 ± 3 (5)	170 ± 12 (5)
	B	114 ± 16 (5)	102 ± 6 (5)	104 ± 5 (5)	83 ± 7 (5)*	126 ± 21 (5)
	C	122 ± 16 (5)	126 ± 24 (5)	122 ± 15 (5)	112 ± 20 (5)	101 ± 8 (5)
	D	111 ± 21 (5)	ND	ND	100 ± 7 (5)	102 ± 8 (5)
Serum protein (g/dl)	A	5.5 ± 0.4 (5)	5.3 ± 0.3 (5)	5.7 ± 0.4 (5)	5.4 ± 0.2 (5)	5.5 ± 0.2 (5)
	B	6.0 ± 0.4 (5)	5.5 ± 0.2 (5)	5.6 ± 0.3 (5)	5.5 ± 0.4 (5)†	5.4 ± 0.4 (5)†
	C	5.6 ± 0.4 (5)	5.1 ± 0.3 (5)	5.6 ± 0.6 (5)	5.3 ± 0.2 (5)	5.4 ± 0.4 (5)
	D	5.7 ± 0.3 (5)	ND	ND	5.8 ± 0.3 (5)	5.7 ± 0.3 (5)
Urine NAG (U/L)	B	14.6 (1)	16.3 ± 6.3 (4)	16.7 ± 5.6 (2)	24.8 ± 13.8 (2)	23.4 ± 8.7 (5)
	C	19.7 ± 11.4 (4)	13.3 ± 4.5 (3)	10.2 (1)	13.7 ± 2.9 (2)	20.5 ± 3.1 (2)
	D	25.3 ± 6.2 (4)	ND	ND	23.8 ± 9.4 (3)	22.2 ± 11.0 (2)
Urine creatinine (mg/dl)	B	45 (1)	43 ± 6 (2)	37 (1)	23 (1)	23 ± 10 (4)
	C	54 ± 35 (4)	36 ± 12 (3)	29 (1)	34 ± 2 (2)	52 ± 3 (2)
	D	92 ± 19 (4)	ND	ND	68 ± 24 (3)	74 ± 7 (2)
Urine NAG/ creatinine ratio	B	0.3 (1)	0.5 ± 0.2 (3)	0.3 (1)	0.6 (1)	1.1 ± 0.3 (4)
	C	0.4 ± 0.1 (4)	0.4 ± 0.0 (3)	0.4 (1)	0.4 ± 0.0 (2)	0.4 ± 0.0 (2)
	D	0.3 ± 0.1 (4)	ND	ND	0.4 ± 0.1 (3)	0.3 ± 0.1 (2)
Urine protein (mg/dl)	B	17 (1)	15 ± 9 (4)	14 ± 6 (2)	49 ± 16 (2)	204 ± 69 (5)
	C	37 ± 43 (4)	23 ± 6 (3)	15 (1)	16 ± 2 (2)	19 ± 5 (2)
	D	87 ± 30 (4)	ND	ND	69 ± 19 (3)	52 ± 8 (2)
Urine glucose (mg/dl)	B	9 (1)	8 ± 4 (4)	9 ± 3 (2)	72 ± 35 (2)	804 ± 543 (5)
	C	9 ± 4 (4)	8 ± 2 (3)	6 (1)	7 ± 0 (2)	9 ± 1 (2)
	D	13 ± 3 (4)	ND	ND	11 ± 2 (3)	14 ± 7 (2)

Values are mean ± SD (n values in parentheses).

A = immediately postexposure; B = 1 day postexposure; C = 4 days postexposure; D = 14 days postexposure; ND = not determined; NAG = N-acetylglucosamidase.

* Statistically significant from control value ($P \leq 0.01$).

† Statistically significant from control value ($P \leq 0.05$).

transferase, alanine aminotransferase, alkaline phosphatase, and gamma glutamyl transpeptidase, were within the range of control values and showed no statistically significant increases (data not shown).

Urinalysis. In some instances, inadequate amounts of urine were available from animals to perform all the desired analyses. This does not appear to be a treatment effect because this occurred in both the control and treated groups at an equal incidence rate. This is further confirmed in that urine samples from the most severely affected rats (those with renal necrosis ratings of moderate) were obtainable.

Urinalysis revealed a concentration-dependent increase in urine glucose 1 day postexposure in male rats exposed to 114 and 202 ppm PIFE. The measured values were 47 mg/dl (in a male with minimal renal necrosis) to 96 mg/dl (in a male with slight renal necrosis) and 188 to 1,680 mg/dl (in 5 males with moderate renal necrosis), in the 114 and 202 ppm exposed males, respectively, compared to 9 mg/dl in the 4 control animals. A marked increase in urine glucose was also noted in 202 ppm exposed female rats at 1 day postexposure (no urine samples were obtained from 114 ppm exposed females). Three females described as having moderate renal necrosis displayed urine glucose values between 336 mg/dl and 2048 mg/dl, compared to a control range of 11–18 mg/dl. No PIFE-exposed rats displayed elevated urine glucose values

Table 3. Serum Chemistry and Urinalysis Values in Female Rats—Mean ± SD (N)

Concentration	Study Day	Control	30 ppm	61 ppm	114 ppm	202 ppm
Serum blood urea nitrogen (mg/dl)	A	13.1 ± 2.4 (5)	12.9 ± 3.5 (5)	12.9 ± 3.5 (5)	14.0 ± 2.5 (5)	19.7 ± 5.6 (5)*
	B	11.2 ± 1.8 (5)	14.1 ± 4.3 (5)	12.1 ± 1.8 (5)	10.4 ± 1.5 (5)	31.1 ± 20.8 (5)*
	C	12.2 ± 2.6 (5)	11.5 ± 2.2 (5)	12.3 ± 1.7 (5)	13.4 ± 1.5 (5)	14.1 ± 2.7 (5)
	D	14.1 ± 2.5 (4)	ND	ND	14.6 ± 2.5 (5)	16.2 ± 5.5 (5)
Serum creatinine (mg/dl)	A	0.4 ± 0.0 (5)	0.4 ± 0.0 (5)	0.4 ± 0.0 (5)	0.4 ± 0.0 (5)	0.6 ± 0.2 (5)
	B	0.4 ± 0.1 (3)	0.4 ± 0.0 (3)	0.4 ± 0.0 (5)	0.4 ± 0.0 (5)	0.9 ± 0.4 (5)*
	C	0.4 ± 0.1 (5)	0.4 ± 0.0 (5)	0.4 ± 0.1 (5)	0.4 ± 0.0 (5)	0.4 ± 0.1 (5)
	D	0.5 ± 0.0 (4)	ND	ND	0.5 ± 0.0 (5)	0.5 ± 0.0 (5)
Serum glucose (mg/dl)	A	157 ± 12 (5)	162 ± 14 (5)	158 ± 25 (5)	155 ± 11 (5)	156 ± 14 (5)
	B	98 ± 14 (5)	91 ± 26 (5)	89 ± 14 (5)	79 ± 9 (5)	101 ± 27 (5)
	C	103 ± 9 (5)	107 ± 14 (5)	93 ± 18 (5)	101 ± 8 (5)	111 ± 8 (5)
	D	121 ± 12 (4)	ND	ND	112 ± 11 (5)	106 ± 11 (5)
Serum protein (g/dl)	A	5.4 ± 0.3 (5)	5.4 ± 0.2 (5)	5.6 ± 0.4 (5)	5.3 ± 0.2 (5)	5.7 ± 0.2 (5)
	B	5.3 ± 0.2 (3)	5.5 ± 0.4 (4)	5.2 ± 0.3 (5)	5.2 ± 0.4 (5)	5.1 ± 0.3 (5)
	C	5.4 ± 0.2 (5)	5.4 ± 0.2 (5)	5.3 ± 0.4 (5)	5.7 ± 0.3 (5)	5.5 ± 0.3 (5)
	D	5.6 ± 0.4 (4)	ND	ND	5.8 ± 0.3 (4)	5.9 ± 0.2 (5)
Urine NAG (U/L)	B	22.5 ± 2.8 (2)	13.2 (1)	NA	NA	20.0 ± 10.8 (3)
	C	25.3 ± 7.0 (2)	19.1 ± 0.2 (2)	15.8 ± 13.4 (2)	NA	23.8 ± 4.9 (2)
	D	10.0 ± 4.0 (2)	ND	ND	21.7 (1)	26.6 (1)
Urine creatinine (mg/dl)	B	38 (1)	22 (1)	NA	NA	22 ± 12 (3)
	C	58 (1)	42 ± 4 (2)	42 ± 31 (2)	NA	51 ± 16 (2)
	D	41 ± 7 (2)	ND	ND	101 (1)	62 (1)
Urine NAG/ creatinine ratio	B	0.5 (1)	0.6 (1)	NA	NA	0.9 ± 0.1 (3)
	C	0.5 (1)	0.5 ± 0.1 (2)	0.4 ± 0.1 (2)	NA	0.5 ± 0.1 (2)
	D	0.3 ± 0.1 (2)	ND	ND	0.2 (1)	0.4 (1)
Urine protein (mg/dl)	B	22 ± 3 (2)	12 (1)	NA	NA	153 ± 85 (3)
	C	23 ± 7 (2)	19 ± 3 (2)	21 ± 9 (2)	NA	25 ± 8 (2)
	D	16 ± 5 (2)	ND	ND	30 (1)	32 (1)
Urine glucose (mg/dl)	B	15 ± 5 (2)	5 (1)	NA	NA	1300 ± 876 (3)
	C	13 ± 1 (2)	9 ± 1 (2)	11 ± 5 (2)	NA	11 ± 1 (2)
	D	7 ± 1 (2)	ND	ND	11 (1)	11 (1)

Values are mean ± SD (n values in parentheses).

A = immediately postexposure; B = 1 day postexposure; C = 4 days postexposure; D = 14 days postexposure; ND = not determined; NAG = N-acetylglucosamidase.

* Statistically significant from control value ($P \leq 0.05$).

at 4 or 14 days postexposure. Urine protein values ranged from 8 to 100 mg/dl in all control animals and the only increase (130–316 mg/dl) above this range was seen in five of five 202 ppm exposed males and two of three 202 exposed female rats at 1 day postexposure who were described as having moderate renal necrosis. If one calculates a protein/creatinine ratio for these animals, a ratio consistently above 1 confirms the presence of renal tubular injury. An increase in the N-acetyl-β-glucoaminidase (NAG)/creatinine ratio was also noted at the same time period in these animals (0.7–1.4 *vs.* 0.2–0.4 in controls) and further supports this. Whereas none of these group mean increases were statistically different from control means the individual animal data provides evidence of impaired renal tubule function. All of these changes were transient and all rats displayed values within the control range by 4 and 14 days postexposure.

Urine sodium and potassium concentrations were within the range of control values and showed no statistically significant increases (data not shown).

Histopathology

Histologic evidence of olfactory epithelial degeneration and desquamation in the nasal turbinates was noted in the male and female rats exposed to 202 ppm PIFE. Desquamation was used to describe the presence of sloughed epithelial cells in the nasal lumen from

Table 4. Incidence and Severity of Renal Necrosis

Severity	Control	30 ppm	61 ppm	114 ppm	202 ppm
Minimal (up to 2% affected tubule cells)					
Male	0	0	0	1/15	3/15
Female	0	0	0	0	2/15
Slight (2–10% affected tubule cells)					
Male	0	0	0	9/15	4/15
Female	0	0	0	2/15	6/15
Moderate (10–30% affected tubule cells)					
Male	0	0	0	0	5/15
Female	0	0	0	0	4/15
Total					
Male	0/15	0/10	0/10	10/15	12/15
Female	0/15	0/10	0/10	2/15	12/15

the animals killed at 1 day postexposure. In the animals examined at 4 and 14 days postexposure, some squamous/squamoid epithelium was present, indicating that there had been loss and replacement of some of the layers of olfactory epithelium.

Renal tubular necrosis in the region of the corticomedullary junction was observed in male and female rats exposed to 114 and 202 ppm PIFE as summarized in table 4. The severity of tubular necrosis in both males and females was considered minimal (up to ~2% affected tubule cells) to slight (~2–10% affected tubule cells) except at the highest exposure concentration (202 ppm) at 1 day postexposure where severity was graded as moderate (~10–30% affected tubule cells) in 9 of 30 rats. Both the numbers of affected animals and the severity were reduced over time (figs. 1 and 2). In the 114 ppm exposed male and female rats there was no evidence of renal injury in animals killed 14 days postexposure. At the highest exposure concentration (202 ppm), 2 of 5 males and 3 of 5 females were described as having only minimal renal injury (up to 2% affected tubule cells) at 14 days postexposure. Males appeared to be more susceptible to renal injury than females. Minimal to slight renal tubular epithelial hyperplasia, indicative of a reparative process, was also observed and was most marked in animals evaluated at 4 days postexposure.

Other microscopic findings appeared either sporadically or with similar incidence in the PIFE and control groups, and did not appear to be related to treatment.

Discussion

This study supports the findings of previous studies in rats associating acute PIFE exposure with renal in

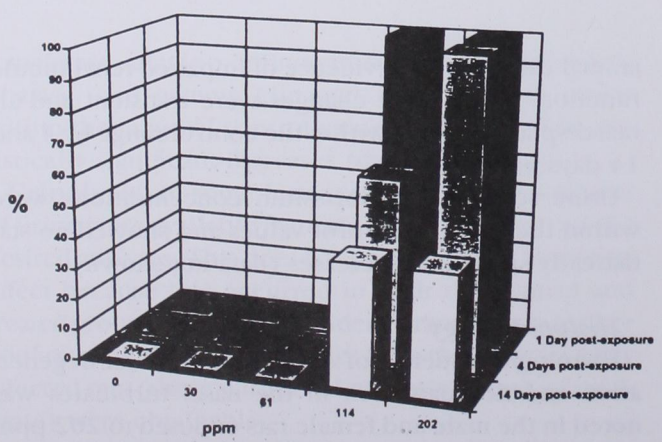

Fig. 1. Incidence of renal necrosis (% of animals) over time—males.

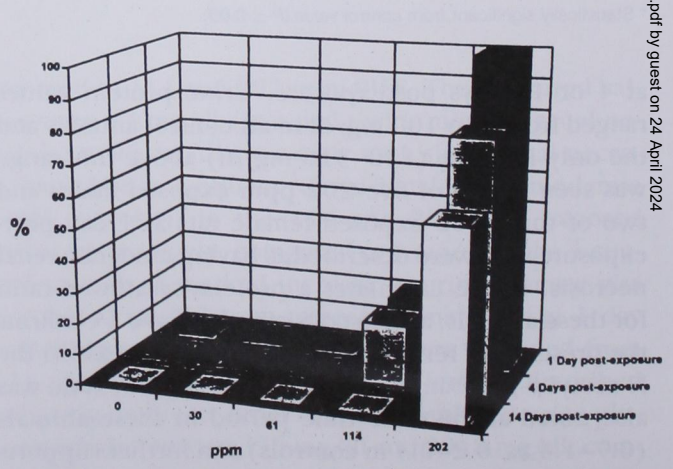

Fig. 2. Incidence of renal necrosis (% of animals) over time—females.

jury. Gonsowski and coworkers[3,4] exposed male Wistar rats to target concentrations of 0, 12.5, 25, 50, 100, 200, 300, 350, or 400 ppm synthetic PIFE for a single 3-, 6-, or 12-h period *via* whole body exposure. All animals in the 3-h 400 ppm exposure group died during the study. One and eight rats exposed to concentrations of 300 and 350 ppm for 3 h, respectively, also died during the study. The 3-h LC_{50} was 331 ppm. Exposure to concentrations of 100 ppm or greater appeared to be irritating to the eyes. Some rats exposed to 350 or 400 ppm displayed convulsive activity. Cerebral and/or hepatic abnormalities were noted at lethal exposure levels and may represent agonal changes or the result of hypoxia in the severely compromised animals.[6] Renal changes, described as cell swelling and/or necrosis of the tubule cells in the corticomedullary junction in this study were noted in both control- and PIFE-treated male rats and this finding was considered, by the authors, to be related to PIFE when found in approximately 1% of the tubule cells or more. After a 3-h exposure, 50 ppm PIFE was reported to be the threshold for these PIFE-related renal changes, described as 1% incidence of affected tubules in 3 of 10 rats killed 24 h postexposure as described by one of two reviewing pathologists. Although Gonsowski *et al.* did not incorporate any clinical pathology measurements in their study, extrapolation from the data presented in this study would suggest that it is doubtful that renal changes of this magnitude would be of any functional and certainly no clinical significance. Increasing the duration of exposure from 3 to 12 h yielded only a minor effect on incidence and severity of necrosis. In a 12-h, 25-ppm exposed group, 4 of 10 rats were reported with 1% affected tubules compared to 4 of 30 and 1 of 30 control rats with 1% and 10% affected tubules, respectively, in the concurrent control group, although it should be noted that one of the two reviewing pathologists reported no affected tubules in these same controls. In a 12-h, 50-ppm exposed group, 3 of 10 rats had 1% affected tubules and 3 of 10 had 1–5% affected tubules.

In the current investigation, changes in renal morphology and function were observed in male and female Sprague-Dawley rats at exposure concentrations of 114 and 202 ppm after 3-h nose-only inhalation. At our lowest effect concentration, 114 ppm, minimal renal necrosis (up to 2% affected tubule cells) was observed in 1 of 15 males, and slight renal necrosis (2–10% affect tubule cells) was observed in 2 of 15 females and 9 of 15 males. The most severe necrosis (moderate, 10–30% affected tubule cells) was observed only in 5 of 15 males and 4 of 15 females exposed to 202 ppm. Rats described as having minimal necrosis had no associated changes in serum chemistry or urine parameters indicative of impaired renal function. Small increases in serum BUN, serum creatinine, and/or urine glucose values were noted in some but not all of the 114 or 202 ppm-exposed rats described as having slight necrosis (2–10% affected tubule cells). More marked changes in the above serum and urine parameters as well as increases in two key indicators of renal impairment, protein/creatinine and NAG/creatinine ratios, were observed only in rats with moderate (10–30% affected tubule cells) necrosis.

The differences in the lowest reported histopathologic effect level between these studies could reflect strain differences in susceptibility between Wistar and Sprague-Dawley rats and/or differences in exposure apparatus and conditions, including whole-body *versus* nose-only inhalation, differences in carrier gas (>95% oxygen *vs.* constant 40% oxygen/60% nitrogen), and chamber temperature (27.8 ± 0.9°C *vs.* 22 ± 1°C). In addition, Gonsowski *et al.*[3] reported reduced weight gain in rats exposed to 100 ppm or greater, whereas we saw no effect on weight gain at concentrations of up to 202 ppm.

We believe that PIFE is most likely acting *via* a well-defined mechanism in rats as described for other fluorinated and chlorinated alkenes.[7–10] Preliminary work supports this proposed mechanism.[11] Other fluorinated alkenes shown to be nephrotoxic in rodents include tetrafluoroethylene,[12] chlorotrifluoroethylene,[13,14] hexafluoropropene,[13,15] and dichlorodifluorethylene.[16] We found no reports in the literature about adverse effects after exposure to fluorinated alkenes in humans. Chlorinated alkenes, including hexachlorobutadiene,[17–19] trichloroethylene,[20] perchloroethylene,[21] and tetrachloroethylene[22] have been associated with mutagenicity and nephrocarcinogenicity in addition to nephrotoxicity in rats. The bioactivation mechanism thought responsible for the production of nephrotoxic metabolites of haloalkenes involves hepatic glutathione S-conjugate formation, metabolism to the corresponding cysteine S-conjugate, translocation to the kidney, and renal bioactivation by cysteine conjugate β-lyase to yield ammonia, pyruvate, and an unstable thiol (fig. 3).[7–9,23] The reactive products derived from the unstable thiol presumably are responsible for the observed nephrotoxicity.

Mitochondria are the primary cellular target for toxicity of these haloalkenes.[24–26] Rats have high β-lyase activity in renal mitochondria,[27,28] which could result in high concentrations of the proposed reactive intermediates. The exact metabolic alterations induced in mitochondria, the timing of injury and their role in the processes leading to cell death appear different both on an interspecies basis and among the S-conjugates formed.[7,8]

The severity and site of renal injury varies among different compounds.[7–9] Renal injury produced by PIFE in the rat appears to resemble most closely the nephrotoxicity induced by hexachlorobutadiene,[29] chlorotrifluoroethylene,[13] and tetrafluoroethylene,[12,30] which is characterized by a distinct band of cellular damage in the outer stripe of the outer medulla, indicating injury confined to the straight portion of the proximal tubules (pars recta, S_3 or P_3 segment). In contrast, compounds such as hexafluoropropene have been reported to induce a more widespread injury involving the S_1 and S_2 segments.[13] Haloalkene-induced renal tubular injury has been associated with functional alterations including decreased urine concentrating ability, glucosuria, proteinuria, enzymuria, elevated plasma urea levels, and decreased renal function.[12,13,19,31–34] There is no example in the literature of a haloalkene producing renal functional changes in rats at exposure concentrations below those associated with the induction of renal morphologic alterations. Finally, haloalkene-induced nephrotoxicity, including that seen in this and other studies[3,4] with PIFE, and in studies with chlorotrifluoroethylene,[13,33] hexafluoropropene,[13] and hexachlorobutadiene[29] appears to be reversible and is characterized by visible regeneration within 3–5 days postexposure and substantial or complete recovery of normal morphology and function by 14 days postexposure, although no long-term follow-up has been conducted in exposed rats.

Haloalkene-induced nephrotoxicity appears to be an acute exposure phenomenon. In a 5-day multiple exposure study, Buckley and coworkers[33] reported regeneration of rat renal tissues after 3 consecutive days of exposure to 395 ppm chlorotrifluoroethylene for 4 h/day and, despite further days of exposure, reported that additional necrosis was minimal. Furthermore, these investigators reported that functional changes observed at 24 h (urine osmolality, urinary lactic dehydrogenase activity) returned to the range of control values before the end of the exposure period. The absence of apparent renal histopathologic changes in rats

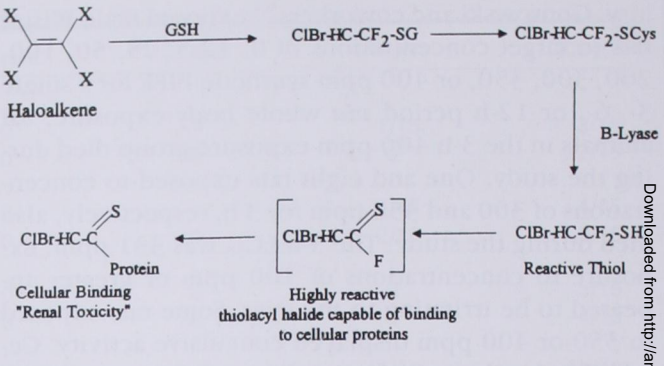

Fig. 3. Biotransformation of haloalkenes.

after exposure to up to 120 ppm PIFE for 3 h/day, 5 days/week for 8 weeks, further supports the conclusion that haloalkenes, including PIFE, do not pose a cumulative health hazard on repeated exposure.[2]

The most salient question that must be asked is what is the relevance of these nephrotoxic findings in rats to the safety of human patients. Direct extrapolation of data in rats to estimates of risk in humans is difficult for a number of reasons. First of all, because a probable mechanism of action has been proposed for the nephrotoxic hazard of PIFE in rats (fig. 3), the likelihood of a similar pathway existing in humans must be investigated (*i.e.*, there must be biologic plausibility, does the bioactivation pathway exist and act similarly in humans *in vivo*?). Secondly, both qualitative and quantitative species differences in the pharmacokinetics of PIFE (*i.e.*, absorption, distribution and excretion) need to be considered. The bioactivation pathway must be present and the relative levels of the bioactivation reactions *in vivo* must be sufficient to initiate adverse effects. Thirdly, in extrapolating a defined hazard into a risk estimate, species-specific exposure conditions including concentration, duration of exposure, and other miscellaneous factors must be taken into account.

The first critical issue to be addressed is whether or not this haloalkene bioactivation pathway exists and acts similarly in humans. The first step in haloalkene bioactivation, glutathione conjugation, is known to occur in both rats and humans. The rate of glutathione conjugation appears to be one of the key determinants of the severity of renal cell injury induced by haloalkenes in rats. For example, chlorotrifluoroethene, a potent rat nephrotoxin, is conjugated 50–100 times

more efficiently by rat hepatic subcellular fractions than tetrafluoroethene, a relatively moderate rat nephrotoxin.[8] It has been reported that hepatic glutathione conjugation of the haloalkene perchloroethylene measured in rats could not be detected in human liver fractions *in vitro*.[21] However, another important consideration is the possibility that renal glutathione conjugation rather than hepatic glutathione conjugation may be more critical in the induction of nephrotoxicity with some haloalkenes. Although the liver, because of the overall concentration of glutathione S-transferase, is generally the primary site of glutathione conjugation,[35] renal conjugation of the haloalkene hexafluoropropene has been demonstrated in bile-duct-cannulated rats.[36] Indeed, hexafluoropropene conjugates formed in the liver and eliminated with bile were not translocated to the kidney, and renal glutathione conjugation was responsible for hexafluoropropene-induced nephrotoxicity. Rat kidney cytosol and microsomal fractions also have been shown to be capable of conjugating hexachlorobutadiene *in vitro* in addition to hepatic conjugation.[18]

The next step in the bioactivation pathway is the sequential metabolism of the glutathione conjugates by γ-glutamyltransferase and dipeptidases to produce the cysteine conjugates. It is known that marked species differences occur in both the activity level and distribution of γ-glutamyltransferase, as well as in the extent of intrahepatic glutathione degradation.[23] Rats and mice have relatively low hepatic and high renal activities of γ-glutamyltransferase compared to humans.[23] This may influence the actual fraction of cysteine S-conjugate substrate reaching the target cells in the kidney and further β-lyase-catalyzed metabolism. The availability of cysteine S-conjugate also may be reduced by N-acetylation activity in the kidney.[8] Cysteine S-conjugates can act as a substrate for N-acetyltransferase as well as β-lyase in the kidney. N-acetylation serves as a detoxification reaction resulting in the formation of excretable mercapturic acids.

The final key step in bioactivation is metabolism of the cysteine S-conjugates to reactive thiols by β-lyase activities in the kidney. Chen and coworkers[37] demonstrated that a glutathione and cysteine conjugate of trichloroethylene, and cysteine conjugates of hexachlorbutadiene, dichlorofluoroethylene, and tetrafluoroethylene induced cytotoxicity in primary cultures of human proximal tubular epithelial cells, the presumed target cell type. These investigators also demonstrated that aminooxyacetic acid, an inhibitor of β-lyase, protected the cells from toxicity. This *in vitro* study demonstrates that human tubule cells contain a form of β-lyase active in haloalkene nephrotoxicity and indirectly, the presence of a transport system allowing entry of the chemicals into renal cells. However, it has also been demonstrated that total cysteine conjugate β-lyase activity in human kidney cytosol is about 10% of that present in rat kidney cytosol.[38] With the cysteine conjugate of perchloroethylene as the substrate, β-lyase biotransformation was up to 30-fold higher in rats than in human kidney cytosol fractions.[21] Hence, the potential for production of reactive thiols by renal β-lyase is significantly lower in the human kidney in comparison to rat.

Thus, it is evident that to some degree all of the various parts of the bioactivation pathway are present in humans. However, the data available suggest that the potential in humans for bioactivation of fluorinated haloalkenes may be less than that observed in rats. Which leads one to the second key consideration in extrapolation of animal data to risk in humans, that is, factoring in species differences in the pharmacokinetics and thus actual exposure to the compound. A lack of correlation between the dose administered by various routes and the concentration of a compound at the target site, which can vary markedly by species, often confounds interspecies extrapolation. After inhalation, actual blood levels of the test article depend on such factors as chemical solubility, ventilation rate, alveolar uptake, blood flow, and other physiologic variables.[39,‖‖] Metabolic rate is inversely related to body size, and relatively small animals have higher minute respiratory volumes per kilogram body weight to supply their relatively larger requirements for oxygen. Thus, rats have a much larger minute volume/kg than humans (0.80 l/min/kg *vs.* 0.29 l/min/kg). Rats also have a much higher heart rate (300–500 beats/min) and cardiac output (50 ml/min). As a general rule, about one third of the rat blood level of a gas or vapor is typically reached in humans after similar exposure concentrations. Unfortunately, adequate pharmacokinetic data in both rat and humans on fluorinated haloalkenes are not available for comparison. However, both rat and human data are available for PIFE's parent

‖‖ United States Environmental Protection Agency: Interim methods for development of inhalation reference concentrations. Review draft. Office of Research and Development, United States Environmental Protection Agency, Washington, DC, August 1990, EPA/600/8-90-066A.

drug sevoflurane and this general rule appears to hold true. Studies have demonstrated sevoflurane plasma concentrations in humans (63 μg/ml[40]) are roughly one third those found in the rat (228 μg/ml; unpublished data) under similar exposure concentrations. All other factors being equal, because sevoflurane and PIFE have similar low partition coefficients (water/gas: sevoflurane = 0.36, PIFE = 0.14 // olive oil/gas: sevoflurane = 47.2–53.0, PIFE = 24.1 [unpublished data; Maruishi Pharmaceutical Co., Ltd.]//blood/gas: sevoflurane = 0.6,[41] PIFE = not available) it is not unreasonable to assume that the human blood concentration of PIFE also will be approximately one third of those achieved in rats. Whereas uptake rates are quite different, elimination of sevoflurane, as a prototype, is rapid and extensive in both the rat and human and therefore is not an important factor in species extrapolation. Potential differences in metabolism/bioactivation/detoxification were discussed earlier.

Concentrations of PIFE in an anesthetic circuit have been closely investigated. Degradation of sevoflurane into PIFE is concentration, temperature and flow dependent. Early forced degradation studies used temperatures up to 120°C.[41] In the clinical setting, however, canister temperatures are much lower, ranging from 30.0 to 41.6°C in recent clinical trials.[42,43] In laboratory simulations, the production of PIFE is dependent on flow, with the highest concentrations observed at the lowest fresh gas flow rates. Recent studies[44–46] have shown that in adult patients, the mean maximum concentration of PIFE measured in the anesthesia circuit in the presence of soda lime was 19 ± 6.38 ppm (maximum concentration = 32 ppm; 0.0032%) at a flow rate of 0.5 l/min and mean high temperature of 46.0 ± 1.7°C. In children, the mean maximum concentration of PIFE measured in the anesthesia circuit was 7.3 ± 3.9 ppm (maximum concentration ± 15 ppm; 0.0015%) at 2 l/min. These investigators did not find any evidence of toxicity when administering sevoflurane for up to 18.23 MAC-hours. Frink and coworkers[47] also reported no evidence of renal changes in patients exposed to sevoflurane for 3–5 h in a low-flow anesthetic circuit. They reported average PIFE concentrations of 20.28 ± 8.6 ppm using Baralyme as the CO_2 absorbant and 8.16 ± 2.67 when using soda lime. The highest reported exposure was 60.78 ppm in a single patient during low-flow anesthesia with Baralyme.[47]

As stated earlier, the interaction of halogenated anesthetics with CO_2 absorbants is not unique to sevoflurane. Halothane, in the presence of CO_2 absorbants, also produces a haloalkene, bromochlorodifluoroethylene,[48] which has been identified as a rat[49] and mouse[50] nephrotoxin. The threshold for renal alterations in mice was reported to be 60 ppm. Unfortunately, dose-response data in rats is not available for comparison. After four decades of use, halothane also has not demonstrated nephrotoxicity in human patients. Although halothane has not often been used in low-flow systems, bromochlorodifluoroethylene is not only a degradant of halothane when mixed with CO_2 but is also a metabolite of halothane and has been identified in the urine of exposed adults and children even in cases using open circuit anesthesia without a CO_2 absorbant.[51–53] In two patients, Sharp and coworkers[1] found bromochlorodifluoroethylene concentrations of 4–5 ppm in the expiratory limb of the circuit. Those levels compare with the reported means of 4.25 ppm[54] and 13 ppm[55] of PIFE found in the expiratory limbs of sevoflurane rebreathing anesthesia circuits in adult patients.

The most important supporting data on the safety of sevoflurane, as used in the clinical setting, comes from human patients. Reports specifically evaluating patients administered sevoflurane in rebreathing conditions (low-flow rate of ≤ 1 l/min) in the literature[44–46,56] and from the Abbott Clinical Program (n = 64) demonstrate no increased risk of renal impairment, regardless of the type of carbon dioxide absorbant (soda lime or Baralyme), CO_2 elimination rate, the fresh gas flow rate, the type of surgical procedure, age, baseline renal function, ASA status, or concurrent medications (*e.g.* aminoglycosides).

Compound A has been shown in this and previous studies to be nephrotoxic in rats, producing transient swelling and/or necrosis and associated renal tubular epithelial hyperplasia in the corticomedullary junction. Differences in lowest reported concentrations associated with histopathologic effects are observed with different rat strains and different methods of administration. The lowest concentration found to induce histopathologic effects in the kidney of Sprague-Dawley rats exposed by nose-only inhalation for 3 h is 114 ppm. Some rats exposed to 114 ppm displayed a small transient increase in urine glucose concentration. The lowest concentration associated with significant alterations in serum BUN, serum creatinine, protein/creatinine, and NAG/creatinine ratios was 202 ppm. This nephrotoxicity is most likely caused by a well-studied mechanism in rats involving bioactivation of haloalkene cysteine S-conjugate metabolites by renal β-lyase into

reactive thiols. The renal injury produced in rats by Compound A is similar to that observed with haloalkenes in that they both have been shown to be threshold dependent, functional changes are observed only at dose levels inducing morphologic changes, injury appears reversible within a short time and injury appears to be an acute phenomenon, posing no additional risk with multiple exposures.

Putting the risk associated with PIFE exposure into perspective based on expected species differences in uptake (expected human blood levels approximately one third of those found in the rat) and threshold for relevant renal toxicity (*i.e.*, concentration associated with changes in urine chemistry) in rats (114 ppm), human patients would have to be exposed to PIFE concentrations well above those observed in clinical use to reach levels that would be required to produce any clinically detectable effects on the kidney. This potential threshold concentration for renal effects in humans is most likely even higher considering the much larger β-lyase activity in the rat kidney (10–30-fold higher) in comparison to human kidney tissue. Expected exposure levels under clinical settings for low-flow anesthesia are clearly well below such concentrations.

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