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Relationship of Metabolism and Cell Proliferation to the Mode of Action of Fluensulfone-Induced Mouse Lung Tumors: Analysis of Their Human Relevance Using the IPCS Framework

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Species-specific lung tumors in the mouse are induced by a number of chemicals. The underlying cause appears to be a high metabolic activity of mouse lung, due to relatively high abundance of Clara cells in mice compared with humans and the mouse-specific cytochrome P450 isoform 2f2 in the Clara cells. The chemicals are activated to reactive intermediates, leading to local cytotoxicity or mitogenicity resulting in increased cell proliferation and tumors. Rats have lower metabolic activity than mice (already below the threshold needed to cause lung tumors upon lifetime exposure) and activity in humans is lower than in rats. The carcinogenic risk for human lung is low for this mode of action (MOA). Fluensulfone has shown an increased incidence of lung adenomas in mice, but not in rats, at high doses. Fluensulfone is not genotoxic. MOA studies were conducted investigating key events of the postulated MOA. Fluensulfone is extensively metabolized by mouse lung microsomes, whereas no metabolic activity is seen with human lung microsomes. Cyp 2f2 is a major contributor in fluensulfone's metabolism and Cyp 2e1 is not involved. Furthermore, administration of fluensulfone to mice led to an early increase in Clara cell proliferation. The International Programme on Chemical Safety (IPCS) MOA and human relevance framework was used to evaluate the collective data on fluensulfone. We concluded that fluensulfone leads to species-specific mouse lung tumors and that these tumors are likely not relevant to human hazard or risk.

Key Words: **fluensulfone; carcinogenicity; lung tumors; human relevance; IPCS; mode of action; cell proliferation.**

Several chemicals have been found to induce lung tumors in the mouse but not in the rat. These include trichloroethylene (Green, 2000), naphthalene, ethylbenzene, alpha-methylstyrene, cumene, divinylbenzene, benzofuran (Cruzan *et al*., 2009), coumarin (Felter *et al*., 2006), and styrene (Cruzan *et al*., 1998, 2001). The metabolic activation and cell proliferative effects by several of these chemicals were recently systematically investigated and found to support a common mode of action (MOA) that is not relevant to the human lung (Cruzan *et al*., 2009, 2012). The involvement of mouse-specific metabolic activation in the lung, namely in the Clara cells by mouse-specific Cyp 2f2, was identified as a key event required for the tumorigenic response (Carlson, 2008; Cruzan *et al*., 2012; Green *et al*., 1997; Li *et al*., 2011). Clara cells are nonciliated nonmucous secretory cells with an abundance of cytochrome P450 monooxygenases. This abundance of metabolic capacity makes the cells susceptible to injury by a wide variety of chemicals, often due to covalent binding of reactive metabolites. Clara cells usually are the origin of lung tumors in mouse carcinogenicity bioassays, mainly bronchiolar papillary tumors, whereas solid tumors arise typically from type II pneumocytes (Kauffman, 1981; Nikitin *et al*., 2004; Thaete and Malkinson, 1991). Clara cells are found in the mouse lung in higher proportion than in rat or human lung (Cruzan *et al*., 2009; Nikitin *et al*., 2004). The expression of P450-isoenzymes is specific for different species and is one basis for species differences in susceptibility toward chemicals. Naphthalene, for example, damages Clara cells in the mouse but not in rats and hamsters, even when approaching the lethal dose (LD_{50}) (Massaro *et al*., 1993). Cytochrome P450 2f2 is a mouse-specific member of the family of mixed-function oxidases. It is specific to the mouse while humans express another orthologue of this enzyme (CYP 2F1). Typical substrates for Cyp 2f2 are naphthalene (Shultz *et al*., 2001), styrene (Carlson, 2008), and trichloroethylene (Green, 2000). Importantly, the isoenzyme expressed in humans (CYP2F1) appears to have low capacity to metabolize the substrates of the mouse orthologue (Cyp 2f2). Although mouse lung microsomal fractions were able to metabolize trichloroethylene to chloral hydrate at significant rates, the rate in rat lung was 23-fold lower, and this reaction could not be detected in human lung microsomes (Green *et al*., 1997). The same was found for styrene, where human lung

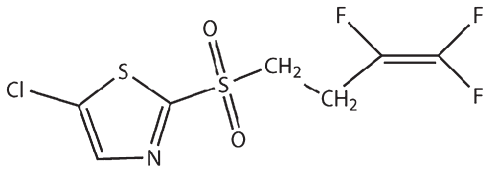

FIG. 1. Chemical structure of fluensulfone (5-chloro-2-[3,4,4-trifluoro-but-3-ene-1-sulfonyl] thiazole).

microsomes converted styrene about 350 times slower than mouse lung microsomes (Carlson, 2008).

This postulated MOA is applicable for mouse lung tumors only; other target organs may have different MOAs for carcinogenic activities and must be evaluated independently in epidemiology and toxicology investigations.

Fluensulfone is a novel nematicide developed for agricultural use (soil incorporation) for the control of root knot nematodes in cucurbits and fruiting vegetables (Fig. 1). An 18-month dietary oncogenicity study in CD-1 mice resulted in an increased incidence of bronchiolo-alveolar hyperplasia and benign lung tumors (bronchiolo-alveolar adenomas) in female CD-1 mice but not in male mice (Harlan Laboratories study report B80190; GLP, unpublished). Lung proliferative lesion incidences are summarized in Table 1 and representative micrographs of these lesions are shown in Figure 2. Other tumor types or preneoplastic lesions in other organs were not elevated in mice, and there were no increased incidences of tumors observed in any tissue or organ in Wistar rats in a parallel 24-month dietary combined oncogenicity/chronic toxicity study at identical dietary concentrations. A genotoxicity testing battery consisting of two bacterial gene mutation assays (Ames tests), two mammalian cell chromosome aberration assays *in vitro* (in V79 Chinese hamster cells and human primary lymphocytes), a mammalian cell gene mutation (HPRT) test *in vitro* in V79 cells, and an *in vivo* mouse bone marrow micronucleus test showed the absence of a mutagenic or clastogenic potential for fluensulfone. A nongenotoxic threshold MOA is therefore likely. Further studies were conducted to elucidate the MOA of the fluensulfone-induced lung proliferative lesions and the relevance of the hyperplasia and adenomas in female mice to humans.

The United States Environmental Protection Agency, Health Canada (organized through an ILSI program) and the International Programme on Chemical Safety (IPCS) have developed and extended the MOA framework to address the human relevance of tumorigenic responses in animal carcinogenicity studies (Boobis *et al*., 2006, 2008; Meek *et al*., 2003; Seed *et al*., 2005; Sonich-Mullin *et al*., 2001), providing a disciplined and rigorous analytical tool for transparent evaluation and structured presentation of the data.

This document (1) summarizes the existing toxicological fluensulfone data, (2) contributes MOA data on induced morphological changes, cell proliferation, and human versus mouse metabolism of fluensulfone, (3) integrates these data with the literature and available MOA studies, and (4) evaluates whether or not these tumors are relevant to humans. We follow the IPCS Mode of Action Human Relevance Framework in this evaluation (Boobis *et al*., 2006, 2008).

MATERIALS AND METHODS

All studies were conducted according to the OECD principles of Good Laboratory Practice. The performing laboratory is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and the animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) as well as the cantonal veterinarian prior to initiation. Fluensulfone (purity between 96.5 and 98.6% in the test batches) was supplied by Makhteshim Chemical Works Ltd., Beer-Sheva, Israel. Other chemicals, if not specified otherwise, were obtained from Sigma Aldrich, St Louis, USA, or Fluka, Buchs, Switzerland.

Comparative* in vitro *metabolism in mouse and human lung microsomes. Untreated CD-1 mice (Harlan Laboratories, Venray, The Netherlands; 12/sex, 6–9 weeks, approximately 28/40 g females/males) were sacrificed by carbon dioxide asphyxiation, the lungs excised, and frozen to −80°C until preparation of the microsomes. The frozen tissue was homogenized in ice-cold HM buffer (85.57 g/l sucrose, 9.32 g/l KCl, 13.61 g/l KH_2PO_4, and 0.29 g/l

TABLE 1
Incidence of Histopathological Findings in the Lungs in the 18-Month Oncogenicity Study in CD-1 Mice With Fluensulfone

	Males				Females			
Fluensulfone (mg/kg diet)	0	30	200	1200	0	30	200	1200
Animals examined	50	50	50	50	50	50	50	50
Bronchiolar hyperplasia	1	—	24**	31**	5	7	43**	48**
Mean grade (0–5)	1.0	—	1.3	1.6	1.0	1.0	1.8	2.6
Alveolar/bronchiolar adenoma	7	9	5	12	2	4	14**	9*
Alveolar/bronchiolar carcinoma	8	3	3	4	2	1	1	4
Combined alveolar/bronchiolar carcinoma and adenoma	15	12	8	16	4	5	15	13

Note. Grading scale for hyperplasia: 1 = minimal, 2 = slight, 3 = moderate, 4 = marked, and 5 = severe.
Fisher's exact test (one-sided): $p < 0.05$*, $p < 0.01$**.

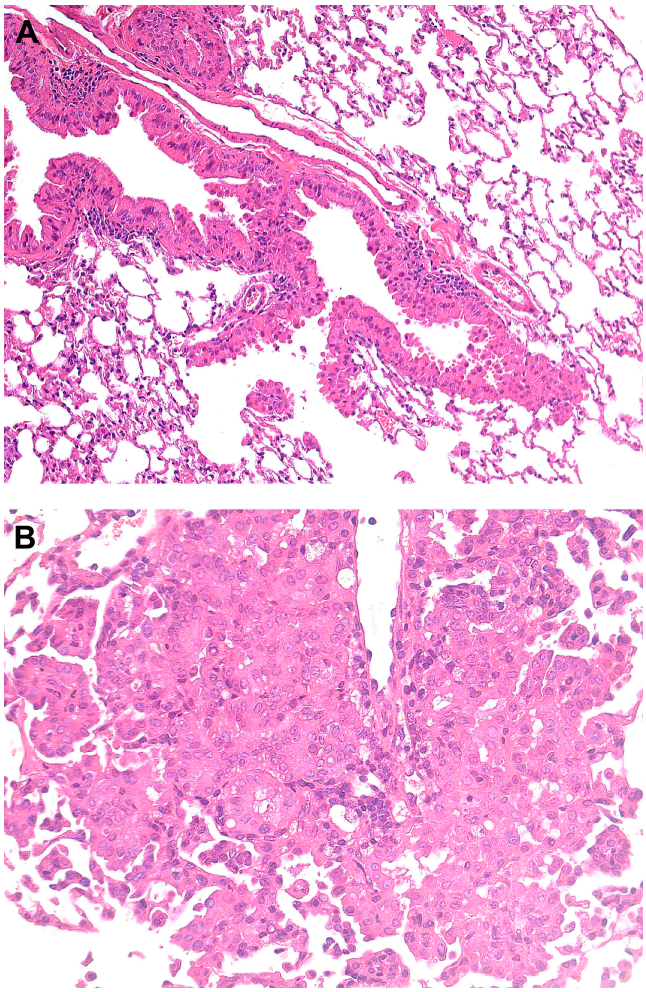

FIG. 2. Representative picture of the bronchiolar-alveolar hyperplasia (A) and adenoma (B) observed in female mice receiving the high dose of fluensulfone (eosin–hematoxylin stained).

EDTA, adjusted to pH 7.4 with NaOH), debris removed by centrifugation at 9000 × g (4°C, 20 min.), microsomes pelleted at 138,000 × g (4°C, 60 min), and resuspended in HM buffer. Human lung microsomes (pool of at least 10 donors, mixed gender from nonsmokers) were obtained commercially (Celsis Invitro Technologies, Baltimore, MD). Protein content was determined against a dilution row of bovine serum albumin according to Bradford (1976) using a commercial kit (Coomassie [Bradford] Protein Assay Kit, Thermo Fisher Scientific, Rockford) according to the manufacturer's recommendations. Two concentrations of fluensulfone (2 and 20μM) were incubated in three replicates each with the different microsomal preparations from human lung tissue and female and male mice lungs (final protein concentration 1.0 mg/ml for humans and 0.5 mg/ml for mice, respectively) in the presence of a nicotinamide adenine dinucleotide (NADH)-regenerating system (4.16 mM glucose 6-phosphate, 1.61 mM NADH, 4.12 mM $MgCl_2$, and 2.5 U/ml of glucose 6-phosphate dehydrogenase) in phosphate-buffer (6.81 g/l KH_2PO_4 in water, pH adjusted to 7.4 using NaOH) with or without addition of the specific inhibitors, 4-methyl pyrazole (CYP 2E1, Cyp 2e1), and 5-phenyl-1-pentyne (Cyp 2f2). The total incubation time was 120 min, and samples were taken at 0, 30, 60, 90, and 120 min from all samples after stopping the enzymatic reaction by the addition of methanol and spiking with the internal standard chlorpropamide. Samples were stored at −20°C and centrifuged at 20,000 × g before analysis. Samples were analyzed by liquid chromatography with mass detection (separation: Agilent 1100 with Luna C18, 2.0 mm ID × 50 mm, 5 μm-column, eluent 0.1% formic acid in a gradient of MeOH/H_2O; detection: electron spray ionization, API 3000 Triple Quadrupole [AB SCIEX]) for the internal standard and unmetabolized fluensulfone. Enzymatic activity of human lung microsomes was confirmed by determination of the conversion of chlorzoxazone to its metabolite 6-hydroxy-chlorzoxazone.

Transmission electron microscopy. Lung tissue obtained from the terminal sacrifice of the 18-month dietary oncogenicity study with fluensulfone in CD-1 mice was stored in 4% neutral phosphate-buffered formalin solution until further routine processing. For electron microscopy, the fixed lung samples were trimmed and refixed in a 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.4, 350 mOsm) for 2 days. From every sample, three representative blocks were dissected. The samples were then extensively washed in cacodylate buffer (0.1 M sodium cacodylate adjusted with HCl to pH 7.4) and post-fixed in 1% (wt/vol) osmium tetroxide (buffered with 0.1 M sodium cacodylate adjusted to 370 mOsm and pH 7.4) for 2 h. Subsequently, they were washed in maleate buffer (0.05 M maleic acid adjusted with NaOH to pH 5.0), block-stained with uranyl acetate (0.5% wt/vol in maleate buffer), dehydrated in ethanol, and embedded in Epon 812. Semi-thin sections, 1 μm thick, were prepared using histodiamond knives, subsequently stained with Toluidine Blue, and analyzed using a Leica Leitz DMR light microscope. Representative areas from the sectioned material were further processed for electron microscopy. Ultrathin sections (80–90 nm in thickness) were obtained with an ultra-microtome (Ultracut; Reichert-Jung, Bensheim, Germany) and floated onto 200-mesh grids. They were stained with uranyl acetate and lead citrate using the Leica EM-stain (Leica, Glattbrugg, Switzerland) and analyzed with a transmission electron microscope (Philips, EM 400).

Cell proliferation (S-phase response) study in mice. Groups of 10 female specific pathogen-free CD-1 mice each (Charles River, Sulzfeld, Germany; 6 weeks of age at start of treatment) were treated with untreated diet, diet containing 1200 mg/kg fluensulfone (high dose in carcinogenicity study), or 1305 mg/kg of isoniazid as a positive control substance for 3 or 7 days, respectively. Two and 14 h before sacrifice, the animals were injected ip with 100 μl of a 10 mg/ml aqueous bromodeoxyuridine (BrdU)-solution (BD Pharmingen). Sacrifice by exsanguination under deep irreversible pentobarbital narcosis was performed early in the morning to assure that the animals were exposed to the test item until shortly before sacrifice. Lungs (and a piece of small intestine as a labeling control) were removed from the mice, fixed in buffered formalin, embedded, cut, and stained with eosin/hematoxylin for light microscopic histopathological evaluation and immunohistochemically stained with anti-BrdU antibody to visualize the BrdU incorporation for determination of labeling index indicative of DNA replication. The microscope slides were evaluated for five animals of each group, and the number of BrdU-positive cells within all positive staining bronchiolar epithelial cells was manually counted to determine the BrdU-labeling index for quantification. Distinction between Clara cells and other bronchial epithelial was not possible by light microscopy on the immunohistochemically stained slides. For this reason, all visible bronchioles on a slide were evaluated to reach at least 500 cells. When there were more cells on the slide due to the lengths of bronchioles, all cells of the respective structures were counted. For comparison, the final counted number of all cells and positive cells was calculated for a ratio with 1000 cells in the denominator.

RESULTS

Transmission Electron Microscopy

During initial evaluation and independent peer-review of the 18-month bioassay in mice, the pathologists agreed that Clara cells are the likely origin of the bronchiolar epithelial hyperplasia and adenomas. The bronchiolar epithelium was multifocally enlarged and appeared to be more basophilic. The

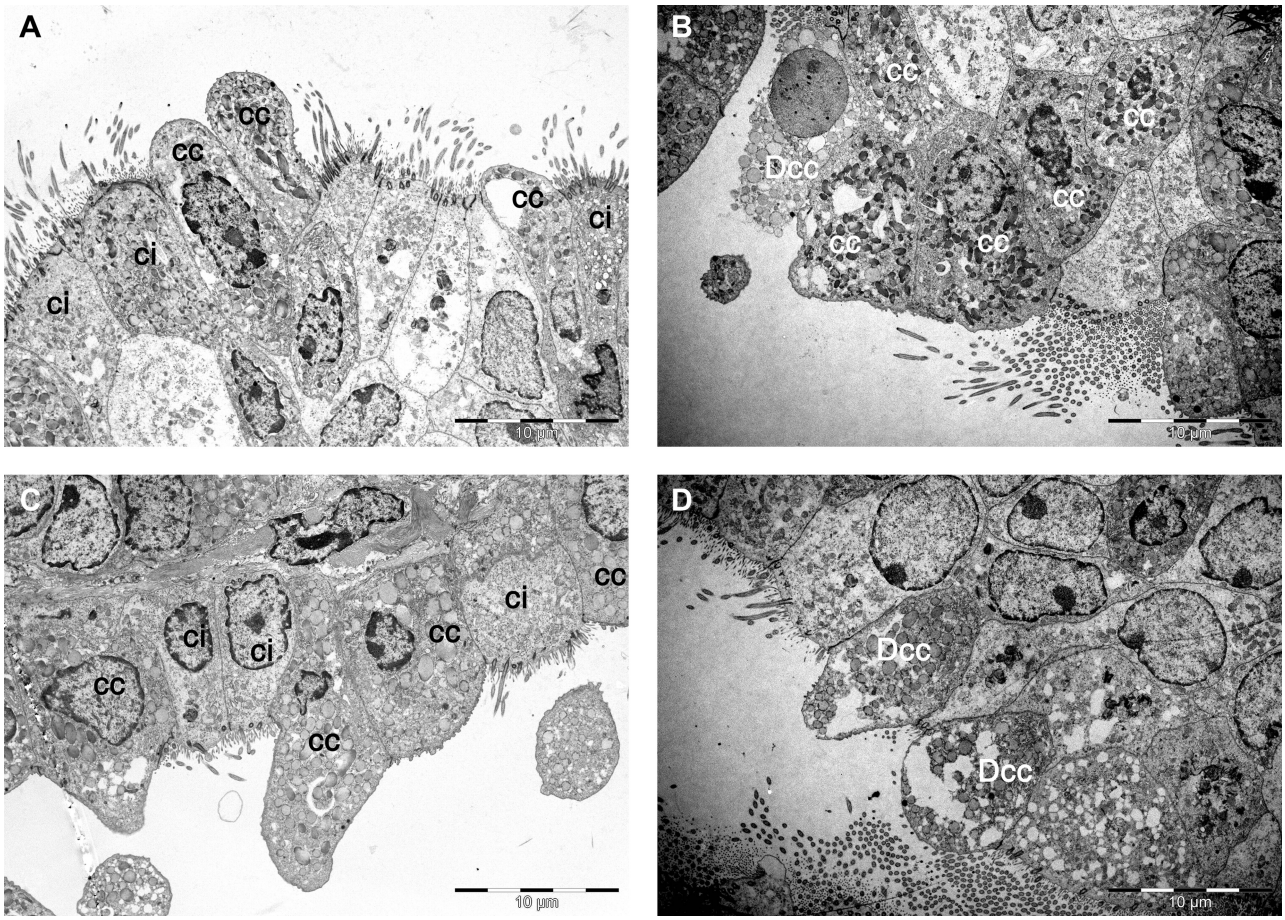

FIG. 3. Transmission electron microscopy of the mouse lungs. CC, Clara cell; Ci, ciliated cuboidal epithelial cell; BC, blood capillary; Dcc, disrupted Clara cells. (A and C): Normal bronchiolar epithelium consisting of ciliated cuboidal epithelial cells and nonepitheliated Clara cells. Clara cells are dome-shaped cells with short microvilli within the simple ciliated epithelium. The cytoplasm contains numerous secretory vesicles. (B and D): Increased number of Clara cells, of which many are disrupted. The layers are pseudostratified indicating hyperplasia. The number of smaller and darker basal cells containing secretory vesicles appears to be increased.

cells composing this epithelium were mainly nonciliated cells. Transmission electron microscopy of tissue obtained at the time of the terminal sacrifice at 18 months was used to verify this postulate. The epithelial hyperplasia in the terminal bronchioles was found to be mainly affecting the nonciliated Clara cells. The normal appearing Clara cells increased in number, causing pseudostratification as an indicator of early hyperplasia and were often disrupted. Small basal cells (most likely Clara cell precursors) were observed close to the basal lamina (Fig. 3). A more detailed description is not provided because the samples are affected by artifacts as a consequence of previous formaldehyde fixation (see the Materials and Methods section).

Cell Proliferation

In an *in vivo* investigation, female mice were treated with fluensulfone (or isoniazid as a positive control) for 3 and 7 days. Quantification of the cell proliferation by manual counting of BrdU-positive and BrdU-negative cells in the bronchiolar epithelium revealed an approximately fourfold increase of cell proliferation upon treatment with fluensulfone and the positive control isoniazid compared with control. Increased cell proliferation was observed at 3 days but had reverted to the control level at day 7 (Table 2, Fig. 4).

TABLE 2
BrdU-Labeling Index of Mouse Bronchioles After Treatment

Duration of exposure (days)	BrdU-positive cells/1000 cells of the bronchiolar epithelium		
	Control	Isoniazid	Fluensulfone
3	25 ± 5	119 ± 69**	120 ± 40**
7	17 ± 10	7 ± 5	13 ± 4

**Fisher's exact test, significant at $p < 0.01$.

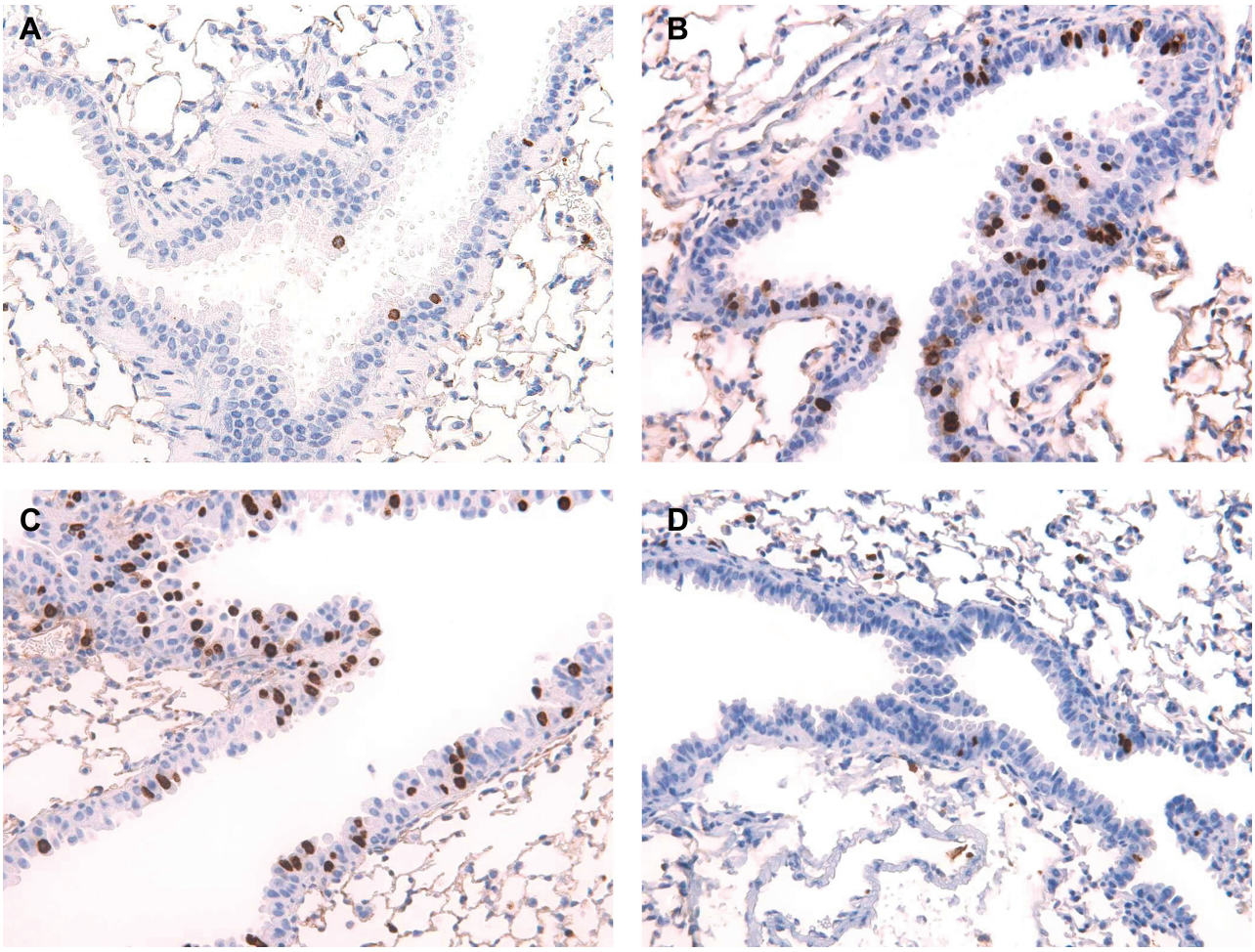

FIG. 4. Representative pictures of bronchioles. The number of BrdU-positive cells (intensive dark staining) in fluensulfone- and isoniazid-treated animals is increased compared with control on day 3, returning to base level after treatment with either substance on day 7. (A) Control, (B) Fluensulfone (day 3), (C) Isoniazid (day 3), and (D) Fluensulfone (day 7).

Metabolic Evaluation

In an *in vitro* MOA study, the metabolic conversion kinetics of fluensulfone were compared in mouse and human lung microsomes. Metabolic capacity of the microsomes of both species was confirmed by their activity in converting chlorzoxazone to hydroxyl-chlorzoxazone (data not shown). Microsomes were incubated with fluensulfone in the presence of a NADH-regenerating system, the reaction stopped at several time points and the amount of remaining (non-metabolized) fluensulfone determined by liquid chromatography with mass detection (LC–MS). Mouse lung microsomes were found to metabolize fluensulfone rapidly and extensively, whereas human lung microsomes had no detectable activity toward fluensulfone (Fig. 5). Addition of inhibitors of CYP 2E1/Cyp 2e1 and Cyp 2f2 indicated that CYP 2e1 played no detectable role in metabolism of fluensulfone by mouse lung microsomes, whereas the Cyp 2f2 inhibitor partly inhibited the metabolism, indicating a significant role of this enzyme in the metabolism for fluensulfone (Fig. 6).

In summary, the present studies provide evidence that the lung hyperplasia and tumors in the 18-month study are of Clara cell origin, there is increased bronchiolar cell proliferation at 3 days of treatment that reverted to control levels by 7 days, and that metabolic activation occurred with mouse microsomes but not with human microsomes. These new data are useful for evaluation of a MOA for the fluensulfone-related lung lesions in female mice.

DISCUSSION

In the oncogenicity study in mice, bronchiolar hyperplasia was observed in the mid and high dose of both sexes. The occurrence of lung adenomas was significantly increased in females at the mid and high dose; however, there was no increase in the incidence of carcinomas. The combined incidence of adenomas and carcinomas followed a trend in females but not in males (Table 1). The MOA and human

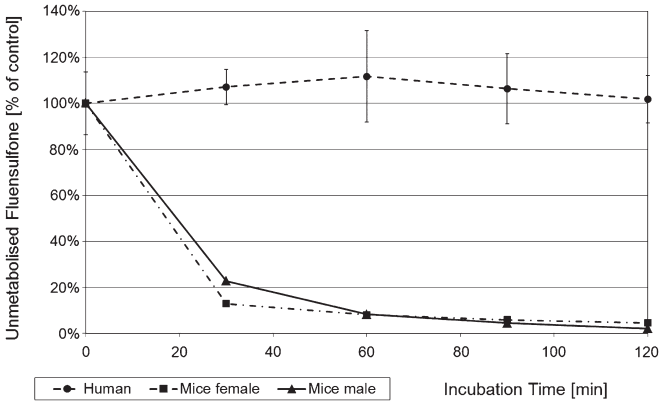

FIG. 5. Time-dependent metabolic conversion of fluensulfone in human and mouse lung microsomes.

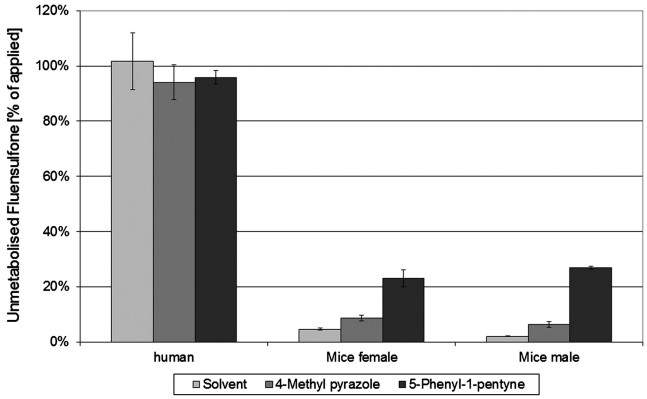

FIG. 6. Metabolic conversion of fluensulfone in mouse lung microsomes at 120 min. The graph represents a simplification of the data by focusing on one time point and one concentration of fluensulfone only—corresponding differences in activity were observed at all time points.

relevance of these lung proliferative lesions were analyzed according to the IPCS framework (Boobis *et al.*, 2006, 2008; Meek *et al.*, 2003; Seed *et al.*, 2005; Sonich-Mullin *et al.*, 2001). The stepwise evaluation follows.

Is the MOA for Fluensulfone-Induced Mouse Lung Tumors Known?

Postulated MOA. Fluensulfone is activated to reactive metabolites by mouse lung Clara cells (mainly by mouse-specific cytochrome P450 2f2). These metabolites produce increased cell replication that leads to lung hyperplasia and neoplasia. The species specificity is partly due to different activity of the sytochrome P450-orthologs in mice and humans and partly due to the lower number of Clara cells in human lungs compared with mouse lungs.

Key events. The key events in the postulated MOA are

- Extensive metabolism of fluensulfone by the mouse lung, predominantly by Cyp 2f2, that produces metabolites that are presumptively reactive.
- Clara cells undergo increased proliferation that results in bronchiolo-alveolar hyperplasia.
- Progression of bronchiolo-alveolar hyperplasia to adenomas and carcinomas.

The *in vitro* studies presented above show that mouse lung microsomes taken from this strain of mice significantly metabolize fluensulfone. This microsomal metabolism appears to be predominantly responsible for the metabolism of fluensulfone by the lungs. Inhibition of cytochrome 2E1/2e1 did not affect the metabolism of fluensulfone.

A key event in the MOA for a nongenotoxic substance inducing cancer above a threshold of exposure is the requirement for an increase in cell proliferation (Cohen and Ellwein, 1990, 1991; Greenfield *et al.*, 1984; Moolgavkar and Knudson, 1981). Increased cell proliferation was shown for fluensulfone in a short-term experiment showing an increase in BrdU-labeling index after 3 days of administration, but a return to control levels by 7 days. Such a transient effect was also evident for isoniazid, another known mouse lung tumorigen (Biancifiori and Ribacchi, 1962; IARC, 1985, 1987). This has also been seen with other nongenotoxic mouse lung carcinogens (Cruzan *et al.*, 2009, 2012). Eventually, there is the development of bronchiolo-alveolar cell hyperplasia, which can be diagnosed microscopically (Boorman and Eustis, 1990). The sequence of events for rodent lung carcinogenesis is the progression of hyperplasia leading to adenomas and ultimately to carcinomas. Although morphologically many of the carcinomas meet the criteria of malignancy, behaviorally, they frequently act in a more benign nature, seldom leading to distant metastases (Boorman and Eustis, 1990; Haschek and Witschi, 1991). Human lung cancer, in contrast, usually metastasizes, frequently early in the course of the disease (Colby *et al.*, 1994).

Morphologically, there was no evidence of hyperplasia at either the 3- or the 7-day sacrifices for either fluensulfone or isoniazid. Similarly, in a previously performed 13-week study at comparable dose levels, there was no evidence of hyperplasia at that time point. However, in the 18-month bioassay, there clearly is the development of hyperplasia some time after 13 weeks with the subsequent development of adenomas and a few carcinomas. Bronchiolar hyperplasia was first recorded in a decedent high dose female mouse on study day 373 and the first adenoma in a mouse that died in study week 56. However, due to spontaneous and induced tumors having the same appearance, it cannot be established if this is the onset of the first induced case of each lesion. Also, it is likely that these lesions were present for some time (weeks) prior to the death of the mice.

The statistically significant increase in incidence of proliferative lung lesions, however, was restricted to hyperplasia and adenoma. The lack of morphologic evidence of hyperplasia through a 13-week period of observation has been reported previously for other mouse lung tumorigens (Boobis *et al.*, 2009). Although there is not a morphologic indication of

hyperplasia, an increase in proliferation as indicated by increased BrdU labeling with fluensulfone and isoniazid frequently (possibly always) occurs prior to 13 weeks.

The transient nature of the increase in proliferation is reminiscent of that seen with cytochrome P450-inducing chemicals such as phenobarbital and their action on the liver in mice and rats (Whysner *et al*., 1996; Yamada *et al*., 2009). In such instances, the increase in cell proliferation is present by 7 days and then returns to control levels by 14 days (Yamada *et al*., 2009). However, this is associated with an increase in liver weight, which is partly due to an increase in the number of hepatocytes. Thus, although the rate of proliferation returns to control levels, the actual number of DNA replications is increased because of the increased number of hepatocytes present. Whether such an increase in the Clara cells is occurring in mouse lung is unknown at this time. A quantitative assessment of the number of Clara cells in the lung is technically difficult. Nevertheless, an increase in cell proliferation in mouse lung is evident early in response to the administration of fluensulfone.

Finally, the evolution of this increased cell proliferation to hyperplasia with subsequent development of adenomas and carcinomas appears to be the usual sequence of events for lung tumorigenesis in rodents (Haschek and Witschi, 1991; Nikitin *et al*., 2004).

It is unclear at this time whether the cell proliferation occurs in response to cytotoxicity with regenerative proliferation or whether it is a direct mitogenic effect. Morphologically, there is no evidence of necrosis in the lungs of the animals treated with fluensulfone or isoniazid, nor is there an inflammatory reaction in the mice. More subtle changes of toxicity that might be detectable by electron microscopy were not evaluated in this study, but there is no evidence that there is an increase in cell death. Thus, it is more likely that the proliferative stimulus is a direct mitogenic effect rather than cytotoxicity and regeneration.

Concordance of dose-response relationships. In the 18-month bioassay, there was an increase in hyperplasia in the mid and high doses, with a slightly greater increase in females than in males. Adenomas were increased in females in the mid- and high-dose groups but not in males. There was not a statistically significant increase in carcinomas in either sex. The dose used in the study for assessing cell proliferation corresponds to the high dose used in the 18-month bioassay. There is concordance between these early and late events. Information is not available for proliferation at the mid dose or lower.

Temporal relationship. The initial key event involves metabolism of the chemical, which will occur immediately upon administration because the enzyme is present at high levels at all times. There is rapid induction of the increase in cell proliferation, as evidenced by the increased BrdU-labeling index by 3 days, and it is only after 13 weeks that the hyperplasia and subsequent adenomas and carcinomas occur. Thus, the temporal sequence of events follows those postulated for the MOA.

Strength, consistency, and specificity of tumor response with key events. The findings in the lung are consistent between studies with the high dose in female mice leading to an increase in proliferation and ultimately an increase in hyperplasia and adenomas. Furthermore, there is consistency with another known mouse lung tumorigen, isoniazid, with a similar sequence of events and correlation between doses for the early events (proliferation) and the ultimate development of hyperplasia and lung tumors. Furthermore, the only response to fluensulfone and isoniazid is the mouse lung, the organ location of the enzyme that is likely responsible for its metabolic activation.

Biological plausibility and coherence. For nongenotoxic agents, an increase in cell proliferation is the common unifying process leading to the development of tumors. This has been delineated on a theoretical, experimental, and epidemiological basis in studies published by Knudson (1971), by Moolgavkar and his associates (Meza *et al*., 2008; Moolgavkar and Knudson, 1981), and by Cohen and his associates (Cohen and Ellwein, 1990, 1991; Greenfield *et al*., 1984). The sequence of increased proliferation leading to an accumulation of cells (hyperplasia) and subsequently to adenoma and carcinoma is the usual sequence of events for lung tumorigenesis in rodents (Nikitin *et al*., 2004).

Other possible MOAs. The main consideration of other possible MOAs is the potential for genotoxicity. As described above, there was a battery of *in vitro* and *in vivo* genotoxicity tests conducted that gave a negative response for genotoxicity. Thus, genotoxicity is not a MOA for fluensulfone-induced mouse lung tumors.

Another possibility is oxidative damage, especially in the setting of the lung with high oxygen tension compared with other tissues. However, this is unlikely because there is no evidence of necrosis or other evidence of cytotoxicity at the early time points of administration, times at which there already are changes of increased proliferation.

Uncertainties, inconsistencies, and possible data gaps. There are no inconsistencies in the data because there is concordance between dose, temporality, and the expected sequence of events for tumorigenicity for nongenotoxic agents in the mouse lung. As always, there are some data gaps. These include the lack of a more extensive evaluation of the effects on fluensulfone on a wider array of cytochrome P450 isozymes and identification of the specific metabolites (Cruzan *et al*., 2009). Furthermore, the molecular target for fluensulfone metabolites is unknown, but these reflect details of mechanism of action versus MOA. A more detailed assessment of the dose-response for the cell proliferation effects at the early time points would be useful, as would a more detailed time sequence for development of hyperplasia.

Assessment of postulated MOA. Metabolic activation of xenobiotics in mouse lung by Cyp 2f2 is a well-described phenomenon and its specificity to the mouse has been well

TABLE 3
Concordance Analysis Between Mice and Humans Regarding Key Events for the MOA of Fluensulfone-Induced Lung Proliferative Lesions in Mice

Key event	Mice	Humans
Metabolic activation by Cyp 2f2	Yes	No (based on *in vitro* microsome analysis, known lack of Cyp 2f2 in human Clara cells and fewer Clara cells in humans)
Increased Clara cell proliferation	Yes	Unlikely
Bronchiolo-alveolar hyperplasia and adenoma	Yes	Unlikely

documented (Cruzan *et al*., 2009, 2012; Li *et al*., 2011) and appears to be valid for fluensulfone. The induction of an increase in cell proliferation leading ultimately to hyperplasia, adenomas, and carcinomas is a described sequence of events for lung tumorigenesis in the mouse (Nikitin *et al*., 2004).

Can Human Relevance of the MOA be Reasonably Excluded on the Basis of Fundamental Qualitative Differences in Key Events Between the Animals and Humans?

There are several aspects of comparison between mice and humans that must be considered in evaluating this question. As part of this evaluation, it is helpful to be able to relate information for species, which do not develop lung tumors, that is, the rat. A concordance table is a useful summary of the comparison of mice to humans and is shown in Table 3.

Several strains of mice are well known to be highly susceptible to the induction of lung tumors, including the strain used in the bioassay (CD-1) of fluensulfone (Shimkin and Stoner, 1975; Nikitin *et al*., 2004). This is evident by the high spontaneous incidence of proliferative lesions of the lung, including hyperplasia, adenoma, and carcinoma (Table 4). This contrasts with the low incidences of lung tumors in most strains of rats (Haschek and Witschi, 1991), including the strain used in the 2-year bioassay with fluensulfone (Han Wistar, Table 4) and the low spontaneous incidence of lung tumors in humans (Colby *et al*., 1994). The reason for the increased susceptibility in mice is not entirely known but appears to be related to the high levels of Cyp 2f2 in mouse lung, particularly in Clara cells (Cruzan *et al*., 2009, 2012; Li *et al*., 2011). Other genes have also been identified that may possibly contribute to this increased susceptibility (Cruzan *et al*., 2009; Manenti *et al*., 2004; Nikitin *et al*., 2004).

Cyp 2f2 is particularly critical for the metabolism of a wide variety of xenobiotics, especially those that have been reported to be lung tumorigens in mice, such as styrene, naphthalene, and others (Cruzan *et al*., 2009, 2012; Li *et al*., 2011). The enzyme is notably high in lung tissue and the nasal epithelium in these mice but not in other tissues such as the liver. This provides an explanation for the tissue specificity for these chemicals in these bioassays. The importance of the cytochrome 2f2 metabolic activation step by this isozyme has been demonstrated by the lack of tumorigenicity of styrene when administered to a strain of mice in which the isozyme has been knocked out (Cruzan *et al*., 2012). Naphthalene loses its lung and nasal toxicity in this same knockout strain (Li *et al*., 2011).

In contrast, the enzyme is present at very low levels in rats and at exceedingly low levels in humans (Cruzan *et al*., 2009). Furthermore, not only is the level low per cell but also there are considerably fewer Clara cells proportionately in humans than in mice (Atkinson *et al*., 2008; Plopper *et al*., 1980, 1992; Stott *et al*., 2003). Thus, the overall implication of these findings is that there is considerably less potential for metabolic activation by this isozyme in human lung compared with the mouse. This is also true as an explanation for the lack of susceptibility in the rat. Although there are Clara cells with the isozyme levels in the rat, these are more than 20 times lower than in the mouse. This lowers the metabolism to a point at which metabolic activation is insufficient to lead to an effect on the lung cells that can produce the tumorigenic effect. Thus, based on metabolic considerations, the human is unlikely to be responsive to the lung tumorigenicity of fluensulfone, similar to the rat.

The proliferative response to fluensulfone is also an indicator that the response is different between the mouse and the human. Data are available on the rat for isoniazid, indicating that a proliferative stimulus does not occur in the rat following isoniazid administration (Cohen, unpublished data), and similarly, there is no lung tumorigenic effect of isoniazid in rats (IARC, 1985, 1987). Isoniazid, like fluensulfone, is a mouse-specific lung tumorigen. Data for fluensulfone in the rat regarding the cell proliferative response are not available.

TABLE 4
Historical Control Range of Neoplastic Lung Lesions in Mice and Rats

Lesion	Mice (CD-1)	Rats (Han Wistar)
Bronchiolar-alveolar adenoma	0–14% (males) 0–6% (females)	0–0% (males) 0–2% (females)
Bronchiolar-alveolar carcinoma	4–12% (males) 0–10% (females)	0–4% (males) 0–2% (females)

Given the proliferative response seen with isoniazid in the mouse lung, which is similar to that produced with fluensulfone, one can then address the relationship of the lung tumors in the mouse as a predictor for lung tumors in humans. Isoniazid is an anti-tuberculosis drug, which has been used for nearly six decades at doses that approximate those administered to the mouse. Extensive epidemiological studies of individuals that have been administered isoniazid have shown that there is no lung tumorigenicity by isoniazid in humans (Clemmesen and Hjalgrim-Jensen, 1979; Costello and Snider, 1980; Hammond *et al*., 1967; IARC, 1985, 1987; Jansen *et al*., 1980; Stott *et al*., 1976). Thus, the mitogenic and tumorigenic response produced by isoniazid in mice is not predictive of a similar response in humans. This is similar to the liver proliferative and tumorigenic effects seen with phenobarbital in mice but not in humans, based on epidemiological investigations (Whysner *et al*., 1996).

Further considerations in extrapolating from the mouse to the human are differences in the structure of the lung in the mouse and the pathogenesis of lung tumors in mice compared with humans (Colby *et al*., 1994; Haschek and Witschi, 1991). The mouse lung differs from the human, partly related to differences in the branching of the bronchiolar network, as well as differences in the proportion of Clara cells in these structures (Massaro *et al*., 1993; Nikitin *et al*., 2004).

The pathogenesis of lung tumors in mice follows a sequence of hyperplasia leading to the production of adenoma and ultimately carcinoma (Nikitin *et al*., 2004). These tumors occur in the periphery of the lung rather than centrally and are not derived from the bronchi. In contrast, human lung tumors arise predominantly from the bronchi, although there is one type of adenocarcinoma of the lung that appears to arise from the periphery bronchiolar-alveolar adenocarcinoma (Colby *et al*., 1994; Kerr *et al*., 2004). However, these tumors in humans do not arise through a sequence of hyperplasia, adenoma, and then the development of carcinoma. Adenomas are exceedingly rare in humans and are not considered preneoplastic (Burke and Flieder, 2004). They are not a precursor lesion of bronchiolar-alveolar carcinomas or of other adenocarcinomas of the lung. Thus, the sequences of events in the mouse lung leading to the induction of adenocarcinoma are different compared with the human. Given all these considerations, metabolic differences, including differences in isozymes, differences in Clara cell numbers and anatomy, and histopathogenesis differences, it can be concluded that the human relevance of this MOA can be reasonably excluded on the basis of these qualitative differences in the key events. This makes the considerations of the quantitative differences, either kinetic or dynamic, less critical. Nevertheless, there are quantitative differences between the isozymes, percentage and numbers of Clara cells, and the overall histopathogenetic sequence of events. Thus, on both qualitative and quantitative bases, the human relevance of this MOA can be reasonably excluded. Finally, the epidemiological studies on isoniazid show that it is not a human carcinogen. Because fluensulfone has the same MOA, it also is not expected to be a human carcinogen.

SUMMARY AND CONCLUSIONS

Fluensulfone induced an increased incidence of pulmonary hyperplasia and adenomas in an 18-month bioassay in mice but not carcinomas. No increased incidences of tumors in other organs in mice were produced, and no increased incidences of tumors were detected in any tissues in the rat. Fluensulfone is metabolized by Cyp 2f2, which is highly specific to the mouse and is a major difference between mice, rats, and humans. This difference in metabolism provides strong evidence for lack of effect in the resistant species, rats, and predicts a lack of effect in humans. Furthermore, an increase in proliferation is seen in mice following administration of fluensulfone, which is transient in nature and similar to isoniazid. The epidemiology of isoniazid clearly indicates that there is no evidence for an increased incidence of lung tumors in treated individuals, even at doses that approximate those in mice. Furthermore, like fluensulfone, isoniazid does not produce tumors in the rat. The significant differences in anatomy and histopathogenesis between mouse lung neoplasms and in humans support the conclusion that the tumors induced by fluensulfone in mice are not predictive of a cancer hazard or risk for humans.

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