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Mode of action analysis for liver tumors from oral 1,4-dioxane exposures and evidence-based dose response assessment

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ABSTRACT

1,4-Dioxane is found in consumer products and is used as a solvent in manufacturing. Studies in rodents show liver tumors to be consistently reported after chronic oral exposure. However, there were differences in the reporting of non-neoplastic lesions in the livers of rats and mice. In order to clarify these differences, a reread of mouse liver slides from the 1978 NCI bioassay on 1,4-dioxane in drinking water was conducted. This reread clearly identified dose-related non-neoplastic changes in the liver; specifically, a dose-related increase in the hypertrophic response of hepatocytes, followed by necrosis, inflammation and hyperplastic hepatocellular foci. 1,4-Dioxane does not cause point mutations, DNA repair, or initiation. However, it appears to promote tumors and stimulate DNA synthesis. Using EPA Guidelines (2005), the weight of the evidence suggests that 1,4-dioxane causes liver tumors in rats and mice through cytotoxicity followed by regenerative hyperplasia. Specific key events in this mode of action are identified. A Reference Dose (RfD) of 0.05 mg/kg day is proposed to protect against regenerative liver hyperplasia based on a benchmark dose (BMD) approach. Based on this RfD, a maximum contaminant level goal of 350 μg/L is proposed using a default relative source contribution for water of 20%.

1. Introduction

1,4-Dioxane is a contaminant of personal care products and cosmetics, and is also used as a solvent for organic products, lacquers, paints, varnishes, oils, waxes, inks, and animal and vegetable oils, among other uses (US EPA, 2013). Recent evaluations of 1,4-dioxane's toxicity are available and general information on its physical properties can be obtained from documents developed by the US EPA (2013), Health Canada (2010), NICNAS (1998) and the Sapphire Group (2007).

Based on weight of evidence assessments, several regulatory bodies have concluded that 1,4-dioxane is not a mutagen and that there is evidence of a threshold dose for the formation of liver tumors (Health Canada, 2010; NICNAS, 1998). NICNAS, 1998 did note there are several possible epigenetic mechanisms (including tumor promotion, cell proliferation, etc.) but inconsistent data from mechanistic studies do not support a clear mechanism. Similarly, the US Environmental Protection Agency (US EPA, 2013) in its recent review of 1,4-dioxane based on the oral route of exposure, alluded to several possible modes of action (MOA) including a regenerative hyperplasia, especially for liver tumors, but then concluded that the available evidence was inadequate to establish a mode of action (MOA) by which 1,4-dioxane or a transient or terminal metabolite induces liver tumors in rats and mice. EPA's (2013) conclusion is based, in part, on apparent uncertainty in the toxic moiety for 1,4-dioxane and the apparent lack of noncancer toxicity data from several mouse bioassays at doses that evoke tumors, or that otherwise appear to have conflicting information concerning non-neoplastic lesions in the liver of rodents exposed orally to 1,4-dioxane. Although there is general agreement among studies on 1,4-dioxane with regard to neoplastic changes, there are substantial differences in the reporting of non-neoplastic lesions in the liver from various repeated exposure studies and carcinogenesis studies. One study (NCI, 1978) reported no non-neoplastic lesions in the livers of mice, at least at the high dose, while other studies reported swelling of the centrilobular hepatocytes, necrosis, and hyperplasia at comparable or lower doses (Kano et al., 2008, 2009; Yamazaki et al., 1994). Since these studies span 3 decades, differences in histologic approaches for

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quantifying and reporting non-neoplastic changes may have been responsible for the differences noted across the studies.

No epidemiology studies, case reports, or clinical trials exist that examine potential adverse health effects of 1,4-dioxane following oral or dermal exposure. However, multiple studies examined the effects of acute inhalation exposure in healthy volunteers (Yant et al., 1930; Fairley et al., 1934; Wirth and Klimmer, 1936; Silverman et al. 1946; Young et al., 1977; Ernstgard et al., 2006). Each of these studies is limited by small sample size and some exposures were very high (i.e., up to 5500 ppm in Yant et al., 1930). Although limited by small sample sizes and limited latency periods, these studies found no increased risk of cancer-related mortality among workers exposed to 1,4-dioxane, Theiss et al. (1976) also found no evidence of liver or kidney disease among a small sample of retired workers. Furthermore, two case reports show that high acute occupational exposure to 1,4-dioxane can result in liver, kidney, and central nervous system toxicity (Barber, 1934; Johnstone, 1959). Exposure estimates are not available from Barber (1934), but Johnstone (1959) reported that the worker was exposed to 208–650 ppm (mean 470 ppm) for 1 week in addition to an unknown dermal exposure.

In experimental animals, 1,4-dioxane from oral exposures caused liver toxicity as evidenced by several histological and/or biochemical changes (e.g., liver enzyme changes, centrilobular swelling, and/or necrosis) at all time points as early as 13 weeks of treatment (Kano et al., 2008), and as nicely summarized by EPA (2013) in a dose-related manner in both sexes of rats and mice after both oral and inhalation exposures.[1] EPA (2013) also showed that this liver toxicity and nasal toxicity (e.g., nuclear enlargement; vacuolar change, and/or squamous cell hyperplasia); precedes tumors in time in both sexes of rats and mice[2] with liver histopathology preceding tumors in dose in both sexes of rats[3] and liver toxicity *as evidenced by biochemistry* occurring at doses similar to those that evoke tumors in either sex of mice.[4] Liver toxicity *as evidenced by histopathology* does not consistently appear to either precede tumors in either sex of mice or necessarily even occur at tumorigenic doses. For example, liver hyperplasia (evidence of a regenerative cell proliferation) is not recorded in high dose female mice (NCI, 1978), although it is shown at 15% in the low dose females. Hepatic cytomegaly and necrosis was also observed for low dose females and for males from both exposure groups, although this latter evidence for male mice is equivocal. Single cell necrosis and hepatocellular hypertrophy were increased in male and female mice given 4000 ppm and above in drinking water for 13 weeks (Kano et al., 2008) and hyperplasia was noted in rats and mice following exposure to 1,4-dioxane in drinking water for 2 years (Yamazaki et al., 1994). The hyperplasia originally reported in the Yamazaki et al. study (1994) was later changed to altered hepatocellular foci including acidophilic, basophilic, and clear cell foci based on certain diagnostic criteria (Kano et al., 2009).

In reviewing this information, the lack of appearance (or consistency) of liver toxicity in mice before tumor occurrence, in terms of dose, seemed at odds with the appearance of liver toxicity before tumor occurrence in both rats and mice at all time points, and the appearance of this toxicity before tumors in dose in rats. For the NCI study, we suspected that the lack of evidence for non-neoplastic lesions had more to do with the common practice of pathologists at this period of time (1978) to record only the most severe endpoint within an organ of an experimental animal (e.g., a tumor) despite the presence of noncancer toxicity. Our suspicion was confirmed by McConnell (2011) who stated that a common practice in the late 1970's was to identify the most severe endpoint, and specifically for these bioassays, to identify and score tumors.

In order to explore these differences in liver histopathology for mice observed across studies and to better understand the sequence of events that may have contributed to the MOA of the observed liver tumors, a blinded reread of the NCI (1978) liver slides for mice was conducted since this study had no reported non-neoplastic lesions in the liver at the high dose. Following the completion of this work, a review of all non-neoplastic lesions in the liver observed in the repeated exposure studies was conducted. In addition, a thorough review of genotoxicity studies was conducted which included DNA replication and promotion bioassays as well as mutation, initiation, and DNA repair studies. Based on the histopathology data as well as the genotoxicity information, a hypothesis concerning the MOA was developed, as shown in Fig. 1, and an MOA analysis was performed.

The EPA (2005) cancer guidelines were utilized to conduct the MOA analysis. This analysis was then used to conduct a dose response assessment, which was similar in many respects to that derived by EPA (2013), but sufficiently different in outcome and direction to warrant additional discussion and review by the scientific community. In brief, our proposed MOA for liver tumors accounts for nearly all of the findings, to the point where other tumor MOAs, such as mutagenicity, can be credibly excluded. This new information and analysis may be valuable to address the potential environmental risk from oral exposures to 1,4-dioxane, and specifically for the development of safe concentrations of this chemical in drinking water. A similar MOA analysis may be needed for other tumors evoked by 1,4-dioxane.

2. Methods

2.1. National Cancer Institute (NCI) data review

Because terminology and practices for reporting liver lesions has changed since the time of the NCI study (1978), and because EPA (2005) is focusing more on an understanding of a chemical's Mode of Action (MOA) prior to any determination of its dose response, a re-review of the liver slides of mice from the NCI study (1978) was performed. This reanalysis was performed at the Experimental Pathology Laboratories (EPL), Research Triangle Park, NC during September through November 2012. The objective of the slide review was to determine if any non-neoplastic lesions in the liver were present in an effort to understand the sequence of events that may have contributed to the MOA of the observed liver tumors in mice. Another reason for the slide review was because at the time of the original slide review (i.e., 1978) the NCI typically recorded only the most severe diagnosis on a given slide, (e.g., adenoma or carcinoma). During this timeframe, the focus of cancer bioassays was to determine the potential carcinogenic activity of the chemical, not its potential chronic toxicity. For example, if an adenoma, carcinoma, and evidence of chronic toxicity (e.g., hepatocellular hypertrophy), were all present on a given slide, only the tumor response was typically recorded. Thus, it was unclear whether non-neoplastic lesions were present in the livers of mice but were not recorded in the NCI carcinogenicity study. An initial review of the livers from five control male mice was conducted followed by five male mice from the high dose. The reason for the initial review was to obtain a baseline for the livers of control animals and to understand the spectrum of lesions that occurred in the high dose

[1] See EPA (2013) Table 4-2 (p. 32), Table 4-5 (p. 41), Table 4-8 (p. 47), Table 4-9 (p. 48), and Table 4-12 (p. 52).

[2] See EPA (2013) Table 4-2 (p. 32), Table 4-16 (p. 58), Table 4-17 (p. 59), and Table 4-22 (p. 69).

[3] Compare EPA (2013) Table 4-5 (p. 41) to its Table 4-6 (p. 42); compare Tables 4-8 & 4-9 (p. 47-48) to Tables 4-10 & 4-11 (p. 50); compare Table 4-20 (p. 64) to Table 4-21 (p. 65).

[4] See EPA (2013) Table 4-25 (p. 85).

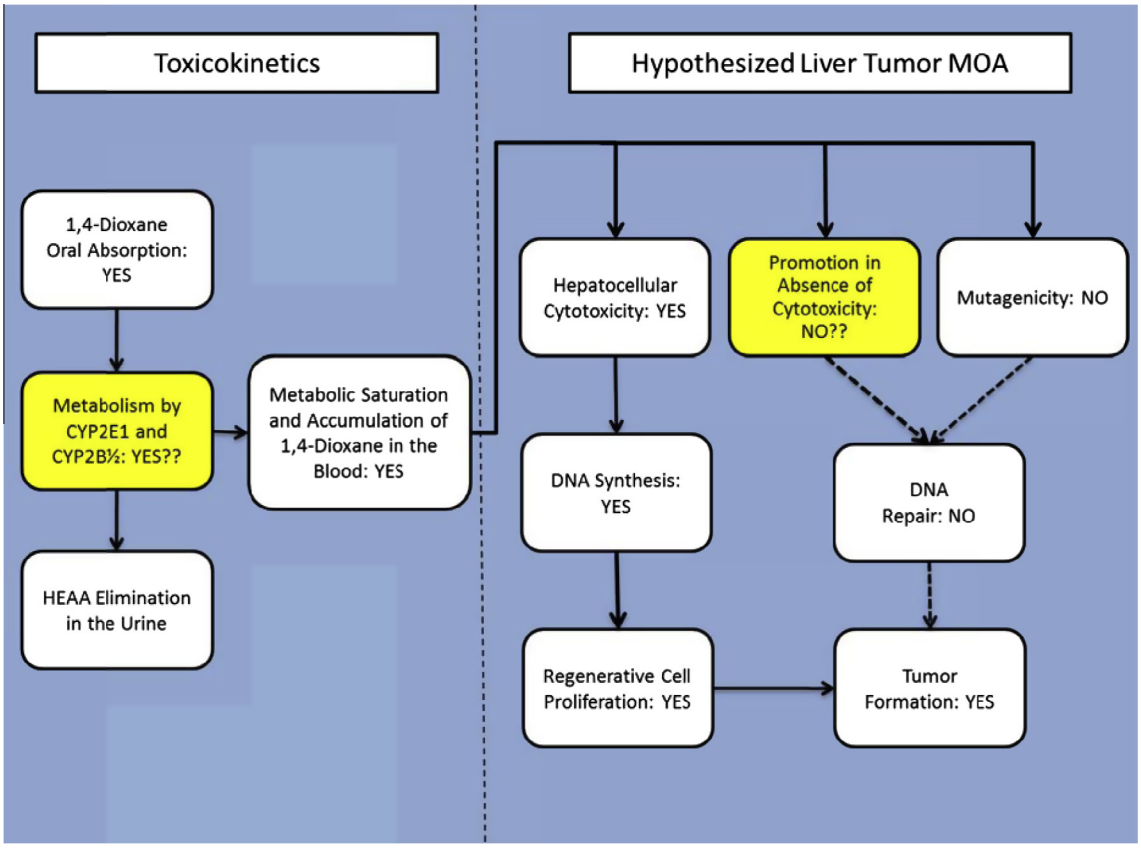

Fig. 1. Proposed Mode of Action (MOA) approach for 1,4-dioxane liver tumors.

animals as a result of exposure to 1,4-dioxane after 2-years of exposure. The remainder of male mice and female mice from the carcinogenicity study were examined in a "blind" fashion with no knowledge of dose. It should be noted that in some cases the liver could not be evaluated for non-neoplastic lesions because not enough tissue was available due to tumor involvement or postmortem autolysis. In such cases these animals were deleted from the sample.

Terminology for this reevaluation included:

- Depletion of hepatocellular glycogen – Homogeneous cytoplasm within a hepatocyte due to the lack of glycogen. Glycogen appears as "empty" spaces in the cytoplasm of normal hepatocytes when stained with H&E. Hepatocellular hypertrophy – Conspicuously larger than normal hepatocytes due to an increase in the amount of cytoplasm. Hepatocytes are also typically more eosinophilic and devoid of recognizable glycogen. If hypertrophy was present, an attempt was made to determine if there was a zonal predilection, e.g., periportal, midzonal, centrilobular or diffuse.
- Necrosis (particularly hepatocellular) – Increased cytoplasmic eosinophilia and disintegration of cytoplasm and cell membranes. Nuclei are often still apparent.
- Inflammation – Focal influx of neutrophils and lymphocytes, primarily in the area of hepatocellular necrosis.
- Fatty infiltration – Individual hepatocytes containing round clear vacuoles.
- Non-neoplastic hyperplasia (e.g., focal hyperplasia of several types: Kupffer cell, bile duct, and basophilic, eosinophilic, clear cell, and mixed cell hyperplastic foci)- Kupffer cell hyperplasia is typically recognized as diffuse proliferation of Kupffer cells. Bile duct hyperplasia is similarly recognized as multifocal proliferation of bile ducts. In contrast, basophilic, eosinophilic, clear-cell and mixed-cell foci are recognized as focal clonal-like accumulations of normal appearing hepatocytes with tinctorial qualities that allow for the specific morphological classifications. Importantly, the various types of hepatocellular foci are considered pre-neoplastic changes.
- Various types of neoplasms – Hepatocellular adenoma and carcinoma, leukemia, lymphoma, etc. are diagnosed using standard morphological criteria.

2.2. Mode of Action (MOA) analysis

US EPA (2005) guidelines for cancer risk assessment state that the MOA should be evaluated in determining the approach for dose response assessment from positive human or experimental animal tumor data. This evaluation is accomplished by first proposing a MOA, including identification of key events. Data on these key events, including available *in vivo*, *in vitro*, and mechanistic studies are then evaluated relative to the modified Hill criteria. When sufficient data are available, a biologically based dose–response (BBDR) model is the preferred method for low dose extrapolation. Absent such data, low dose extrapolation usually proceeds via a linear model (if the chemical acts via a direct DNA-reactive MOA or the MOA is not known) or a non-linear model (for a non-DNA-reactive MOA) based on one or more combinations of relevant tumors. Afterwards, determination of the human equivalent dose from the experimental animal dose is accomplished by a comparison of human and experimental animal kinetics or a default procedure.

These guidelines were followed by first describing our methods for analyzing mutagenicity and growth stimulation MOAs. EPA guidelines (2005) were then followed to model tumors by either

a mutagenic (linear at low dose) or a threshold (not linear at low dose) MOA, or to "consider the respective contribution of each mode of action in different dose ranges."

The MOA framework within the cancer risk assessment guidelines of the US Environmental Protection Agency (2005) is built in part on the work of Meek et al. (2003). In accordance with these guidelines, we consider whether each hypothesized MOA is sufficiently supported by the existing human or experimental animal data, and whether the available evidence suggests these MOAs are relevant to humans. Based on the data describing the key precursor events, we also consider life stage susceptibility for dose–response analysis. Furthermore, as per US EPA (2005) guidelines, the model used for extrapolation to low doses is determined based on the most relevant MOA(s) given our current understanding of the science.

US EPA (2005) lists several potential MOAs. We specifically investigated two of them discussed by EPA (2013) for the liver and nasal tumors evoked by oral 1,4-dioxane exposures; these MOAs were:

- A heritable mutation to liver and/or nasal cell DNA.
- Liver and nasal cytotoxicity followed by regenerative cell proliferation and stimulation of endogenously mutated DNA.

The mutagenicity data was analyzed to determine the extent to which a mutagenic MOA is responsible for the liver and nasal tumors observed in the 1,4-dioxane bioassays. To make that evaluation, one is interested in the consistency of, or concordance between, the pattern of tumor response, on the one hand, and the pattern of the selected genotoxicity measures. This comparison is ideally done between the tumor data and a marker of mutagenicity in the same species, sex, and tissue.

If such mutagenicity data are negative for 1,4-dioxane, then relevant available *in vivo* genotoxicity data are reviewed for possible relevance for tumor development. Concordance of the tumor and genotoxicity patterns is based on comparison of the dose–response curves describing the observed tumor and genotoxicity data sets.

We also evaluate data to determine the extent to which a regenerative cell proliferation mode of action is responsible for the liver and nasal tumors observed in the bioassays. For this MOA, we used US EPA Guidelines for Cancer Risk Assessment (2005) and specifically, the modified Hill criteria in the determination of sufficiency of evidence.

2.3. Modeling tumors via mutagenic and regenerative cell proliferation MOAs

The US EPA approach for quantitatively characterizing cancer risk begins by modeling the available data and defining a Point of Departure (POD). The POD is a dose–response point that is estimated for a specified response, the Benchmark Response (BMR), near the low end of the dose–response data (US EPA, 2005). The 1,4-dioxane analysis focuses on experimental animal data, thus the approaches described here are those most relevant to animal studies. Risk levels of 1–10% are commonly used for the BMR, and the US EPA Guidelines for Cancer Risk Assessment (2005) specify that the POD should be the lowest POD that is adequately supported by the data. The dose at the BMR, called the Benchmark Dose (BMD), is usually defined in terms of extra risk. For a specified BMR, the BMD is then the dose that satisfies the following formula for extra risk:

$$\mathrm{BMR} = \frac{P(\mathrm{BMD}) - P(0)}{1 - P(0)}$$

As per EPA (2005) guidelines, uncertainties were represented in the estimation of risk as the best estimate and its upper bound. A lower bound on risk is often desired to show the full range of uncertainty, but current EPA (2012) BMD software does not provide a way to estimate this risk in many of its models. For policy reasons, the POD is usually defined using the upper bound on the risk, which is associated with the lower 95% confidence bound on the prescribed dose, referred to as the lower bound on the benchmark dose, or BMDL. However, as noted above, US EPA (2005) also recommends presenting the best estimate POD, referred to as the Benchmark Dose, or BMD, to improve the uncertainty description. Another common term is the Slope Factor (SF). This value is calculated from the BMD or BMDL and represents the slope at the given parameter; the SF can be used to determine risk at lower doses.

The next step depends on the MOA that has been determined to apply to the tumor type of interest. For a mutagenic MOA, the modeling assumption, in the absence of more definitive data, is a one molecule-threshold dose, and low-dose linearity. A line connects the POD to the origin, corrected for background. The slope of the line is used to estimate a risk per incremental increase in dose. Using a BMD or BMDL based on extra risk, one calculates the SF directly from the desired BMR level. The stability of the slope estimate is gauged by evaluating it for different BMR and BMD values. For example, if the BMD at 0.10 excess risk equals 0.83, then:

$$\mathrm{SF} = \mathrm{BMR}/\mathrm{BMD} = 0.10/0.83 = 0.0012$$

When the chemical acts via a regenerative hyperplasia MOA, which is expected to exhibit a threshold in response, US EPA describes a nonlinear modeling approach for quantitatively characterizing cancer risk. In this case, the POD (based on either tumors or a precursor endpoint) is used to develop a Reference Dose or Reference Concentration for oral or inhalation exposures, respectively, following the procedures prescribed by US EPA for non-cancer toxicity, with the BMDL commonly divided by the appropriate combination of uncertainty factors (US EPA, 2002).

However, if supporting data exists, US EPA (2005) guidelines also allow the separate evaluation of MOAs in different parts of the dose response range. In this case, the guidelines are not prescriptive, but an approach consistent with the guidelines would be to select a model within US EPA's array that best fits the most relevant data from a MOA(s) perspective. Afterwards, different approaches may be used at points of the dose response curve associated with the appropriate MOA(s). An example of this latter approach has been published for acrylamide exposures by Dourson et al. (2008).

2.4. Maximum contaminant level goal

The maximum contaminant level goal (MCLG) is the level of a contaminant in drinking water at or below which there is no known or expected risk to health. The Maximum Contaminant Level (MCL) is the highest level of a contaminant that is allowed in drinking water. Typically MCLs are set as close to MCLGs as feasible taking the best available treatment technology and cost into consideration. The main difference between the MCLG and MCL is that the MCLG is a non-enforceable public health goal and the MCL is an enforceable standard. The first step in determining a MCL is an evaluation of the adverse effects caused by the chemical and the doses needed to cause such effects.

The MCLG can be derived for chemicals with a cancer endpoint based on the SF or a non-cancer endpoint based on the RfD. The MCLG is derived by converting the SF or RfD to a water concentration. If an RfD is utilized, then EPA (2005) recommends the use of a margin of safety to protect against adverse effects. This is done by calculating the daily average intake for humans using the RfD and adjusting it using the average body weight of 70 kg and a water consumption of 2 liters/day. The MCL would then be based on this water concentration and a default assumption that the water is

only 20% of the total source of 1,4-dioxane. This is known as the relative source contribution (RSC). However, the RSC can be based on actual data from the various sources of exposure, such as water, food, air and consumer products. Based on the available data for 1,4-dioxane, a data-derived RSC was considered.

3. Results

3.1. Liver tumors and the re-review of the NCI mouse liver slides

Tables 1 through 5 show the results of the re-review of the liver slides from mice from the NCI study conducted in 1978. Fig. 2 shows photomicrographs of select lesions observed during the pathology review of the slides. These results and photomicrographs were summarized in a study report (McConnell, 2013). Lesions that were noted with increased incidence included the following:

- Depletion of hepatocellular glycogen (Table 1): Depletion, shown here as an average severity score where the lower the score the more the depletion, was noted in many control mice, probably due to various causes, e.g., inanition and chronic disease of various types. Despite this high control incidence, exposed mice appeared to have a higher depletion of glycogen in the 1,4-dioxane-exposed mice in a dose–response manner.
- Hepatocellular hypertrophy (Tables 2 and 5): There was a very clear dose–response for this endpoint, shown here as both an increase in incidence and average severity score where the higher the severity score the greater the hypertrophy. In affected livers, most of the hepatocytes were diffusely enlarged. In cases with minimal hypertrophy, the affected hepatocytes were more apparent in the central lobular areas near the central vein.
- Necrosis (Table 2) (particularly hepatocellular): Dose-related hepatocellular necrosis was apparent in most of the exposed animals, manifested as isolated diffusely scattered necrotic hepatocytes. Most of the necrotic hepatocytes were centrilobular, particularly near the central veins.
- Inflammation (Table 2): Inflammation was micro-focal and was primarily in reaction to the necrosis of individual hepatocytes (described above). The appearance was somewhat unusual in that enlarged hepatocytes with almost normal appearing nuclei appeared to be invaded by neutrophils and lymphocytes. There was a definite dose–response.
- Fatty infiltration: Fatty change was only rarely observed and there was no evidence of a treatment related effect (data not shown).
- Non-neoplastic hyperplasia (e.g., focal hyperplasia of several types):
- Kupffer cell (Table 2): An increase in Kupffer cell hyperplasia was found in male mice and high dose female mice, and the response appeared to be dose related.
- Bile Duct Hyperplasia: Bile duct hyperplasia was only found in a few animals and only in exposed animals (data not shown). There were not enough affected mice to make any definite conclusions regarding dose-effects for this endpoint.
- Basophilic, eosinophilic, clear cell and mixed cell foci (Table 3): These specific types of hyperplastic foci were observed in a dose-related pattern, especially when the various types of foci are combined. The incidence of foci decreases at the high dose as the proportion of experimental animals with adenomas and carcinomas increases. This suggests that tumors arise from these foci. However, an alternative explanation might be that the available area of adjoining normal liver in the tissue sample that were taken was less due to the tumors, and that if more tissue samples were taken from areas without tumors, more hepatic foci would have been found.[5] Interestingly, the hepatocytes in these clonal expansions are generally of normal size, i.e., not enlarged (hypertrophic), as are the hepatocytes surrounding the foci.
- Various types of neoplasms (Table 4), e.g., hepatocellular adenoma and carcinoma, leukemia, lymphoma, etc. were observed with an increased incidence in the treated mice. The tumor incidence closely matched the incidence reported in the NCI study (1978).

Table 1
Glycogen incidences in male and female B6C3F1 mice from NCI (1978).

Dose	*n*	2 = Normal (%)	1 = Decreased/ minimal (%)	0 = No glycogen (%)	Average score
Male					
Control	44	14 (32)	19 (43)	11 (25)	1.1
Low	43	5 (12)	6 (14)	32 (74)	0.4
High	42	1 (2.4)	6 (14)	35 (83)	0.2
Female					
Control	46	8 (17)	20 (44)	18 (39)	0.8
Low	37	7 (19)	13 (35)	17 (46)	0.7
High	30	3 (10)	6 (20)	21 (70)	0.4

In addition to the increase in incidence in many of these endpoints, the average severity of these endpoints also generally increased as shown in Table 5. In general, the non-neoplastic lesions in the male mice were more apparent than in the females, but this may be due to the fact that the low dose female mice had only about ½ of the dose of the low dose male mice. The high doses in both sexes gave roughly comparable results, except for the incidence of Kupffer cell hyperplasia.

The appearance of the liver toxicity follows the pattern where glycogen depletion occurs either concurrently with, or preceding, hypertrophy in both sexes. This was followed closely by necrosis and inflammation in males, but a high control incidence of necrosis and inflammation clouded this overall pattern found in females. This female control incidence may have been due to an infection of murine hepatitis virus that was known to occur in all mice at the time of the bioassay (as per EEMc).

The incidence of hypertrophy, foci of hepatocyte hyperplasia, adenoma and carcinoma followed the pattern shown in Fig. 3 for pooled male and female average responses. In terms of dose–response behavior, hypertrophy preceded the formation of foci, which appeared to precede formation of tumors. This pattern was also evident in an individual animal analysis (data not shown, but available upon request).

3.2. Description of key events in the proposed MOA for liver tumors

Evaluation of genotoxicity studies in the scientific literature indicate that 1,4-dioxane does not cause point mutations, DNA repair, or initiation. However, 1,4-dioxane appeared to promote tumors and stimulate DNA synthesis. Using a Mode of Action (MOA) analysis and human relevance framework (US EPA, 2005), the weight of the evidence supports a non-linear mode of action with 1,4-dioxane causing liver tumors in rats and mice through a MOA involving cytotoxicity followed by regenerative hyperplasia and stimulation of endogenously formed mutations as the cause

[5] One approach to make this distinction would be to nominalize the amount of non-tumor tissue among experimental groups and then express foci per square cm liver tissue examined (excluding the obvious adenomas and carcinomas). Of course if a hepatic focus is still present at the end of a 2-year NCI bioassay, it is not a particularly aggressive lesion and its relationship to becoming a cancer seems highly unlikely.

Table 2
Incidences of selected nonneoplastic lesions in male and female B6C3F1 mice from NCI (1978).

Dose	0-No lesion (%)	1-Minimal (%)	2-Mild (%)	3-Moderate (%)	4-Marked (%)	Total (%)
Male						
Hypertrophy						
Control (0 mg/kg day)	41 (93)	2 (4.5)	1 (2.3)	0	0	**3/44 (6.8)**
Low (720 mg/kg day)	2 (4.7)	17 (40)	24 (56)	0	0	**41/43 (95)**
High (830 mg/kg day)	1 (2.4)	13 (31)	27 (64)	1 (2.4)	0	**41/42 (98)**
Necrosis						
Control (0 mg/kg day)	44 (92)	4 (8.3)	0	0	0	**4/48 (8.3)**
Low (720 mg/kg day)	4 (9.8)	16 (39)	16 (39)	5 (12)	0	**37/41 (90)**
High (830 mg/kg day)	7 (18)	20 (50)	10 (25)	3 (7.5)	0	**33/40 (83)**
Inflammation						
Control (0 mg/kg day)	44 (92)	4 (8.3)	0	0	0	**4/48 (8.3)**
Low (720 mg/kg day)	4 (9.8)	17 (41)	16 (39)	4 (9.8)	0	**37/41 (90)**
High (830 mg/kg day)	8 (20)	19 (48)	10 (25)	3 (7.5)	0	**32/40 (80)**
Kupffer cell hyper						
Control (0 mg/kg day)	41 (93)	2 (4.5)	1 (2.3)	0	0	**3/44 (6.8)**
Low (720 mg/kg day)	14 (33)	20 (47)	8 (19)	1 (2.3)	0	**29/43 (67)**
High (830 mg/kg day)	11 (26)	15 (36)	13 (31)	3 (7.1)	0	**31/42 (74)**
Female						
Hypertrophy						
Control (0 mg/kg day)	46 (100)	0	0	0	0	**0/46 (0)**
Low (380 mg/kg day)	20 (54)	14 (38)	3 (8.1)	0	0	**17/37 (46)**
High (860 mg/kg day)	1 (3.3)	14 (47)	12 (40)	3 (10)	0	**29/30 (97)**
Necrosis						
Control (0 mg/kg day)	19 (41)	25 (54)	2 (4.3)	0	0	**27/46 (59)**
Low (380 mg/kg day)	20 (54)	14 (38)	2 (5.4)	1 (2.7)	0	**17/37 (46)**
High (860 mg/kg day)	2 (11)	12 (63)	5 (26)	0	0	**17/19 (90)**
Inflammation						
Control (0 mg/kg day)	20 (44)	24 (52)	2 (4.3)	0	0	**26/46 (57)**
Low (380 mg/kg day)	20 (54)	14 (38)	2 (5.4)	1 (2.7)	0	**17/37 (46)**
High (860 mg/kg day)	3 (16)	11 (58)	5 (26)	0	0	**16/19 (84)**
Kupffer cell hyper						
Control (0 mg/kg day)	46 (100)	0	0	0	0	**0/46 (0)**
Low (380 mg/kg day)	36 (97)	1 (2.7)	0	0	0	**1/37 (2.7)**
High (860 mg/kg day)	21 (70)	5 (17)	2 (6.7)	1 (3.3)	1 (3.3)	**9/30 (30)**

Table 3
Foci incidences in male and female B6C3F1 mice from NCI (1978); B = basophilic; E = eosinophilic; CC = clear cell; MC = mixed cell.

Dose	n	B Focus (%)	E Focus (%)	CC Focus (%)	MC Focus (%)	Total Foci (%)
Male						
Control	44	2 (4.5)	0	2 (4.5)	0	4/44 (9.1)
Low	43	6 (14)	2 (4.7)	2 (4.7)	3 (7.0)	13/43 (30)
High	42	2 (4.8)	0	4 (9.5)	1 (2.4)	7/42 (17)
Female						
Control	46	1 (2.7)	1 (2.2)	0	0	1/46 (2.2)
Low	37	1 (3.3)	5 (14)	2 (5.4)	2 (5.4)	10/37 (27)
High	30		2 (6.7)	4 (13)	1 (3.3)	8/30 (27)

Table 4
Incidences of animals with neoplasms in male and female B6C3F1 mice from NCI (1978); parentheses indicate percentage.[a]

Dose	Adenoma		Carcinomas		Adenomas/carcinomas
	NCI	Recount	NCI	Recount	Recount
Male					
Control	6/49	2/44 (4.5)	2/49	4/44 (9)	5/44 (11)
Low	1/50	1/48 (2)	18/50	16/48 (33)	17/48 (35)
High	4/47	3/48 (6)	24/47	21/48 (43)	22/48 (45)
Female					
Control	0/50	0/49	0/50	0/49	0/49
Low	9/48	7/45 (16)	12/48	7/45 (16)	14/45 (31)
High	6/37	11/37 (30)	29/37	23/37 (62)	29/37 (78)

[a] In some cases the "*n*" between foci and tumor counts differ and in some cases the "*n*" between the tumor counts shown here and with NCI differ. In former case this is due to the fact that some tissues did not have enough non-tumor related tissue to make a judgment on foci. In the latter case this is due to the fact that the reread of a few slides was not possible, or in one case the reread allowed a larger "*n*".

of tumors occurring in the liver and nasal cavity of rodents exposed to 1,4-dioxane. See Fig. 1. The specific key events in this mode of action include: (1) accumulation of parent compound, (2) liver cell hypertrophy and necrosis, (3) DNA synthesis, (4) regenerative cell proliferation, and (5) promotion of endogenously-initiated tumors.

Table 5
Average severity score in male and female B6C3F1 mice from NCI (1978).

Dose	Hypertrophy	Necrosis	Inflammation	Kupffer cell hyperplasia
Male				
Control	0.1	0.08	0.08	0.1
Low	1.5	1.5	1.5	0.9
High	1.7	1.2	1.2	1.2
Female				
Control	0	0.6	0.6	0
Low	0.5	0.6	0.6	0.03
High	1.6	1.2	1.1	0.5

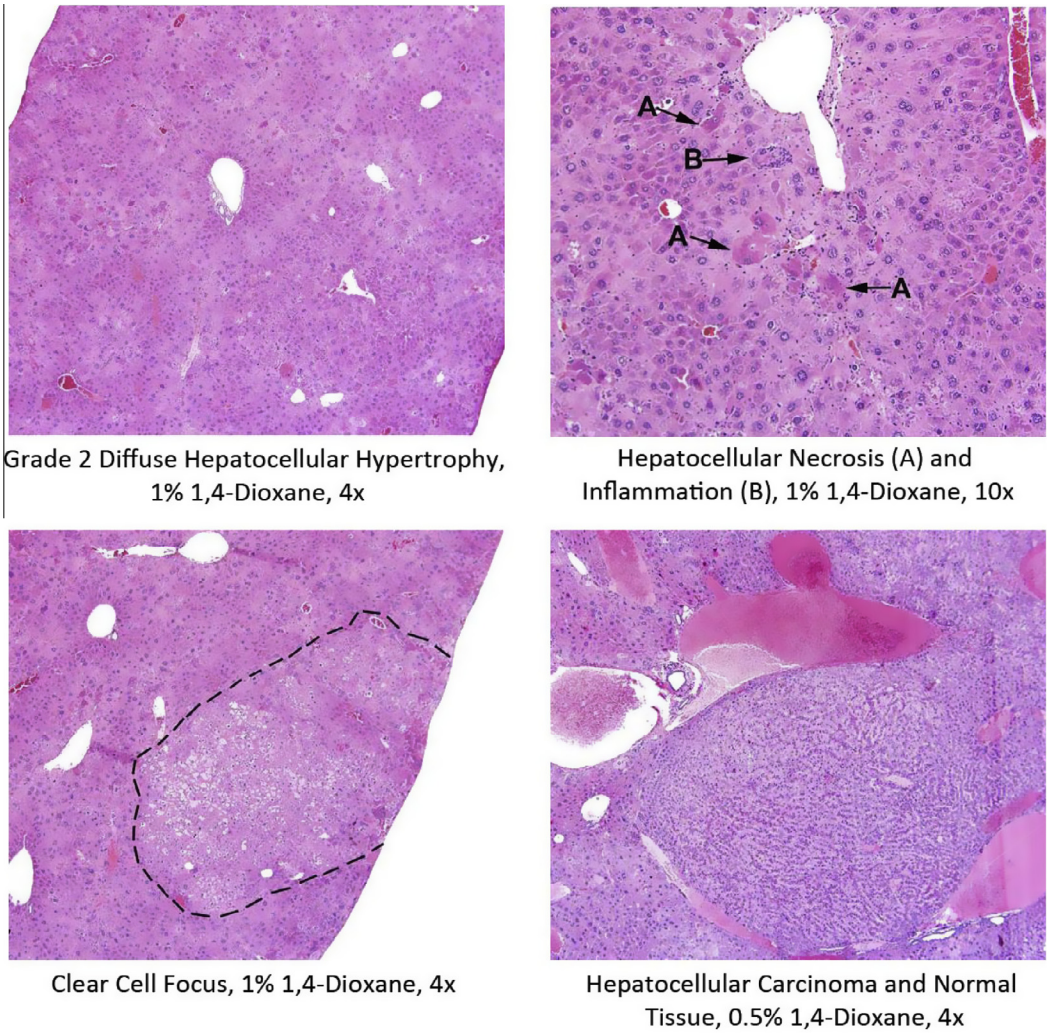

Fig. 2. Photomicrographs from the re-review of the original NCI (1978) study slides.

3.2.1. Key event 1: Accumulation of parent compound

Humans, rats, and mice extensively metabolize 1,4-dioxane (Young et al., 1978; Kociba et al., 1975; Sweeney et al., 2008). Evidence supports the conclusion that cytochrome P450 (CYP450) oxidases are the major pathway for 1,4-dioxane oxidation, although the precise CYP450 has not been unequivocally identified. In rats, and by inference in humans as well, metabolism is a capacity-limited process (Young et al., 1978). As doses of 1,4-dioxane increase, a transition occurs from linear first-order pharmacokinetics to nonlinear Michaelis–Menten kinetics. This transition appears to occur at plasma concentrations in the range of 30–100 μg/mL. At doses yielding plasma concentrations below this level, 1,4-dioxane, is rapidly and efficiently detoxified. When dose of 1,4-dioxane approaches or exceeds the metabolizing capacity, the unmetabolized fraction of the dose increases and target organ toxicity occurs (Kociba et al., 1975; Young et al., 1978). Thus, there appears to be a threshold below which metabolism and elimination are rapid and with less or perhaps without toxicological effects. In rat studies as long as 2 years, administration of 0.01% 1,4-dioxane in drinking water produces plasma levels that are below the saturating threshold and no discernible effects is observed (Kociba et al., 1974, 1975). Several studies, including the 1978 NCI study, administered high doses (0.5% and 1.0% in drinking water) that would be expected on the basis of 1,4-dioxane's pharmacokinetic disposition to yield plasma concentrations well above 100 μg/mL. At these doses the parent compound would be eliminated more slowly and accumulate, resulting in pathology. Likewise, the higher-end doses used in most chronic and sub-chronic studies (i.e., 1–2% in drinking water) are well in excess of the metabolizing capacity and would be expected to result in toxicological manifestations (Argus et al., 1965, 1973; Kociba et al., 1974; NCI, 1978). However, Kano et al. (2008) gave numerous doses over 13 weeks in both rats and mice, including several doses that appeared to be within metabolic capacity, with few if any non-cancer effects noted.

Human environmental exposures to 1,4-dioxane are unlikely to approach doses that saturate metabolizing enzymes and which produce liver and nasal tumors in rats. Saturation of the CYP450 enzymes that metabolize 1,4-dioxane would be predicted to occur at the high 1,4-dioxane doses used in chronic cancer bioassays, resulting in elevated urinary and respiratory excretion of the parent compound. Conversely, at the environmentally- and occupationally-relevant concentration of 50 ppm (which is the ACGIH threshold limit value for 1,4-dioxane), humans extensively metabolize 1,4-dioxane with over 99% metabolism of a 50 ppm/6 h inhalation exposure. The elimination half-life in rats exposed to 50 ppm 1,4-dioxane for 6 h was calculated to be 1.01 h (Young et al., 1978). Hence, since humans, like rats, efficiently metabolize 1,4-dioxane at low doses, enzyme saturation is negligible at low exposure levels.

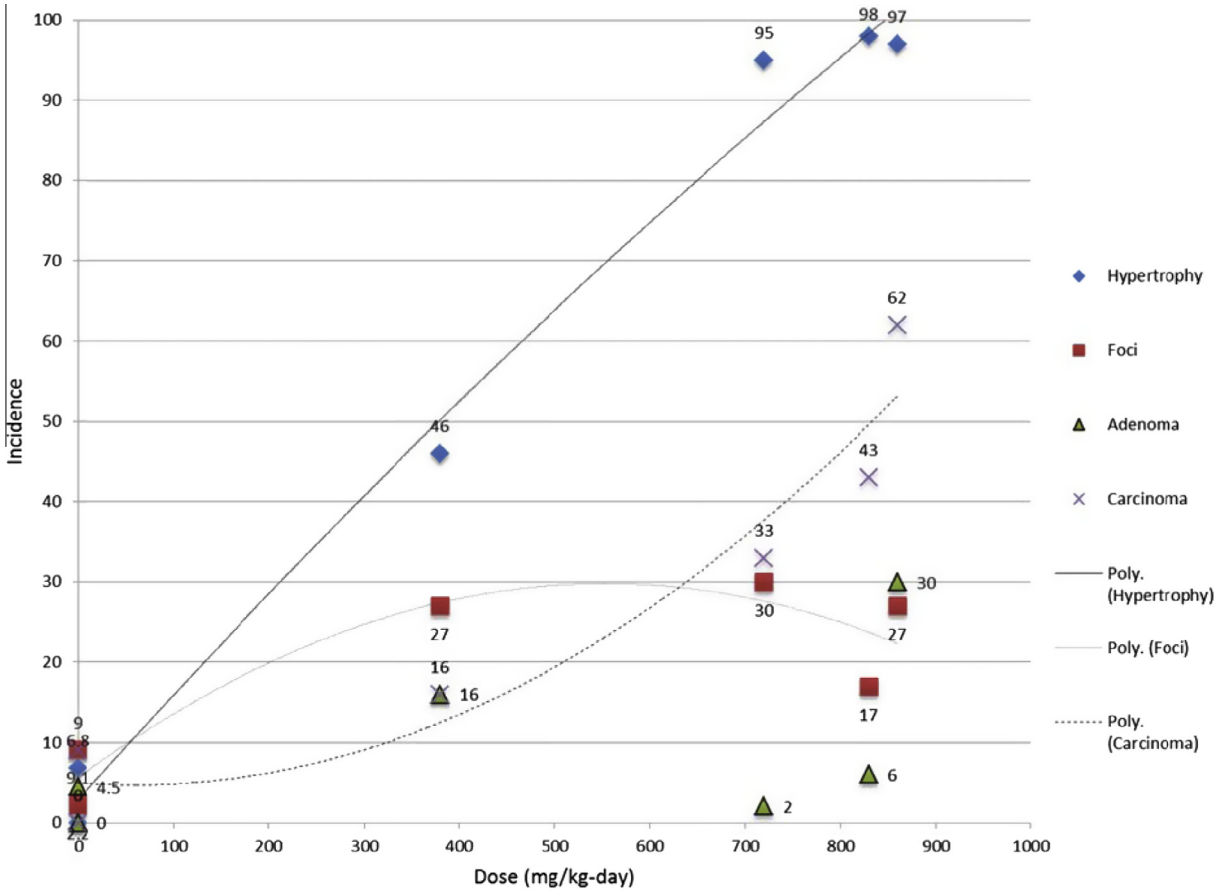

Fig. 3. Pooled incidence for 4 effects in B6C3F1 male and female mice given 1,4-dioxane (NCI, 1978).

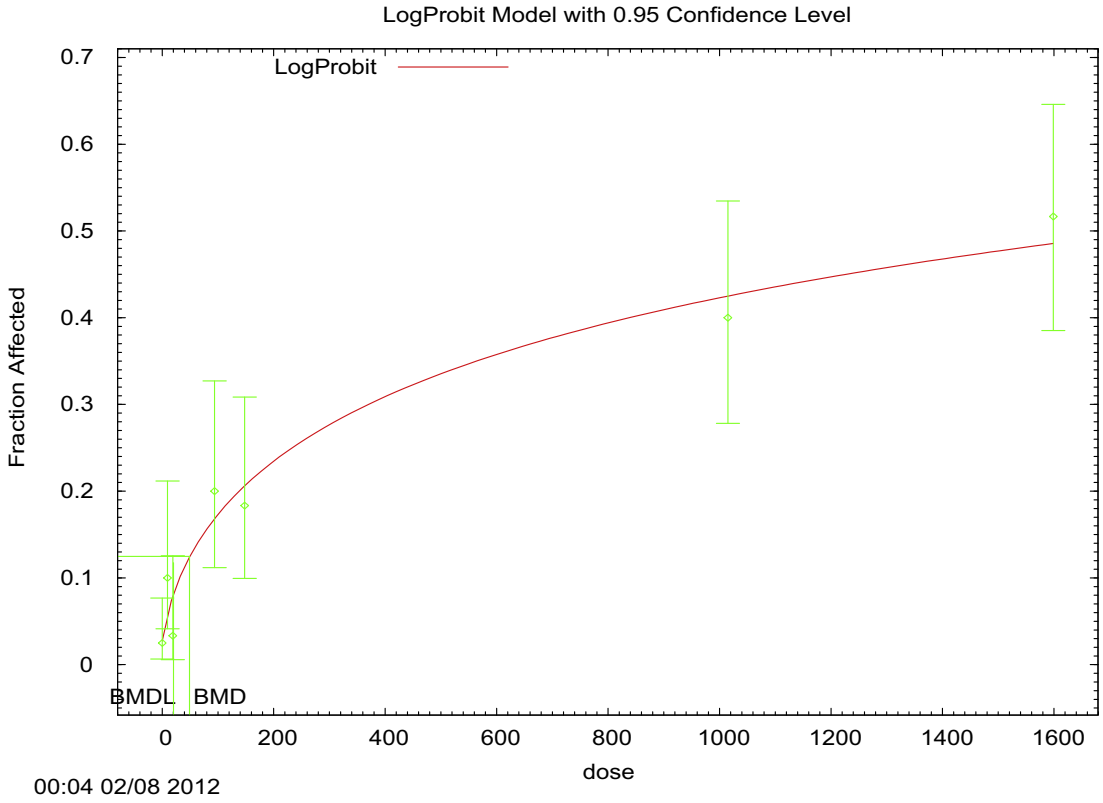

Fig. 4. Benchmark dose (BMD) in mg/kg day from log-probit model plot for the hepatic necrosis endpoint with a benchmark response of 10. Results of EPA (2012) modeling software.

3.2.2. Key event 2: Liver cell hypertrophy and necrosis

Liver cell hypertrophy and necrosis are key events in the 1,4-dioxane MOA leading to regenerative cell proliferation and, with chronic exposures, liver tumors. Liver changes including centrilobular swelling, single cell necrosis coincide exclusively with saturating doses of 1,4-dioxane and occur in as little as 11 weeks (Stott et al., 1981; Kano et al., 2008; Kasai et al., 2008, 2009). The mechanism by which 1,4-dioxane, or its metabolites, are hepatotoxic has not been rigorously investigated. Hence, it has been proposed that liver toxicity due to uncharacterized metabolites cannot be ruled out (US EPA, 2013). However, evidence suggests this scenario is highly unlikely: (1) phenobarbital-mediated induction of 1,4-dioxane metabolism does not increase liver toxicity (Nannelli et al., 2005); and (2) pretreatment with inducers of CYP450 also did not significantly change the extent of covalent binding in subcellular fractions (Woo et al., 1977). The macromolecule(s) to which 1,4-dioxane bound was not identified, but it is reasonable to expect that such covalent binding would be related to 1,4-dioxane toxicity. These data suggest that an unknown, highly toxic intermediate does not play a role in liver toxicity. Although there remains a remote possibility that a non-CYP450-mediated pathway yields a reactive intermediate at high doses, such an intermediate, if present, would not be a key event in the formation of tumors at low, environmentally relevant doses. The pharmacokinetic description of 1,4-dioxane disposition, however, supports the parent molecule as the toxic entity.

Evidence of hepatocellular damage preceding evidence of hepatocellular tumors caused by higher doses of 1,4-dioxane has been provided by several studies. Kociba et al. (1971, 1974) reported hepatocellular degeneration and necrosis in rats receiving 1.0% or 0.1% 1,4-dioxane. In 13 week studies, both Kasai et al. (2008) and Kano et al. (2008) reported a dose–response increase in plasma AST and ALT in male and female rats (Kasai et al.) and in both rats and mice (Kano et al.) with concurrent liver lesions (e.g., single-cell necrosis and centrilobular swelling). These changes temporally precede the pre-neoplastic and neoplastic changes characterized in subsequent 2-year studies (Kasai et al., 2009; Kano et al., 2009). Similarly, Stott et al. (1981) also reported signs of liver cytotoxicity at a dose of 1000 mg/kg/day but not 10 mg/kg/day following 11 weeks of 1,4-dioxane exposure.

3.2.3. Key event 3: DNA synthesis

EPA (2013) reported that 1,4-dioxane does not cause DNA repair activity in five standard *in vitro* and *in vivo* bioassays that tested for the presence of DNA repair in various model systems.[6] Conversely, 1,4-dioxane does cause DNA replication as evidenced by *in vitro* bioassays in rat hepatocytes. Specifically, three out of five studies, all conducted in rats, reported that 1,4-dioxane stimulated DNA synthesis in hepatocytes at doses at or above 1000 mg/kg. Of the two remaining studies, one was negative for replication in nasal epithelial cells (1500 mg/kg day for 2 week) and one was equivocal (2000 mg/kg).[7] Both the negative findings for DNA repair and the positive findings for DNA synthesis are consistent with our proposed MOA.

In general 1,4-dioxane does not appear to be genotoxic; nor do DNA and RNA synthesis appear to be genotoxic events. Rather, DNA synthesis appears to be a key event for a regenerative cell proliferation and/or tumor promotion and can occur in either the presence or absence of cytotoxicity. In light of the extensive toxicology evoked by 1,4-dioxane in the long-term animal bioassays (*v. infra*), DNA synthesis provides evidence that 1,4-dioxane promotes cell proliferation through cytotoxicity.

[6] See also EPA (2013) Table 4-23 (p. 76).

[7] See also EPA (2013) Table 4-24 (pages 79).

3.2.4. Key event 4: Regenerative cell proliferation

According to the proposed MOA, regenerative hyperplasia is anticipated to accompany hepatotoxicity. Dose–response and temporal data support the occurrence of cell proliferation and hyperplasia prior to the development of liver tumors in the rat model. For example, Kociba et al. (1971, 1974) showed evidence of hepatic regeneration as indicated by hepatocellular hyperplastic nodule formation in rats receiving 1.0% or 0.1% 1,4-dioxane. Using replicative DNA synthesis as a surrogate marker of cell proliferation, increased hepatocyte proliferation is a common finding at tumorigenic doses of 1,4-dioxane (Goldsworthy et al., 1991; Miyagawa et al., 1999; Stott et al., 1981; Uno et al., 1994). Cell proliferation appears to be an early response with significant changes (1.5- to 2-fold) occurring in rats with as little as 2 weeks of exposure (Goldsworthy et al., 1991; Stott et al., 1981). Similarly, tumor promotion studies have shown that 1000 mg/kg 1,4-dioxane promotes formation of GGT-positive liver foci in rats (Lundberg et al., 1987) and enhances the growth of previously initiated skin cells in mice (King et al., 1973). Given time, proliferative changes manifest as pre-neoplastic foci in studies where the histopathology of such changes are reported (Kano et al., 2008; Lundberg et al., 1987; Kasai et al., 2008). Thus, it is highly plausible that doses of 1,4-dioxane exceeding the capacity to be metabolized are linked to the occurrence of liver and respiratory tumors in rodents. This is further supported by evidence from Koissi et al. (2012) showing that the oxidation products of 1,4-dioxane do not elicit preneoplastic liver foci in rats when administered orally for 12 weeks (Koissi et al., 2012).

3.2.5. Key event 5: Promotion of endogenously-initiated tumors

Three studies relevant to tumor initiation and promotion establish that 1,4-dioxane does not cause initiation in standard *in vivo* bioassays, in agreement with the absence of observed mutagenic or genotoxic activity (Bull et al., 1986; King et al., 1973; Lundberg et al., 1987).[8] Although not an initiator, 1,4-dioxane does causes promotion of tumors, as evidenced by positive results in two standard initiation/promotion bioassays (King et al., 1973; Lundberg et al., 1987). Both studies have deficiencies that limit interpretation, however.[9] Lundberg et al. (1987) evaluated tumor promotion of 1,4-dioxane after initiation and partial hepatectomy in terms of significant GGT-positive foci and lipid accumulation only. Following initiation, statistically increased foci volume was observed in the highest dose group given 1000 mg/kg. In this dose group significantly increased foci volume was associated with hepatocyte lipid accumulation, an indicator of liver toxicity. Therefore, toxicity may be an important component for a significant increase in this pre-neoplastic lesion. The study by King et al. (1973) presented limited data, as it was a preliminary report of interim results. The complex study design evaluated tumor initiation and promotion by oral and dermally administered 1,4-dioxane. From the limited data presented, this study provides evidence that 1,4-dioxane is not a complete carcinogen or initiating agent; it does support other studies that 1,4-dioxane has tumor promoting activity. In both studies, tumor promotion was

[8] See also EPA (2013) page 76, Table 4-23.

[9] The publication by King et al. (1973) was a preliminary report with several significant shortcomings. For example, it was reported as an ongoing study. However, final results were never published; drinking water treatment groups were on study eight weeks longer than the controls to which they were compared; profound mortality was associated with dermal administration of 1% 1,4-dioxane to rats (the only concentration tested dermally); there is no control for 1,4-dioxane alone without initiation. Thus, the King et al. study provides unreliable evidence regarding 1,4-dioxane as a tumor promoter and no evidence regarding initiation. The primary limitation of Lundberg et al. (1987) is that it focuses only on GGT-positive liver foci and lipid accumulation and does not report whether any other pathology was observed.

associated with significant toxicity in rats when administered by either dermal or oral routes (King et al., 1973; Lundberg et al., 1987).

3.2.6. Alternative MOA hypothesis

In several respects 1,4-dioxane acts as if it were a mutagenic carcinogen. It evokes multiple tumors in both sexes of two different species, and some of these tumors are malignant. Thus, it is reasonable to explore a mutagenic MOA for this chemical.

For example, 1,4-dioxane has been tested for genotoxicity using *in vitro* assay systems with prokaryotic organisms, non-mammalian eukaryotic organisms, and mammalian cells, both with and without metabolic activation. Based on the data presented by EPA (2013), all fifteen mutagenicity tests reported (8 without activation and 7 with metabolic activation) were negative. In addition, 22 *in vitro* genotoxicity assays, and 9 *in vivo* genotoxicity assays were negative. Eight genotoxicity assays were noted to be positive but only at high or noted cytotoxic doses. EPA (2013) concluded that the evidence supports the possibility that 1,4-dioxane is non-genotoxic or weakly genotoxic. Our conclusion is similar in that 1,4-dioxane does not cause mutations, but differs from EPA (2013) in that if mutations are caused by 1,4-dioxance, it is only at high cytotoxic doses. Furthermore, we note that replicative DNA and RNA synthesis reflect cell division, rather than being direct or even indirect measures of DNA damage (unlike reparative DNA synthesis, which is an indirect measure of DNA damage). DNA synthesis is a key event for hyperplasia and tumor promotion, and can occur in either the presence or absence of cytotoxicity. The DNA synthesis evidence supports the possibility that 1,4-dioxane promotes cell proliferation, possibly through mitogenesis or cytotoxicity.

Based on testing results, we conclude that 1,4-dioxane does not cause mutagenicity as evidenced by uniformly negative results in standard *in vitro* and *in vivo* genotoxicity bioassays at levels that are not overtly toxic, but it may be a clastogen *in vivo*, in light of the mixed results in the micronucleus assays. It follows that mutations needed for tumor formation are then likely from the known endogenously available pool of mutations, and that a regenerative hyperplasia evokes more of these endogenous mutations to form tumors. Mutation potentially caused by 1,4-dioxane at high doses is precluded as a key event in tumor formation.

3.3. Derivation of a drinking water level

3.3.1. Dose–response assessment for key events for liver toxicity/tumors

EPA (2013) used Kociba et al. (1974) for the development of its RfD. We agree with EPA in its choice of study and critical effect, but based our assessment on the laboratory report of this published study, kindly provided by The Dow Chemical Company (Kociba et al., 1971), which showed additional quantitative data for the liver and kidney effects. After a review of this laboratory report, we selected histopathology diagnoses reflective of key events in the process of tumor formation in the liver of Sherman rats for the purposes of benchmark dose (BMD) modeling (Fig. 4). Specifically, hepatocellular cytoplasmic vacuolar degeneration occurred first in the dose scale, with hepatocellular necrosis following. The incidence and severity of both lesions was roughly similar between male and female rats, and thus, we combined these data into a joint analysis in order to derive more confident BMD modeling.

However, males had what appeared to be a hormetic effect for the degeneration, and BMD modeling did not result in any single model that met EPA's criterion of p value greater than 0.1.[10] The combined rat male and female data also failed this modeling criterion for hepatic degeneration. In contrast, data for individual male and female rats, and joint modeling for hepatocellular necrosis yielded successful outcomes.

Table 6
Kociba et al. (1971, 1974) Rat hepatocellular necrosis data used for BMD analysis.

Dose (mg/kg day)	No. of animals	Hepatocellular necrosis incidence[a]
Male rats		
0	60	2
9.6	60	6
94	60	12
1015	60	24
Female rats		
0	60	1
19	60	2
148	60	11
1599	60	31

[a] Incidence data for both sexes was combined for the BMD analysis.

Thus, BMD modeling was conducted for hepatocellular necrosis for rats as shown in Table 6. Risk assessment experts use the following 5 criteria from EPA (2002, 2012) in BMD model choice:

(1) Models with p-values greater than 0.1,
(2) Models where the visual fit of the data is good, especially in the range of the BMD,
(3) Models that have the lowest residuals near the range of the BMD,
(4) Models that have the lowest AIC, and
(5) Models that have the smallest BMD/BMDL ratio.

Table 7 shows results of this modeling. The log-probit model has a slightly better fit on several criteria and was selected to provide the point of departure for development of a Reference Dose (RfD). But actually any of these models would be a good choice and lead to a similar RfD.

Nasal toxicity was clearly not the critical effect as demonstrated by EPA (2013). Thus, since any assessment based on liver effects would clearly be protective of nasal effects and tumors evoked in both organs appear to have the same regenerative hyperplasia MOA, we did not further analyze these tumors.

Renal toxicity as evidenced by both incidence and severity was also evident at these doses and BMD modeling was also conducted for selected endpoints. However, since these toxicities did not lead to tumor formation, and were not more sensitive than the liver effects, this modeling was not selected for further analysis (data not shown but available upon request).

3.3.2. Developing a Reference Dose (RfD)

We find that the most appropriate choice of the point of departure for development of an RfD for 1,4-dioxane is $BMDL_{10}$ for hepatic necrosis from the joint analysis of males and females of 20 mg/kg day (human equivalent concentration of 5.2 mg/kg day). This choice is supported because among chronic rat studies, Kociba et al. (1971, 1974) quantifies early events in the formation of liver tumors (Table 8). Moreover, the choice of rat versus mouse specie is reasonable because the administered doses to mice are higher (compare Tables 8 and 9), and this difference is maintained even after adjustment for the human equivalent concentration. Finally, the choice of this endpoint is protective, since liver toxicity, resulting in liver tumors, is the clear apical effect of greatest intensity in the available array of toxic effects. This key event is considered to have a threshold as suggested by the MOA analysis shown in this text.

It would be most appropriate to apply a Chemical Specific Adjustment Factor to this adjusted $BMDL_{10}$ of 5.2 mg/kg day for

[10] EPA's BMD models assume monotonically increasing responses and are generally incapable of modeling hormetic effects.

Table 7
Selected BMD modeling results from sherman rats in Kociba et al. (1971).

Model	BMD mg/kg day	BMDL mg/kg day	*p*-value (>0.1)	Scaled residual[b]	Visual fit[a]	AIC	BMD/BMDL ratio
Gamma	48	17	0.41	−1.5	Good	376	2.9
LogLogistic	50	19	0.38	−1.4	Good	376	2.5
LogProbit	**49**	**20**	**0.33**	**−1.3**	**Good**	**376**	**2.4**
Weibull	49	18	0.40	−1.5	Good	376	2.7

[a] Visual fit rating is classified as poor, moderate or good.
[b] Scaled residue near the range of the BMD is selected.

Table 8
Dose response, temporality concordance table for dioxane-induced liver tumors in rats.

	Dose mg/kg day (Author reported dose)	Temporal[a]					
		Key event 1	Key event 2		Key event 3	Key event 4	Liver adenomas/carcinomas
		Non-linear metabolism Hours to days	Hypertrophy Weeks	Necrosis Weeks	DNA synthesis Weeks	Hyperplasia/pre-neoplastic foci Months	Years
Stott et al. (1981)[b]	10	nd	–	–	–	–	–
(11 weeks; oral)	1000	nd	+	+	+	–	–
Kano et al. (2008)[c]	52 (m)/83 (f) (640 ppm)	nd	–	–	nd	–	–
(13 weeks; oral)	126 (m)/185 (f) (1600 ppm)	nd	+(m)/−(f)	–	nd	–	–
	274 (m)/427 (f) (4000 ppm)	nd	+(m)/−(f)	–	nd	–	–
	657 (m)/756 (f) (10000 ppm)	nd	+	–	nd	–	–
	1554 (m)/1614 (f) (25000 ppm)	nd	+	+[d]	nd	–	–
Kasai et al. (2008)[e] (13 week;	584 (800 ppm)	nd	nd	–	nd	–	–
Inhalation)	1168 (1600 ppm)	nd	nd	–	nd	–	–
	2336 (3200 ppm)[f]	nd	nd	+	nd	+[g]	–
Kano et al. (2009)[h] (2 year;	11 (m)/18 (f) (200 ppm)	nd	nd	nd	nd	–	–
oral)	55 (m)/83 (f) (1000 ppm)	nd	nd	nd	nd	+(m)/−(f)	–
	274 (m)/429 (f) (5000 ppm)	nd	nd	nd	nd	+	+
Kociba et al. (1974)[i] (2 year;	9.6 (m)/19 (f) (0.01%)	–	nd	–	nd	–	–
oral)	94 (m)/148 (f) (0.1%)	+[j]	nd	+[k]	nd	+	–
	1015 (m)/1599/1078 (f) (1%)	+[k]	nd	+	nd	+	+
Kasai et al. (2009)[l] (2 year;	36 (50 ppm)	nd[m]	nd	–	nd	–	–
inhalation)	181 (250 ppm)	nd	nd	–	nd	–	–
	909 (1250 ppm)	nd	nd	+	nd	+	+
NCI, 1978[n] (2 year; oral)	240 (m)/350 (f)	nd	–	–	nd	−(m)/+(f)[o]	−(m)/+[p](f)
	550 (m)/640 (f)	nd	–	–	nd	+[o]	−(m)/+[p](f)

[a] Abbreviations and symbols: +, key event observed; −, key event not present;+/−, equivocal; nd, not determined/reported.
[b] Sprague-Dawley rats were dosed daily for 11 weeks (7 days/week) via drinking water with 10 or 1000 mg dioxane/kg body weight.
[c] Fifty male and female fisher 344 rats were administered 1,4-dioxane in drinking water for 13 weeks.
[d] Statistically significant ($p \leqslant 0.01$) single cell necrosis, which was consistent with increased plasma AST/ALT levels (male rats) and AST (females). The most sensitive sign of toxicity was centrilobular swelling of hepatocytes in male rats given 1,600 ppm for 13 weeks. No foci were observed at any dose levels.
[e] Thirteen-week inhalation of 1,4-dioxane in male and female F344 rats vapor for 6 h/day and 5 days/week. Inhalation exposures were mg/kg doses assuming a minute volume as 561 ml/min/kg body weight for rats and an uptake ratio of 1,4-dioxane of 100%. Authors included dose groups ranging from 3200 to 100 ppm with doubling dilutions, but since these groups were negative for the occurrence of key events they have not been included in the table.
[f] A 6400-ppmexposure was also tested but is not relevant to this mode of action analysis because all animals in this group died at the first week of the 13-week exposure period.
[g] GST-P-positive liver foci were observed in 3/10 males exposed to 3200-ppm; 2/10 females exposed to 3200-ppm; and 4/10 females exposed to 1600-ppm; no GST-P-positive foci could be found in any of the 800- and 1600-ppm-exposed males and 800-ppm-exposed females and control groups of both sexes.
[h] 1,4-dioxane was administered in drinking-water to F344/DuCrj rats (50 of each sex/treatment group) for 2 years.
[i] Sixty male and female Sherman strain rats,6-8 weeks old, were administered 1,4-dioxane in their drinking water for up to 716 days. Female rats during days 114–198 consumed a dose of 1,4-dioxane ranging from 914–1229 mg/kg/day, but consumed less (1019–1176 mg/kg/day) days 446–460. Male rats receiving the 1% exposure has similar consumption during the same exposure periods.
[j] Kociba et al. (1975) 1,4-dioxane: correlation of the results of chronic ingestion and inhalation studies with its dose-dependent fate in rats. In proceedings of the 6th Annual Conference on Environmental Toxicology (pp. 345–354). Wright-Patterson Air Force Base, OH: Wright-Patterson Air Force Base, Air Force Systems Command, Aerospace Medical Division, Aerospace Medical Research Laboratory.
[k] Kociba et al. (1974) reported that the occurrence of hepatocellular degeneration and necrosis, as well as hyperplastic nodule formation, are significantly increased by doses of 1,4-dioxane $\geqslant$ 0.1%; the incidence for these changes are provided in Kociba et al. (1971).
[l] 2 year inhalation exposure of male fisher 344 rats (50 animals per dose group). Internal exposure from 6-h inhalation exposure was approximated by the authors assuming the minute volume as 561 ml/min/kg body weight for rats and an uptake ratio of 1,4-dioxane of 100%.
[m] Kasai et al. (2008) demonstrate steady-state proportionality between dose and plasma blood levels for the top 4 exposure levels ($\geqslant$400 ppm). Based on the pharmacokinetics of 1,4-dioxane, these plasma concentrations are predicted to be associated with saturation-limited metabolism.
[n] Groups of 35 rats of each sex administered 1,4-dioxane at concentrations of either 0.5% or 1.0% (v/v) in the drinking water for 110 weeks.
[o] Hyperplasia in female rat liver was 7/31 (23%), 11/33 (33%) and 17/53% (53%) for the control, low- and high-dose groups, respectively. In male rats the incidents were 5/31 (16%), 3/32 (9%) and 11/33 (33%) for control, low- and high-dose groups, respectively.
[p] Adenoma only in female rats and no tumors in male rats.

Table 9
Dose response, temporality concordance table for dioxane-induced liver tumors in mice.

		Temporal[a]					
	Dose mg/kg day (Author reported dose)	Key event 1	Key event 2		Key event 3	Key event 4	Liver adenomas/ carcinomas
		Non-linear metabolism Hours to days	Hypertrophy Weeks	Necrosis Weeks	DNA synthesis Weeks	Hyperplasia/pre-neoplastic foci Months	Years
Kano et al. (2008)[b] (13 weeks; oral)	86 (m)/170 (f) (640 ppm)	nd	–	–	nd	–	–
	213 (m)/387 (f) (1600 ppm)	nd	–	–	nd	–	–
	585 (m)/898 (f) (4000 ppm)	nd	+[c]	+[d]	nd	–	–
	882 (m)/1620 (f) (10000 ppm)	nd	+	+	nd	–	–
	1570 (m)/2669 (f) (25000 ppm)	nd	+	+	nd	–	–
Kano et al. (2009)[e] (2 year; oral)	49 (m)/66 (f) (500 ppm)	nd	nd	nd	nd	nd[f]	–(m)/+(f)
	191 (m)/287 (f) (2000 ppm)	nd	nd	nd	nd	nd	+[f]
	677 (m)/964 (f) (8000 ppm)	nd	nd	nd	nd	nd	+
NCI, 1978 re-read (2 year; oral)[g]	380 (f)	nd	+	±[h]	nd	+	+
	720[i] (m)	nd	+	+	nd	+	+
	830 (m)/860 (f)	nd	+	+	nd	+	+

[a] Abbreviations and symbols: +, key event observed; –, key event not present; ±, equivocal; nd, not determined or reported; m, male only; .f, female only.
[b] Four-week-old Crj:BDF$_1$ mice of both sexes (n = 60, 10 animals per control or treatment group) were administered 1,4-dioxane in drinking water for 13 weeks.
[c] Mouse hepatic lesions were characterized by both single cell necrosis and centrilobular swelling of hepatocytes occurring at 4,000 ppm and above.
[d] Hepatocellular damage indicated by increases in plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were noted in male and female mice dose with 25,000 ppm dioxane, and ALT was increased in female mice at 10,000 ppm.
[e] 1,4-dioxane was administered to 50 Crj:BDF$_1$ mice of each sex in the drinking-water for 2 years.
[f] Hepatic hyperplasia of mice diagnosed in the previous report Yamazaki et al. (1994) was re-examined histopathologically and changed to hepatocellular adenomas and altered hepatocellular foci.
[g] McConnell (2013). Review of liver slides from the National Cancer Institute's bioassay of 1,4-dioxane for possible carcinogenicity conducted in 1978 (NCI, 1978). Groups of 50 mice of each sex administered 1,4-dioxane at concentrations of either 0.5% or 1.0% (v/v) in the drinking water for 90 weeks.
[h] The occurrence of necrosis in low-dose female mice was equivocal with an incidence similar to the elevated control level but with increased severity of centrilobular necrosis. Both incidence and severity were increased at the high-dose (Tables 2 and 5 of this text).
[i] It is noteworthy that the dose of 1,4-dioxane consumed by the high and low doses males in the 1978 NCI study was similar and in the words of the authors, "did not reflect the 2-fold difference in concentration between the low and high doses". Thus, histologic pathology between the low and high males is generally similarity.

development of the RfD. Guidelines for the development of such factors exist (IPCS, 2005). Unfortunately, the available comparative toxicodynamic data between experimental animals and humans, and comparative toxicokinetic and toxicodynamic data within humans, are limited and do not allow the development of these preferred factors. Thus, a default value of 3-fold was used for the toxicodynamic extrapolation of experimental animals to humans, and a factor of 10-fold was used for within human variability. An additional 3-fold factor was used for the lack of a 2-generation reproductive study, based on EPA (2002) guidelines and prior analysis of this area of uncertainty by Dourson et al. (1992). This latter factor addresses the general lack of toxicity data on 1,4-dioxane for various lifestages, especially in younger experimental animals. The composite factor is thus 100-fold (i.e., 3 × 10 × 3 = 100).[11] The use of this composite factor is similar to that described by EPA (2013), and for similar reasons.

The resulting RfD is 0.05 mg/kg day. This RfD is similar to that derived by ATSDR (2007), and for similar reasons.

3.3.3. Maximum contaminant level goal (MCLG)

The Relative Source Contribution (RSC) for 1,4-dioxane is derived by application of the Exposure Decision Tree approach published in US EPA's Methodology for Deriving Ambient Water Quality Criteria for the Protection of Human Health (US EPA, 2000). The purpose of the RSC is to account for identified sources and routes of non-occupational exposures to a particular chemical and to apportion allowable amounts from each source so that an individual would not have a total (aggregate) exposure greater than the RfD. RSCs are calculated for chemicals that are non-carcinogens or threshold carcinogens. Exposure data for various pathways are available for 1,4-dioxane.

Exposures to 1,4-dioxane are expected to be local and limited. The relevant potential exposure sources are identified as consumer products, groundwater, and air. Potential pathways include ingestion of water; dermal contact with consumer products, water used in bathing, and inhaled air. Exposure data (Sapphire Group, 2007) was evaluated for each of these pathways to determine the RSC for groundwater. Although the data found in Table 10 are only a snapshot, they suggest that contributions from water are only around 10%. In such situations, it is common to use the default value of 20% for the RSC. Thus, based on the data available data, we use the default RSC of 20%.

Based on the proposed RfD of 0.05 mg/kg day and applying a RSC of 20%, the MCLG would be 0.35 mg/L (0.05 mg/kg day × 70 kg ÷ 2 L of water per day = 0.35 mg/L or 350 μg/L).

4. Discussion

In making decisions about potential MOA, the animal tumor findings often give important clues. Some of the factors EPA (2005) recommends in a review of such findings include tumor types, number of studies and of tumor sites, similarity of metabolic activation and detoxification, influence of route of exposure on the spectrum of tumors, effect of high dose exposures on the target organ or systemic toxicity that may not reflect typical physiological conditions, presence of proliferative lesions, effect of dose and time on the progression of lesions, ratio of malignant to benign tumors as a function of dose and time, time of appearance of tumors, development of tumors, tumors at organ sites with high or low background historical incidence, biomarkers in tumor cells, and shape of the dose–response curve in the range of tumor observation.

In considering these criteria, 1,4-dioxane oral exposure appears to be a mutagenic carcinogen in some respects. It evokes multisite and multispecies tumors that are not restricted to one sex

[11] As per convention, 3-fold factors are considered as ½ of the default value of 10-fold; thus multiplying two 3-fold factors is equivalent to a value of 10-fold.

Table 10
Sources of 1,4-dioxane from the environment.

Exposure pathway	Estimated intakes		Average intake mg/kg day	RSC%
	Sapphire Group (2007)	Health Canada (2010)		
Ambient air (mg/m^3)	Median value: 0.00026	Max: 0.000646	0.00013	7.3
Indoor air (mg/m^3)	Median value: 0.00024	Max: 0.00085	0.00012	6.8
Drinking water (mg/L)	Average:0.002	Det limit:0.01	0.00017	9.7
Food (mg/kg food)	0.0012	Det. Limit: 0.002	0.00003	1.5
Dermal (mg/kg bw-day)	0.00019	Absorbed: 0.000061	0.00013	7.1
Consumer products (mg/kg bw-day)		100% absorption: 0.0012	0.00120	68

suggesting an influence that is not restricted to gender, strain, or species. In addition, tumors evoked by 1,4-dioxane are both benign and malignant. For example, statistically significant peritoneal mesotheliomas are found in male, but not female, F344 rats at high dose. This sex difference is likely due to the occurrence of tunica vaginalis mesotheliomas (TVM), a commonly occurring tumor in these male rats, which grow into the peritoneal cavity. A previous analyses of such tumors evoked by other chemicals might be helpful in understanding the underlying MOA for this tumor caused by 1,4-dioxane. Such an analysis has been done by Haber et al. (2009) for acrylamide. These investigators explored several MOAs, and perhaps more importantly, show that the risk calculated from the observed response in rats is at large variance with the observed incidence of this tumor in humans. In brief, the quantitative difference between male rats and male humans is expected to be in the range of 100- to 1000-fold where humans are less sensitive (Haber et al., 2009). While TVMs from exposures to 1,4-dioxane and acrylamide may be evoked by different MOAs, it is not scientifically reasonable to assume that such tumors in rats are relevant to humans based on this quantitative disparity as described by Haber et al. (2009) without further support.

Statistically significant mammary tumors are also found in both sexes of rats. These tumors are of high dose and uniformly benign. EPA (2005) considers the evaluation of such uniformly benign tumors on a case-by-case approach. In the case of 1,4-dioxane, neither the mammary tumors nor peritoneal tumors occur with the frequency and severity of the liver tumors, nor do dose response assessments based on these tumors, have the same impact as assessments based on liver tumors (US EPA, 2013). Therefore, it does not appear to be scientifically reasonable to base a dose response assessment on this mammary endpoint either. A separate MOA analysis might be done to further support this, or other conclusion.

As briefly described earlier, 1,4-dioxane from oral exposures also causes nasal toxicity as evidenced by histology at all time points and in a dose related manner in both sexes of rats and mice as nicely summarized by EPA (2013).[12] This toxicity also precedes tumors in time in both sexes of rats and mice, and also precedes tumors in dose in both sexes of rats and mice.[13] The MOA for the development of nasal tumors has the same appearance as that for liver tumors, although the nasal tumor response, like the response for both TVM and mammary tumors, is not as severe as that for liver.

In contrast to some data that suggest 1,4-dioxane as a mutagenic carcinogen, all but one of the tumor types (for nasal tumors) are at sites with a high historical background incidence. In addition, extensive toxicity is seen in the primary tumors sites (liver and nose) suggesting a growth-promoting, and specifically, a regenerative cell proliferation, mode of action. This latter MOA is supported by positive findings in promotion bioassays and DNA replication bioassays, and is also supported by the negative findings in mutagenicity bioassays, initiation bioassays, and DNA repair bioassays. The modified Hill criteria of EPA (2005) for the our proposed regenerative cell proliferation MOA hypothesis for liver tumors was evaluated and determined to be met for strength, consistency, biological plausibility, and coherence. Moreover, dose response and temporal concordance for noncancer precursors to tumors were clearly evident in the reread of the NCI (1978) mouse liver slides that utilized current histopathology reporting practices.

Our histopathology findings from the reread of the slides of the NCI study (1978) are supported by changes in liver enzymes in mice as shown in (Table 9),[14] and by a repeated exposure study of 13 weeks (Kano et al., 2008), where the earliest histopathology key event in the MOA was single cell liver necrosis and centrilobular swelling at 4000 ppm and higher doses. Also, altered hepatocellular foci stained positive with the anti-GST-P antibody were observed in this 13-week study at the highest exposure dose. While the non-neoplastic lesions found in the NCI (1978) slide reread that we show in this paper were not reported in mice from one long-term study (Kano et al., 2009), the same Japanese investigators did report hepatic hyperplasia (later changed to altered hepatocellular foci) in an earlier report of this same 2-year study (Yamazaki et al., 1994). Moreover, mice in the Kano et al. (2009) study showed hepatocellular injury as evidenced by an enhanced cytolytic release of liver enzymes (e.g., GOT, GPT, LDH, and ALP) at doses of about 140–1400 mg/kg day [unpublished results].

Based on the current findings from the comprehensive reread of the NCI study (with support from other repeated exposure studies on 1,4-dioxane), the two principal modified Hill criteria of EPA (2005), that of temporal and dose concordance, are met in that chronic bioassays show liver toxicity preceding the development of tumors in both dose and time for both rats and mice (Tables 8 and 9). The additional modified Hill criteria for strength, consistency, biological plausibility and coherence are also met. Only the criterion of specificity of association is not met. This is because a regenerative hyperplasia is a common MOA for chemicals causing tumors.

As to other tumors, peritoneal mesotheliomas, which are only found in F344 male rats, are not unexpected, but as discussed by Haber et al. (2009), this tumor endpoint does not have relevance to humans quantitatively. We therefore did not use this tumor in a quantitative evaluation of risk to humans. Mammary tumors are also found in both sexes of rats from oral exposure, but these tumors are at high dose and uniformly benign. Using EPA (2005) we judge that a dose response assessment based on these mammary tumors should not be conducted using linear extrapolation and that a nonlinear assessment based on this endpoint will not be lower than the calculated RfD for liver toxicity, so that any risk from this endpoint is prevented. However, a full MOA assessment of these mesotheliomas and benign mammary tumors might be a useful exercise for future evaluations.

[12] See EPA (2013) Table 4-2 (p. 34), Table 4-8 (p. 47), Table 4-9 (p. 48), Table 4-12 (p. 52), and Table 4-13 (p. 52), Table 4-17 (p. 59), and Table 4-22 (p. 69).

[13] Compare EPA (2013) Table 4-5 (p. 41) and NCI (1978) Tables C1 & C2, (pages 61 & 65) to EPA (2013) Table 4-6 (p. 42). Also compare EPA Tables 4-8 & 4-9 (p. 47-48) to EPA Tables 4-10 & 4-11 (p. 50). Also compare EPA Table 4-20 (p. 64) to EPA Table 4-21 (p. 65).

[14] See also EPA (2013) Table 4-25 (p. 85).

5. Conclusion

Using a Mode of Action (MOA) analysis and human relevance framework (US EPA, 2005), the weight of the evidence supports a non-linear mode of action with 1,4-dioxane causing liver tumors in rats and mice through a MOA involving cytotoxicity followed by regenerative hyperplasia and the promotion of endogenous mutations and resulting in increased tumors. This mode of action appears to also be occurring for tumors in the nasal cavity of rodents exposed to 1,4-dioxane. The specific key events in this mode of action include: (1) accumulation of parent compound, (2) liver cell hypertrophy and necrosis, (3) DNA synthesis, (4) regenerative cell proliferation, and (5) promotion of endogenously-initiated tumors.

Evidence for this sequence includes both temporal and dose concordance through the integration of studies across different durations and the incorporation of new information from the re-read of the NCI (1978) mouse liver slides. This reread supports the view that clearly identifiable dose-related non-neoplastic changes exist in the liver of mice exposed to 1,4-dioxane; specifically, a dose-related effect in the hypertrophic response of hepatocytes, followed by necrosis, inflammation and hyperplastic hepatocellular foci. As per EPA (2005) guidelines, "a *nonlinear approach* should be selected when there are sufficient data to ascertain the MOA and conclude that it is not linear at low doses and the agent does not demonstrate mutagenic or other activity consistent with linearity at low doses." We conclude that the available data for 1,4-dioxane, and specifically for its liver effects, necessitates such a selection. The RfD developed from the key event of liver necrosis for the liver tumor endpoint is 0.05 mg/kg day. An MCLG developed from this RfD is 0.35 mg/L.

Conflict of interest statement and Acknowledgments

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