Update: Mode of Action (MOA) for Liver Tumors Induced by Oral Exposure to 1,4-Dioxane

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Abstract

Previous scientific studies show that the weight of evidence supports the hypothesis that 1,4-dioxane causes liver tumors in rodents through cytotoxicity and subsequent regenerative hyperplasia. Questions regarding a lack of concordant findings for this mode of action (MOA) in mice have not been resolved, however. In the current work, a reanalysis of data from two chronic mouse cancer bioassays on 1,4-dioxane, one 13-week mouse study, seven rat cancer bioassays, coupled with other data demonstrating negative mutagenicity, lack of up-regulated DNA repair, and the appearance of liver tumors with a high background incidence, support the conclusion that rodent liver tumors, including those in mice, are evoked by a regenerative hyperplasia MOA. The initiating event for this MOA is metabolic saturation of 1,4-dioxane. Above metabolic saturation, higher doses of the parent compound cause an ever increasing toxicity in the rodent liver as evidenced by higher blood levels of enzymes indicative of liver cell damage and associated histopathology that occurs in a dose and time related manner. Importantly, alternative modes of action can be excluded. The observed liver toxicity has a threshold in the dose scale at or below levels that saturate metabolism, and generally in the range of 9.6 to 42 mg/kg-day for rats and 57 to 66 mg/kg-day for mice. It follows that threshold approaches to the assessment of this chemical's toxicity are supported by the non-mutagenic, metabolic saturation kinetics, and cytotoxicity-generated regenerative repair information available for 1,4-dioxane promoted rodent liver tumors.

Introduction

Differences in the evaluation and interpretation of toxicological data for 1,4-dioxane (CAS number 123-91-1) has led to contrasting approaches for extrapolating

results from experimental animals to humans for assessment of cancer risk. Some investigators, such as Health Canada (2005), Neumann et al. (1997), NICNAS (1998), Netherlands (1999), and Stickney et al. (2003), rely on a threshold approach for this extrapolation, while others, such as the U.S. Environmental Protection Agency (U.S. EPA, 2013) and Office of Environmental Health Hazard Assessment (2002), default to a non-threshold or linear low-dose extrapolation approach for their toxicological assessment. Despite these differences, however, none of these groups consider 1,4-dioxane to be mutagenic, a hallmark of a non-threshold approach (U.S. EPA, 2005), nor to cause DNA repair. Importantly, all groups describe data that support alternative modes of action (MOA), such as a regenerative hyperplasia.

The source of this inconsistency stems from apparently conflicting data from rat and mouse bioassays, specifically, in findings for dose-related non-neoplastic liver lesions in rats from multiple studies that support a cytotoxicity, regenerative repair, in contrast to the general lack of non-neoplastic (or noncancer) histopathology findings in the livers of mice from two chronic studies. As one step to resolve this apparent conflict, U.S. EPA's external peer review panel for 1,4-dioxane suggested a re-read of liver slides from the first mouse study, by the National Cancer Institute (NCI, 1978).[1] This suggestion was based on the fact that NCI pathologists in 1978 generally recorded the most severe pathology for individual experimental animals, and when tumors were found, did not always record, or otherwise were not able to record available non-neoplastic toxicity (McConnell, 2011). Evidence of this practice is found in the NCI (1978) report, where female mice are shown to have liver hyperplasia in the low dose group, but this effect was not recorded for the high dose group where most animals had liver tumors. Thus, because of early practices that overlooked other histological findings in the presence of liver tumors, important histology data went unreported in the original reports while certain histological data are critical for developing and establishing the MOA.

Based on this suggestion, we previously worked with scientists from the National Toxicology Program to re-read the 1978 NCI slides (Dourson et al., 2014). The older mouse liver slides were re-stained and then re-read in a blinded protocol. The findings from the re-read were in stark contrast to the minimal noncancer liver findings in the original NCI report. Specifically, noncancer toxicity was evident at all doses and in a manner (i.e., hypertrophy, necrosis, inflammation, foci, adenoma, carcinoma) that was consistent with a regenerative hyperplasia MOA for the development of liver tumors. This published reanalysis of the NCI (1978) mouse slides was supported by the pathology report by McConnell (2013).

The second long-term oral mouse bioassay and a 13-week precursor were conducted by the Japan Bioassay Research Center (JBRC, 1990a,b) and subsequently

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[1] Specifically: "The EPA should explore the possibility that slides from the NCI studies on 1,4-dioxane are available and in adequate condition to evaluate possible linkages between toxic effects and tumor outcome in the drinking water carcinogenicity studies in rats and mice." PEER REVIEWER COMMENTS. External Peer Review on the *Toxicological Review of 1,4-Dioxane* (CASRN No. 123-91-1). Versar, Inc. Contract No. EP-C-07-025 Task Order 118 (May 2012)

published as Kano et al. (2008, 2009). Similar to the NCI (1978) bioassay, little noncancer toxicity in the mouse liver was reported by JBRC (1990a) after long-term exposure. The lack of reported noncancer toxicity is perhaps not surprising given a similar underreporting in the NCI (1978) bioassay. However, these findings conflict with noncancer liver toxicity reported by JBRC (1990b) in the 13-week study.

The objective of this work was to perform a detailed evaluation of the findings reported in the original Japanese (JBRC, 1990a,b) rat and mouse bioassays and to integrate these findings with other lines of evidence to determine whether a regenerative hyperplasia MOA for hepatic tumor formation is supported. Evaluation of these findings expands the scope of our previous work and allows for a more comprehensive MOA analysis.

Methods

Because the JBRC reports (1990a,b) were not available in English, a consortium of government and nongovernment scientists requested full access to the lab reports and had them translated.[2] These reports were graciously received during 2014 and then translated in early 2015. Taken together, these translated reports include additional noncancer effects in the liver of rats and mice, which were otherwise not available in the published versions (Kano et al., 2008, 2009). Unfortunately, histopathology slides from these studies were not available for a re-reading.

The U.S. EPA (2005) guidelines for cancer risk assessment state that the MOA should be evaluated in determining the quantitative 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 as shown in Figure 1, which is adapted from U.S. EPA (2013) and Dourson et al. (2014). Data on these key events, including available *in vivo, in vitro*, and mechanistic studies are then evaluated as per U.S. EPA (2005). 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 threshold model based on one or more combinations of relevant tumors for a non-DNA-reactive MOA. Finally, the human equivalent dose is determined from the experimental animal dose by comparing human and experimental animal kinetics or a default procedure (U.S. EPA, 2011). Adverse outcome pathway (AOP) frameworks are also emerging for expanding the use of mechanistic toxicological data for risk assessment and regulatory applications (NRC, 2007). The development of an AOP for 1,4-dioxane might prove useful for future investigations.

[2] The full translations of these Japanese findings can be obtained http://allianceforrisk.org/14-dioxane-analysis/ (TERA, 2015).

The U.S. EPA (2005) cancer risk assessment guidelines were followed by Dourson et al. (2014) in their analysis of two potential MOAs for liver tumor development from exposure to 1,4-dioxane: a heritable mutation to liver and/or nasal cell DNA, or liver cytotoxicity followed by regenerative cell proliferation. The analyses reported by Dourson et al. (2014) were performed on the basis of pooled results[3] from both male and female mice for hepatocellular necrosis because the incidences of this effect were similar between the sexes. In the current work, we again utilized a pooled approach for data analysis, and we specifically enhanced the investigation of the MOA on regenerative cell proliferation by performing a detailed evaluation of the translated Japanese study reports (JBRC 1990a,b).

Results

The translated study reports from the JBRC (1990a,b) confirm information found in the publications of Kano et al. (2008 and 2009) and add some information relevant to the hypothesized MOA not found in the published articles. From the Japanese studies, the NCI (1978) bioassay, the re-read of the mouse liver slides from the NCI (1978) study by McConnell (2013), and other relevant information, we have further developed the hypothesized regenerative hyperplasia MOA, to the point where we conclude that consistent non-cancer effects are observed in both rats and mice preceding tumor development, with the level of documentation of these observations more evident in the rat studies.

Review of the Japanese Translations and Integration with Other Findings: Rats

Figure 2 shows hyperplasia preceding the development of liver foci (generally basophilic and mixed cell) in rats in a dose related fashion, and both of these effects are shown to precede the development of liver adenomas and carcinomas. The inset shows the relationship of hyperplasia and foci more clearly. Figure 3 shows the pooled incidence of two additional effects in rats, namely centrilobular swelling and single cell liver necrosis from the 13-week studies overlaid on Figure 2.[4] These two effects precede the development of other effects in both dose and time. Liver enzyme changes in the blood of rats shown in Supplemental Figure 1 as ALT and AST pattern the histology

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[3] Data are considered "pooled" when individual group level information is maintained in any analysis, such as the development of a dose response curve. In contrast, data are considered combined, when individual group level information is combined at the same or similar dose for subsequent analysis.

[4] Here, the doses from the 13-week studies have been reduced by a 3-fold factor to address the well-known differences in effect level among durations (Dourson and Stara, 1983). Some might argue that a 10-fold uncertainty factor would be more appropriate here. If so, the use of this factor would shift the data points for centrilobular swelling and single cell liver necrosis to the left, making the pattern of toxicity proceeding the development of tumors more apparent. Perhaps more appropriately, the use of the area under the curve might be able to better adjust doses among studies of different durations. We are open to doing this given sufficient data.

shown in Figures 2 and 3 with slight increases at lower doses followed by dramatically larger increases in these enzymes at doses above 200 mg/kg-day.[5] Figure 4 shows the histopathology results from the NCI (1978) study in rats (note scale change in the y-axis; corresponding liver enzyme changes were not monitored in this study). Although the overall incidences of the various effects are lower in the NCI (1978) rat bioassay, the dose sequence of these effects match the findings in rats from the JBRC (1990).

All of these findings in rats (including some not shown in these figures) show the expected changes in the liver due to a regenerative cell proliferation to promote liver tumors. That is, liver cell swelling, hypertrophy and liver weight increase occur at doses of 42-55 mg/kg-day; this precedes necrosis at doses of 94-219 mg/kg-day; which has a lower overlapping range of hyperplasia and foci development found at 55-389 mg/kg-day; which precedes in dose the development of adenomas and carcinomas at doses of 274-1015 mg/kg-day. Changes in liver AST and ALT enzymes also follow the expected pattern with increases seen at doses in excess of about 200 mg/kg-day. Importantly, the observed effects are in the expected dose sequence, and several of these effects occur in the expected time sequence (the data are limited in this respect because only two time points were monitored). This sequence in dose also matches the findings from the laboratory study report of Kociba et al. (1971), which was subsequently published by Kociba et al. (1974) (see supplemental figures 2 and 3 based on the laboratory report that is available by request from The Dow Chemical Company).

Review of the Japanese Translations and Integration with Other Findings: Mice

The information from the Japanese translated study reports and publications on mice are displayed on Figure 5, with information from the 13-week studies also plotted as adjusted by 3-fold uncertainty factor.[4]; Centrilobular liver cell swelling hypertrophy and liver weight increase appear between 190-200 mg/kg-day, and overlap necrosis in the same dose range, but hyperplasia and foci are nearly absent and adenomas and carcinomas appear early in the dose sequence, as low as at doses of 66 mg/kg-day in females. The corresponding changes in mouse liver enzymes from the Japanese work occur at or around 200 mg/kg-day (where the 13-week doses are adjusted by 10-fold uncertainty factor), and follow the pattern of liver cell swelling and necrosis, but not the adenoma and carcinoma sequence (see supplemental Figure 4). Specifically, the lack of noncancer histopathology in the chronic mouse study is not consistent with the changes in liver enzymes in this same chronic study, nor is the lack of noncancer findings expected based on the histopathology of the precursor 13-week study. Nor does the tumor response in the low dose female mice of JBRC (1990a) match the tumors findings in the McConnell (2013) re-read of NCI (1978).

In contrast, Figure 6 shows the results of a sequence of effects in mice found in the McConnell (2013) reread of NCI (1978) and as reported in Dourson et al. (2014). Here, hypertrophy and necrosis at doses between control and less than 400

[5] Here, the doses from the 13-week studies have been divided by a 10-fold uncertainty factor; caveats as in the previous footnote still apply.

mg/kg-day precede in dose the development of fewer foci (of various types) at similar and higher doses, which precedes in dose the development of tumors at higher doses. These findings are similar to the dose-related effects pattern found in the rat data.

When the data for mice from both chronic bioassays are overlaid, the results are mixed (see supplemental Figure 5). Centrilobular liver cell swelling, hypertrophy and necrosis more clearly precede tumor development in mice from the NCI (1978) study as re-read by McConnell (2013), and these results in mice are consistent with the sequence observed in rat studies. In contrast, the Japanese histopathology findings in mice (JBRC 1990a,b) are not consistent in sequence with either McConnell (2013) or rat studies. This difference may be due to a change in histopathological analysis, as stated by Kano et al. (2009, page 2777):

> "The hepatic hyperplasia of rats and mice diagnosed in the previous report (Yamazaki et al., 1994) [*authors note: which was a presentation of the JBRC, 1990a*] was re-examined histopathologically and changed to hepatocellular adenomas and altered hepatocellular foci including acidophilic, basophilic and clear cell foci in the present studies, according to the current diagnostic criteria of liver lesions in rats and mice."

This statement suggests that results from the JBRC (1990a) study report were modified prior to publication as Kano et al. (2009). However, the translation of this Japanese laboratory report does not show any dose-related hepatic hyperplasia in mice. Specifically, the report shows an incidence of hyperplasia of 5, 7, 5, 6 out of 50 males at each dose, and of 2, 2, 1, 1, out of 50 females, for control, low, medium and high doses, respectively. Foci are likewise nearly absent in mice in the JBRC (1990 a,b) reports and in the publication (Kano et al., 2009). Therefore, it is uncertain from reading this report as to what has been specifically changed in the mouse findings from the original JBRC report compared to the later publication. Additional pictures of a sufficient number of mouse liver slides to solve this dilemma were not available.

Saturation Kinetics

Metabolism of 1,4-dioxane in humans and experimental animals is well characterized and extensive. Workers exposed to 1,4-dioxane at low concentrations (~ 2ppm) showed a metabolite to parent ratio in the urine of 118:1 (Young et al., 1976). Higher concentrations (~50 ppm) by Young et al. (1977) also showed a linear elimination of 1,4-dioxane in both plasma and urine indicating that, at low levels, 1,4-dioxane metabolism is a nonsaturated, first-order process, leading to the principle metabolite β-hydroxyethoxy acetic acid (HEAA) with a pH dependent reversal to 1,4-dioxane-2-one.

However, higher doses of 1,4-dioxane in experimental animals show that the metabolism of 1,4-dioxane is saturable. For example, rats given *i.v.* exposures demonstrated a dose-related shift from linear, first-order metabolism to nonlinear, saturable metabolism of 1,4-dioxane in the range of 30 to 100 mg/kg (Young et al., 1978a,b). Similarly, rats given gavage doses of 10, 100, or 1,000 mg/kg singly showed that the percent urinary excretion of the radiolabel decreased significantly with increasing dose while radiolabel in expired air

increased, again indicating saturable kinetics in the dose range of 100 mg/kg. For mice this saturation appears to start at 200 mg/kg (Sweeney et al., 2008). The point of saturation for rats is consistent with effects being caused by the accumulation of the parent compound; the point of saturation found in mice is mostly consistent with effects caused by the accumulation of the parent compound (tumors found in the low dose female mice of the Japanese study being the exception).

Studies on whether 1,4-dioxane or one or more of its metabolites was the toxic moiety were pursued. Nannelli et al. (2005) investigated the role of CYP450 isozymes in the liver toxicity of 1,4-dioxane by inducing hepatic CYPB1/2 and CYP2E1 levels with phenobarbital or fasting. No change in glutathione content or ALT activity was observed when compared with control, suggesting that potentially highly reactive and toxic intermediates did not play a role in the liver toxicity of 1,4-dioxane. Pretreatment with inducers of mixed-function oxidases also did not significantly change the extent of covalent binding in subcellular fractions (Woo et al., 1977), again indicating that metabolites were not toxicologically active. Furthermore, a comparison of the pharmacokinetic profile of 1,4-dioxane with the toxicology data from a chronic drinking water study (Kociba et al., 1975) showed that liver toxicity did not occur unless clearance pathways were saturated and elimination of 1,4-dioxane from the blood was reduced. Koissi et al. (2012) also found that a major metabolite of 1,4-dioxane, namely 1,4-dioxane-2-one, fails to induce pre-neoplastic hepatic foci in orally treated rodents. Taken together, these data collectively support the hypothesis that the parent compound, 1,4-dioxane, and not a metabolite, is the toxic moiety. After metabolic saturation, when more of the parent chemical is available, liver toxicity occurs with sufficient frequency to be recorded. Such saturation occurs at an oral dose in the range 30 to 100 mg/kg in rats and at approximately 200 mg/kg in mice, although after induction, these saturation doses may be higher.

The reanalysis of rodent data on 1,4-dioxane that we highlight here can be used to evaluate the strength of the hypothesized MOA as suggested by (U.S. EPA, 2005; Boobis et al., 2008; Meek et al., 2014). Tables 1 and 2 show these data arranged in dose, time, and severity of effect, following the hypothesized regenerative hyperplasia MOA shown in Figure 1.

Rat Toxicity Data

Table 1 shows the key event sequence for the available rat data. The hypothesized key event 1 is metabolic saturation resulting in accumulation of parent compound. Key event 2 is shown to be cellular swelling, hypertrophy and liver weight increases. These occur at administered 13-week doses as low as 126 mg/kg-day (chronic dose equivalent of 42 mg/kg-day), or 2-year doses as low as 55 mg/kg-day. Key event 3, necrosis and/or inflammation, is shown to occur at administered 13-week doses as low as 657 mg/kg-day (chronic dose equivalent of 219 mg/kg-day), or 2-year doses as low as 94 mg/kg-day. Key event 4a, increased DNA synthesis as measured by [3H]-thymidine incorporation, is shown to occur at administered 11-week doses as low as 1000 mg/kg-day (chronic dose equivalent of 330 mg/kg-day). Key event 4b, hyperplasia, is also shown to occur at administered 11-week doses as low as 1000 mg/kg-day (chronic dose

equivalent of 330 mg/kg-day, and is seen at administered 2-year doses as low as 55 mg/kg-day. Key event 4c, pre-neoplastic foci, is seen at administered 13-week doses as low as 1168 mg/kg-day (chronic dose equivalent of 389 mg/kg-day), or 2-year doses as low as 55 mg/kg-day. Finally, the apical effect, adenomas and/or carcinomas is not seen at 13 weeks, but does occur after two years at doses as low as 274 mg/kg-day.

Thus, the dose sequence of these key events is:

- Key event 1,metabolic saturation at 30 to 100 mg/kg;
- Key event 2, cellular swelling, hypertrophy and liver weight increases at 42-55 mg/kg-day;
- Key event 3, necrosis and/or inflammation at 94-219 mg/kg-day;
- Key event 4
  a. increased DNA synthesis at 330 mg/kg-day;
  b. hyperplasia at 55-330 mg/kg-day; and
  c. basophilic and mixed cell (when measured) foci at 55-389 mg/kg-day
- Apical effect, adenomas and carcinomas at 274-1015 mg/kg-day.

This sequence of key events from seven rat bioassays, when coupled with 1,4-dioxane's negative mutagenicity, its lack of induction of DNA repair (indicating no DNA damage), and the appearance of background/spontaneous liver tumors (U.S. EPA, 2013), leads to the conclusion that rat liver tumors are evoked by a regenerative hyperplasia MOA. Regenerative hyperplasia is due to nonmutagenic toxicity in the rat liver that occurs in a dose and time related manner throughout the animal lifespan after metabolic saturation of 1,4-dioxane metabolism as shown in Table 1. Findings include similarities in toxicity between shorter term/high dose and longer term/lower dose, which is typical for other chemicals. Thus, the expectation that the shorter-term higher dose liver toxicity shown in Kano et al. (2008) would occur at lower doses with longer exposures as in Kano et al. (2009) is evident in Figure 2b for rats. Here the adjustment of the shorter-term exposures by a 3-fold uncertainty factor matches the doses in the chronic study, and shows similar findings.

Mouse Toxicity Data

Table 2 shows the key event sequence for the available mouse data. As before, the hypothesized initiating event is metabolic saturation resulting in accumulation of parent compound. Key event 1 is shown to be cellular swelling, hypertrophy and liver weight increases, which occur at administered 13-week doses as low as 585 mg/kg-day (chronic dose equivalent of 195 mg/kg-day) or 2-year doses as low as 191 mg/kg-day. Key event 2, necrosis and/or inflammation, is also shown to occur at administered 13-week doses as low as 585 mg/kg-day (chronic dose equivalent of 195 mg/kg-day), or 2-year doses as low as 191 mg/kg-day. Information on Key event 3, DNA synthesis, was not reported in mice. Key event 4a, hyperplasia, is not shown to occur in the sole 13 week study, but is seen in the 2-year dose of 380 mg/kg-day (interestingly this effect is not recorded for the high dose of the NCI bioassay, see previous discussion). Key event 4b, pre-neoplastic foci, was also not reported in the 13-week doses, but is found at administered 2-year doses as low as 380 mg/kg-day in the McConnell re-read of the NCI (1978) bioassay, but

was generally not found in JBRC (1990a) nor its publication by Kano et al. (2009). Finally, the apical effects, adenomas and/or carcinomas are not seen at 13-weeks, as expected, but does occur after two years at doses between 66-964 mg/kg-day.

Thus, the dose sequence of these key events is:

- Key event 1, metabolic saturation at ~200 mg/kg;
- Key event 2, cellular swelling, hypertrophy and liver weight increase, in the range of 190-200 mg/kg-day;
- Key event 3, necrosis and/or inflammation in the same range of 190-200 mg/kg-day;
- Key event 4,
  a. DNA synthesis has not been evaluated in mice;
  b. hyperplasia at doses as low as 380 mg/kg-day in one study but not the other, and
  c. foci development at doses as low as 380 mg/kg-day in the longer-term but not the shorter-term study
- Apical effect, adenomas and carcinomas at doses of 66-1015 mg/kg-day.

This sequence of key events from two chronic mouse studies and a subchronic mouse study generally support the hypothesized regenerative hyperplasia MOA. The collective results are not any stronger than this, however, due to the varying interpretations of liver lesions in the chronic mouse study of JBRC (1990a) versus that of Kano et al. (2009). Specifically, tumors in female mice from JBRC (1990a) are reported at the lowest dose of 66 mg/kg-day, which is lower than doses from suggested key events. Although it might be appropriate to adjust 13-week mouse study doses by a 10-fold factor to estimate the chronic dose equivalent (rather than a 3-fold factor), which would allow a sequence in doses of the key events in mice to be more similar to that found in the rat studies, the underlying reality is that the results of the two chronic mouse bioassays are simply different. This difference may be due in part to the change in the diagnostic criteria used to record the liver lesions reported by Kano et al. (2009).

Discussion

As discussed more extensively by U.S. EPA (2005) animal tumor findings give important clues in making decisions about potential MOAs. Often, animal cancer bioassays and their supporting sub-chronic and *in-vitro* data provide the only mechanistic/key event insights for a MOA serving to support the application of animal cancer data in risk assessment. Thus, all lines of evidence need to be explored when developing a rodent liver tumor MOA. Some of this evidence includes the number of studies conducted, the similarity of metabolic activation and detoxification among species, the influence of route of exposure on the spectrum of tumors, the effects of high dose exposures on the target organ or systemic toxicity that may not reflect typical physiological conditions, the presence of proliferative lesions, the effect of dose and time on the progression of lesions, the ratio of malignant to benign tumors as a function of dose and time, the time of appearance of tumors, the spectrum of tumors developed, the

number and incidence of tumors at organ sites with high or low background historical incidence, the biomarkers in tumor cells, and the shape of the dose-response curve for key events and tumors.

In considering this evidence Dourson et al. (2014) stated that in some respects 1,4-dioxane appears to be a mutagenic carcinogen. It evokes multisite and multispecies tumors that are not restricted to one sex suggesting an influence that is not restricted to gender, strain, or species, and, tumors evoked by 1,4-dioxane are both benign and malignant. However, all but one of the tumor types (i.e., nasal tumors) are at sites with a high historical background incidence, and findings in mutagenicity bioassays, initiation bioassays, and DNA repair bioassays are predominantly negative as described by U.S. EPA (2013). Woo et al. (1977) also found that covalent binding of radiolabeled 1,4-dioxane within hepatocytes was greatest in the cytosolic fraction, followed by the microsomal, mitochondrial, and nuclear fractions, but not to DNA. U.S. EPA (2005) concludes that: "The results from *in vitro* and *in vivo* assays do not provide overwhelming support for the hypothesis of a genotoxic MOA for 1,4-dioxane carcinogenicity." Thus, a MOA involving mutagenicity, which has been addressed by U.S. EPA (2013) and Dourson et al. (2014), is not further analyzed here since new information is not available. Both groups concluded that a mutagenic MOA is not likely.

Alternative MOAs include infection, receptor mediated processes, oxidative damage and cytotoxicity with compensatory hyperplasia. None of the available studies recorded infections, and since all of the studies showed a dose related response in tumors, infection was not the likely MOA. Data for receptor mediated processes or DNA binding are also generally unavailable or otherwise negative (e.g., Woo et al., 1977; U.S. EPA, 2013). Data for oxidative damage as a potential MOA are limited, but otherwise negative, with enhanced metabolism of 1,4-dioxane not showing any greater toxicity as discussed above.

In contrast, extensive toxicity is seen at the primary tumor sites (liver and nose) suggesting a growth-promoting, and specifically, a regenerative cell proliferation MOA. A regenerative hyperplasia MOA is also supported by positive findings in promotion bioassays and DNA replication bioassays suggesting growth stimulation. We re-evaluated the regenerative cell proliferation MOA hypothesis for liver tumors, as reported by Dourson et al. (2014), in light of the translations of JBRC (1990a,b) laboratory reports, and reaffirm that the U.S. EPA (2005) criteria for evaluation are met for strength, consistency, biological plausibility, and coherence. Moreover, dose response and temporal concordance for noncancer precursors to tumors are clearly evident for rats (Table 1), and generally supportive for mice (Table 2). Furthermore, 1,4-dioxane appears to be able to induce its own metabolism via CYP2E1. If so, 1,4-dioxane might share some characteristics with ethanol or phenobarbital-induced liver neoplasia.

The reason that the findings in mice are not more supportive of the regenerative hyperplasia MOA is because the histopathological characterizations of McConnell (2013) and of JBRC (1990a) in mice do not agree. McConnell (2013) found extensive liver toxicity as demonstrated by histopathology and fewer tumors than JBRC (1990a). JBRC (1990a) reported more tumors and nearly an absence of liver noncancer histopathology in

the chronic study. The lack of liver noncancer histopathology in JBRC (1990a) is unexpected, especially since an increase in liver enzymes associated with cell damage is found in this same study. Also, the JBRC (1990b) 13-week study showed extensive liver noncancer histopathology at suitably adjusted-to-chronic doses. Unfortunately, this internal inconsistency is not resolvable because slides or pictures from a sufficient number of experimental animals are not available for the current reanalysis.

During the course of this analysis, we obtained the opinions of several pathologists on the contrasting findings of the chronic mouse bioassays. Collectively these pathology opinions support the hypothesized MOA discussed in U.S. EPA (2013) and Dourson et al. (2014) that liver tumors from oral exposure to 1,4-dioxane occur after metabolic saturation, accumulation of the parent 1,4-dioxane molecule, liver toxicity and a regenerative hyperplasia. While additional live experimental animal testing might add confirmatory findings, a threshold for these tumors is expected if metabolism of the parent compound is not saturated, since subsequent liver toxicity does not occur. See Supplemental materials.

When the many lines of evidence are taken together, the reevaluation of the Japanese studies show consistent findings in rats and consistent findings in mice other than liver histopathology perhaps not being fully recorded in the chronic study. However, based on the number of studies conducted, the well-established metabolic saturation of 1,4-dioxane metabolism in humans and experimental animals, the effects of higher dose exposures on target organ toxicity, the presence of proliferative lesions, the effect of dose and time on the progression of lesions, the time of appearance of tumors, the spectrum of tumors developed, the number and incidence of tumors at organ sites with high or low background historical incidence, and the shapes of the dose-response curve for key events and tumors, all lead to the conclusion that a regenerative hyperplasia MOA is operating with 1,4-dioxane induced liver tumors. Furthermore, Tox21 dataset provides additional support for a non-genotoxic mode of action for 1,4-dioxane as more fully described in PubChem Tox21 (2015). These data include inactive outcomes of quantitative high-throughput ELG1-luciferase reporter gene assay that identifies compounds blocking DNA replication, and no activation of any biological pathways in the high-throughput screening assays.

Thus, the available lines of evidence collectively indicate that a nonlinear approach to dose-response assessment will protect against these tumorigenic effects. It might also be added that mouse liver tumors by themselves are often difficult to match to corresponding human disease and many groups have suggested reliance on other animal models such as the rat (e.g., U.S. FDA, 1997). While this issue does not directly address the likely MOA for 1,4-dioxane induced liver tumors, it suggests that reliance of this MOA in rats may be more appropriate, because the MOA is more clear in rats than in mice. Future work along these lines might be to describe this MOA within the emerging AOP framework described earlier.

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