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An integrated assessment of the 1,4-dioxane cancer mode of action and threshold response in rodents

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ABSTRACT

1,4-Dioxane is an environmental contaminant that has been shown to cause cancer in rodents after chronic high dose exposures. We reviewed and integrated information from recently published studies to update our understanding of the cancer mode of action of 1,4-dioxane. Tumor development in rodents from exposure to high doses of 1,4-dioxane is preceded by pre-neoplastic events including increased hepatic genomic signaling activity related to mitogenesis, elevation of Cyp2E1 activity and oxidative stress leading to genotoxicity and cytotoxicity. These events are followed by regenerative repair and proliferation and eventual development of tumors. Importantly, these events occur at doses that exceed the metabolic clearance of absorbed 1,4-dioxane in rats and mice resulting in elevated systemic levels of parent 1,4-dioxane. Consistent with previous reviews, we found no evidence of direct mutagenicity from exposure to 1,4-dioxane. We also found no evidence of CAR/PXR, AhR or PPARα activation resulting from exposure to 1,4-dioxane. This integrated assessment supports a cancer mode of action that is dependent on exceeding the metabolic clearance of absorbed 1,4-dioxane, direct mitogenesis, elevation of Cyp2E1 activity and oxidative stress leading to genotoxicity and cytotoxicity followed by sustained proliferation driven by regenerative repair and progression of heritable lesions to tumor development.

1. Introduction

1,4-Dioxane (1,4-DX) is a cyclic ether that has been used primarily as an industrial solvent in the production of chemicals and as a stabilizer for some chlorinated organic solvents. It can also be generated as a by-product during the manufacture of other chemicals. The International Agency for Research on Cancer (IARC) classified 1,4-DX as "possibly carcinogenic to humans" (group 2B) based on sufficient evidence in experimental animals and inadequate evidence in humans (IARC, 1999). 1,4-DX has been identified as "reasonably anticipated to be a human carcinogen" by the National Toxicology Program (NTP) based on sufficient evidence of carcinogenicity in experimental animals (NTP, 2016). Most recently, 1,4-DX was classified as "likely to be carcinogenic to humans" by the US EPA based on evidence of carcinogenicity in animal bioassays at multiple sites, in multiple species, and following both inhalation and oral routes of exposure (US EPA, 2020, 2013, 2010).

Although use as a solvent has largely been phased out, 1,4-DX has been detected in a wide range of media during environmental investigations, including drinking water sources throughout the United States, that has led to regulation by state and federal authorities. As such, an understanding of the cancer mode of action (MOA) is important since this can serve as the basis for determining action levels that protect human health.

After more than 60 years of research, understanding how exposure to 1,4-DX leads to tumors is still evolving. Thanks to the application of new tools, such as various "omics" and systems biology concepts, the pace of progress has increased significantly. Fundamentally, the MOA conceptually describes the key events (KEs), and the essentiality and relationship of those events, in the progression from exposure to tumor formation (Simon et al., 2014). Establishment of a MOA for rodent

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tumors also allows the determination of the human relevance of those tumors and their application in the risk assessment process. The decision regarding the cancer MOA for a substance directly affects the dose-response model that is used to determine human health protective exposures (i.e., linear no-threshold vs. threshold model). We note that currently there is no harmonized agreement among regulatory agencies on the MOA and appropriate risk model for 1,4-DX. Most, such as Health Canada, the World Health Organization, the European Chemicals Agency, and the Australian National Occupational Health and Safety Commission concluded the threshold MOA is appropriate (ECHA, 2021; Health Canada, 2021; NICNAS, 1998; WHO, 2005) while the US Environmental Protection Agency (US EPA) following their Cancer Risk Assessment Guidelines (US EPA, 2005) chose to default to the linear low-dose *(i.e.* no threshold) extrapolation model (US EPA, 2020).

Our goal in conducting this integrated assessment was to identify and analyze information on development of tumors from available 1,4-DX cancer bioassays, pre-neoplastic findings from all studies including shorter-term studies, along with information from more recent *in vivo* and *in vitro* mechanistic investigations. For the purposes of this assessment, we chose to focus on liver tumors (hepatic carcinomas and adenomas) because these were the tumors most commonly reported across studies and are the most responsive to development of tumors from 1,4-DX exposures. Tumors in other tissues, such as the nasal and respiratory epithelium, have also been reported. Where possible, we included information on these tissues as well. However, most of the subsequent studies investigating the biological activity of 1,4-DX target the liver. In our analysis, we reasoned the cancer MOA developed from information generated in liver would be transferable to other tissues. We then integrated the assembled information into an analysis of potential MOAs, highlighting those MOAs that are most consistent with the weight-of-evidence from the assembled information. We conclude by proposing an updated MOA informed by this analysis.

2. Assessment methods

For this assessment we searched the publicly available literature for studies published on the apical and mechanistic effects of exposures to 1,4-DX. Our assessment did not include a systematic review *per se*, but included a comprehensive literature search designed to build upon the systematic review conducted by the US EPA for the risk evaluation of 1,4-DX under the Toxic Substances and Control Act (TSCA) with reports published since the TSCA systemic review was completed (US EPA, 2020). We used similar health hazard search terms used by the US EPA for the TSCA risk assessment analysis (US EPA, 2017). We augmented those with additional search terms to identify topics such as nuclear receptor or cytochrome P-450 function as they relate to 1,4-DX. We retained peer-reviewed publications utilizing rodent species or human tissues. We considered limitations such as small numbers of subjects or sampling or dose groups, inadequate description of study design or dosing and statistical analysis in each of the studies reviewed and included these factors in our analysis and interpretation.

There have been seven cancer bioassays of 1,4-DX of various designs and exposures conducted on rats and two with mice. See Tables 1 and 2 in the Supplementary Information for a summary of the species and strains, durations of exposure, exposures and estimates of nominal doses of these studies. We converted exposure concentration values (% or ppm in drinking water or volumetric ppm in inhalation studies) to dose units (mg/kg/d or mg/kg) when possible to enable more direct comparison of dose-response across the exposure routes and study designs.

In our assessment of the two chronic inhalation studies (Kasai et al., 2009; Torkelson et al., 1974), as well as an available sub-chronic inhalation study (Kasai et al., 2008), we found inconsistencies in methods for converting air concentrations to estimates of delivered alveolar dose. This made dose-response comparisons across studies difficult. To resolve this, we recalculated the dose estimates for all the inhalation studies by reapplying the methods and assumptions used by the US EPA (US EPA, 2020). We used the published air concentrations and exposure durations from each study, usually published as volume concentration in ppm, converted to mass concentrations then applied the normalized alveolar ventilation rate (QPC) from Sweeney et al. (2008) along with a pulmonary retention factor of 1 (US EPA, 2020). The results of these conversions and the calculations used are included in the Supplemental Information accompanying this assessment.

In addition to variabilities in expressing inhalation exposures, there is also variability in estimating exposures when 1,4-DX exposure is from drinking water. Differences in body weights and water consumption of individual animals in an exposure group contribute to the variability in estimating dose. Most publications included some description of these variables, which we used to express doses as a mean and a range of dose estimates for each exposure group. We considered these ranges of dose in our analysis of the dose and response from exposure to 1,4-DX.

3. Development of tumors

The tumor findings have been reviewed and assessed numerous times by others (ATSDR, 2012; US EPA, 2013; Health Canada, 2021; IARC, 1999; 1,4-Dioxane. Report on Carcinogens : Carcinogen Profiles, 2011; US EPA, 2010). Nine rodent bioassays, two by inhalation and seven by oral (drinking water) exposures, have demonstrated that chronic high levels of exposure to 1,4-DX causes hepatocellular adenomas and carcinomas in rats following both oral and dermal inhalation exposures. See Tables 1 and 2 for a summary of the incidence and associated doses causing tumors in rats and mice, respectively. We have included the tumor incidence data from these legacy cancer bioassays and estimated doses in our assessment as an enabler for comparing dose-response of tumor incidence with other responses relevant to determining the cancer MOA.

We plotted the tumor incidence data across studies to enable more direct comparison and evaluation of consistency of responses. Fig. 1A and B show rat liver tumor incidences (combined hepatocellular adenomas and carcinomas, as well as cholangiosarcomas), while Fig. 1C and D show nasal cavity squamous cell carcinoma incidences for male and female rats, respectively.[2] As can be seen, the onset of liver and nasal tumor responses shows remarkable similarity regardless of whether exposure was from the oral or inhalation routes.

Fig. 2A and B show the dose-response plots for mouse tumors. Unlike rats, only treatment-related liver tumors have been reported in mice. Some strains of mice are particularly sensitive to hepatotoxicity and the development of liver tumors and have high background rates of liver tumors (Grisham, 1997; Maronpot, 2009) both of which can complicate the interpretation of mouse liver tumor data and comparisons across species. There is a clear and positive trend in dose-response in both studies and for both sexes. However, the tumor responses in Kano et al. (2009) are higher in the controls (especially males) and tend to show a higher tumor incidence per unit dose compared to those observed by the NCI (1978). The incidence of spontaneous liver tumors and sensitivity to hepatocarcinogens in various Charles River colonies and sub-strains of mice (including the Crj:BDF1 used in Kano et al. (2009), should be kept in mind when interpreting these data and comparing to data from other mouse stains (Engelhardt et al., 1993; Katagiri et al., 1998; Low--Marcheilli, 2017; Yamate et al., 1990).

The findings from the Kano mouse study (2009) deserve additional comment because they are controversial (for reasons described below) and are inconsistent with findings from the NCI study (NCI, 1978) as well as in comparison to conclusions from rat studies. The Kano et al., 2009 study reported a 70% combined liver tumor response rate in mice dosed for a lifetime at 66 mg/kg/day. There are three important

[2] The data presented from the Kociba et al. (1974) study were obtained from an unpublished 1971 study report (Kociba et al., 1971) provided by The Dow Chemical Company.

Table 1
Summary of findings from rats treated with 1,4-dioxane.

Citation	Duration	Sex	Target Organ and Endpoint[a]	No Observed Effect Level (mg/kg/d)[b]		Lowest Observed Effect Level (mg/kg/d)[b]	
Inhalation							
Torkelson et al. (1974)	104 Weeks	M	No adverse findings	50	(33–67)		
		F	No adverse findings	52	(37–67)		
Kasai et al. (2009)	2 Years	M	Increased liver weights	97	(65–130)	488	(327–649)
		M	Hepatic hypertrophy	97	(65–130)	488	(327–649)
		M	Hepatic hyperplasia	97	(65–130)	488	(327–649)
		M	Serum AST	97	(65–130)	488	(327–649)
		M	Serum ALT	97	(65–130)	488	(327–649)
		M	Serum ALP	97	(65–130)	488	(327–649)
		M	Serum γ-GTP	97	(65–130)	488	(327–649)
		M	Liver nuclear enlargement	97	(65–130)	488	(327–649)
		M	Liver acidophilic foci	97	(65–130)	488	(327–649)
		M	Liver Basophilic foci	97	(65–130)	488	(327–649)
		M	Hepatocellular adenomas/carcinomas	97	(65–130)	488	(327–649)
Kasai et al. (2008)	13 Weeks	M	Increased liver weight	158	(109–208)	316	(217–416)
			Centrilobular swelling	633	(435–832)	1271	(878–1663)
			Serum ALT	633	(435–832)	1271	(878–1663)
			Single cell necrosis	633	(435–832)	1271	(878–1663)
		F	Increased liver weight	167	(125–208)	334	(253–416)
			Centrilobular swelling	669	(507–832)	1342	(1021–1663)
			Serum AST	669	(507–832)	1342	(1021–1663)
			Serum ALT	669	(507–832)	1342	(1021–1663)
Drinking Water							
Kociba et al. (1974)	2 Years	M	Increased liver weights	94	(59–114)	1015	(914–1229)
		M	Hepatic Hypertrophy	79	(28–130)	395	(141–649)
		M	Hepatic Hyperplasia	10	(10–12)	94	(59–114)
		M	Hepatocellular degeneration/necrosis	10	(10–12)	94	(59–114)
		M	Hepatocellular adenomas/carcinomas	94	(59–114)	1015	(914–1229)
		M	Nasal cavity squamous cell carcinomas	94	(59–114)	1015	(914–1229)
		F	Increased liver weights	148	(130–168)	1599	(1416 to 2149)
		F	Hepatic hypertrophy	79	(28–130)	395	(141–649)
		F	Hepatic hyperplasia	19	(18–20)	148	(130–168)
		F	Hepatocellular adenomas/carcinomas	148	(130–168)	1599	(1416 to 2149)
		F	Nasal cavity squamous cell carcinomas	148	(130–168)	1599	(1416 to 2149)
NCI (1978)	110 Weeks	M	Nasal cavity squamous cell carcinomas			240	(130–380)
		F	Hepatocellular adenomas/carcinomas			350	(200–580)
		F	Nasal cavity squamous cell carcinomas			350	(200–580)
(JBRC, 1990; Kano et al., 2009)	2 Years	M	Increased liver weights	55	(52–58)	274	(256–292)
		M	Hepatic hypertrophy	79	(28–130)	395	(141–649)
		M	Serum AST (GOT)	55	(52–58)	274	(256–292)
		M	ALT (GPT)	55	(52–58)	274	(256–292)
		M	Serum LDH	55	(52–58)	274	(256–292)
		M	Serum ALP	55	(52–58)	274	(256–292)
		M	Serum γ-GTP	55	(52–58)	274	(256–292)
		M	Hepatocellular adenomas/carcinomas	55	(52–58)	274	(256–292)
		M	Respiratory epithelium nuclear enlargement	55	(52–58)	274	(256–292)
		M	Olfactory epithelium nuclear enlargement	55	(52–58)	274	(256–292)
		M	Respiratory epithelium squamous cell metaplasia	55	(52–58)	274	(256–292)
		F	Increased liver weights	83	(69–97)	429	(360–498)
		F	Hepatic hypertrophy	79	(28–130)	395	(141–649)
		F	Serum AST (GOT)	83	(69–97)	429	(360–498)
		F	Serum ALT (GPT)	83	(69–97)	429	(360–498)
		F	Serum ALP	83	(69–97)	429	(360–498)
		F	Serum LDH	83	(69–97)	429	(360–498)
		F	Serum γ-GTP	83	(69–97)	429	(360–498)
		F	Hepatocellular adenomas/carcinomas	83	(69–97)	429	(360–498)
		F	Respiratory epithelium nuclear enlargement	83	(69–97)	429	(360–498)
		F	Nasal squamous cell carcinomas	83	(69–97)	429	(360–498)
(Argus et al., 1965)	63 Weeks	M	Hepatocellular adenomas/carcinomas			640	
(Argus et al., 1973)	13 Months	M	None reported	1032			
(Kano et al., 2008)	13 Weeks	M	Increased liver weight	274	(264–284)	657	(627–687)
			Single cell necrosis	126	(110–142)	274	(264–284)
			Centrilobular swelling	126	(110–142)	274	(264–284)
			Hepatic vacuolization	657	(627–687)	1554	(1463 to 1645)
			Serum AST (GOT)	657	(627–687)	1554	(1463 to 1645)
			Serum ALT (GPT)	657	(627–687)	1554	(1463 to 1645)
			Respiratory epithelium nuclear enlargement	52	(43–61)	126	(110–142)
			Olfactory epithelium nuclear enlargement	126	(110–142)	274	(264–284)
			Tracheal epithelium nuclear enlargement	126	(110–142)	274	(264–284)
		F	Increased liver weight	83	(56–110)	185	(143–227)
			Single cell necrosis	756	(700–812)	1614	(1493 to 1735)
			Centrilobular swelling	427	(266–588)	756	(700–812)
			Hepatic vacuolization	756	(700–812)	1614	(1493 to 1735)

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Table 1 (*continued*)

Citation	Duration	Sex	Target Organ and Endpoint[a]	No Observed Effect Level (mg/kg/d)[b]		Lowest Observed Effect Level (mg/kg/d)[b]	
			Serum AST (GOT)	756	(700–812)	1614	(1493 to 1735)
			Respiratory epithelium nuclear enlargement	83	(56–110)	185	(143–227)
			Olfactory epithelium nuclear enlargement	185	(143–227)	427	(266–588)
			Tracheal epithelium nuclear enlargement	185	(143–227)	427	(266–588)
			Bronchial epithelium nuclear enlargement	756	(700–812)	1614	(1493 to 1735)
(Gi et al., 2018)	16 Weeks	M	Increased liver weight	222		562	
			Increased DNA synthesis	222		562	
Stott et al. (1981)	11 Weeks	M	Increased DNA synthesis	10		100	
Mnaa et al. (2016)	42 Days	M	Oxidative stress biomarkers[c]			100	
			Serum ALP			100	
Furihata et al. (2018)	4 Weeks	M	Increased signaling apoptosis			500	
			Increased signaling stress Response			500	
Nannelli et al. (2005)	10 Days	M	Induction hepatic Cyp2E1 activity			2000	
			Induction sasal Cyp2E1 activity			2000	

[a] Entries in parentheses are historical names used for the enzymes evaluated.
[b] Vaules in parentheses identify the range of potential NOELs or LOELs where data are available from the publication or could be calculated from information in the publication. Blanks cells indicate insufficient information from publication to estimate a value.

Table 2
Summary of findings from mice treated with 1,4-dioxane.

Citation	Duration	Sex	Target Organ and Endpoint[a]	No Observed Effect Level (mg/kg/d)[d]		Lowest Observed Effect Level (mg/kg/d)[d]	
NCI (1978)	90 Weeks	M	Hepatocellular adenomas/carcinomas			720	(530–990)
		F	Hepatocellular adenomas/carcinomas			380	(180–620)
(JBRC, 1990; Kano et al., 2009)	2 Years	M	Increased liver weights	49	(44–54)	191	(170–212)
			Serum AST (GOT)	49	(44–54)	191	(170–212)
			Serum ALT (GPT)	49	(44–54)	191	(170–212)
			Serum LDH	49	(44–54)	191	(170–212)
			Serum ALP	49	(44–54)	191	(170–212)
			Serum γ-GTP	49	(44–54)	191	(170–212)
			Hepatocellular adenomas/carcinomas	49	(44–54)	191	(170–212)
			Olfactory epithelium nuclear enlargement	191	(170–212)	677	(603–751)
			Respiratory epithelium nuclear enlargement	49	(44–54)	191	(170–212)
		F	Increased liver weights	66	(56–76)	278	(238–318)
			Serum AST (GOT)	66	(56–76)	278	(238–318)
			Serum ALT (GPT)	66	(56–76)	278	(238–318)
			Serum LDH	66	(56–76)	278	(238–318)
			Serum ALP	66	(56–76)	278	(238–318)
			Serum γ-GTP	66	(56–76)	278	(238–318)
			Hepatocellular adenoma or carcinoma			66	(56–76)
			Olfactory epithelium nuclear enlargement	66	(56–76)	278	(238–318)
			Respiratory epithelium nuclear enlargement	278	(238–318)	964	(876–1052)
Dourson et al. (2014) [b]	90 Weeks	M	Hepatocellular hypertrophy			720	(530–990)
			Hepatic hyperplasia[c]			720	(530–990)
			Hepatic glycogen depletion			720	(530–990)
			Hepatocellular necrosis			720	(530–990)
			Hepatic inflammation			720	(530–990)
			Hepatocellular adenomas/carcinomas			720	(530–990)
		F	Hepatocellular hypertrophy			380	(180–620)
			Hepatic hyperplasia[c]	380	(180–620)	860	(450–1560)
			Hepatic glycogen depletion	380	(180–620)	860	(450–1560)
			Hepatocellular adenomas/carcinomas			380	(180–620)
Kano et al. (2008)	13 Weeks	M	Increased liver weights	882	(735–1029)	1570	(1431 to 1709)
		M	Hepatic single-cell necrosis	86	(69–103)	231	(165–297)
		M	Hepatic centrilobular swelling	86	(69–103)	231	(165–297)
		M	Olfactory epithelium nuclear enlargement	86	(69–103)	231	(165–297)
		M	Bronchial epithelium nuclear enlargement	86	(69–103)	231	(165–297)
		M	Tracheal epithelium nuclear enlargement	86	(69–103)	231	(165–297)
		M	Degeneration bronchial epithelium	882	(735–1029)	1570	(1431 to 1709)
		M	Serum AST (GOT)	882	(735–1029)	1570	(1431 to 1709)
		M	Serum ALT (GPT)	882	(735–1029)	1570	(1431 to 1709)
		F	Increased liver weights	1620	(1448 to 1792)	2669	(2408 to 2930)
		F	Hepatic single-cell necrosis	170	(119–221)	387	(304–470)
		F	Hepatic centrilobular swelling	170	(119–221)	387	(304–470)
		F	Olfactory epithelium nuclear enlargement	170	(119–221)	387	(304–470)
		F	Respiratory epithelium nuclear enlargement	898	(695–1101)	1620	(1448 to 1792)
		F	Degeneration bronchial epithelium	898	(695–1101)	1620	(1448 to 1792)
		F	Tracheal epithelium nuclear enlargement	387	(304–470)	898	(695–1101)
		F	Bronchial epithelium nuclear enlargement	170	(119–221)	387	(304–470)
		F	Serum AST (GOT)	1620	(1448 to 1792)	2669	(2408 to 2930)
		F	Serum ALT (GPT)	898	(695–1101)	1620	(1448 to 1792)

(*continued on next page*)

Table 2 (*continued*)

Citation	Duration	Sex	Target Organ and Endpoint[a]	No Observed Effect Level (mg/kg/d)[d]		Lowest Observed Effect Level (mg/kg/d)[d]	
Lafranconi et al. (2021)	90 Days	F	Increased liver weights	364	(337–391)	979	(895–1063)
		F	Hepatic hypertrophy	364	(337–391)	979	(895–1063)
		F	Hepatic single-cell necrosis	364	(337–391)	979	(895–1063)
		F	Hepatic vacuolization	116	(106–126)	364	(337–391)
		F	Hepatic hypertrophy	364	(337–391)	979	(895–1063)
		F	Increased DNA synthesis	364	(337–391)	979	(895–1063)
Chappell et al. (2021)	90 Days	F	Increased mitotic signaling	364	(337–391)	979	(895–1063)
		F	Increased GHS conjugation signaling	116	(106–126)	364	(337–391)
Charkoftaki et al. (2021)	4 Weeks	F	Increased liver weights	96	(83–109)	927	(892–962)
		F	Increased xenobiotic metabolism signaling	96	(83–109)	927	(892–962)
		F	Increased nicotine degradation III	96	(83–109)	927	(892–962)
		F	Increased glutathione conjugation signaling	96	(83–109)	927	(892–962)
		F	Increased NRF2 signaling	96	(83–109)	927	(892–962)
Chen et al. (2022) [c]	3 Months	F	Increased liver weights			651	(520–782)
		F	Hepatic single-cell necrosis			651	(520–782)
		F	Serum AST (GOT)			651	(520–782)
		F	Serum ALT (GPT)			651	(520–782)
		F	Hepatic GSH/GSSG ratio			651	(520–782)
		F	Hepatic Cyp2E1			651	(520–782)
Roy et al. (2005)	5 days	M	Increased DNA synthesis	1500		2500	

1,4-dioxane was administered in drinking water in all of the studies included in this table.

[a] Entries in parentheses are historical names used for the enzymes evaluated.

[b] Re-read of mouse liver slides from 1978 NCI study. Since no statistics were conducted on the tumor incidence, professional judgement (a doubling or more of control incidence) was used to identify a no effect or lowest effect level from this study.

[c] Identified as Kupffer cell hyperplasia.

[d] Vaules in parentheses identify the range of potential NOELs or LOELs where data are available from the publication or could be calculated from information in the publication. Blanks cells indicate insufficient information from publication to estimate a value.

considerations for this reported response that, taken together, undermine the usefulness of mouse liver tumor incidence findings from this study.

1) The Kano et al. (2009) study in Crj:BDF1 female mouse demonstrated a near maximum liver tumor response (e.g., 70%) at the lowest dosage tested (66 mg/kg/d) that increased modestly to 92% at the highest dosage (964 mg/kg/d). In contrast, the 1978 NCI study in B6C3F1 female mice demonstrated a more abrupt increase in treatment-related liver tumors, where tumor incidence increased from 44% at 380 mg/kg/d to 95% at 860 mg/kg/d.
2) The 13-week mouse drinking water study (Kano et al., 2008) reported non-neoplastic liver pathology that was inexplicably not reported in the 2-year study. In addition, similar non-neoplastic findings were also observed in the re-read of the liver slides from the NCI study (Dourson et al., 2014) indicating that the reporting of pre-neoplastic findings from the chronic study by Kano was incomplete.
3) The diagnostic criteria used in the original JRBC report (JBRC, 1990) and associated conference proceeding (Yamazaki et al., 1994) changed in the subsequent peer-reviewed publication of the same study (Kano et al., 2009).

> "*Diagnostic improvement from the hepatic hyperplasia in our preliminary report* (Yamazaki et al., 1994) *to the altered hepatocellular foci and hepatocellular adenomas in the present studies according to the current criteria (*Mohr, 1997*; Deschl* et al*., 2001) was found to increase the incidences of hepatocellular adenomas in the 1,4-DX-dosed groups, resulting in the definite dose-hepatocarcinogenic response relationships as compared with those in our preliminary report*." (pg. 2783, Kano et al., 2009).

It is uncertain whether the evolving pathological assessment of the Japanese mouse drinking water findings inadvertently counted altered hepatic foci as tumors, thereby inflating the number of liver tumors reported across the dose groups in Kano et al. (2009). Unfortunately, the slides from this study are no longer available for a pathology peer-review reassessment (Dourson et al., 2014).

4. Pre-neoplastic histology, biochemical and genomic findings

Pre-neoplastic changes including changes in cell organization and structure as well as biochemistry and genomics are often associated with tumor development (Felter et al., 2022; Wolf et al., 2019). Some events are slower to develop, and the duration of exposure plays a role in the onset of these events. Pre-neoplastic observations provide an opportunity to observe events and establish temporal relationships to identify key events for the cancer MOA for chemical carcinogens.

Evidence for adaptive and pre-neoplastic changes in histology have been reported in rodent studies following exposures to 1,4-DX by inhalation and ingestion (drinking water). Unfortunately, pre-neoplastic histology findings are not consistently reported from cancer bioassays. Historically, the focus of the pathology evaluations from cancer bioassays was on tumor findings. Other observations were frequently not reported (Dourson et al., 2014). Sub-chronic studies typically report on a broader array of observations including pre-neoplastic histology and, by study design, are better suited to identify early events.

Liver cells and nasal or respiratory epithelium are targets for pre-neoplastic events. These findings are summarized in Table 1 (Rats) and Table 2 (Mice). For ease of comparison across studies, we have included findings from chronic, including tumor incidence, and sub-chronic studies along with exposure estimates.

4.1. Histology

The most common findings from chronic studies with rats and mice include hepatic hypertrophy, nuclear enlargement in the liver and nasal/respiratory epithelium, hepatic hyperplasia and single-cell necrosis in the liver. Dourson et al. (2014) conducted a re-evaluation of the mouse liver slides from the NCI study using NCI's contemporary diagnostic criteria. This re-evaluation revealed pre-neoplastic lesions in livers of both male and female mice not recorded in the original NCI study report. Findings include a decrease in glycogen, an increase in the incidence of hypertrophy, necrosis, inflammation, Kupffer cell hyperplasia and basophilic, eosinophilic, clear cell, and mixed cell foci in both sexes.

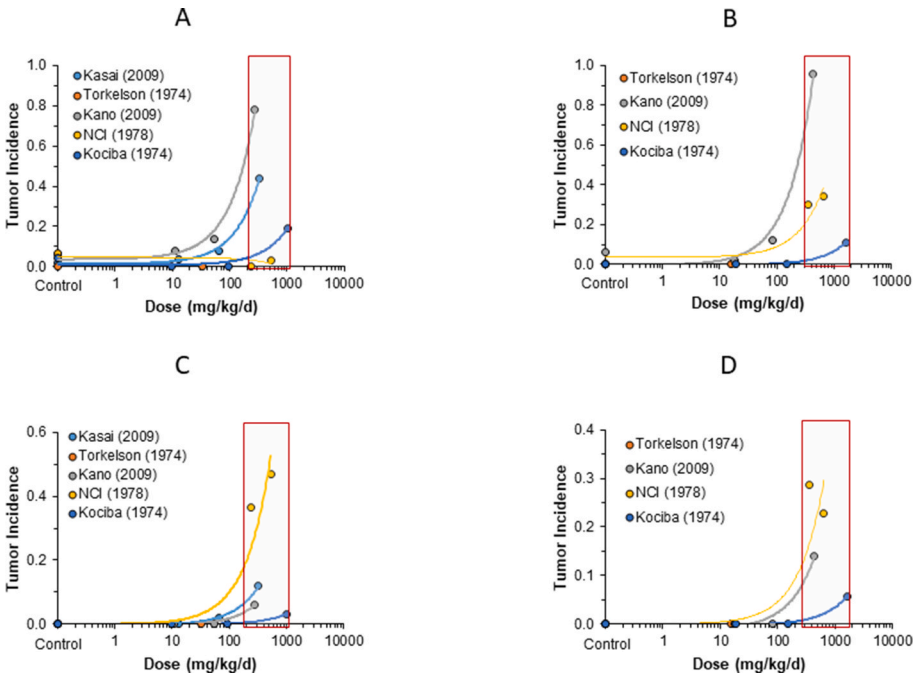

Fig. 1. Rat liver tumor indidence (A. male, B. female) and nasal tumor incidence (C. male, D. female) after chronic inhalation or drinking water exposures. The lines are meant to help identify data points from each study. They were not mathematically fitted to the data using dose-response modeling procedures. Boxes indicate range of exposures resulting in significant incidence of tumors.

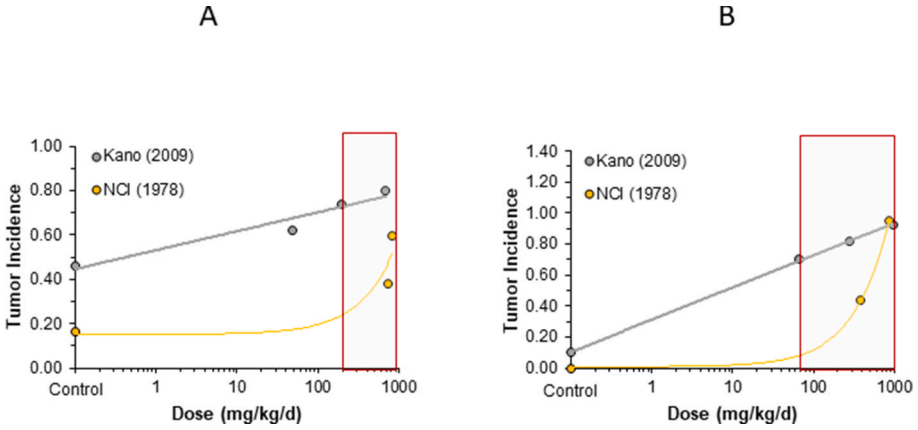

Fig. 2. Mouse liver tumor incidence (A. male, B. female) after chronic drinking water exposure.
The lines are meant to help identify data points from each study. There were not mathematically fitted to the data using dose-response modeling procedures. Boxes indicate range of exposures resulting in significant incidence of tumors.

The target organs from sub-chronic studies were similar to those observed after chronic exposures with findings primarily in the liver and the nasal and respiratory epithelium in both rats and mice. As expected, sub-chronic studies revealed more pre-neoplastic findings compared to the chronic studies because the expanded scope of examination. In addition to the increases in liver weight observed in both chronic and sub-chronic studies, there were findings of hepatic centrilobular swelling. There were also dose-dependent increases in the frequency of single-cell necrosis, presumably a sign of apoptosis. In the respiratory tract there were nuclear enlargement findings in both rats and mice.

Increased liver weight was the most consistent observation reported across studies. This was observed in both chronic and sub-chronic studies with increases observed as early as 13 weeks in rats after onset of exposure (Kano et al., 2008) and as early as 7 days in mice (Lafranconi et al., 2021). The lowest LOEL for increased liver weights was 274 mg/kg/d in male rats and 429 mg/kg/d in female rats after chronic exposure (Kano et al., 2009) and as low as 185 mg/kg/d in female rats from a sub-chronic (13-week) study (Kano et al., 2008). In mice, the lowest LOEL for increased liver weight in chronic studies was 191 mg/kg/d for males and 278 mg/kg/d for females (Kano et al., 2009). There was also evidence of hyperplasia reported occasionally with the lowest LOEL of 94 mg/kg/d in male rats and 148 mg/kg/d in female rats (Kociba et al., 1974). In mice, there was histological evidence of Kupffer cell hyperplasia from the report by Dourson et al. (2014) at the lowest doses used in the NCI studies of 720 mg/kg/d in males and 860 mg/kg/d for females. (Dourson et al., 2017).

Likewise, histological changes in the nasal and respiratory tract were common across studies and species. The lowest LOEL was 126 and 185 mg/kg/d for nuclear enlargement of respiratory epithelium in male and female rats, respectively after 13 weeks of exposure to 1,4-DX in drinking water (Kano et al., 2008). The lowest LOEL was 191 and 964 mg/kg/d for nuclear enlargement of the respiratory epithelium in male and female mice, respectively, after chronic exposure to 1,4-DX in drinking water (Kano et al., 2009). The lowest LOEL from sub-chronic

exposure was 387 mg/kg/d in female mice after 13 weeks of exposure (Kano et al., 2008). Note, we did not consider histopathology findings of the respiratory tract from the inhalation studies in this analysis because the exposure to the respiratory epithelium was by direct contact from the inhaled 1,4-DX and not comparable to exposures and effects generated from systemic circulation.

4.2. Biochemistry

Biochemical findings were included in one chronic inhalation study (Kasai et al., 2009), one chronic drinking water study (JBRC, 1990) and seven sub-chronic studies (Chen et al., 2022; Gi et al., 2018; Kano et al., 2008; Kasai et al., 2008; Lafranconi et al., 2021; Nannelli et al., 2005; Stott et al., 1981). The findings are summarized in Table 1 (rats) and Table 2 (mice) along with estimates of the corresponding NOAEL or LOAEL for each effect.

Looking across all studies, 1,4-DX exposures often caused changes in circulating liver enzymes, DNA synthesis, and cytochrome P450 activity. Elevations of serum enzymes occurred with doses as low as 274 and 429 mg/kg/d in male and female rats, respectively from chronic drinking water exposures (Kano et al., 2009) and 100 mg/kg/d after 42 days of drinking water exposure in male rats (Mnaa et al., 2016). Similar results were observed in rats following inhalation exposure as well (Kasai et al., 2009). The lowest LOEL for elevated serum enzymes in mice was 191 and 278 mg/kg/d for male and female mice, respectively after chronic exposure to 1,4-DX in drinking water (Kano et al., 2009).

Indicators of increased DNA synthesis, such as increased incorporation of tritiated thymidine or bromodeoxyuridine (BrdU) immunohistochemical staining, occurred in sub-chronic studies at doses as low as 100 mg/kg/d in male rats (Stott et al., 1981) and 979 mg/kg/d in female mice after 90 days of exposure to 1,4-DX in drinking water (Lafranconi et al., 2021). Cytochrome P450 Cyp2E1 induction has been characterized by studies in rats and mice. Cyp2E1 activity increased approximately three-fold in male rats after 10-days of exposure to 2000 mg/kg/d in drinking water (Nannelli et al., 2005). Chen et al. (2022) reported a doubling (approximate[3]) of Cyp2E1 activity in female mice at a dose of 651 mg/kg/d after 12 weeks of exposure to 1,4-DX in drinking water.

It is noteworthy that changes in hepatic glycogen stores have been reported in mice from chronic exposure to 1,4-DX. There was a decrease in hepatic glycogen deposits at 380 and 860 mg/kg/d in male and female mice, respectively as determined from the re-read of the mouse liver slides obtained from the NCI study (Dourson et al., 2014). It is possible that the decrease in glycogen may be related to the metabolic stress from the increased energy demands caused by the biotransformation of 1,4-DX. It is also noteworthy that decreased glycogen has been associated with increased metabolic demands of cancer cells (Liu et al., 2021).

4.3. Genomics

More recently, transcriptomics has been employed to characterize changes at the genomic level of pre-neoplastic biological responses from exposures to 1,4-DX. Transcriptomics techniques aims to identify and quantify the changes in molecular transcripts (e.g., mRNA, non-coding RNA, and small RNA) of a cell or tissue (Wang et al., 2009).

Several studies have used transcriptomic analyses to evaluate effects from 1,4-DX. Furihata et al. (2018) evaluated liver gene expression change of 11 hepatic marker genes selected to differentiate between genotoxic and non-genotoxic hepatocarcinogens. They exposed male F344 rats to 5000 ppm 1,4-DX in drinking water for 4 weeks for an estimated dose of 500 mg/kg/d and compared the results to two genotoxic hepatocarcinogens: 0.001% *N*-nitrosodiethylamine (DEN) and 0.015% 3,3′-dimethylbenzidine·2HCl (DMB) in water, and a non-genotoxic hepatocarcinogen, 1.2% di (2-ethylhexyl)phthalate (DEHP) in feed. Using principal component analysis (PCA), liver responses for 1,4-DX did not cluster with the other hepatocarcinogens investigated. The authors concluded 1,4-DX had an intermediate expression profile to those they studied; however, this is only based on its transcriptomic fingerprint compared to two genotoxic hepatocarcinogens and a single non-genotoxic carcinogen (DEHP), which is a peroxisome proliferator and may not represent the fingerprint of other non-genotoxic hepatocarcinogens. Of the 11 marker genes tested, 9 were not significantly different compared to control animals as a result of 1,4-DX treatment while *Bax*, *Btg2*, *Cdkn1a*, *Lrp1* and *Plk2* gene expression changes were significantly different (Tukey test) between the two genotoxic hepatocarcinogens studied and 1,4-DX. This study did reveal enhancement of signaling associated with two of the marker genes: apoptosis (*Aen*) and cyclin G1 (*Ccg1*) involved in growth promotion after cellular stress (Kimura et al., 2001). Overall, this study demonstrated a genetic response from 1,4-DX exposure that did not cluster with genotoxic hepatocarcinogens indicating a dissimilar MOA.

Chappell et al. (2021) conducted a transcriptomic analysis on the livers of female B6D2F1/Crl mice exposed to 40, 200, 600, 2000, or 6000 mg/L 1,4-DX in drinking water for 7, 28, or 90 days. Treatment-related changes in genomic endpoints occurred only after 90-days of exposure to 2000 ppm (337–391 mg/kg/d) and above – see Table 2. The gene changes reported were related to mitotic cell cycle checkpoints and Phase II Metabolism. Consistent with results from Tox21 high-throughput screening assays performed with 1,4-DX, Chappell et al. (2021) found no evidence of enriched gene expression related to DNA damage response or repair in liver tissue at any of the exposure levels.

Charkoftaki et al. (2021) performed a transcriptomic study in female BDF-1 mice exposed to 0 or 5000 ppm 1,4-DX in drinking water for 4 weeks (estimated dose of 927 mg/kg/d). The authors reported a small enrichment of pathways of oxidative stress response, nuclear factor erythroid 2-related factor 2 (Nrf2) signaling, glutathione (GSH) redox & detox reactions, xenobiotic detoxification pathways and DNA repair at the 5000 ppm exposure level. A similar pattern was observed in mice exposed for 3 months to 5000 ppm (estimated dose of 651 mg/kg/d) 1,4-DX in drinking water or one week of gavage treatment at 1000 mg/kg/d (Chen et al., 2022). In this latest study, the authors reported a compensatory Nrf2 anti-oxidative response at the early stage (one week) in both WT and GSH-deficient mice. This was followed up by a persistent induction of Nrf2 transcription factor that regulates the expression of detoxifying and antioxidant defense genes in the liver after three months of exposure via drinking water; the effects of oxidative stress with the elevations in oxidative DNA damage and DNA repair response were exaggerated in GSH-deficient mice.

5. Integrated analysis of pre-neoplastic findings

Numerous pre-neoplastic changes in histology, biochemistry and genomics occur in response to 1,4-DX exposure. In studies with multiple doses tested, each of these effects occurred at the high doses tested, but not at lower doses. In studies where only one dose was tested, the only dose was consistent with the threshold dose. For example, of the four genomic studies, Chappell et al. (2021) evaluated multiple doses and presented a threshold for effects. The three other genomic studies (Charkoftaki et al., 2021; Chen et al., 2022; Furihata et al., 2018) used a single dose which was above the threshold reported by Chappell et al.

The primary target organ is the liver in both rats and mice and, to a lesser extent, the nasal and respiratory epithelium but this may simply reflect the selection of tissues targeted for study. It is reasonable to assume that these same events occur at some level in other tissues but responses may be blunted by differences in toxicokinetic/exposure parameters like blood flow, or metabolic capabilities such as cytochrome P450 activity. There appears to be a difference in sensitivity among rats

[3] Inferred from Fig. 4C of publication.

and mice with rats responding to lower exposures of 1,4-DX than mice across all endpoints evaluated in both species. There does not appear to be a consistent difference between male and females in response to pre-neoplastic events.

Finally, the genomic profiling available aligns with the apical findings. There were shifts in signaling for xenobiotic biotransformation processes, increases in signaling for metabolic stress and evidence of proliferative (mitotic) processes in response to 1,4-DX exposure.

6. Genotoxicity

The genotoxicity of 1,4-DX has been widely studied using *in vitro* and *in vivo* methods and more recently with genomics and characterization of DNA adducts. Reviews by authoritative bodies have concluded that 1,4-DX is not likely to be mutagenic and weakly genotoxic (ATSDR, 2012; ECHA, 2002; US EPA, 2013; Health Canada, 2021; NICNAS, 1998; US EPA, 2020). There is no evidence that 1,4-DX is reactive with DNA nor does it appear to be metabolized to reactive electrophiles capable of causing mutations or causing nicks or breaks in DNA. 1,4-DX has been shown to be negative in the majority of *in vitro* genotoxicity studies including bacterial mutagenicity assays, cytogenetic assays with non-mammalian eukaryotes and with mammalian cells. On the other hand, the findings from *in vivo* studies have been mixed with the weight of evidence demonstrating some genotoxic activity only at high doses (where dose response data are available; See Table 3 *for a summary of genotoxicity findings*) and no evidence of mutagenicity (ATSDR, 2012; ECHA, 2002; US EPA, 2013; Health Canada, 2021; NICNAS, 1998; US EPA, 2020).

From US EPA TSCA Risk Assessment (US EPA, 2020);

> "Based on the weight of scientific evidence, EPA concluded that there is some evidence for genotoxicity *in vivo* at high doses, but there is insufficient evidence to conclude that 1,4-dioxane is mutagenic or induces cancer through a mutagenic mode of action."

Other agencies reached the same conclusion in separate analyses of the muatagnic poential of 1,4-DX (ECHA, 2021; Health Canada, 2021; WHO, 2005; NCI, 1998). The information generated to date has linked these *in vivo* genotoxic events as the secondary events to elevated generation of Reactive Oxygen Species (ROS) and subsequent oxidative DNA damage; this AOP has recently been proposed and is discussed below (Cho et al., 2022).

6.1. DNA adducts

DNA reactivity *in* vivo was first investigated with the help of [^{14}C] 1,4-DX given acutely or repeated (11-week) oral administration of up to 1000 mg/kg/d to male Sprague-Dawley rats (Stott et al., 1981). In this series of experiments, [^{14}C]1,4-DX did not cause detectable alkylation of hepatic DNA following single or repeated exposures of up to daily limit dose of 1000 mg/kg/d 1,4-DX compared to the treatment with the concurrent dimethylnitrosamine genotoxic carcinogen control (data not shown). The limit of detection in this study was reported as $\geq$ 1 alkylation per 10^6 nucleotides.

Totsuka et al. (2020) likewise investigated 1,4-DX associated rat liver DNA lesions from their 16-week oral drinking water study (Gi et al., 2018) utilizing their adductome analysis technique. They reported two separate clusters of DNA lesions with their high-resolution accurate-mass mass spectrometry combined with a principal component analysis-discriminant analysis (PCA-DA). These two clusters are:

1. Endogenous lesions in the 0 ppm and low-dose of 20 ppm treatment groups (*estimated dose 0 and* 2 mg*/kd/d, respectively)* with a small number of DNA adducts detected;

Table 3
Summary of genotoxicity findings from rodents treated with 1,4-dioxane.

Citation	Assay	Finding	No Observed Effect Level (mg/kg/d)[a]	Lowest Observed Effect Level (mg/kg/d)[a]	
Totsuka et al. (2020)	Adductome analysis	Statistically significant increase in 8-oxo-dG DNA and oxidative dT Lesions	19	440	
		Statistically significant increase in dC/dU Lesions	2	19	
Chen et al. (2022) [c]	ELISA DNA adduct	8-OHdG (KO only)		651	(520–782)
(Kitchin and Brown, 1990) [f]	Western blot & ICH	DNA Strand Breaks	840	2550	
Gi et al. (2018)	Alkaline elution	DNA Strand Breaks	92	440	
	HPLC adduct detection	8-OHdG	440		
	DNA repair activity	MGMT direct repair	92	440	
		BER, NER, MMR Excision repair	440		
Charkoftaki et al. (2021) [c]	Immuno-histochemical analysis	γH2AX (KO- Chen only & WT) DSB repair		927	(892–962)
Chen et al. (2022) [c]					(520–782)
Furihata et al. (2018) [c]	Genotoxic marker gene analysis	1,4-DX not associated with either genotoxic or non-genotoxic signal patterns	500		
Gi et al. (2018)	Transgenic rodent assay	*gpt* delta mutation frequency	92	440	
		A:T-to-G:C transitions and A:T-to-T:A transversions	92	440	
		A:T-to-T:A transversion mutations	18.7	92	
Morita and Hayashi (1998) [b]	*In vivo* micronucleus	Peripheral blood micronuclei	3000		
		Hepatocyte micronuclei	1000	2000	
Mirkova (1994) [b]	*In vivo* micronucleus	Bone marrow micronuclei	450	900	
Roy et al. (2005) [d]	*In vivo* micronucleus	Bone marrow micronuclei		1500	
		Hepatocyte micronuclei	1500	2500	
Itoh and Hattori (2019)	*In vivo* micronucleus	Hepatocyte micronuclei in adults and juvenile animals[e]		1000	
		Bone marrow micronuclei[b]	3000		
	Gene mutation	Peripheral blood *Pig*-a[b]	3000		

[a] Vaules in parentheses identify the range of potential NOELs or LOELs where data are available from the publication or could be calculated from information in the publication. Blanks cells indicate insufficient information from publication to estimate a value.
[b] Single oral gavage dose.
[c] Single dose study.
[d] Five-day oral gavage dose (mg/kg/d).
[e] Two-day oral gavage dose (mg/kg/d).
[f] Two oral gavage doses within one day (mg/kg).

2. Treatment-related cluster of DNA adducts in mid- and high-dose group animals of 200 and 5000 ppm, respectively (*estimated dose of 19 and* 440 mg/kg/d*, respectively*).

The three types of treatment-related adducts from the second cluster are: 8-oxo-dG, a well-known oxidative damage biomarker; lesion(s) at thymine (dT) reported as a novel, but least abundant, adduct; and, lesion (s) at cytidine (dC) and/or uracil (dU), a deaminated dC base (Table 3). The 1,4-DX associated 8-oxo-dG lesions have been shown in this study to have highest intensity compared to other two identified types of adducts. While 8-oxo-dG lesion is the most abundant and best recognized biomarker of oxidative DNA damage, the full spectrum of oxidative lesions in mammalian DNA exceeds more than 100 different types (Croteau and Bohr, 1997). The authors conclude that there is an "apparent threshold" in 1,4-DX treatment-related adductome between low vs. mid/high-treatment groups and that these biomarker adducts "may not require direct binding action with DNA" (Totsuka et al., 2020). The absence of direct DNA reactivity of 1,4-DX is consistent with the initial findings of (Stott et al., 1981).

6.2. DNA strand breaks

A small but significant increase in DNA strand breaks has been seen in rat hepatocytes after two oral doses of ≥2550 mg/kg/d administered within the same day (Kitchin and Brown, 1990); it is noteworthy that the highest dose of 4200 mg/kg/d was set at the LD50 value. Importantly, no increased strand breakage was seen at the two lower doses of 168 and 840 mg/kg/d in the same study. Chappell et al. (2021) were unable to identify an enrichment of genes associated with known DNA repair activities except for a dose-responsive trend in the expression of Rad51 after 90 days of the high 1,063 mg/kg/d study dose in the mouse livers. In humans, RAD51 plays a role in homologous recombination repair of double strand breaks (DSBs), DNA damage response (DDR) as well as in DNA replication and processing of stalled replication forks (Bhattacharya et al., 2017; Jiang et al., 2021; Laurini et al., 2020).

Increased strand breakage, including DSBs, has been reported in a number of rodent investigations with 1,4-DX (Charkoftaki et al., 2021; Itoh and Hattori, 2019; Kitchin and Brown, 1990; Roy et al., 2005). See Table 3. Small, but statistically significant increases in phosphorylated γ-H2AX foci, an indicator of DSBs in DNA, at top exposure concentration of 5000 ppm 1,4-DX in mouse hepatocytes after 1- and 4-week treatments as well as in non-hepatocyte endothelial, Kupffer, and stellate liver cells after 1-week treatment have been reported (Charkoftaki et al., 2021).

Strand breaks can also be intermediates of DNA excision repair pathways. Towards this, Gi et al. (2018) probed the transcripts of key enzymes in base excision, nucleotide excision and mismatch repair pathways after treating male F344 rats with 1,4-DX at up to 5000 ppm in drinking water for 16 weeks. However, they reported no significant transcript changes compared to the control levels (Table 3).

6.3. Mutations

Gi et al. (2018) also conducted a transgenic rodent assay with *gpt* delta transgenic F344 rats. In this study, 1,4-DX was administered in the drinking water for 16 weeks at 0, 0.2, 2, 20, 200, 1000, 2000 or 5000 ppm over three separate experiments; the base substitution mutation information was obtained from experiment 1 with 1,4-DX exposures of 0, 200, 1000 and 5000 ppm. They found an increased mutation frequency (MF) and significantly increased base pair substitution mutations at the A:T base pair in the high-dose treatment group of 5000 ppm (442 mg/kg/d). A-to-T transversions were also significantly increased in the 1000 ppm (92 mg/kg/d) treatment group but without concomitant increase in MF. The NOAEL Point-of-Departure value for the induction of mutation identified by the authors was 200 ppm 1,4-DX.

The authors suggested that the increased expression of methylguanine methyltransferase (MGMT) repair protein at the high dose of 5000 ppm is the key line of evidence for a mutagenic MOA. MGMT predominantly removes alkyl adducts from the O^6-position of guanine (dG) (Wyatt and Pittman, 2006; Zak et al., 1994). However, this should be interpreted with caution. MGMT prevents G-to-A mutations, and those transitions were not increased in the Gi et al. (2018) study. Instead, they found mutations at the A:T base pair, specifically A-to-G and A-to-T mutations, predominating as a result of 1,4-DX treatment (Gi et al., 2018) – see Table 3. MGMT is one of the many DNA repair genes linked to the DDR pathways (Jiang et al., 2021) and would have likely been increased as a result of alkylation of DNA from lipid peroxidation as noted by Chen et al. (2022). In addition, oxidative stress and lipid peroxidation have been linked to the 1,4-DX treatment (Charkoftaki et al., 2021; Chen et al., 2022). Numerous DNA repair pathways have been linked to the repair of lesions caused by oxidative stress and lipid peroxidation including homologous repair and direct repair (Winczura et al., 2012). Hence, the increased MGMT expression may result from such indirect DNA damage caused by oxidative stress.

6.4. Chromosomal aberrations

Positive liver micronucleus assays in mice were reported at exposure concentrations of ≥2000 mg/kg/d (Morita and Hayashi, 1998; Roy et al., 2005; see Table 3). However, mixed results were also obtained in the bone marrow of *in vivo* micronucleus tests (MNT) with two out of five MNT assays reported; the positive findings were reported after gavage 1, 4-DX doses of ≥900 mg/kg/d (Mirkova, 1994; Roy et al., 2005; see Table 3). Again, the positive *in vivo* outcomes were associated with high exposure concentrations and concomitant with cytotoxicity (IARC, 1999; US EPA, 2013).

Roy et al. (2005) investigated the types of hepatic and erythrocyte micronuclei formed following oral gavage of 1,4-DX at 1500–3500 mg/kg for 5 days to mice. They reported that the majority of micronuclei induced by 1,4-DX were formed as a result of chromosome breakage.

More recent study by Itoh and Hattori (2019) conducted a *Pig-a* gene mutation and MNT assays with male rats orally treated with 1,4-DX up to two times at doses between 1000 and 3000 mg/kg/d. For the liver MNT, the authors utilized juvenile animals or two partial hepatectomy methods. After the treatment, the authors reported statistically significant increases in liver micronuclei that were dose-responsive as a result of 1,4-DX treatment at all treatment doses starting at 1000 mg/kg/d; lower exposures were not investigated by the authors. This finding was consistent with previous murine investigations (Morita and Hayashi, 1998; Roy et al., 2005); however, no increase in MF was reported in the same study in the bone marrow MNT or *Pig-a* gene mutation assays up to the highest 1,4-DX dose tested of 3000 mg/kg/d (Itoh and Hattori, 2019).

6.5. Role of Reactive Oxygen Species in 1,4-DX genotoxicity

Oxidative stress and lipid peroxidation have been shown to also cause secondary alkylating DNA damage (Bartsch and Nair, 2006; Gi et al., 2018; Winczura et al., 2012; Zhai et al., 2020). Numerous recent investigations have reported an association between 1,4-DX treatment and oxidative stress resulting in increased generation of ROS and lipid peroxidation, which are in turn culprits for secondary oxidative and alkylating DNA damage, respectively. These observations were recently reviewed (Ginsberg et al., 2022; Wang et al., 2022). Moreover, recently an adverse outcome pathway (AOP) has been published that links oxidative DNA damage with mutation and chromosomal aberration outcome (Cho et al., 2022).

Charkoftaki et al. (2021) found evidence of oxidative stress in the mouse liver following treatment with 5000 ppm 1,4-DX in drinking water for 4-weeks (Table 2). This was based on the enrichment of Nrf2-mediated oxidative stress response pathway and GSH-related pathways in their transcriptomic analysis. A follow-up investigation

with the same exposure protocol using GSH-deficient glutamate-cysteine ligase modifier subunit (*Gclm*)-null mice confirmed the occurrence of oxidative stress (Chen et al., 2022). Limited dose-response information is available from these studies. For example, Charkoftaki et al. (2021) administered three concentrations of 1,4-DX via drinking water (0, 50, 500 and 5000 mg/L) but did not report results for the 50 mg/L and the 500 mg/L treatment group that showed no difference from controls. The oxidative stress endpoints were increased in this study only in the highest exposure group (5000 mg/L) which corresponds to an estimated dose of 1000 mg/kg/d.

In a follow-up to the study by Gi et al. (2018), the same investigators concluded that oxidative stress may explain the increased frequency of A:T mutations in the liver of *gpt* delta rats treated with ≥200 ppm drinking water exposure (19 mg/kg/d) of 1,4-DX (Totsuka et al., 2020, 2021; Gi et al., 2018). Moreover, while 8-oxo-dG lesion is the most abundant and best recognized biomarker of oxidative DNA damage, the full spectrum of oxidative lesions in mammalian DNA exceeds more than 100 different types (Croteau and Bohr, 1997). In addition, the adductome analysis conducted by this group shows a non-linear dose response in the formation of adducts and a proportional dose-response in oxidative DNA adducts.

These findings are consistent with the formation of breaks that most likely result from increased oxidative DNA damage and ROS production observed at the higher dosages of 1,4-DX that overwhelm the adaptive response capabilities of the exposed rodents. In combination with increased cellular proliferation, such unrepaired DNA breaks would become DSBs during DNA replication at stalled replication forks; these forks and DSBs would then need to be resolved with help of homologous recombination repair (involving previously mentioned Rad51) and cell cycle signaling pathways as well as DSB DNA repair visualized by the γ-H2AX foci staining. Together, 1,4-DX-induced oxidative DNA damage would lead to 1,4-DX indirectly increasing mutations and chromosomal aberrations, consistent with the entire genotoxicity dataset for this substance (Cho et al., 2022).

Collectively, these findings present a compelling case for oxidative stress, resulting from high exposures to 1,4-DX, leading to dysregulation of the ROS system and causing genotoxic events such as DNA strand breaks and DNA adducts.

7. Metabolism and toxicokinetics

The biological fate of 1,4-DX has been studied in rodents and humans and reviewed numerous times including most recently by the US EPA (US EPA, 2020), European Chemicals Agency (ECHA, 2021) and Health Canada (2021). In summary, studies conducted in rodents and humans demonstrated that 1,4-DX is readily absorbed, distributed and metabolized after ingestion or inhalation.

7.1. Metabolism

When 1,4-DX is administered by inhalation, oral administration, or directly into systemic circulation by intravenous injection, the majority of the absorbed 1,4DX is biotransformed to β-hydroxyethoxyacetic acid (HEAA) and excreted in the urine (Braun and Young, 1977; Göen et al., 2016; Take et al., 2012; Young et al., 1978; Woo et al., 1977c). See Fig. 3.

The cytochrome P450 enzyme system, particularly Cyp2B1/2 and Cyp2E1, is associated with the biotransformation of absorbed 1,4-DX to HEAA (Braun and Young, 1977; Nannelli et al., 2005). Pretreatment of rats with known inducers of cytochrome P450 enzymes (phenobarbital and polychlorinated biphenyls) significantly increased the total amount of HEAA excretion while P450 inhibitors reduced urinary excretion of HEAA (Woo et al., 1977c).

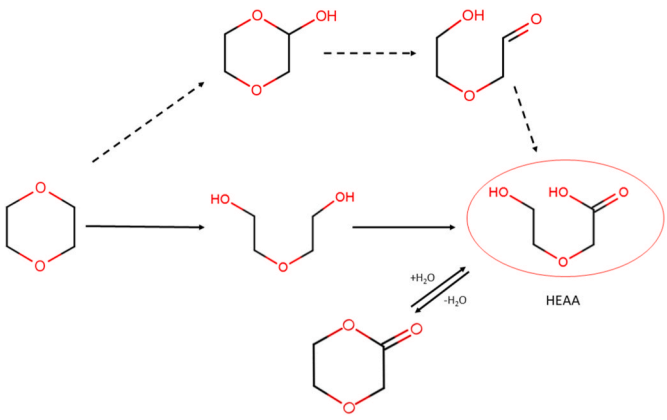

Fig. 3. Biotransformation of 1,4-dioxane to β-hydroxyethoxyacetic acid (HEAA)
Adapted from Woo et al. (1977c). Solid arrows represent primary metabolic pathways while dashed arrows are potential alternative pathways.

7.2. Toxicokinetics

The toxicokinetics of 1,4-DX metabolism and elimination as HEAA following oral and inhalation exposure is dose-dependent and biphasic. At low levels of exposure, metabolism is a linear first-order process in which the rate of metabolism and elimination is proportional to dose. However, at higher exposures the kinetics shift and show characteristics of saturation.

Elimination of 1,4-DX from plasma in male Sprague Dawley rats administered 3 and 10 mg/kg 1,4-DX by intravenous injection followed first-order linear Michaelis-Menten kinetics (Young et al., 1978). However, with intravenous doses at or above 30 mg/kg, the rate of elimination plateaus with a maximum rate (V_{max}) of about 10 mg/h kg[4] regardless of the administered dose.

Mice show a similar biphasic toxicokinetic profile. Male B6C3F1 mice treated with a single dose of 20 mg/kg by oral gavage had no measurable plasma concentrations of 1,4-DX 1 h after dosing (Sweeney et al., 2008). In contrast, doses of 200 and 2000 mg/kg resulted in the appearance of 1,4-DX in plasma with area under the time-concentration curves proportional to doses administered (Sweeney et al., 2008). See Table 4.

The route of exposure also can have an effect on metabolic saturation. The primary studies demonstrating metabolic thresholds, administered 1,4-DX by intravenous or oral routes. We were able to find only two studies that investigated toxicokinetics by inhalation. Exposure of male rats to 50 ppm (approximately 28 mg/kg) 1,4-DX by inhalation for 6 h resulted in a linear reduction of plasma levels of 1,4-DX and appearance of HEAA in urine (Young et al., 1978; data not shown). Since this study included only one exposure level, no exposure threshold was identified. In a 13 week inhalation study with male and female rats exposed to 400–3200 ppm 1,4-DX (approximate dose range 109–1663 mg/kg/d), the levels of 1,4-DX in plasma increased linearly in samples collected approximately 1 h after the cessation of exposure in week 12 of the 13 week study (Kasai et al., 2008; data not shown). The authors commented that induction may account for the apparent absence of a metabolic threshold with the exposures used in this study. However, there was no time-course sampling to enable detection of a possible saturation threshold response. In addition, substances absorbed by inhalation enter directly into systemic circulation and are not subject to first pass metabolism by the liver. Inhalation exposure also continuously contributes to systemic 1,4-DX concentrations with every breath.

[4] Calculated by normalizing the estimated Vmax of 4 mg/h by the body weight (0.25 kg) of rats from the study by Young et al. (1978). $VmaxC = \frac{Vmax}{BW^{0.7}}$

Table 4
Clearance Thresholds identified in single dose and repeat dose studies in rodents.

Species	Sex	Route	Dose Frequency	1,4-DX Compartment	Threshold (mg/kg)	Reference
Rat	Male	Intravenous	Single	Blood	30–100	Young et al. (1978)
Rat	Male	Gavage	Single	Blood	100	(Kociba et al., 1974)
Rat	Male	Gavage	Single	Urine	100	(Kociba et al., 1974)
Rat	Male	Inhalation	2 year	Blood	None identified	Kasai et al. (2009)
Mouse	Male	Gavage	Single	Blood	200	Sweeney et al. (2008)
Mouse	Female	Drinking Water	90-day	Blood	364<>979	Lafranconi et al. (2021)

Together, these factors can contribute to potentially masking detection of the saturation threshold from exposure by inhalation.

We also note that Young et al. (1978) estimated that blood levels between 30 and 100 μg/ml in male rats represented the metabolic saturation threshold. The plasma levels reported in the 13-week inhalation study by Kasai et al. (2008) achieved average values of 48 and 80 μg/ml at the lowest exposure level of 400 ppm for males (approximately 158 mg/kg) and females (approximately 167 mg/kg), respectively. The other exposures used in this study, 800, 1,600, and 3200 ppm, all generated blood levels exceeding the estimated metabolic threshold and are in the range of exposures that caused tumors in the two year inhalation study by the same authors (Kasai et al., 2009). Inhalation exposures of 50, 250 and 1250 ppm (approximately 19, 97, 488 mg/kg/d) were used in a two-year follow-up study by the same investigators (Kasai et al., 2009). Based on the plasma levels generated in the 13-week study, it is reasonable to expect the highest exposure, 1,250 ppm, would generate blood levels above the metabolic saturation threshold. Unfortunately, blood or plasma levels of 1,4-DX were not reported to confirm this.

7.3. Metabolic induction

1,4-DX appears to induce its own metabolism. Treatment of male rats with 1,000 mg/kg for 17 days resulted in an enhanced rate of elimination of plasma 1,4-DX compared to rats treated with a single dose (Young et al., 1978). In addition, treatment of male rats at up to 2000 mg/kg by gavage or intraperitoneal injection or with 1.5% (15000 ppm) 1,4-DX in drinking water for 10 days (approximate dose 400 mg/kg/d)[5] resulted in increased cytochrome P450 dependent activity associated with isozymes Cyp2B1/2, and Cyp2E1 (Nannelli et al., 2005). In male mice, treatment with 100000 ppm in drinking water (approximately 1000 mg/kg/d) for one week or 5000 ppm in drinking water (approximately 651 mg/kg/d) for three months increased liver Cyp2E1 protein and activity compared to controls (Chen et al., 2022).

As shown in Table 4, the metabolic or clearance saturation threshold for 1,4-DX covers a range of exposures depending on duration of exposure. The energetics involved in the biotransformation of 1,4-DX places a high demand on intermediary metabolism to provide the necessary cofactors to support the metabolism of 1,4-DX. It is likely that this additional metabolic stress can, in time, degrade the capability of the cells to thrive, resulting in decreased capacity to metabolize and clear absorbed 1,4-DX. The appearance of hepatic enzymes in the serum of male rats treated for two years by inhalation (Kasai et al., 2009) at 1250 ppm (approximate dose 488 mg/kg/d) or through ingestion of drinking water (NCI, 1978) at 5000 ppm (approximate dose of 274 mg/kg/d) is an indication of hepatic damage and, presumably, a reduction in the vital capacity of the liver to support metabolism of 1,4-DX. Similar effects on hepatic enzymes were observed in both male and female mice (Kano et al., 2009) treated for 2 years with 2000 ppm 1,4-DX in drinking water (approximate dose of 191 mg/kg/d males and 278 mg/kg/d females). Unfortunately, there are no data available on blood levels of 1,4-DX or HEAA from animals exposed beyond 90-days that can be used to test this hypothesis.

7.4. 1,4-Dioxane reactivity

From the available evidence, it is reasonable to conclude that metabolism of 1,4-DX does not generate reactive intermediates. While cytochrome P450 systems are capable of generating reactive electrophiles from certain substrates, investigations into reactive intermediates of 1,4-DX have failed to generate evidence of DNA reactivity and repair, protein binding, or enhancement of cytotoxicity after induction of xenobiotic biotransformation (Goldsworthy et al., 1991; Stott et al., 1981; Y. Woo et al., 1977b). Pretreatment with inducers of mixed-function oxidases also did not significantly change the extent of covalent binding in subcellular fractions (Woo et al., 1977b); again indicating that metabolites were not toxicologically active.

1,4-Dioxane-2-one, a potentially reactive metabolite of 1,4-DX, has also been reported as a possible metabolite (Woo et al., 1977c). However, as has been pointed out by various investigators, 1,4-dioxane-2-one is an analytical artifact resulting from tautomerization of HEAA under acid sample preparation and analysis conditions (Braun and Young, 1977; Health Canada, 2021; US EPA, 2020; Woo et al., 1977a, 1977b, 1977c).

8. Dose-response analyses of effects

Analysis of the dose-response for 1,4-DX is challenging due to study-specific design limitations. Some of the cancer bioassays and many of the sub-chronic studies included a single dose-group for comparison to controls. However, when viewed collectively with multi-exposure studies, the available responses, and their corresponding doses, do provide a certain consistency that helps refine our understanding of the cancer MOA of 1,4-DX.

Across the rat bioassays (Torkelson et al., 1974; Kasai et al., 2009; Kociba et al., 1974; NCI, 1978; Kano et al., 2009), the results show that the incidence of all treatment-related tumors (i.e., nasal cavity squamous cell carcinoma, hepatocellular adenoma and carcinoma, differed significantly from controls starting at lifetime average daily doses in excess of 30 mg/kg. This point of departure aligns with current understanding that 1,4-DX metabolism/clearance saturation occurs somewhere between 30 and 100 mg/kg/d (Young et al., 1978).

This is also true for the dose-response analysis of tumor formation for mice in which the point of departure, determined by the LOEL, exceeds the metabolic/clearance threshold of 200 to >400 mg/kg/d projected for mice (Lafranconi et al., 2021; Sweeney et al., 2008). In mice, doses resulting in tumors after chronic exposure to 1,4-DX typically are in excess of 200–400 mg/kg/d.

We recognize that in one study (Kano et al., 2009), hepatic tumors in female mice have been reported at exposures predicted to be below the estimated metabolic saturation. In the Kano bioassay study, there was a significant increase in combined hepatocellular adenomas and carcinomas in female mice exposed to 500 ppm (approximately 66 mg/kg/d) 1,4-DX in drinking water for two years. This observation has not been reconciled with results from other rodent cancer bioassays and remains an outlier as we note in the Tumor Findings section. Health Canada did not use the results from the Kano study as the basis of its cancer risk

[5] As reported in the publication.

assessment due to the numerous limitation and uncertainties in the data generated by this study (Health Canada, 2021).

9. Mode of action model

The information collected and analyzed in this analysis reinforces the conclusions made in previous reviews that the metabolism of 1,4-DX is biphasic and saturable. This review further demonstrates that the apical, biochemical, and genomic responses to 1,4-DX exposure align with its toxicokinetic profile in that effects develop when exposures are above the metabolic or clearance thresholds. Previous reviews proposed regenerative hyperplasia dependent upon exposures exceeding the metabolic or clearance threshold and accumulation of 1,4-DX leading to cytotoxicity and regenerative hyperplasia (Dourson et al., 2014, 2017). Evidence supporting this model was largely derived from chronic rodent bioassays, sub-chronic, toxicokinetic studies and the recognition that 1,4-DX is not likely mutagenic and only weakly genotoxic.

Recent studies have largely reinforced the MOA proposed earlier and expanded recognition of additional early events relevant to the rodent cancer MOA resulting from chronic, high exposures to 1,4-DX. Evidence uncovered by these more recent investigations include 1,4-DX-induced mitosis identified through histological examination of female mouse livers and supported by genomic analyses (Chappell et al., 2021; Lafranconi et al., 2021). Another early event is induction of hepatic cytochrome P450, particularly Cyp2E1, in rats and mice (Nannelli et al., 2005; Wang et al., 2022). Evidence is also accumulating that biochemical stress, particularly oxidative stress, is also an early event in the cancer MOA of 1,4-DX (Charkoftaki et al., 2021; Chen et al., 2022). Importantly, these newly recognized events occur prior to the development of cytotoxicity and the regenerative repair that is a cornerstone of the regenerative hyperplasia MOA.

Based on the collected information, a better picture is emerging about the cancer MOA resulting from exposures to 1,4-DX. We find that the weight of evidence shows that the key initiating event continues to be exposure to high doses of 1,4-DX that overwhelm clearance adaptive response capabilities. This corresponds with increased circulating and tissue levels of parent 1,4-DX, mitosis, induction of Cyp2E1 activity, increased biochemical stressors resulting in oxidative stress, genotoxicity and cytotoxicity followed by sustained proliferation driven by regenerative repair and progression of heritable lesions to tumor development. This progression is graphically outlined in Fig. 4.

10. Analysis of other potential mode of action models

We also analyzed the information relevant to other MOA models and concluded none were supported by the available information. A variety of other MOAs for rodent liver carcinogens have been identified and can be organized into genotoxic or cell proliferation MOAs (Cohen, 2010; Wolf et al., 2019). Both types of MOA first require exceedance of the metabolic threshold such that parent 1,4-DX accumulates in the key target organs (e.g., liver), which then enables subsequent events.

10.1. Genotoxicity

The genotoxic MOA can be separated into a mutagenic and/or clastogenic events. The mutagenic MOA is initiated by the formation of pro-mutagenic lesions, e.g., alkylation of specific nucleophilic nucleotide locations by electrophilic parent compounds or metabolites, which are subsequently fixed into mutation upon DNA replication of the unrepaired or mis-repaired lesion or these pro-mutagenic lesions have insufficient repair times in response to direct mitogenic or regenerative stimuli which could be more relevant for 1,4-DX. This is followed by clonal expansion of liver foci which lead to the formation of liver tumors (Becker et al., 2017; Moore et al., 2018; US EPA, 2005). Importantly, there have been no alkylating 1,4-DX DNA lesions identified following treatment (Stott et al., 1981). Moreover, it is becoming recognized that for certain substances capable of reacting with DNA, non-linear mutagenic responses are observed (Hartwig et al., 2020). As pointed out above in our review of the genotoxic potential of 1,4-DX, and consistent with the conclusions of other reviews, (ATSDR, 2012; ECHA, 2002; US EPA, 2013; Health Canada, 2021; NICNAS, 1998), 1,4-DX is not directly mutagenic and mutagenicity is not an early KE for 1,4-DX-mediated carcinogenesis, specifically at low doses that are not associated with increased oxidative stress or cytotoxicity. Mutations following ≥1000 ppm 1,4-DX sub-chronic exposures (Gi et al., 2018) are likely related to accumulation of oxidative damage and repair intermediates such as DNA breaks, and mitotic regeneration (discussed more below).

10.2. Proliferation

Increased mitogenesis that is directly stimulated or secondary to regeneration following chemically induced cytotoxicity, combined with clonal expansion altered cells, e.g., hepatic foci, are hallmarks of carcinogenesis and are important in both mutagenic and non-mutagenic

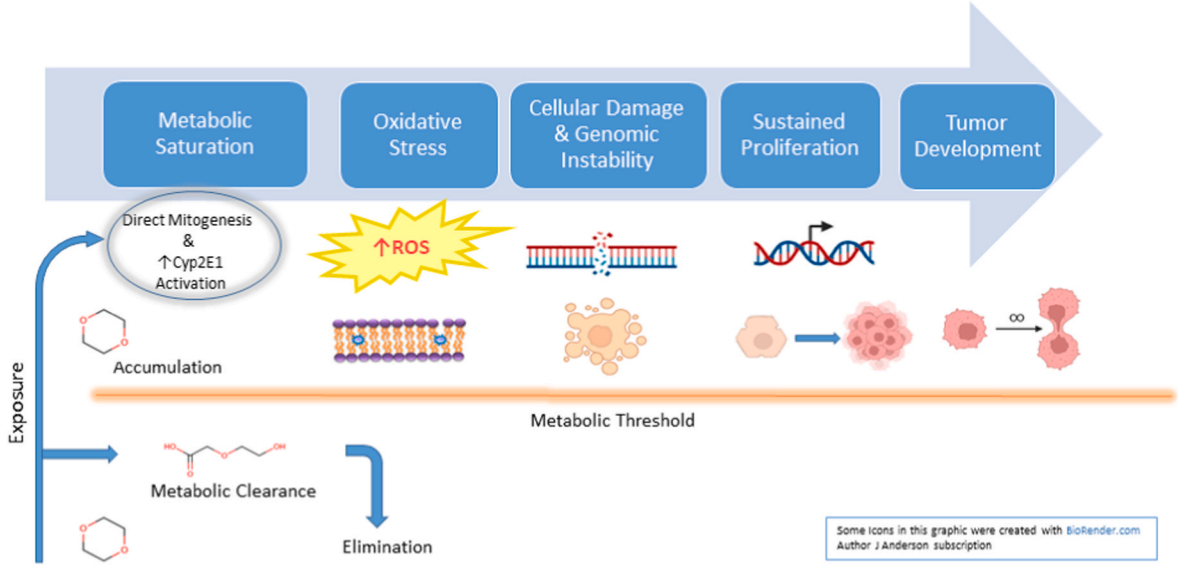

Fig. 4. Updated cancer mode of action for 1,4-dioxane.

carcinogenesis (Hanahan and Weinberg, 2011). Increased cellular DNA replication has been consistently observed within the 1,4-DX dataset and is relevant to its cancer MOA.

Lafranconi et al. (2021) evaluated both the dose-response and time course of hepatic events of female B6D2F$_1$ mice treated with 20, 40, 200, 600, 2000 or 6000 ppm 1,4-DX in drinking water for 7, 28 or 90 days. Liver weight increases after 90 days of exposure were accompanied by evidence of increased pan-lobular hepatocellular proliferation as determined by increased BrdU incorporation. Other than limited evidence of single-cell necrosis typical of apoptosis, there was no histological or biochemical evidence of cytotoxicity at any of the exposures used in this study. There was evidence of changes in genomic signaling only at 2000 ppm (337–391 mg/kg/d) and 6000 ppm (895–1063 mg/kg/d) from whole transcriptome analyses consistent with mitotic events (Chappell et al., 2021).

10.3. Regenerative repair

Previously, Dourson et al. (2014, 2017) summarized and analyzed the evidence supporting regenerative repair MOA for liver tumors in both mice and rat studies requiring cytotoxic events leading to regeneration of damaged cells. However, the current compilation of data sets from mice and rats demonstrate that 1,4-DX causes an early and direct mitogenic response absent cytotoxicity; this reduces the need for cytotoxicity-driven regenerative repair in the MOA sequence.

A number of studies report an increase in DNA synthesis and hepatic cell proliferation in mice and rats -see Table 1. One of the more recent mouse studies reported a 4.3% background proliferative rate that increased approximately 5-fold–20.8% - this occurred somewhere between 28 and 90-days of drinking water consumption at the highest dosage (6000 ppm) where 1,4-DX metabolic saturation to HEAA occurred (Lafranconi et al., 2021). Typically in young adult mice, between 1 and 3% of hepatocytes, or less, are proliferating so this 5-fold increase in replicating hepatocytes is important (Chang et al., 2008). 1,4-DX's induced proliferative response is within the range reported for other mouse liver mitogens including phenobarbital (Geter et al., 2014), nitrapyrin (LaRocca et al., 2017) dieldrin (Wang et al., 2020) and pronamide (LeBaron et al., 2014) among others. Rats also show an early stage increase in DNA synthesis at high dosages of 1,4-DX. Stott et al. (1981) observed a 1.5-fold increase in ^{3}H-methyl thymidine DNA labeling in male Sprague Dawley rats after 11 weeks of drinking water consumption delivering 100 mg/kg/d. The increased DNA-labeling was accompanied by minimal centrilobular hepatocyte swelling. Rat hepatocytes harvested 24, 39, and 48 h following a single 200 mg/kg 1,4-DX gavage dose produced an approximate 3-fold increase in "replicative DNA synthesis (RDS)" at 24 h as measured by ^{3}H-methyl thymidine while being accompanied by a slight but statistically significant reduction in cell viability (Uno et al., 1994). This RDS response was replicated in a follow-up dose-response study with 1,4-DX showing a 24 h post-dosing increase in DNA labeling (Miyagawa et al., 1999). Curiously, Roy et al. (2005) reported a decrease in hepatocyte proliferation index in male mice receiving a single oral dose 1,500, 2500 and 3500 mg/kg. At these high doses, cell proliferation may have been hindered by DNA DSBs, acute cytotoxicity and increases in apoptosis or other events caused by the high exposures.

Since the mitogenic response from 1,4-DX occurs early after exposure and prior to cytotoxicity/necrosis and regeneration activities, it appears that 1,4-DX directly stimulates hepatocyte entry and cycling through the cell cycle. We note this is not an isolated case. Phenobarbital (Elcombe et al., 2014) and piperonyl butoxide (Lake et al., 2020) are two examples in which a substance causes cell proliferation leading to liver tumors without causing hepatic necrosis. Based in part on these examples, direct mitogenic response is recognized as a cancer MOA (US EPA, 2005).

Evidence from rat studies consistently supports a regenerative-repair MOA involving cytotoxicity. In male F344 rats centrilobular necrosis, spongiosis hepatis, centrilobular nuclear enlargement, and a variety of foci were significantly increased following inhalation exposures of 1250 ppm but not 250 ppm (Kasai et al., 2009). A similar increase in hepatic foci was reported in male and female F344/DuCrj rats after two years of ingesting 5000 ppm 1,4-DX but not at the next lower water concentration of 1000 ppm (Kano et al., 2009). Cytotoxicity, as a driver of regenerative proliferation, has also been supported by the re-read of the 1978 NTP 1,4-DX cancer bioassay (Dourson et al., 2014). The reassessment of control and high dose liver samples found evidence of necrosis accompanied by inflammation and centrilobular hypertrophy that was not originally reported (reviewed in Dourson et al., 2014; Dourson et al., 2017).

It should be noted that the analyses in the Dourson papers included cytotoxicity as a key driver of regenerative repair from chronic exposure in rats and mice. The evidence of cytotoxicity from shorter-term studies is less compelling and suggest cytotoxicity is a late developing KE in the cancer MOA of 1,4-DX. Combining the old and newer cell proliferation data (Chappell et al., 2021; Charkoftaki et al., 2021; Gi et al., 2018; Lafranconi et al., 2021) strengthens the MOA by adding a direct mitogenic KE and reduces the reliance on cytotoxicity as a driver for proliferation.

10.4. Activation of nuclear receptors and transcription factors

Among the potential MOAs, the category of non-genotoxic rodent carcinogens includes activators of the nuclear receptor (NR) family, Constitutive Androstane Receptor (CAR also known as NR113), Pregnane X Receptor (PXR, also known as NR112), Peroxisome proliferator-Activated Receptor alpha (PPARα, also known as NR1C1) and Aryl Hydrocarbon Receptor (AhR). All of these potential MOA schemes involve biological processes resulting in increased tissue proliferation, discussed above, which magnifies inherent defects in DNA fidelity eventually leading to clonal expansion of tumors (Doe et al., 2019; LeBaron et al., 2013; US EPA, 2005). Activation of transcription factors have been used to characterize the MOA for some carcinogens (US EPA, 2005; Wolf et al., 2019). Linking a transcription factor to a MOA usually involves a weight of evidence approach relying on histology, biomarkers, and most recently genomics.

The involvement of NR activation in the MOA of 1,4-DX has been evaluated in studies utilizing genomics and various biomarkers. A common feature of activation of transcription factors is the induction of cytochrome P450s (LeBaron et al., 2013; Peffer et al., 2018). While 1,4-DX induces its own metabolism through induction of cytochrome P450s, the level of induction is not consistent with patterns generated through activation of nuclear receptors. When evaluating gene expression as a biomarker of NR activation (i.e., specific isoforms of cytochrome P450s), a prototypical carcinogenic NR activator generally induces its respective cytochrome P450 by hundreds of fold (LeBaron et al., 2013). 1,4-DX does not increase signals of gene expression for cytochrome P450 isozymes to levels typical of NR activation (Chappell et al., 2021; Chen et al., 2022; Nannelli et al., 2005). Additionally, liver tissue from Lafranconi et al. (2021) was interrogated in a whole transcriptome analysis (Chappell et al., 2021). That analysis did not identify changes in signals prototypical for NR activation factors. Nonetheless, evidence specific to 1,4-DX activation of several of the canonical NRs is reviewed in more detail below.

10.5. CAR/PXR

Ligands that activate (either directly or indirectly) the CAR and/or PXR nuclear receptors have been shown to have a significant amount of crosstalk, hence are often investigated together. The MOA and KE for CAR activation is well-established (Elcombe et al., 2014) and has been applied to a number of chemicals (Yamada et al., 2021). The KEs include CAR activation, altered gene expression specific to CAR, increased cell proliferation, clonal expansion leading to altered foci, and eventually

liver tumor formation. Importantly, robust induction of Cyp2b enzymes and hepatocellular hypertrophy, histologically centrilobular or pan lobular or increased liver weight, are considered associated events and are considered characteristic of the CAR MOA. Recent publications have established a best-practice, minimum dataset required to establish a CAR-mediated MOA in rodent hepatocarcinogenesis (Peffer et al., 2018). Other publications have identified a panel of gene transcripts specific to CAR/PXR activation, which includes Cyp2b/Cyp3a, as well as other CAR/PXR-related genes (Corton et al., 2020).

The available information does not indicate 1,4-DX as a CAR or PXR activator. In fact, although Cyp3a (PXR activation) was the most highly induced of the Cyps correlated to NR activation and rodent hepatocarcinogenesis, Cyp3a4 was induced less than 2-fold at the 90-day time point at the highest administered concentration and not appreciably induced at any other time point or concentration (Chappell et al., 2021). In comparison, Cyp2b10 has been shown to be induced hundreds-of-fold for a carcinogenic dose of phenobarbital (Geter et al., 2014) and other CAR/PXR inducers (Elcombe et al., 2014; LeBaron et al., 2014; Peffer et al., 2007, 2018). In addition, the histological and gross hepatic phenotype after 1,4-DX administration was not as would be expected from a CAR/PXR activator due to a considerably lower magnitude of liver weight increase, hepatocyte hypertrophy, and cytoplasmic eosinophilia.

10.6. AhR

While the AhR is technically a ligand-activated transcription factor and not a prototypical nuclear receptor, AhR activation has been associated with rodent liver tumors (Budinsky et al., 2014; Cohen, 2010). The MOA for AhR-induced rodent liver tumors has a different KE sequence than that of CAR- and PPARα nuclear receptor-mediated induction (described below). The KEs for the AhR MOA are sustained Cyp activation, typically of Cyp1a1, altered focal cell growth/homeostasis, and pre-neoplastic focal tissue changes (Budinsky et al., 2014). It is well-established that Cyp1a induction is a robust biomarker of the activation of the AhR pathway and is considered an associative event (AE) to the first KE of sustained AhR activation. However, the data for 1,4-DX demonstrate that AhR is not appreciably activated, with inconsistent-to-nonexistent increases of Cyp1a1, compared to those generated by prototypical AhR activators (Nannelli et al., 2005).

10.7. PPARα

PPARα activation, which can lead to rodent hepatocarcinogenesis, has been studied for decades and activators include many human pharmaceuticals (e.g., fibrate class of lipid-lowering drugs) as well as other environmental and endogenous ligands (Corton et al., 2018; Klaunig et al., 2003). The KEs are similar to the CAR/PXR pathway and include PPARα activation, alteration in cell growth pathways/perturbation of growth and survival, and selective clonal expansion of pre-neoplastic foci leading to tumor formation (Corton et al., 2014). Transcriptional upregulation of PPARα-specific cytochromes (Cyp4a) is considered an associative event that is directly related (and measurable) to the first KE of PPARα activation, although other gene transcript biomarkers have been identified (Corton et al., 2020; LeBaron et al., 2014).

The gene expression data from the most recent MOA study for 1,4-DX did not indicate appreciably altered Cyp4a transcript at any of the time points or dose levels (Chappell et al., 2021). Furthermore, the histological alterations of the 1,4-DX-treated livers were not consistent with a PPARα activator, specifically the lack of histologically identified smooth endoplasmic reticulum (SER) proliferation or magnitude of hepatic hypertrophy, either histologically or by substantial liver weight increases (Lafranconi et al., 2021). Finally, rats pretreated with 1,4-DX by oral gavage (2000 mg/kg) or in drinking water for 10 days (400 mg/kg/d) failed to enhance palmitoyl CoA oxidase activity (Nannelli et al., 2005), an indicator of peroxisome proliferation of peroxisome proliferation (Hawkins et al., 1987; Roberts, 1999).

Taken together, the lack of NR-related histological, gross organ weight, and molecular changes (including gene expression) in the livers of 1,4-DX-treated mice indicate that 1,4-DX does not act as a robust NR activator. The aforementioned data are not indicative of a NR-mediated rodent hepatocarcinogen but clearly indicate the dose- and temporal-threshold nature of hepatocellular proliferation, along with shifts in metabolism.

10.8. Cyp2E1 induction

Recently, an AOP for liver carcinogenesis involving Cyp2E1 induction has been developed for the Organization for Economic Co-operation and Development's AOP program (Webster et al., 2021). This AOP consists of three KE. KE#1 consists of oxidative stress induced by the inefficiency and decoupling of Cyp2E1-mediated metabolism generating ROS capable of causing cellular damage. Sustained Cyp2E1 activity fosters an increased free radical environment in hepatocytes. KE#2 is hepatotoxicity arising from constant, elevated ROS and/or hepatotoxicity formed from potentially reactive, but as yet unidentified intermediates generated by Cyp2E1. KE#3 is the proliferative response stimulated by ROS and/or reactive intermediate-induced liver injury.

Up-regulation of Cyp2E1 activity has been reported following 1,4-DX treatment (Chen et al., 2022; Nannelli et al., 2005). This increase in Cyp2E1 activity is in part, the result of post-translational events since up-regulation of Cyp2E1 mRNA is not observed (Chappell et al., 2021; Wang et al., 2022). At this time, it is reasonable to include Cyp2E1 activity increases as a KE in the MOA model for 1,4-DX.

11. Uncertainty

While our understanding of the cancer MOA of 1,4-DX has benefitted from recent investigations, there are still areas where additional research would be beneficial to fill the more detailed mechanistic information and dose-response gaps. First amongst these are refinements in the dose-response of early events. Many of the investigations of early events cited in this review are based on *in vivo* exposures to a single high dose or short-term repeated exposures without the additional information necessary for characterizing dose-response. Investigations using lower doses that frame the range of exposures around the metabolic threshold appropriate for the model used would enable better definition of many of the KEs and aid in the risk assessment of 1,4-DX.

There is also uncertainty in the role metabolic induction plays in defining the clearance threshold for 1,4-DX. The key studies establishing the toxicokinetics of 1,4-DX metabolism used acute, or short-term repeated dosing and in a single sex. Toxicokinetic studies after repeated exposures to 1,4-DX and investigations into sex differences in the toxicokinetics of 1,4-DX have not been conducted.

An additional area of mechanistic uncertainty is the source of the stressors induced by 1,4-DX. Is the elevation of Cyp2E1 activity the only event to elevate ROS above constituent levels? Is the effect of 1,4-DX on intermediary metabolism a significant contributor to the development of oxidative stress or is it having some other effect that leads to an imbalance of ROS? Generation of ROS is a common end stage outcome from various types of cellular stressors (Azad and Iyer, 2014; Circu and Aw, 2010; Rossman, 2009). In addition, there is little known with respect to dose-response for the generation of ROS in response to 1,4-DX exposure.

12. Conclusion

There has been significant progress in developing an understanding of the cancer threshold MOA for 1,4-DX. The findings from this integrated assessment reinforces the threshold nature of responses to 1,4-DX exposure and further shows that 1,4-DX does not cause cancer via

mutagenic or direct genotoxic mechanism or activation of nuclear transcription factors. Proliferative responses, either from direct mitogenic stimulus and/or in response to biochemical stressors such as ROS, play a significant role in the cancer MOA of 1,4-DX. Our current understanding includes these successive events;

1. Metabolic or clearance saturation and accumulation of 1,4-DX in blood and tissues – all subsequent events are dependent on this as the KE which is, by default, the threshold event for tumor development.
2. Mitotic responses and induction of Cyp2E1 activity.
3. Development of biochemical stress including oxidative stress and DNA strand breaks.
4. Genotoxicity as a result of the oxidative stress.
5. Subsequent events including cytotoxicity and genomic instability, sustained proliferation driven by regenerative repair and progression of heritable lesions to tumor development.

Together, these data support the use of a threshold dose-response model for development of a regulatory value protective of human health.

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CRediT authorship contribution statement

Mark Lafranconi: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Janet Anderson:** Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Robert Budinsky:** Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Lisa Corey:** Investigation, Data curation, Writing – original draft, Writing – review & editing. **Norman Forsberg:** Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Joanna Klapacz:** Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Matthew J. LeBaron:** Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization.

Declaration of competing interest

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Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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