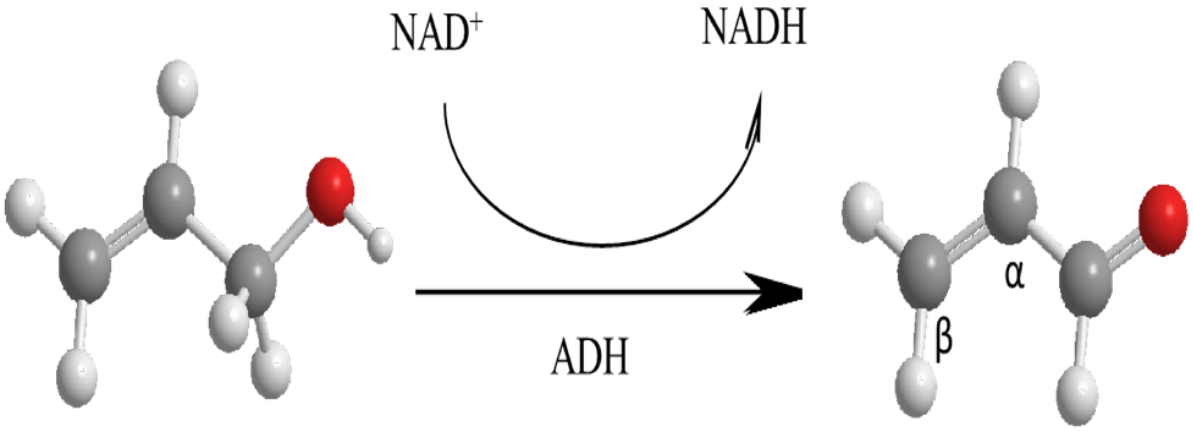
Graphical Abstract



Read-Across of 90-Day Rat Oral Repeated-Dose Toxicity: A Case Study for Selected β-olefinic Alcohols

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Abstract: There are no *in vivo* repeated-dose data for the vast majority of β-olefinic alcohols. However, there are robust and consistent *ex vivo* data suggesting many of these chemicals are metabolically transformed, especially in the liver, to reactive electrophilic toxicants which react in a mechanistically similar manner to acrolein, the reactive metabolite of 2-propen-1-ol. Hence, an evaluation was conducted to determine suitability of 2-propen-1-ol as a read-across analogue for other β-olefinic alcohols. The pivotal issue to applying read-across to the proposed category is the confirmation of the biotransformation to metabolites having the same mechanism of electrophilic reactivity, via the same metabolic pathway, with a rate of transformation sufficient to induce the same *in vivo* outcome. The applicability domain for this case study was limited to small (C3 to C6) primary and secondary β-olefinic alcohols. Mechanistically, these β-

unsaturated alcohols are considered to be readily metabolised by alcohol dehydrogenase to polarised α, β-unsaturated aldehydes and ketones. These metabolites are able to react via the Michael addition reaction mechanism with thiol groups in proteins resulting in cellular apoptosis and/or necrosis. The addition of the non-animal *in chemico* reactivity data (50% depletion of free glutathione) reduced the uncertainty so the read-across prediction for the straight-chain olefinic β-unsaturated alcohols is deemed equivalent to a standard test. Specifically, the rat oral 90-day repeated-dose No Observed Adverse Effect Level (NOAEL) for 2-propen-1-ol of 6 mg/kg body weight bw/d in males based on increase in relative weight of liver and 25 mg/kg bw/d in females based on bile duct hyperplasia and periportal hepatocyte hypertrophy in the liver, is read across to fill data gaps for the straight-chained analogues.

Keywords: read across, No Observed Adverse Effect Level (NOAEL), β-olefinic alcohols, weight-of-evidence (WoE), uncertainty

1 Introduction

1.1 Read-across

The basis for a toxicological read-across are chemicals which are similar in molecular structure, display similar chemical properties and in so doing have similar toxicokinetic and toxicodynamic properties. As a consequence, experimentally-derived toxicological properties from one compound, the source chemical, can be read across to fill the data gap for a second compound, the target chemical, which has been shown to be similar. This type of data gap filling may find particular use, for instance, for cosmetics ingredients where *in vivo* testing is prohibited by legislation in some geographic regions [1].

Read-across as a predictive tool has been used by industry and regulators for decades [2]. However, with the advances in non-animal test methods over the past 15 years, read-across arguments today are being held to a different standard than a decade ago, with greater expectation in terms of the certainties required from, and justification of, the read-across argument [3]. This is especially true for sub-chronic and chronic health effects.

In order to facilitate the development of better guidance on how to formulate a high quality read-across, a series of case studies have been conducted by the authors. This investigation describes a case study that has been designed to illustrate specific issues associated with an analogue approach [4] of the scenario where metabolism is the primary consideration in determining molecular similarity. The case study is intended to illustrate how non-animal data may be used to reduce uncertainties, as well as add to mechanistic plausibility and weight-of-evidence (WoE) to a read-across argument.

One of the crucial issues in toxicological read-across is addressing substances that are altered metabolically to more toxic species [5]. The toxic metabolites of these indirect acting toxicants

may be identical or different in structure within a group. In the former case, after *in vivo* dosing the various organs and systems of the animal are exposed to the same metabolite, thus toxicodynamic similarity may be assumed. In the latter case, after dosing the various organs and systems are exposed to metabolites with minor differences in chemical structure which may, or may not, elicit the same toxicological effects. This second situation adds complexity to the read-across justification because of the burden of establishing both toxicokinetic and toxicodynamic similarity.

The purpose of this investigation was to demonstrate the utility of non-animal methods to provide data and information that reduce uncertainties and add to the WoE associated with read-across predictions of *in vivo* data. The proposed use of the estimations from this read-across is quantitative data gap filling with sufficiently low uncertainty that the predictions may be used in risk assessments. As such, the predicted NOAEL values are accompanied by sufficient non-animal test data so the uncertainties are equal to do a test using a protocol similar to OECD TG 408. In the present study, a previously reported 'strategy' [6] was employed to assess similarities and overall completeness of the read-across. The initial category included a wide variety of β-unsaturated alcohols. Based on consideration of a common metabolic pathway the tertiary alcohols and the β-acetylenic alcohols (β-alkynols) were eliminated from further consideration. Subsequently the olefinic β-unsaturated alcohols were evaluated using relevant *ex vivo*, *in chemico* and *in silico* information.

1.2 β-Olefinic alcohols considered in this study and toxicological evidence

Olefinic β-unsaturated alcohols vary in molecular structure. These structural variations impact both reactivity and toxicity. While all olefinic alcohols contain a C=C moiety, they can be sub-divided further according to whether the olefinic group is β-, or non-β-oriented to the hydroxyl group. Additionally, they can be subdivided based on whether the hydrocarbon moiety is straight-chain or branched and whether the alcohol is primary, secondary or tertiary. These subdivisions are important for the toxicity effect as the overall structure of the parent alcohol determines the metabolic pathway and the specific metabolite formed.

Only primary and secondary β-olefinic alcohols can undergo first step oxidation to α, β-unsaturated aldehydes or α, β-unsaturated ketones, respectively [7, 8]. While all of these oxidative metabolites have the capability to be reactive with biological macromolecules as Michael acceptors, they exhibit different levels of reactivity and toxicity [9-11].

Among the β-olefinic alcohols, 2-propen-1-ol (i.e., 1-propen-3-ol; allyl alcohol) is the most studied derivative with a wide variety of toxicological data and information being reported. There is strong evidence that the mode of toxic action for 2-propen-1-ol involves metabolism via cytosolic alcohol dehydrogenase (ADH) to acrolein, an electrophile which elicits covalent cellular effects [12]. Overall, currently available data suggest that the kidney, liver and lung are potential targets for 2-propen-1-ol, following repeated oral or inhalation exposure. In oral repeated-dose toxicity testing, exposure to 2-propen-1-ol may lead to liver fibrosis [13, 14]. Liver fibrosis is a reversible response to acute or chronic hepatocyte injury [15-17]. The mechanistic rational is that the parent alcohol is relatively non-toxic; however its metabolite acrolein, a unique α, β-unsaturated aldehyde, is a Michael-type soft electrophile. Such electrophiles preferentially interact covalently with thiol groups in proteins leading to necrotic or apoptotic cell death [18]. During the *in vivo* response to cell death, stellate cells in the liver are

activated, for example by transforming growth factor beta (TGF-β) and connective tissue is formed [19].

Historically, the hepatotoxic action of 2-propen-1-ol to rodent liver is related to oxidative metabolism to acrolein which, in turn, can bind covalently to proteins in periportal hepatocytes [20, 21]. The covalent binding of acrolein to hepatic proteins was also confirmed by a study on radiolabelled ^{14}C 2-propen-1-ol and its deuterated derivative [22]. Two-propen-1-ol exerts a dose-dependent toxicity on cells, which is inversely related to the concentration of cellular GSH [23]. After severe depletion of GSH, the reactive metabolite of 2-propen-1-ol – acrolein - can bind to essential sulfhydryl groups in the cellular macromolecules, leading to cellular damage [13]. The toxicity of 2-propen-1-ol can be prevented by inhibitors of ADH and augmented by the aldehyde dehydrogenase (ALDH) inhibitor disulfiram [23].

In vivo oral exposure to 2-propen-1-ol leads to periportal necrosis and subsequent connective tissue development [12, 14]. Histopathological studies of 2-propen-1-ol exposed to repeatedly dosed rat livers showed signs of necrosis around the portal triad, with relatively little damage around the central vein. In addition, ductular proliferation, connective tissue accumulation and cirrhosis were evident.

2 Method and Materials

This evaluation of selected β-olefinic alcohols followed a read-across workflow proposed by Schultz et al (2015) [6]. It is in accord with the guidance proposed by Organization for Economic Co-Operation and Development (OECD) (2015) [24]. *In vivo* data used in the assessment were taken from the literature, including ECHA REACH Registered Substances database [25].

Mechanistic relevance, as well as, toxicokinetic and toxicodynamic similarity of the category analogues was established using relevant non-animal data.

2.1 Target and Source Substances

The analogues that were evaluated are listed in Table 1 and include 15 potential target substances and one source chemical (noted in bold). This list is not meant to be all inclusive, rather it represents existing industrial organic materials that are likely to be found in a governmental or industrial inventory (e.g., OECD High Production Volume Chemicals). Additional substance identifier information, such as chemical structures and molecular formulas are available in the Table 1 of the supplemental information. Based on extended structural fragments, the β-olefinic alcohol category includes five sub-groups (Figure 1). These sub-groups can be clustered into two sub-categories – straight-chained and branched β-olefinic alcohols.

Table 1. Potential category analogues for β-olefinic alcohols.

ID	Name	CAS No	SMILES	Type of Alcohol	
			Straight-chained		
1	**2-Propen-1-ol (allyl alcohol)**	**107-18-6**	**OCC=C**	**prim. allylic**	**terminal OH & C=C**
2	2-Buten-1-ol	6117-91-5	OCC=CC	prim. allylic	terminal OH, internal C=C
3	2-Penten-1-ol	20273-24	OCC=CCC	prim. allylic	terminal OH, internal C=C
4	2-Hexen-1-ol	2305-21-7	OCC=CCCC	prim. allylic	terminal OH, internal C=C
5	1-Buten-3-ol	598-32-3	C=CC(O)C	sec. allylic	internal OH, terminal C=C
6	1-Penten-3-ol	616-25-1	C=CC(O)CC	sec. allylic	internal OH, terminal C=C
7	1-Hexen-3-ol	4798-44-1	C=CC(O)CCC	sec. allylic	internal OH, terminal C=C
8	3-Penten-2-ol	1569-50-2	CC(O)C=CC	sec. allylic	internal OH & C=C
9	3-Hexen-2-ol	42185-97-7	CC(O)C=CCC	sec. allylic	internal OH & C=C
10	4-Hexen-3-ol	4798-58-7	CCC(O)C=CC	sec. allylic	internal OH & C=C
			Branched-chained		
11	2-Methyl-2-propen-1-ol	513-42-8	OCC(C)=C	prim. allylic	terminal OH & C(C)=C

12	2-Methyl-2-buten-1-ol	4675-87-0	OCC(C)=CC	prim. allylic	terminal OH, internal C(C)=C
13	2-Methyl-2-penten-1-ol	1610-29-3	OCC(C)=CCC	prim. allylic	terminal OH, internal C(C)=C
14	3-Methyl-2-buten-1-ol	556-82-1	OCC=C(C)C	prim. allylic	terminal OH, internal C=C(C)
15	3-Methyl-3-penten-2-ol	2747-53-7	CC(O)C(C)=CC	sec. allylic	internal OH & C(C)=C
16	4-Methyl-3-penten-2-ol	4325-82-0	CC(O)C=C(C)C	sec. allylic	internal OH & C=C(C)

CAS No - chemical abstracts service number
SMILES - simplified molecular-input line-entry system

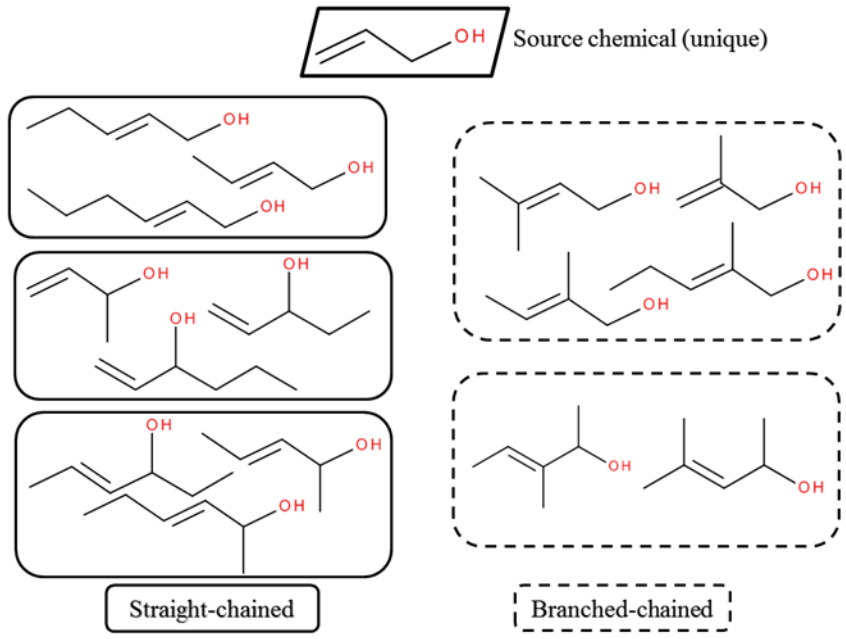

Figure 1. Five sub-categories of β-olefinic alcohols.

2.2 Endpoint

The NOAEL for the 90-day rat oral repeated-dose is the single endpoint for which this analogue approach to read-across is applied. The 90-day oral repeated-dose data for 2-propen-1-ol (allyl alcohol) are well suited for reading across; the multiple reported NOAELs are highly similar both qualitatively and quantitatively, based on experimental results from 4-or 5-dose exposure scenarios, and follow test guidelines similar to OECD TG 408.

2.3 Hypothesis of the category

The premise for this read-across case study is:

- Primary and secondary β-olefinic alcohols of short chain length (i.e., C3 to C6) are indirect-acting toxicants (i.e., metabolism is critical factor in toxicity) with the same covalent mechanism of action (i.e., Michael addition electrophilicity) and similar reactive potency.
- Within the C3 to C6 derivatives, C-atom chain length or branching does not significantly affect oral bioavailability.
- Whilst all short-chain β-olefinic alcohols are rapidly and nearly completely absorbed from the gut; only the primary and secondary alcohols are capable of being metabolised, primarily in the liver, via ADH.
- Oxidative metabolism of primary and secondary β-olefinic alcohols results in the corresponding α, β-unsaturated aldehyde or α, β-unsaturated ketone.
- These α, β-unsaturated aldehydes or α, β-unsaturated ketones are the definitive electrophilic toxicants and their *in vivo* potency is related to relative thiol reactivity; thus, only β-unsaturated alcohols with metabolism similar to 2-propen-1-ol and reactive potency similar to acrolein may be read across for 2-propen-1-ol with reasonable certainty.

3 Results

3.1 Read across justification

In order to conduct a read-across there is the requirement for high quality *in vivo* data for the endpoint under consideration [6, 24] which, in this case, is 90-day oral repeated dose-toxicity for rat in the form of a NOAEL value.

3.1.1 Rodent repeated-dose toxicity for 2-propen-1-ol

In general, toxicological data on 2-propen-1-ol demonstrate significant toxicity. The oral LD50 for rat is 37 mg/kg for 2-propen-1-ol [26], while the rat oral LD50 for the saturated isomer 1-propanol is 1870 mg/kg [27].

Several 90-day oral repeated-dose toxicity evaluations of 2-propen-1-ol have been conducted according to test guidelines similar to OECD TG 408. Male and female Long-Evans rats were exposed orally to 0, 0.17, 0.94, 7.3, 13.2, 34.0, 43.7, and 67.4 mg/kg bw/d (females) and 0, 0.13, 0.62, 5.9, 11.6, 25.5, 41.0, and 72.0 mg/kg bw/day (male) for 13 weeks [29]. The No Observed Adverse Effect Level (NOAEL) of 13.2 mg/kg bw/d (for females) and 11.6 mg/kg bw/d for male were reported. This observation was based on increases in relative kidney (both sexes) and liver weights (males) [28].

In another study, male and female Wistar rats were exposed orally to 0, 4.8, 8.3, 14.0 and 48.2 mg/kg bw/d (males) and 0, 6.2, 6.9, 17.1 and 58.4 mg/kg bw/d (females) for 15 weeks [29]. The reported NOAEL, based on increases in relative kidney weight and decrease in water intake and body weight, was 4.8 and 6.2, mg/kg bw/d for male and female respectively.

In a third study, mixed sexes of F344/N rats and B6C3F1 mice were exposed to 2-propen-1-ol by gavage to 0, 1.5, 3, 6, 12, or 25 and 0, 3, 6, 12, 25, or 50 mg/kg bw/d, respectively for 14 weeks and the clinical chemistries were examined in detail [30]. The major toxic response in both mice and rats occurred in the forestomach and the NOAEL values derived from this toxic effect were

3 and 6 mg/kg bw/d for mice and rats, respectively. However, the treatment with the highest evaluated dose, 25 mg/kg, significantly increased the incidences of bile duct hyperplasia and periportal hepatocellular hypertrophy in female rats but not in males. For male rats, the increase in relative weight of liver was observed at a dose of 6 mg/kg. The sex difference in 2-propen-1-ol hepatotoxicity in rats was also reported by Rikans and Moore [31] and was explained by the greater alcohol dehydrogenase activity in female rats than in male rats. Also in mice, females were more responsive than males, and increased incidences of portal cytoplasmic vacuolisation occurred in 12 mg/kg or greater in females; whereas in males, this lesion was first observed at 25 mg/kg [30]. However, these differences in hepatotoxic responses between male and female rats seem not to be relevant to this case study as they should be observed for other β-olefinic alcohols. Based on the effects in the liver, the NOAEL values of 6 and 25 mg/kg bw/day in male and female rats, respectively, have been established.

3.1.2 Rodent repeated-dose toxicity for other β-olefinic alcohols

The second β-olefinic alcohol tested in acute toxicity tests as well as the 90 days repeat dose assay is 3-methyl-2-buten-1-ol. The LD50 for the rat after oral administration of 3-methyl-2-buten-1-ol is 810 mg/kg with symptoms such as: apathy, dyspnoea, redness of eyes and ears [32].

To our knowledge, the findings of only one 90-day oral repeated-dose toxicity evaluation of 3-methyl-2-buten-1-ol are publicly available [33-35]. Following OECD Test Guideline 408, 3-methyl-2-buten-1-ol was administered to groups of 10 male and 10 female Wistar rats via drinking water at concentrations of 14.4, 65.4 and 243.8 mg/kg bw/day for male and 21.0, 82.1 and 307.2 mg/kg bw/day for female for three months [33]. Substance related effects were seen at the high and mid dose levels. In the mid dose groups, decreased food and water consumption in

male rats and reduced water consumption in female rats were noted. Body weight was significantly impaired at the high dose in male and female rats. In the mid and high dose, the mean absolute liver weights in male rats were significantly decreased, but not the relative liver weights. There were no other treatment related significant changes in clinical examinations. As reduction in food and water consumption resulted in significant decrease of body weight only at the high dose level, the NOAEL was assessed to be 65.4 mg/kg bw/day in male rats and 82.1 mg/kg bw/day in female rats.

It is noted that two more sub-acute oral studies in rats do not show any other effects of 3-methyl-2-buten-1-ol. Specifically, a 14-days drinking water study with rats (3/sex/dose) exposed to 250, 500, 750 and 1500 mg/kg bw/d reported acute toxic effects at 1500 mg/kg bw/d; reduced food and water intake was observed at 250 mg/kg bw/d [32]. So there is good concordance with 90-day test results. In a 14-day gavage test with rats exposed to 250, 500 and 750 mg/kg bw/d no treatment related effects were observed [36].

In summary, while protocols vary, three studies have experimentally evaluated 2-propen-1-ol and one study evaluated 3-methyl-2-buten-1-ol in 90-day, oral repeated-dose testing schemes. Repeated-dose toxicity data on 2-propen-1-ol indicate liver and kidney are the target organs. For the 3-methyl-2-buten-1-ol, only the reduction in food and water consumption was observed. The 90-day NOAEL values for oral administration are between 3 and 15 mg/kg bw/d for 2-propen-1-ol and 60 -85 mg/kg bw/d for 3-methyl-2-buten-1-ol (see Table 8 of the supplemental information). These ranges of NOAEL values are 10-100 times smaller than those reported for saturated derivatives (data not shown).

3.1.3 Applicability domain

After elimination of tertiary alcohols and β-alkynols, the applicability domain was limited to subclasses of β-olefinic aliphatic alcohols with carbon chain lengths from C3 to C6. Specifically, these included primary (external hydroxyl group) and secondary (internal hydroxyl group) with a β-positioned vinylic moiety (Table 1).

3.1.4 Purity/impurities

A purity/impurity profile for the analogue listed in Table 1 is not reported. No effort was made to take into account impurities based on production. However, since the category is structurally limited, the potential impact of any impurities on the endpoint being evaluated is considered very limited. The most likely impurities are saturated derivatives or isomers (e.g. *cis* vs. *trans* conformations or S/R stereoisomers).

3.2 Data matrices for assessing similarity

In order for a read-across prediction to be accepted there is the requirement to establish similarity between the source and target substance [6, 24]. While structural similarity is a minimum, toxicokinetic similarity, especially for metabolism, and toxicodynamic similarity, especially in regard to mechanistic plausibility is required for chronic endpoints such as 90-day oral repeated dose-toxicity [6, 24].

3.2.1 Structural similarity

As demonstrated in Table 1 and Table 3 of the supplemental information, all the β-olefinic alcohols included in the category are structurally similar (e.g., C3-C6). Specifically, they: 1) belong to a common chemical class, β-unsaturated alcohols, 2) the subclass β-olefinic alcohols,

and 3) possess one of two molecular scaffoldings, primary with an external hydroxyl or secondary with an internal hydroxyl configuration. Structural similarity is complicated by the presence or absence of alkyl substituents (i.e., methyl groups) on the allylic moiety. The potential source substance, 2-propen-1-ol, is a unique β-olefinic alcohol, one with both a terminal hydroxyl and terminal vinyl group. In contrast, two other potential category members, 3-methyl-2-buten1-ol and 4-methyl-3-penten-2-ol, are dissimilar as they have an alkyl substituent on the olefinic carbon that can inhibit the protein binding site of the vinyl group.

3.2.2 Chemical property similarity

As demonstrated in Table 2 of the supplemental information, all the β-olefinic alcohols included in the category have a very narrow value range for their physico-chemical properties. Specifically, all category members exhibit molecular weights from 58 to 100 g/mol. While hydrophobicity (log Kow) increases with number of C-atoms from 0.17 to 1.66, density is constant at 0.8 +/- 0.1 g/cm^3. Vapour pressure and water solubility decrease with molecular size and therefore vary only slightly within the category. All category members are liquids over the typical temperature range as melting points are all well below 0 °C and boiling points are all around or above 100 °C.

3.2.3 Chemical constituent similarity

As demonstrated in Table 3 of the supplemental information, all the β-olefinic alcohols include in the category have common constituents in the form of: 1) a single polar substituent, -OH, 2) a β-positioned olefin (C=C) moiety. Other structural fragments are limited to -H, -CH_3 and -CH_2- groups.

3.2.4 Toxicokinetic similarity

As shown in Table 4 of the supplemental information, the toxicokinetic understanding of primary and secondary β-olefinic alcohols is incomplete. The oxidation of primary alkanols and primary olefinic alcohols to the corresponding aldehydes is catalysed by NAD+/NADH-dependent ADH [37]. Olefinic alcohols were better substrates for human liver ADH than the corresponding saturated alcohols. A comparison of the alcohol structure with the enzyme binding affinity of alcohol dehydrogenase indicates that increased binding (lower Km) occurs with increasing chain length (C3-C6) of the alcohols and the presence of unsaturation. Specifically, binding affinities with human liver ADH were measured *in vitro* for 2-propen-1-ol, 2-buten-1-ol, 3-methyl-2-buten-1-ol and 2-hexen-1-ol and they are: 0.05, 0.01, 0.0045 and 0.003 mM, respectively [37]. With the exception of 2-propen-1-ol, the Km values of unsaturated alcohols are 14-20 times lower than for the corresponding saturated alcohols (Km = 0.10, 0.14, 0.07 and 0.06 for 1-propanol, 1-butanol, 3-methyl- 1-butanol and 1-hexanol, respectively) [37]. The maximum rates of oxidation were essentially constant, regardless of the alcohol structure, suggesting that alcohol-enzyme binding is not the rate-limiting step for oxidation [38]. The activity of the enzyme appears to be dependent on the lipophilic character of the alcohol. Another study on biotransformation of 2-propen-1-ol by rat lung and liver preparations showed that 80 % of alcohol was metabolised to acrolein when liver supernatant and cytosol fractions were used [39]. 2-propen-1-ol did not appear to be metabolised to acrolein when lung fractions were used.

Fontaine et al. [40] studied the enzymatic formation of 2-butenal from the β-olefinic alcohol, 2-buten-1-ol. This is analogous to the manner in which allyl alcohol is converted *in vivo* to its toxic oxidative product, acrolein. In kinetic studies it was found that 2-buten-1-ol was readily oxidised

by equine liver ADH, with electrospray-mass spectrometry confirming that 2-butanal was the main metabolite formed. It was also reported that in mouse hepatocytes, 2-buten-1-ol produced marked time- and concentration-dependent cell killing as well as pronounced glutathione depletion. Most importantly, both cytotoxicity and glutathione loss were eliminated with the addition of the ADH inhibitor 4-methylpyrazole, indicating the ADH-mediated pathway is responsible for producing these effects. In keeping with expectations that Michael addition adducts would feature prominently during protein modification, Fontaine and co-workers [40] note that exposure to 2-buten-1-ol resulted in marked carbonylation of a range of cell proteins. Damage to a subset of small proteins (e.g., 29, 32, 33 kDa) is closely correlated with the severity of cell death. This cytotoxicity, as well as glutathione depletion, were eliminated by the addition of 4-methylpyrazole. Collectively, these results demonstrate that for the model β-olefinic alcohol, 2-buten-1-ol, toxicity via Michael addition is accompanied by ADH-mediated metabolism.

Further oxidation of the aldehyde produces the corresponding acid. However, Patel et al. [39] showed that only 15% of the acrolein was metabolised to acrylic acid. The corresponding carboxylic acid may enter the β-oxidation pathway and be subsequently metabolised to CO_2 via the tricarboxylic acid pathway or be glucuronidated prior to excretion in the urine. However, this detoxification is not considered to be relevant to repeated-dose toxicity.

Secondary alcohols are expected to be excreted via conjugation or oxidised to ketones, which cannot be further oxidised. Additionally, they can be excreted unchanged or undergo hydroxylation of the carbon chain, which in turn may give rise to a metabolite that can be more readily excreted.

3.2.5 Metabolic similarity

As demonstrated in Table 5 of the supplemental information, all of the β-olefinic alcohols included in the category are predicted by *in silico* tools to be metabolised via oxidation to the corresponding α, β-unsaturated aldehydes or α, β-unsaturated ketones. Other biotransformation pathways, such as hydroxylation and epoxidation are also predicted. These soft electrophiles subsequently react with GSH and protein thiols in hepatocytes [40, 41].

From a structural standpoint, only primary and secondary β-olefinic alcohols are able to be activated by ADH to form polarised α, β-unsaturated electrophiles [7]. The availability of H-atoms on the C-atom with the hydroxyl OH group is crucial to the metabolic activation and subsequent expression of relative toxic potency. Primary alcohols have one alkyl-group; thus, two H-atoms are available for metabolism. Secondary alcohols have two alkyl-groups and one H-atom available for alcohol dehydrogenase attack. Tertiary alcohols are substituted with three alkyl-groups on the α-carbon; thus, no H-atoms are available for metabolism. Since at least one H-atom must be freely available for cleavage by ADH, tertiary alcohols are not metabolised to Michael acceptor electrophilic derivatives by ADH [42]. It follows that primary β-olefinic alcohols are likely to be more readily converted to reactive metabolites than secondary ones.

The finding of Moridani et al. [43] suggests that the primary β- acetylenic alcohol, 2-propyn-1-ol, induces cytotoxicity via metabolic activation by CYP 2E1 to form 2-propynal which in turn causes hepatocyte lysis as a result of GSH depletion and lipid peroxidation. Specifically, 2-propyn-1-ol-induced cytotoxicity was marked by enhanced CYP 2E1-induced hepatocytes and prevented by various CYP 2E1 inhibitors. Moreover, the authors further reported that cytotoxicity of 2-propyn-1-ol was only slightly affected when ADH was inhibited with 4-methylpyrazole or when liver catalase was inactivated with azide or aminotriazole. However,

cytotoxicity was prevented when lipid peroxidation was inhibited with antioxidants, desferoxamine or dithiothreitol. Additionally, the authors found out that hepatocyte GSH depletion preceded cytotoxicity and can be inhibited by cytochrome P450 inhibitors but not by liver catalase and ADH inhibitors. Two-propyn-1-ol –induced cytotoxicity and reactive oxygen species formation were markedly increased in GSH-depleted hepatocytes [43]. Therefore, based on metabolic similarity, the read-across category is limited to primary and secondary β-olefinic alcohols which differ from β- acetylenic alcohols in the activation pathway.

3.2.6 Toxicophore similarity

As demonstrated in Tables 6A and 6B of the supplemental information, based on *in silico* predictions, only the metabolites of β-olefinic alcohols and not the parent compounds triggered the OECD DNA and protein binding profilers within the OECD QSAR Toolbox v3.3.5. With the exception of 3-methyl-2-butenal and 4-methyl-3-penten-2-one, all relevant metabolites of β-olefinic alcohols are associated with Michael addition mechanisms. The potency of protein binding varies consistently between the five sub-structural groups that can be accounted for by sub-categorisation of β-olefinic alcohols.

3.2.7 Mechanistic plausibility similarity

Reactivity with biological molecules consists of a variety of conjugation, substitution, and addition reactions, which have their foundation in the principles of organic reactions [8]. As summarised in Table 7 of the supplemental information, the β-olefinic alcohols included in the category are associated via covalent interaction with thiols. This mechanism is based on ADH-induced Michael addition [8].

As noted by Richarz et al. [44], the over-arching toxic pathway involves metabolic activation to soft electrophilic derivatives which prefer to covalently interact with thiol-containing cellular nucleophiles (e.g., glutathione). Cellular events include dose-dependent necrosis or mitochondrial-based apoptosis; whereas liver and kidney are the target organs.

Landesmann et al. [45] reported a preliminary adverse outcome pathway (AOP) leading from the molecular initiating event of covalent protein binding to the adverse effect of liver fibrosis. They noted a number of key intermediate events including:

- Hepatocyte injury and death
- Activation of Kupffer cells (liver macrophages)
- Inflammation
- Oxidative stress
- Activation of TGF- β
- Activation of stellate cells (mesenchymal stem cells)
- Collagen synthesis and accumulation
- Alteration in connective tissue extracellular matrix

This AOP was constructed, in large part, from data on 2-propen-1-ol and its metabolite - acrolein. The molecular initiating event of this pathway is covalent binding to thiols. More specifically, upon reaching the liver, the non-reactive parent alcohol is converted enzymatically to the corresponding α, β-unsaturated aldehyde or α, β-unsaturated ketone. These reactive species, in turn, bind to thiols such as GSH. Once GSH is dissipated, the α, β-unsaturated substrates react with other cellular thiols, especially in mitochondrial proteins. This denaturing of proteins leads to apoptosis or necrosis of hepatocytes and subsequent events along the AOP.

The short-term isolated perfused liver represents an *ex vivo* model which is close to the *in vivo* condition. The major advantages are that the three-dimensional architecture of the liver and the metabolic capabilities of the hepatocytes are preserved. Strubelt et al. [18] studied acute toxicity and metabolism in a series of short-chain alcohols. Specifically, the effects of 23 alcohols at a single concentration (65.1 mmol/L) in isolated rat livers perfused at 60 ml/hr for two hours were examined. The authors demonstrated that, for straight-chain saturated primary alcohols, hepatic cell injury measured by the release of three cytosolic enzymes into the perfusate and reduction in oxygen consumption were directly related to chain length. In most cases, hepatic ATP concentrations decreased in a similar manner to hepatic cell injury and oxygen consumption [18] *Ex vivo* toxicity profiles for selected β-unsaturated alcohols are reported in Table 2.

Table 2. *Ex vivo* toxicity profiles for β-olefinic alcohols.

ID	**Name**	**LDH (U/l)**	**O_2 consumption (μmol/g x min)**	**ATP (μmol/g)**	**GSH (μmol/g)**
	Control	1109 ± 265	1.54 ± 0.07	1.25 ± 0.20	2.52 ± 0.29
			Straight-chained		
1	2-Propen-1-ol	27747 ± 2756	0.10 ± 0.01	0.07 ± 0.01	0.28 ± 0.12
2	2-Buten-1-ol	10977 ± 2433	0.47 ± 0.06	0.11 ± 0.01	0.02 ± 0.01
3	2-Penten-1-ol				
4	2-Hexen-1-ol				
5	1-Buten-3-ol	25756 ± 1355	0.19 ± 0.04	0.09 ± 0.00	0.03 ± 0.00
6	1-Penten-3-ol				
7	1-Hexen-3-ol				
8	3-Penten-2-ol				
9	3-Hexen-2-ol				
10	4-Hexen-3-ol				
			Branched-chained		
11	2-Methyl-2-propen-1-ol	15552 ± 3282	0.45 ± 0.01	0.15 ± 0.01	0.04 ± 0.02
12	2-Methyl-2-buten-1-ol				
13	2-Methyl-2-penten-1-ol				
14	3-Methyl-2-buten-1-ol	7738 ± 1460	0.84 ± 0.24	0.55 ± 0.22	0.26 ± 0.07

15	3-Methyl-3-penten-2-ol				
16	4-Methyl-3-penten-2-ol				
			β-acetylenic		
17	2-Propyn-1-ol	13743 ± 2457	0.19 ± 0.05	0.14 ± 0.02	0.08 ± 0.05
			Saturated		
18	1-Propanol		4731 ± 1867	1.66 ± 0.13	0.98 ± 0.19
19	1-Butanol		8946 ± 2411	0.98 ± 0.40	0.88 ± 0.09
20	1-Pentanol		28959 ± 4142	0.06 ± 0.01	0.22 ± 0.03
21	2-Methyl-1-propanol		11499 ± 2898	0.88 ± 0.10	0.53 ± 0.05
22	3-Methyl-1-butanol		8680 ± 1216	0.22 ± 0.07	0.10 ± 0.01
23	2-Methyl-2-butanol		9353 ± 2582	1.13 ± 0.33	0.62 ± 0.23
24	2-Methyl-3-butyn-2-ol		2078 ± 1524	1.20 ± 0.20	0.68 ± 0.07

LDH – lactate dehydrogenase; ATP - adenosine triphosphate; GSH – reduced glutathione

Testing using isolated perfused liver demonstrated that saturated alcohols elicited no change in GSH levels. In contrast, unsaturated straight-chain alcohols, including allyl alcohol caused significant reductions in GSH [18].

The major weakness of the Strubelt study is the lack of dose-response data. However, the results of the Strubelt study support the premise that 1-alken-3-ols, 2-alken-1-ols, and 2-methyl-2-alken-1-ols are metabolised and give rise to a metabolite of similar potency to 2-propen-1-ol and thus are very likely to cause similar repeated-dose toxicity. The data in Table 2 also support the structural selectivity of the category as tertiary β-unsaturated alcohols, as well as alkanols, do not reduce GSH (i.e., are not metabolised to reactive electrophiles). Moreover, they do not elicit the same repeated-dose effects. The structural saturated analogue of 2-propen-1-ol – 1-propanol was tested in rats for four months at the dose of 3000mg/kg bw/d [46]. Food consumption, body weight gain, and liver histopathology were comparable to those of the control group. Additionally, the 90 day oral repeat-dose toxicity NOEL for 2-propanol in rat was reported as 870 mg/kg bw/d, based on the relative organ weights of liver, kidneys, and adrenals [47].

3.2.8 Other endpoint similarity

The basic structure-activity relationships for chemical reactivity via Michael addition to thiols are pivotal for understanding both *in vitro* and *in vivo* hepatotoxic potency.

Acrolein is unique amongst the α, β-unsaturated carbonyl compounds as it is the only molecular structure having both a terminal vinyl group and a terminal carbonyl group. These structural features associated with relative reactivity of polarised α, β-unsaturated molecules, especially where an olefinic moiety conjugated to a carbonyl group, towards the model nucleophile glutathione, have been examined [10]. This α, β-unsaturated structure conveys the capacity to undergo a covalent interaction with the thiol group of cysteine in the form of Michael addition [8]. Quantitatively, reactivity of the α, β-unsaturated carbonyl compounds with glutathione is reliant upon the specific molecular structure, with several trends being observed and reported [8, 10]. *In chemico* reactivity data (RC_{50} values) in the form of the depletion of GSH after 120-minutes by selected α, β-unsaturated carbonyl compounds are reported in Table 3.

Table 3. *In chemico* reactivity profiles for α, β-unsaturated aldehydes and ketones.

ID	Alcohol	Metabolite	Metabolite SMILES	GSH RC_{50}
		Straight-chained		
1	2-Propen-1-ol	2-Propenal (acrolein)	O=CC=C	0.085
2	2-Buten-1-ol	2-Butenal (crotonaldehyde)	O=CC=CC	0.22
3	2-Penten-1-ol	trans-2-Pentenal	O=CC=CCC	0.35
4	2-Hexen-1-ol	trans-2-Hexenal	O=CC=CCC	0.42
5	1-Buten-3-ol	Methyl vinyl ketone	C=CC(=O)C	0.070
6	1-Penten-3-ol	Ethyl vinyl ketone	C=CC(=O)CC	0.051
7	1-Hexen-3-ol	Propyl vinyl ketone	C=CC(=O)CCC	0.059
8	3-Penten-2-ol	3-Penten-2-one	CC(=O)C=CC	0.15
9	3-Hexen-2-ol	3-Hexen-2-one	CC(=O)C=CC	not tested
10	4-Hexen-3-ol	4-Hexen-4-one	CCC(=O)C=CC	0.34

		Branched-chained		
11	2-Methyl-2-propen-1-ol	2-Methyl acrolein	O=CC(C)=C	not tested
12	2-Methyl-2-buten-1-ol	2-Methyl-2-butenal	O=CC(C)=CC	12
13	2-Methyl-2-penten-1-ol	2-Methyl-2-pentenal	O=CC(C)=CCC	21
14	3-Methyl-2-buten-1-ol	3-Methyl-2-butenal	O=CC=C(C)C	13
15	3-Methyl-3-penten-2-ol	3-Methyl-3-penten-2-one	CC(=O)C(C)=CC	10
16	4-Methyl-3-penten-2-ol	4-Methyl-3-penten-2-one	CC(=O)C=C(C)C	26
		Saturated		
17	1-Propanol	1-Propanal/1-Propionic acid	CCC=O/CCC(=O)O	not reactive at 1000 mg/l
18	1-Butanol	1-Butanal/1-Butyric acid	CCCC=O/CCCC(=O)O	not reactive at 1000 mg/l
19	1-Pentanol	1-Pentanal/1-Pentanoic acid	CCCCC=O/CCCCC(=O)O	not reactive at 1000 mg/l
20	2-Methyl-1-propanol	2-Methyl-1-propanal/2-Methyl-1-propionic acid	CC(C)C=O/CC(C)C(=O)O	not reactive at 1000 mg/l
21	3-Methyl-1-butanol	3-Methyl-1-butanal/2-Methyl-1-butyric acid	CC(C)CC=O/CC(C)CC(=O)O	not reactive at 1000 mg/l
22	2-Methyl-2-butanol	2-Methyl-2-butanone	CC(C)C(=O)C	not reactive at 1000 mg/l
23	2-Methyl-3-butyn-2-ol	not metabolised	-	not reactive at 500 mg/l

Specifically, it has been reported that for α, β-unsaturated carbonyl compounds, such as those derived from hepatic oxidative metabolism of β-olefinic alcohol, the: 1) terminal vinyl-substituted derivatives (H_2C=C-) were more reactive than the internal vinylene-substituted ones (-CH=CH-); 2) methyl-substitution on the vinyl carbon atoms diminishes reactivity, 3) methyl-substitution on the carbon atom farthest from the carbonyl group (C(=O)C=C(C) causes a larger reduction than methyl-substitution on the carbon atom nearest to the carbonyl group (C(=O)C(C)=C), and 4) derivatives with a carbon-carbon double bond at the end of the molecule (i.e., vinyl ketones) were more reactive than ones with the carbon-oxygen double bond at the end of the molecule (i.e., aldehydes).

The results from the measurement of thiol reactivity (see Table 3) suggest that the ability of α, β-unsaturated carbonyl compounds other than acrolein (and thus, β-olefinic alcohol other than 2-

propen-1-ol) to elicit kidney and liver targeted toxicity may be reduced, especially for branched alcohols with alkyl substitutions on the vinyl carbon atoms.

In fish, the mode of (acute) toxic action involves metabolism of the parent alcohol to the corresponding α, β-unsaturated aldehyde or ketone via alcohol dehydrogenase [42, 48]. The conventional thinking is that, whilst the parent aliphatic alcohols elicit baseline toxic action through narcosis, the metabolites are electrophilic toxicants. Specifically, the metabolites are polarised α, β-unsaturated chemicals which undergo a Michael-type addition to soft nucleophilic sites in proteins [8]. Bradbury and Christensen [7] confirmed the role of alcohol dehydrogenase activity in metabolic activation and enhanced toxicity in fish. Specifically, the alcohol dehydrogenase in the gill epithelial cells metabolises the appropriate alcohol to the corresponding aldehyde (or ketone), which in turn reacts with cellular proteins. The end result is death of the gill epithelia cells, which results in the loss of the ability to extract oxygen causing subsequent hypoxia and fish mortality. This mechanism was described for model electrophiles by respiratory and cardiovascular responses in trout [49].

Acute toxicity studies with the fathead minnow (*Pimephales promelas*) found that primary and secondary allylic alcohols and primary and secondary propargylic alcohols exhibit potency in excess of that predicted by saturated alcohols and baseline narcosis QSAR models [48, 50]. However, tertiary olefinic and tertiary acetylenic alcohols exhibit fish toxic potency consistent with baseline narcosis models. The enhanced toxicity of acetylenic alcohols is thought to be due to metabolic activation to electrophilic α, β-unsaturated propargylic aldehydes or ketones. For primary and secondary homopropargylic alcohols, an activation step involving biotransformation to an allenic electrophile intermediate was proposed [42]. The results from fish acute toxicity experiments support the premise that the basic structure-activity relationships for chemical

reactivity via Michael additions to thiol is key for understanding mammalian repeated-dose toxic potency of β-unsaturated alcohols.

3.3 Uncertainty in similarities

Data uncertainty and weight-of-evidence associated with the fundamentals of chemistry, as well as toxicokinetic and toxicodynamic similarity of category members is presented in Table 4.

3.3.1 Uncertainty in chemical similarities

The similarities in physico-chemical properties are reduced by the narrow structural range (i.e., C3 to C6) of the category. Moreover, the differences in chemical property values are not considered to be toxicologically relevant. In terms of structure, the complex extended fragment of the applicability domain of this category results in moderate similarity across the analogues in Table 1. The key feature, being a primary or secondary β-olefinic alcohol of short-chain length is common within the category and is relevant to the toxicity read across. The extended fragment differences among the category members are best presented with their 2D structure. These differences are related to the location of hydroxyl group: external (primary alcohols) and internal (secondary alcohols); the position of the unsaturated moiety, which can be either internal or external and the substitution of vinyl group carbon atoms with alkyl group (e.g., methyl group). Amongst the category members, the source substance, 2-propen-1-ol, is a structurally unique β-olefinic alcohol with both a terminal vinyl group and a terminal hydroxyl. Additionally, the presence of geometric isomerism or stereoisomerism among the different category members reduces the chemical similarity and can affect the reproducibility of test results as well as metabolism and reactivity.

Structural differences within the β-olefinic alcohols lead to 1) different likely metabolite (e.g., aldehyde or ketone), 2) different *ex vitro* metabolism (i.e., free GSH levels) and 3) different rates of *in chemico* reactivity (i.e., GSH reactivity). However, it is uncertain if these short-term (i.e., 2-hour) differences are relevant to repeated-dose toxicity.

Table 4. Data uncertainty and weight-of-evidence associated with the fundamentals of chemistry, transformation/toxicokinetic and toxicodynamic similarities

Similarity Parameter	**Data Uncertainty** [a]	**Strength of Evidence** [b]	**Comment**
Substance Identification, Structure and Chemical Classifications	Low	High	All category members are discrete organic substance of simple structure. They all have CAS numbers, similar 2D structure and belong to the same chemical class and one of five noted subclasses. The presence of stereoisomerism in some substances was noted.
Physio-Chem & Molecular Properties	Empirical: low Modelled: low	High	All category members are appropriately similar with respect to key physicochemical and molecular properties. There is a high degree of consistency between measured and model estimated values.
Substituents, Functional Groups, & Extended Structural Fragments	Low-to-medium	High	Substituents and functional groups are consistent across all category members. There is a complex extended structural fragment (see Table 1) which is accounted for in sub-categorisation
Transformation/Toxicokinetics and Metabolic Similarity	Empirical: *In vivo*: none *In vitro*: low Simulated: low	Medium	Due to the small size range, bioavailability is not considered a factor in these predictions. Based on high quality data for two category members, there is evidence for similar toxicokinetics and metabolic pathways. There is metabolic evidence suggesting some methyl-substitution affects the rate of metabolites. *In vivo* data suggests the rate of metabolism affects chronic toxicity. This can be accounted for sub-categorisation.
Potential Metabolic Products	Simulated: low	High	Based on *in silico* metabolic simulations, metabolites from oxidation are predicted to be produced by the category members.
Toxicophores /Mechanistic alerts	Medium	High	Based on *in silico* profilers, category members contain established toxicophores for protein and DNA binding via metabolic activation. However, the potency of protein binding varies between the five sub-structure groups. Potency differences can be accounted for sub-categorisation.

Similarity Parameter	Data Uncertainty [a]	Strength of Evidence [b]	Comment
Mechanistic plausibility and AOP-Related Events	Medium	High	The available AOP leads to the hypothesis that the mode of toxic action of all category members is related to oxidative metabolism to corresponding α, β-unsaturated electrophilic aldehydes or α, β-unsaturated ketones.
other relevant, *in vivo*, *in vitro* and *ex vivo* endpoints	Low	High	Fish *in vivo* data and *in vitro* data for cellular effects are in agreement with the electrophilic reactivity hypothesis for rodent repeated-dose toxicity.

[a] Uncertainty associated with underlying information/data used in the exercise (empirical, modelled; low, medium, high)

[b] Consistency within the information/data used to support the similarity rational and prediction (low, medium, high)

3.3.2 Uncertainty in toxicokinetic similarity

The narrow range of carbon atoms of the applicability domain limits the impact of analogues on absorption and distribution (i.e., bioavailability). The most likely metabolic pathway of all analogues under consideration is considered to be metabolism via ADH oxidation to similar, but not identical, reactive derivatives, which elicit the same mechanism of chemical reactivity (i.e., Michael addition). This metabolic activation is supported indirectly by the results of the liver profusion studies by Strubelt et al. [18]. However, other metabolic mechanisms, such as ROS formation or P450 activation, while unlikely, are not completely ruled out by the information presented in this study.

3.3.3 Uncertainty in toxicodynamic similarity

Primary and secondary β-olefinic alcohols are experimentally associated with the pro-electrophilic mode of toxic action. This mode of action is well-studied, and the molecular mechanism, soft electrophilic reactivity, is well understood. There is a qualitative Adverse Outcome Pathway (AOP) available linking electrophilic reactivity via ADH-mediated

metabolism to cellular necrosis and/or apoptosis [45]. It is evident that oral repeated-dose toxicity of primary and secondary β-olefinic alcohols is related to this molecular mechanism. However, there is conflicting evidence as to whether the mode of action results in liver fibrosis. This conflicting evidence is the major source of uncertainty associated with toxicodynamic uncertainty.

In an effort to further reduce uncertainties, the category was examined within and between structural sub-categories. Results for selected compounds representing each of the five sub-structural groups from the *ex vivo* assay, the 2-hour rat isolated perfused liver, are consistent with the mechanistic hypothesis of metabolic activation via ADH to soft electrophiles. Specifically, all primary and secondary β-olefinic alcohols tested exhibit a dramatic reduction (90-99%) in glutathione (GSH) as compared to controls. *In chemico* reactivity data, in the form of the concentration eliciting a 50% reduction in free GSH after 2 hours exposure for selected α, β-unsaturated carbonyl compounds (i.e., potential reactive metabolites of β-olefinic alcohols) also support the applicability domain of this chemical category. All α, β-unsaturated carbonyl compounds such as those derived from hepatic metabolism of primary and secondary β-olefinic alcohol readily react with GSH. Specifically, α, β-unsaturated carbonyl derivatives of straight-chain alcohols: allyl alcohol, 1-alken-3-ols and 2-alken-1-ols exhibit 2-hour RC_{50} values between 0.05 and 0.40 mM, while those of branched alcohols: 2-methyl-2-alken-1-ols, 3-methyl-2-alken-1-ols, 3-methyl-3-alken-2-ols and 4-methyl-3-alken-2-ols exhibit RC_{50} values between 12-22 mM.

The *ex vivo* and *in chemico* data (see Tables 2 and 3) support the premise that the single source substance, 2-propen-1-ol is potentially one of the most potent analogues and can be read across to other β-alkenols, especially primary ones.

Endpoint specific factors affecting the prediction include the uncertainty associated with how exactly the molecular structure impacts repeated-dose toxicity. These uncertainties are considered low to moderate since the most likely metabolites are well-studied Michael acceptors, either a β-unsaturated aldehyde or a β-unsaturated ketone. Since results from cytotoxicity, fish toxicity and skin sensitization studies reveal similar structure-activity relationships, no endpoint non-specific factors affecting the predictions are identified.

The *in chemico* data, but not the *ex vivo* data, support the argument for sub-categorisation. In the sub-categorisation scheme 2-propen-1-ol can be read-across to the other straight-chained alcohols, such as 1-alken-3-ols and 2-alken-1-ols, with less uncertainty (i.e., greater confidence) than to the branched ones, 2-methyl-2-alken-1-ols, 3-methyl-2-alken-1-ols, 3-methyl-3-alken-2-ols and 4-methyl-3-alken-2-ols.

As reported in Table 3, high quality *in chemico* data exist for 14 of the 16 category members based on the proposed α, β-unsaturated metabolites and their reactivity with GSH. These 14 derivatives include more than one representative of four of the five structural sub-groups (the other group has only a single analogue). All 14 analogues exhibit GSH reactivity and there is consistent potency within the two sub-categories: straight-chained and branched. Specifically, the results showed that β-olefinic alcohols with a methyl group substituted on a vinyl C-atom are 100 times less reactive than the non-methyl-substituted β-olefinic alcohol. However, this difference in *in chemico* reactivity between substituted and unsubstituted β-olefinic alcohols is not exhibited *ex vivo* in liver profusion tests. In order to reduce the toxicodynamic uncertainty to an acceptable level, without the need for further information or testing, it is recommended the read-across prediction only be applied to the straight-chain sub-category (i.e., alcohols 2-10 in Table 1).

3.3.4 Uncertainty in mechanistic relevance and completeness

Assessment of uncertainty associated with mechanistic relevance and completeness of the read-across is presented in Table 5. Uncertainty associated with mechanistic relevance and completeness of the read-across is judged to be medium. Briefly, uncertainty associated with this read-across stems from the facts that: 1) the single source substance, allyl alcohol, is a unique β-olefinic alcohol and is metabolised to a unique electrophile, acrolein, 2) the most likely mode-of-action, liver fibrosis is not consistently supported by the rat oral repeated-dose toxic data, and 3) ADH metabolic activation is central to the hypothesis; however other transformation mechanisms, such as autooxidation, ROS formation or P450 activation, cannot be overlooked.

Table 5. Assessment of uncertainty associated with mechanistic relevance and completeness of the read-across.

Factor	**Uncertainty[a]**	**Comment**
The problem and premise of the read-across	Low-to-medium; limited by lack of experimental support for mechanistic plausibility	The endpoint to be read across, oral 90-day repeated-dose toxicity for primary and secondary β-olefinic alcohols is not well-studied. The scenario of the read-across hinges on metabolic similarity and the formation of electrophilic α, β-unsaturated aldehydes and α, β-unsaturated ketones which elicit similar reactive potency leading to hepatic and renal effects related to apoptosis and necrosis.
***In vivo* data read across**		
Number of analogues in the source set	Medium; 1 of 10	There is only one suitable category member (2-propen-1-ol) with *in vivo* apical endpoint data. This source substance represents the straight-chained sub-category
Quality of the *in vivo* apical endpoint data read across	Low	High quality empirical data for the stated regulatory endpoint exists from multiple studies for 2-propen-1-ol. These data are consistent in regards to qualitative and quantitative descriptions of effects.
Severity of the apical *in vivo* hazard	Low	Potency data for the *in vivo* apical endpoint are NOAELs for 2-propen-1-ol include 6 mg/kg body weight bw/d in males based on increase in relative weight of liver, and 25 mg/kg

		bw/d in females based on bile duct hyperplasia and periportal hepatocyte hypertrophy in the liver.
Evidence to the biological argument for RA		
Robustness of analogue data set	Low-to-medium; *ex vivo* and *in chemico* endpoints reveal the same structure-activity relationships.	The available data from *ex vivo* studies of category members are of high quality but limited to one representative compound of the five structural sub-groups. The available data from *in chemico* studies for the category members are robust, representing multiple chemicals in four of the five structural sub-groups. All the tests were judged to be reliable and conducted under the appropriate conditions.
Concordance with regard to the intermediate and apical effects and potency data	Medium	While data are limited, there appears to be good agreement between the sequences of biochemical and physiological events leading to the *in vivo* toxicity. There is consistency and high specificity for the association between *in vivo* symptoms, and the *ex vivo* data as well as the structural domain of the category. There is general agreement among the dose-response relationships of the tested category members for the relevant *in chemico* event. Limiting the final domain to straight-chain derivatives markedly improves the concordance.
Weight of Evidence	Low-to-medium	Overall the available information is consistent with the stated premise. The variation in structural (i.e., complex extended fragment) of the initial category weakens the WoE. While the toxicokinetics data are limited, the high quality *ex vivo* data (i.e., profused liver) support metabolism being a key factor to the category and add to the WoE. The fact that there is consistent relevant *in chemico* data for most if not all the category members strengthens the WoE. The lack of consistency between the *in chemico* data and the *ex vivo* data detracts from the WoE. Limiting the final domain to straight-chain derivatives markedly improves the WoE.

[a] Uncertainty: low, medium, high

4 Discussion

The overall chemical similarity of the β-olefinic alcohols considered is limited by the complexity of the extended fragment but enhanced by clustering into sub-categories. Within the primary and secondary β-olefinic alcohols data similarity and WoE associated with the fundamentals of toxicokinetic is the major weakness as there is uncertainty associated with the metabolite

pathway and rate of metabolism. This uncertainty is reduced when the *ex vivo* liver perfusion data (see Table 2) are considered. Within the primary and secondary β-olefinic alcohol category data similarity and WoE associated with toxicodynamics is a secondary weakness. This is, in large part, due to having a single source substance and the disparity in *in vivo* data for 2-propen-1-ol and 3-methyl-2-buten-1-ol (see Table 8 of the supplemental information). While 2-propen-1-ol was administered via gavage in a protocol similar to OECD TG408, in the 3-methyl-2-buten-1-ol study, rats were exposed via drinking water and decreased water consumption was only noted. The administration via drinking water reduces the alcohol dosage, which in turn is likely to reduce toxicity. Uncertainties associated with mechanistic relevance and completeness of the read-across (i.e., uncertainty in the predictions) are reduced with the addition of *in chemico* data (see Table 3), as well as sub-categorisation.

In order to reduce the uncertainties further, there is a need to secure further information by targeted testing. Of particular value would be data from *in vitro* assays quantifying hepatocyte metabolism and fibrosis-related activities. For example, an *in vitro* model consisting of hepatic organoids (3D co-culture) of human hepatocyte-like cells (HepaRG and primary human hepatic stellate cells (HSC)) can be used [51]. This system has been shown to maintain good hepatocyte functionalities and maintain HSCs in a quiescent-like state for 3 weeks. During this period, the 3D HepaRG/HSC co-culture model has been validated for drug-induced toxicity and fibrosis assays using compounds such as methotrexate and allyl alcohol [51]. Another *in vitro* method, with potential application to this case study uses the HepG2 BAC-GFP reporter system [52]. Briefly, stress response activation of SRXN1, a target of the transcription factor NRF2, which is activated upon oxidative stress, and stress response activation of p21 and BTG2, both targets of the transcription factor p53, which is activated upon DNA damage, were evaluated [52]. Stress

response activation is evaluated at various times after exposure using Nikon confocal microscopy. HepG2 cells can be cultured in conventional 2D monolayer and 3D hydrogel-based assays; green fluorescent protein (GFP) pixel intensity can be measured per single cell for 2D monolayer or measured per spheroid in 3D [53].

5 Conclusions

While a submission for regulatory purposes may take on a different format, the present case study illustrates the key issues associated with modern day read-across and the use of non-animal data to support the prediction. In the end, the applicability domain for this case study is limited to small (C3 to C6), straight-chain, primary and secondary β-olefinic alcohols. The oral 90-day repeated-dose NOAEL of 6 and 25 mg/kg bw/d, in male and female rats, respectively, reported for 2-propen-1-ol can be read across to untested straight-chained β-olefinic alcohols (i.e., 1-alken-3-ols and 2-alken-1-ols) with acceptable uncertainty as a worst case scenario. Greater uncertainty is associated with read-across to the branched primary and secondary β-olefinic alcohols.

The mechanistic argument is consistent with primary and secondary β-olefinic alcohols being readily metabolised by ADH to polarised α, β-unsaturated aldehydes and ketones, which react via Michael addition interaction with thiols in proteins resulting in cellular apoptosis and/or necrosis. Upon oral repeated-dose exposure, the latter may, as in the case of 2-propen-1-ol, lead to *in vivo* toxicity involving the kidney and liver. The main route of exposure for β-olefinic alcohols is oral with immediate absorption from the upper gastrointestinal tract. They are distributed unbound in the blood and are subsequently readily enzymatically oxidised, especially in the liver to reactive metabolites.

The key element of uncertainty in accepting read-across predictions is rooted in metabolism. Specifically, the pivotal issues for establishing category membership include: 1) are the β-olefinic alcohols transformed to metabolites having the same mechanism of electrophilic reactivity, 2) is the metabolic pathway the same, 3) are the rates of transformation sufficient so the reactive metabolites are the definitive toxicant for the endpoint being read across, and 4) are the metabolites similar in reactive potency.

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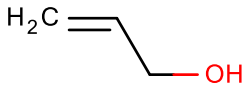
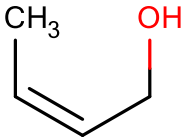
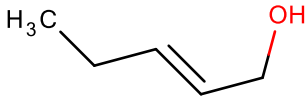
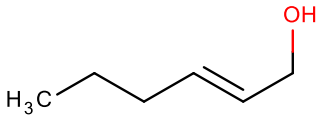
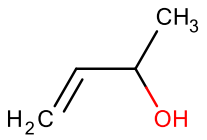
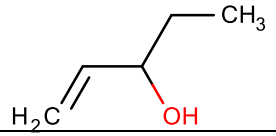
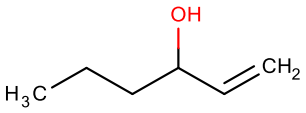
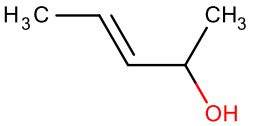
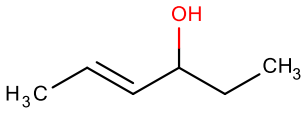
Supplementary material

Read-Across of 90-Day Rat Oral Repeated-Dose Toxicity: A Case Study for Selected β-olefinic Alcohols

Tables for Assessing Similarity of Analogues and Category Members for Read-Across

Table 1. Comparison of Substance Identification, Structure and Chemical Classifications

ID	Name	CAS No:	SMILES	2D Structure	Molecular Formula:
1	2-propen-1-ol	107-18-6	C(CO)=C		C_3H_6O
2	2-buten-1-ol	6117-91-5	OCC=CC		C_4H_8O
3	2-penten-1-ol	20273-24-9	CCC=CCO		$C_5H_{10}O$
4	2-hexen-1-ol	2305-21-7	CCCC=CCO		$C_6H_{12}O$

ID	Name	CAS No:	SMILES	2D Structure	Molecular Formula:
5	1-buten-3-ol	598-32-3	CC(C=C)O		C_4H_8O
6	1-penten-3-ol	616-25-1	CCC(C=C)O		$C_5H_{10}O$
7	1-hexen-3-ol	4798-44-1	CCCC(C=C)O		$C_6H_{12}O$
8	3-penten-2-ol	1569-50-2	CC=CC(C)O		$C_5H_{10}O$
9	3-hexen-2-ol	42185-97-7	CC(O)C=CCC		$C_6H_{12}O$
10	4-hexen-3-ol	4798-58-7	CCC(C=CC)O		$C_6H_{12}O$

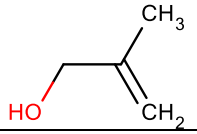
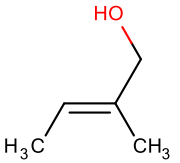
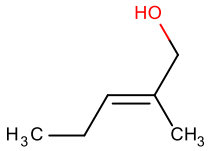
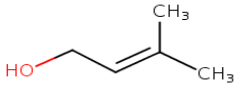
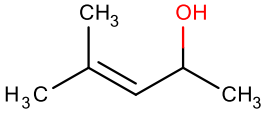
ID	Name	CAS No:	SMILES	2D Structure	Molecular Formula:
11	2-methyl-2-propen-1-ol	513-42-8	CC(=C)CO		C_4H_8O
12	2-methyl-2-buten-1-ol	4675-87-0	CC=C(C)CO		$C_5H_{10}O$
13	2-methyl-2-penten-1-ol	1610-29-3	CCC=C(C)CO		$C_6H_{12}O$
14	3-methyl-2-buten-1-ol	556-82-1	CC(=CCO)C		$C_5H_{10}O$
15	3-methyl-3-penten-2-ol	2747-53-7	CC(O)C(C)=CC		$C_6H_{12}O$
16	4-methyl-3-penten-2-ol	4325-82-0	CC(O)C=C(C)C		$C_6H_{12}O$

Table 2. Comparison of Physico-Chemical and Molecular Properties[1]

ID	Name	**Molecular Weight** [g/mol]	**Log Kow**[a]	**Vapor Pressure**[b] [Pa at 25 deg C]	**Density**[d] [g/cm^3]	**Melting Point** [b] [deg C]	**Water Solubility**[c]	**Boiling Point**[b] [deg C]	**pKa**[e]
1	2-propen-1-ol	58.08	0.21 0.17 (M)	3.12x10^3 3.48x10^3(M)	0.8±0.1	-76.37 -129 (M)	3.177 x10^5 1 x10^6 (M)	88.13 97 (M)	14.43
2	2-buten-1-ol	72.11	0.63	794	0.8±0.1	-62.76 <-30 (M)	1.272 x10^5 1.66 x10^5 (M)	121.10 123 (M)	14.7
3	2-penten-1-ol	86.13	1.12	351	0.8±0.1	-50.48	4.572 x10^4	143.87 138 (M)	14.7
4	2-hexen-1-ol	100.16	1.61	121	0.8±0.1	-38.47	1.6 x10^4	165.73 157 (M)	14.45
5	1-buten-3-ol	72.11	0.63	3.29x10^3	0.8±0.1	-77.70	1.259 x10^4	89.94 96-97 (M)	14.49
6	1-penten-3-ol	86.13	1.12	1.22x10^3	0.8±0.1	-65.08	4.526 x10^4 9.01 x10^4 (M)	113.89 115 (M)	14.49
7	1-hexen-3-ol	100.16	1.61	437	0.8±0.1	-52.76	1.58 x10^4 2.52 x10^4 (M)	136.94 134 (M)	14.49
8	3-penten-2-ol	86.13	1.04	802	0.8±0.1	-64.13	5.283 x10^4 8.92 x10^4 (M)	122.82	14.77
9	3-hexen-2-ol	100.16	1.53	231	0.8±0.1	-51.87	1.849 x10^4	145.52	14.77
10	4-hexen-3-ol	100.16	1.53	231	0.8±0.1	-51.87	1.849 x10^4 3.81 x10^4 (M)	145.52	14.77
11	2-methyl-2-propen-1-ol	72.11	0.76	199	0.8±0.1	-72.59	9.757 x10^4 1.94 x10^5 (M)	105.69	14.49
12	2-methyl-2-buten-1-ol	86.13	1.17	356	0.8±0.1	-59.25	4.094 x10^4	137.75	14.87
13	2-methyl-2-penten-1-ol	100.16	1.66	66.7	0.8±0.1	-47.16	1.433 x10^4	159.86 167.5 (M)	14.86

14	3-methyl-2-buten-1-ol	98.1	1.17	314	0.8±0.1	-59.25	4.094 x10^4	137.75 140 (M)	14.83
15	3-methyl-3-penten-2-ol	100.16	1.59	325	0.8±0.1	-60.63	1.655 x10^4	139.41	14.94
16	4-methyl-3-penten-2-ol	100.16	1.59	325	0.8±0.1	-60.63	1.655 x10^4	139.41	14.9

[1]Values typically derived from EPISuite v4.1; [a] KOWWIN Program (v1.68); [b] MPBPWIN v1.43; [c] at 25 deg C (mg/L) Kow (WSKOW v1.42); [d] ACD/Lab Percepta Platform - PhysChem Module (from ChemSpider); [e] ACD (Advanced Chemistry Development Inc., Toronto, Canada) (M): measured: Hansch, C et al. (1995); Yalkowsky, SH & Dannenfelser, RM (1992); Beilstein database.

Table 3. Comparison of Substituents, Functional Groups, and Extended Structural Fragments

ID	Name	Key Substituent(s)	Functional Group(s)		Chemical Class:	Chemical Sub-Class:
1	2-propen-1-ol	β-Olefin (C=C)	External hydroxyl	External C=C	β-unsaturated alcohols	primary allylic
2	2-buten-1-ol	β-Olefin (C=C)	External hydroxyl	Internal C=C	β-unsaturated alcohols	primary allylic
3	2-penten-1-ol	β-Olefin (C=C)	External hydroxyl	Internal C=C	β-unsaturated alcohols	primary allylic
4	2-hexen-1-ol	β-Olefin (C=C)	External hydroxyl	Internal C=C	β-unsaturated alcohols	primary allylic
5	1-buten-3-ol	β-Olefin (C=C)	Internal hydroxyl	External C=C	β-unsaturated alcohols	secondary allylic
6	1-penten-3-ol	β-Olefin (C=C)	Internal hydroxyl	External C=C	β-unsaturated alcohols	secondary allylic
7	1-hexen-3-ol	β-Olefin (C=C)	Internal hydroxyl	External C=C	β-unsaturated alcohols	secondary allylic
8	3-penten-2-ol	β-Olefin (C=C)	Internal hydroxyl	Internal C=C	β-unsaturated alcohols	secondary allylic
9	3-hexen-2-ol	β-Olefin (C=C)	Internal hydroxyl	Internal C=C	β-unsaturated alcohols	secondary allylic
10	4-hexen-3-ol	β-Olefin (C=C)	Internal hydroxyl	Internal C=C	β-unsaturated alcohols	secondary allylic
11	2-methyl-2-propen-1-ol	β-Olefin (C=C) Methyl	External hydroxyl	External C(C)=C	β-unsaturated alcohols	primary allylic
12	2-methyl-2-buten-1-ol	β-Olefin (C=C) Methyl	External hydroxyl	Internal C(C)=C	β-unsaturated alcohols	primary allylic
13	2-methyl-2-penten-1-ol	β-Olefin (C=C) Methyl	External hydroxyl	Internal C(C)=C	β-unsaturated alcohols	primary allylic
14	3-methyl-2-buten-1-ol	β-Olefin (C=C) Methyl	External hydroxyl	Internal C(C)=C	β-unsaturated alcohols	primary allylic
15	3-methyl-3-penten-2-ol	β-Olefin (C=C) Methyl	Internal hydroxyl	Internal C(C)=C	β-unsaturated alcohols	secondary allylic
16	4-methyl-3-penten-2-ol	β-Olefin (C=C) Methyl	Internal hydroxyl	Internal C(C)=C	β-unsaturated alcohols	secondary allylic

Table 4. Comparison of Abiotic Transformation and Toxicokinetics

ID	Name	Abiotic Transformation	Toxicokinetics
1	2-propen-1-ol	Photodegradation: half-life = 4.32 hrs; rate constant = 2.59x10-11 cm3/molecule-sec[a]	Rapidly metabolised to acrolein by alcohol dehydrogenase; can be further oxidised to carboxylic acids and finally to CO_2; Tmax = 30-60 min[a] Km= 0.05 mM (binding affinities for human alcohol dehydrogenase), V= 10.3 (turnover no. X active site^{-1} X min^{-1})[b]
2	2-buten-1-ol		Km= 0.01 mM (binding affinities for human alcohol dehydrogenase), V= 13.0 (turnover no. X active site-1 X min-1)[b]
3	2-penten-1-ol		
4	2-hexen-1-ol		Km= 0.003 mM (binding affinities for human alcohol dehydrogenase), V= 15.5 (turnover no. X active site-1 X min-1)[b]
5	1-buten-3-ol		
6	1-penten-3-ol		
7	1-hexen-3-ol		
8	3-penten-2-ol		
9	3-hexen-2-ol		
10	4-hexen-3-ol		
11	2-methyl-2-propen-1-ol		
12	2-methyl-2-buten-1-ol		
13	2-methyl-2-penten-1-ol		
14	3-methyl-2-buten-1-ol		Km= 0.0045 mM (binding affinities for human alcohol dehydrogenase), V= 13.0 (turnover no. X active site-1 X min-1)[b]

15	3-methyl-3-penten-2-ol		
16	4-methyl-3-penten-2-ol		

[a] OECD SIDS Allyl Alcohol; [b] Pietruszko, R., Crawford, K. & Lester, D. 1973. Arch. Biochem. Biophys., 159, 50-60

Table 5. Comparison of Potential Metabolic Products

ID	Name	Liver metabolism simulator Toolbox v3.3.5		MetaPrint2D-React software	Meteor Nexus
		Rat liver S9	Skin metabolism		
1	2-propen-1-ol	Oxidation (1)	Oxidation (1)	Epoxidation Oxidation	Epoxidation (1) Oxidation (1)
2	2-buten-1-ol	Hydroxylation (1) Oxidation (1)	Oxidation (1)	Hydroxylation Oxidation Acylation	Hydroxylation (1) Oxidation (1) Epoxidation (1)
3	2-penten-1-ol	Hydroxylation (1) Oxidation (1)	Hydroxylation (1) Oxidation (1)	Oxidation Acylation	Hydroxylation (2) Oxidation (1) Epoxidation (1)
4	2-hexen-1-ol	Hydroxylation (2) Oxidation (1)	Hydroxylation (2) Oxidation (1)	Hydroxylation Oxidation Acylation	Hydroxylation (3) Oxidation (1) Epoxidation (1)
5	1-buten-3-ol	Oxidation (1)	Hydroxylation (1)	Epoxidation Epoxidation/Hydrolysis	Oxidation (1) Hydroxylation (1) Epoxidation (1)
6	1-penten-3-ol	Hydroxylation (1) Oxidation (1)	Hydroxylation (2)	Hydroxylation	Hydroxylation (2) Oxidation (1) Epoxidation (1)
7	1-hexen-3-ol	Hydroxylation (2) Oxidation (1)	Hydroxylation (2)	Hydroxylation Oxidation Acetylation	Oxidation (1) Hydroxylation (3) Epoxidation (1)
8	3-penten-2-ol	Oxidation (1)	Hydroxylation (1)	Hydroxylation Oxidation Epoxidation	Oxidation (1) Hydroxylation (2) Epoxidation (1)
9	3-hexen-2-ol	Hydroxylation (1) Oxidation (1)	Hydroxylation (2)	Hydroxylation Oxidation Alkylation	Oxidation (1) Hydroxylation (3) Epoxidation (1)

10	4-hexen-3-ol	Hydroxylation (2) Oxidation (1)	Hydroxylation (2)	Hydroxylation Oxidation Acetylation	Oxidation (1) Hydroxylation (3) Epoxidation (1)
11	2-methyl-2-propen-1-ol	Oxidation (1)	No metabolism	No metabolism	Oxidation (1) Hydroxylation (1)
12	2-methyl-2-buten-1-ol	Oxidation (1)	Hydroxylation (1)	Acetylation Acylation	Hydroxylation (2) Oxidation (1) Epoxidation (1)
13	2-methyl-2-penten-1-ol	Hydroxylation (1) Oxidation (1)	Hydroxylation (1)	Hydroxylation Oxidation Acetylation Acylation Dehydroxylation	Hydroxylation (3) Oxidation (1) Epoxidation (1)
14	3-methyl-2-buten-1-ol	Hydroxylation (1) Oxidation (1)	No metabolism	Hydroxylation Oxidation Alkylation Acylation	Hydroxylation (2) Oxidation (1) Epoxidation (1)
15	3-methyl-3-penten-2-ol	Oxidation (1)	Hydroxylation (2)	Hydroxylation Oxidation Acetylation	Oxidation (1) Hydroxylation (3) Epoxidation (1)
	4-methyl-3-penten-2-ol	Oxidation (1)	Hydroxylation (1)	Hydroxylation Oxidation Alkylation	Oxidation (1) Hydroxylation (3) Epoxidation (1)

() - The number of metabolites for specific transformation.

Table 6A. Comparison of Toxicophores for β-unsaturated alcohols

ID	Name	Toxicophores[1]	Structural alerts[1,2]				
			DNA binding by OECD[1]	Protein binding by OECD[1]	Protein binding potency (GSH)[1]	In vivo mutagenicity (Micronucleus) alerts by ISS[1]	Mitochondria toxicity[2]
1	2-propen-1-ol	Cramer Class III	No alert	No alert	Not classified	No alert	Alert C=CCO
2	2-buten-1-ol	Cramer Class I	No alert	No alert	Not classified	No alert	Alert C=CCO
3	2-penten-1-ol	Cramer Class I	No alert	No alert	Not classified	No alert	Alert C=CCO
4	2-hexen-1-ol	Cramer Class I	No alert	No alert	Not classified	No alert	Alert C=CCO
5	1-buten-3-ol	Cramer Class III	No alert	No alert	Not classified	No alert	Alert C=CCO
6	1-penten-3-ol	Cramer Class III	No alert	No alert	Not classified	No alert	Alert C=CCO
7	1-hexen-3-ol	Cramer Class III	No alert	No alert	Not classified	No alert	Alert C=CCO
8	3-penten-2-ol	Cramer Class II	No alert	No alert	Not classified	No alert	Alert C=CCO
9	3-hexen-2-ol	Cramer Class II	No alert	No alert	Not classified	No alert	Alert C=CCO
10	4-hexen-3-ol	Cramer Class II	No alert	No alert	Not classified	No alert	Alert C=CCO
11	2-methyl-2-propen-1-ol	Cramer Class III	No alert	No alert	Not classified	No alert	Alert C=CCO
12	2-methyl-2-buten-1-ol	Cramer Class I	No alert	No alert	Not classified	No alert	Alert C=CCO

ID	Name	Toxicophores[1]	Structural alerts[1,2]				
			DNA binding by OECD[1]	Protein binding by OECD[1]	Protein binding potency (GSH)[1]	In vivo mutagenicity (Micronucleus) alerts by ISS[1]	Mitochondria toxicity[2]
13	2-methyl-2-penten-1-ol	Cramer Class I	No alert	No alert	Not classified	No alert	Alert C=CCO
14	3-methyl-2-buten-1-ol	Cramer Class I	No alert	No alert	Not classified	No alert	Alert C=CCO
15	3-methyl-3-penten-2-ol	Cramer Class I	No alert	No alert	Not classified	No alert	Alert C=CCO
16	4-methyl-3-penten-2-ol	Cramer Class I	No alert	No alert	Not classified	No alert	Alert C=CCO

[1] OECD QSAR Toolbox 3.3.5; [2] COSMOS profiler available at: http://knimewebportal.cosmostox.eu/

Table 6B. Comparison of Toxicophores for metabolites

ID	Name	Toxicophores[1]	Structural alerts[1]				
			DNA binding by OECD	Protein binding by OECD	Protein binding potency (GSH)	Carcinogenicity (genotox and nongenotox) alerts by ISS	*In vivo* mutagenicity (Micronucleus) alerts by ISS
1	2-propenal (acrolein)	Cramer Class II	Michael addition	Michael addition, Schiff Base Formers	Extremely reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
2	2-butenal (crotonal dehyde)	Cramer Class I	Michael addition	Michael addition, Schiff Base Formers	Highly reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
3	trans-2-pentenal	Cramer Class I	Michael addition	Michael addition, Schiff Base Formers	Highly reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
4	trans-2-hexenal	Cramer Class I	Michael addition	Michael addition, Schiff Base Formers	Highly reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
5	methyl vinyl ketone	Cramer Class II	Michael addition	Michael addition	Extremely reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls

ID	Name	Toxicophores[1]	Structural alerts[1]				
			DNA binding by OECD	Protein binding by OECD	Protein binding potency (GSH)	Carcinogenicity (genotox and nongenotox) alerts by ISS	*In vivo* mutagenicity (Micronucleus) alerts by ISS
6	ethyl vinyl ketone	Cramer Class II	Michael addition	Michael addition	Extremely reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
7	propyl vinyl ketone	Cramer Class II	Michael addition	Michael addition	Extremely reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
8	3-penten-2-one	Cramer Class I	Michael addition	Michael addition	Highly reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
9	3-hexen-2-one	Cramer Class I	Michael addition	Michael addition	Highly reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
10	4-hexen-4-one	Cramer Class I	Michael addition	Michael addition	Highly reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls

ID	Name	Toxicophores[1]	Structural alerts[1]				
			DNA binding by OECD	Protein binding by OECD	Protein binding potency (GSH)	Carcinogenicity (genotox and nongenotox) alerts by ISS	*In vivo* mutagenicity (Micronucleus) alerts by ISS
11	2-methyl acrolein	Cramer Class II	Michael addition	Michael addition, Schiff Base Formers	Moderately reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
12	2-methyl-2-butenal	Cramer Class I	Michael addition	Michael addition, Schiff Base Formers	Moderately reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
13	2-methyl-2-pentenal	Cramer Class I	Michael addition	Michael addition, Schiff Base Formers	Moderately reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
14	3-methyl-2-butenal	Cramer Class I	No alert	Schiff Base Formers No MA alert	Moderately reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls
15	3-methyl-3-penten-2-one	Cramer Class I	Michael addition	Michael addition	Highly reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls

ID	Name	Toxicophores[1]	Structural alerts[1]				
			DNA binding by OECD	Protein binding by OECD	Protein binding potency (GSH)	Carcinogenicity (genotox and nongenotox) alerts by ISS	*In vivo* mutagenicity (Micronucleus) alerts by ISS
16	4-methyl-3-penten-2-one	Cramer Class I	No alert	No alert	Highly reactive	Genotoxic carcinogenicity, α,β-unsaturated carbonyls	α,β-unsaturated carbonyls

[1] OECD QSAR Toolbox 3.3.5

Table 7. Comparison of Mechanistic Plausibility and AOP-Related Event Data

ID	Name	Mechanistic Plausibility	Adverse Outcome Pathway or Mode of Toxic Action:	Molecular Initiating Event:	Key Event 1 etc.:	Key Event Relationship 1 etc.:	Other Mechanistically-Relevant Events
1	2-propen-1-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
2	2-buten-1-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
3	2-penten-1-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
4	2-hexen-1-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
5	1-buten-3-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
6	1-penten-3-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
7	1-hexen-3-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
8	3-penten-2-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
9	3-hexen-2-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity

ID	Name	Mechanistic Plausibility	Adverse Outcome Pathway or Mode of Toxic Action:	Molecular Initiating Event:	Key Event 1 etc.:	Key Event Relationship 1 etc.:	Other Mechanistically-Relevant Events
10	4-hexen-3-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
11	2-methyl-2-propen-1-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
12	2-methyl-2-buten-1-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
13	2-methyl-2-penten-1-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
14	3-methyl-2-buten-1-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
15	3-methyl-3-penten-2-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity
16	4-methyl-3-penten-2-ol		Apoptosis or necrosis	Covalent binding of reactive metabolite with thiols			Hepatotoxicity

Table 8. Comparison of Toxicologically Relevant *In Vivo*, *In Vitro* and *Ex Vivo* Data

Name / Endpoint	2-propen-1-ol	2-buten-1-ol	2-penten-1-ol	2-hexen-1-ol	1-buten-3-ol	1-penten-3-ol	1-hexen-3-ol	3-penten-2-ol	3-hexen-2-ol	4-hexen-3-ol	2-methyl-2-propen-1-ol	2-methyl-2-buten-1-ol	2-methyl-2-penten-1-ol	3-methyl-2-buten-1-ol	3-methyl-3-penten-2-ol	4-methyl-3-penten-2-ol
NOAEL (Repeat dose toxicity)	3-11.6 (mg/kg bw/day) 20-400 (ppm) 12 (mg/m^3) [1-5]													65.4-82.1 (mg/kg bw/day) [9]		
NOEL (Repeat dose toxicity)	1.37 (mg/kg/day) [6]													14.4-21 (mg/kg bw/day) [9]		
LOAEL (Repeat dose toxicity)	47 (mg/m^3) 6-34 (mg/kg/day) [1, 4, 7]													243.8-307.2 (mg/kg bw/day) [9]		
LOEL (Repeat dose toxicity)	4.8-87.1 (mg/kg/day) [2, 6, 8]															
NOAEL (Reproductive toxicity)	8 (mg/kg/day) 48.2-58.4 (mg/m^3) [2, 3]															
NOAEL (Teratogenicity)	10 (mg/kg/day) [10]															
	10 (mg/kg/day)															

Name / Endpoint	2-propen-1-ol	2-buten-1-ol	2-penten-1-ol	2-hexen-1-ol	1-buten-3-ol	1-penten-3-ol	1-hexen-3-ol	3-penten-2-ol	3-hexen-2-ol	4-hexen-3-ol	2-methyl-2-propen-1-ol	2-methyl-2-buten-1-ol	2-methyl-2-penten-1-ol	3-methyl-2-buten-1-ol	3-methyl-3-penten-2-ol	4-methyl-3-penten-2-ol
LOAEL (Maternal toxicity)	[7]															
NOEL (Reproductive toxicity)	40 (mg/kg/day) [3]															
Carcinogenic/ Genotoxicity	5 x Negative [11-13]															
LC50 (Acute toxicity)	140-2130 (mg/m^3) 500 (mg/m^3/2H 75 (ppm/8H) 50->400 (ppm) [1, 5, 14-16]															
LD50 (Acute toxicity)	37 -105 (mg/kg) [1,14, 16-20]	1084 -793 (mg/ kg) [21, 37]		3500 (mg/ kg) [22]	50 (ppm) [17]	70 (mg/ kg) [23]	450 (mg/ kg) [24]				2924 (ppm) 2-500 (mg/ kg) [25]		3 (mL/ kg) 4920 (mg/ kg) [26]	810-3900 (mg/kg) [27-30]		
	64-76 (U/L)															

Name / Endpoint	2-propen-1-ol	2-buten-1-ol	2-penten-1-ol	2-hexen-1-ol	1-buten-3-ol	1-penten-3-ol	1-hexen-3-ol	3-penten-2-ol	3-hexen-2-ol	4-hexen-3-ol	2-methyl-2-propen-1-ol	2-methyl-2-buten-1-ol	2-methyl-2-penten-1-ol	3-methyl-2-buten-1-ol	3-methyl-3-penten-2-ol	4-methyl-3-penten-2-ol
hepatic fibrosis	[31]															
Genotoxicity (AMES, Chromosomal aberration, gene mutation)	6x Negative 5 x Positive [11, 13, 32-36]													Negative [30]		

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