Read-across of 90-Day Oral Repeated-Dose Oral Toxicity for β-Olefinic Alcohols: A Case Study of Compounds with Similar Metabolism

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Executive Summary:

The elicitation of a common metabolite is one of the means of establishing similarity between compounds to allow for read-across. Beta-olefinic alcohols provide an excellent example of such an instance where the primary issue for establishing similarity for the chemical category and the key element of uncertainty in accepting read-across predictions is rooted in metabolism. This – read-across case study has been developed for the repeat dose toxicity of short chain primary and secondary β-olefinic alcohols. The pivotal issues in the applying read-across to this proposed category whether: 1) all the β-olefinic alcohols considered are transformed to metabolites having the same mechanism of electrophilic reactivity; 2) the metabolic pathway is the same for all compounds in the category; 3) the rates of transformation are sufficient so that the reactive metabolites are the definitive toxicant for the endpoint being read across; and 4) the metabolites are similar in reactive potency. The applicability domain for this case study was limited to small (i.e., carbon (C) atom chain lengths from C3 to C6) primary and secondary β-olefinic alcohols. Mechanistically, these β-unsaturated alcohols are considered to be readily metabolised by alcohol dehydrogenase (ADH) 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 main route of exposure for β-olefinic alcohols is oral with direct absorption from the upper gastrointestinal tract. They are distributed unbound in the blood and are subsequently readily enzymatically oxidised, especially in the liver, to form reactive metabolites.

The category considered in this case study was confined to selected subclasses of β-olefinic alcohols from C3 to C6. In addition to the structurally unique 2-propen-1-ol (i.e., 1-propen-3-ol; allyl alcohol), the category included: straight-chain primary alcohols (e.g., 2-alken-1-ols), straight-chain secondary alcohols (e.g., 1-alken-3-ols and 3-alken -2-ols), branched primary alcohols (e.g., 2-methyl-2-alken-1-ols and 3-methyl-2-alken-1-ols) and branched secondary alcohols (e.g., 3-methyl-3-alken-2-ols and 4-methyl-3-alken-2-ols). There were only 90 day repeated-dose toxicity test results for 2-propen-1-ol and 3-methyl-2-buten-1-ol. The reported 90-day oral repeated dose toxicity No Observed Adverse Effect Levels (NOAELs) in rats for 2-propen-1-ol were 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. The 90-day oral repeated dose NOAELs for 3-methyl 2-buten-1-ol in rat were reported based on the decreased food and water consumption: 65.4 mg/kg bw/day in males and 82.1 mg/kg bw/day in females.

Compounds representing each of five sub-structural groups of β-olefinic alcohols were tested in an *ex vivo* model, the 2-hr rat isolated perfused liver assay, are consistent with metabolic activation to soft electrophiles. Specifically, all tested primary and secondary β-olefinic alcohols 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 olefinic α, β-unsaturated carbonyl compounds such as those derived from hepatic metabolism of primary and secondary olefinic β-unsaturated alcohols readily react with GSH. Specifically, α, β-unsaturated carbonyl derivatives of straight-chain alcohols:1-alken-3-ols, 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.

A human cell-based hepatic organoid *in vitro* model was used to assess fibrosis of selected β-unsaturated alcohols. 2-propen-1-ol, 2-buten-1-ol, 1-buten-3-ol, 3-methyl-2-buten-1-ol, as well as the β-acetylenic alcohols 2-methyl-3-butyn-2-ol and 2-propyn-1-ol were evaluated. Briefly, mRNA expression of the hepatic stellate cells (HSC) activation markers COL1A1, COL3A1 and LOXL2 were checked in the hepatic organoids upon exposure to the different alcohols. Strong induce (i.e., >3-4 fold up-regulation) of at least two out of three HSC markers was observed with the four olefinic alcohols. This pattern was not observed with the two β-acetylenic alcohols.

The *in vivo*, *ex vivo*, *in vitro* and *in chemico* data support the read-across premise. Specifically, all the category members are highly likely 1) transformed to metabolites having the same mechanism of electrophilic reactivity (i.e., Michael acceptors), 2) metabolized via the same pathway (i.e., ADH-mediated), and 3) to have rates of transformation sufficient so the reactive metabolites are the definitive toxicant for repeated-dose. However, the category members have metabolites with different reactive potencies (i.e., GSH RC50 values). In order to reduce the uncertainty associated with reactivity, the category is sub-categorised into straight-chained and branched derivatives. With acceptable uncertainty, the rat oral 90-day repeated-dose toxicity data for 2-propen-1-ol may be read across to fill data gaps for straight-chained analogues and the rat oral 90-day repeated-dose toxicity data for 3-methyl-2-buten-1-ol may be read across to fill data gaps for branched analogues.

Introduction

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 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 case study has been designed to illustrate specific issues associated with the read-across approach and stimulate discussion on how to best address them. It is not intended to be related to any regulatory discussions on this group of chemicals.

One of the crucial issues in toxicological read-across is addressing substances that are altered metabolically to more toxic species (Berggren et al., 2015).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.

In the present study, the "Strategy for Structuring and Reporting a Read-Across" (Schultz et al., 2015) was applied to undertake this proof-of-concept study. The read-across scenario is chemical similarity as a result of metabolism to the same toxic metabolite. Therefore, the parent compounds are not the ultimate toxicants; rather, the metabolite(s) which acting by a common mode of toxic action are considered as the definitive toxicants.

The present case study is for olefinic β-unsaturated alcohols where the read-across based on *in vivo* data was supplemented by appropriate *ex vivo*, *in vitro*, *in chemico* and classic structure-activity relationship information. The goal was to reduce uncertainty associated with the *in vivo* prediction by the increased weight-of-evidence provided by the alternative methods data, which make reduce uncertainty and hence potentially make the prediction more acceptable in the regulatory context.

Olefinic β-unsaturated alcohols vary in molecular structure with these structural variations impacting on reactivity and toxicity. While all olefinic alcohols contain a C=C moiety, they can be sub-divided further according to whether the olefinic alcohol is beta-, or non-beta- 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 (Bradbury and Christensen, 1991; Schwöbel et al., 2011). 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 (Mekenyan et al., 1993; Schultz et al., 2007; Yarbrough and Schultz, 2008).

Among the β-olefinic alcohols, 2-propen-1-ol (1-propen-3-ol; allyl alcohol) is the best studied chemical with the greatest quantity of toxicological data and information. 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 (Badr et al., 1986). 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 leads to liver fibrosis (Atzori et al., 1989; Jung et al., 2000). Liver fibrosis is a reversible response to acute or chronic hepatocyte injury (Bataller and Brenner, 2005; Lee and Friedman, 2011; Trautwein et al., 2015). The mechanistic rational is that the parent alcohol is relatively non-toxic however its metabolite acrolein, an α, β-unsaturated aldehyde, is a Michael-type soft electrophile. Such electrophiles preferentially covalently interact with thiol groups in proteins leading to necrotic or apoptotic cell death (Strubelt et al., 1999). 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 (Friedman, 2008).

Historically, the hepatotoxic action of 2-propen-1-ol to rodent liver is related to oxidative metabolism into acrolein, which in turn can bind covalently to proteins in periportal hepatocytes (Reid, 1972; Serafini-Cessi, 1972). 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 (Patel et al., 1983). Two-propen-1-ol exerts a dose-dependent toxicity on cells, which is inversely related to cellular glutathione (GSH) (Ohno et al., 1985). 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 (Atzori et al., 1989). The toxicity of 2-propen-1-ol can be prevented by inhibitors of ADH and augmented by the aldehyde dehydrogenase (ALDH) inhibitor disulfiram (Ohno et al., 1985).

In vivo oral exposure to 2-propen-1-ol leads to periportal necrosis and subsequent connective tissue development (Badr et al., 1986; Jung et al., 2000). 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 are evident.

3-methyl-2-buten-1-ol is far less studied (ECHA Study report 2002; OECD 2003; Mc Ginty et al., 2010) with fewer toxicological data. In 90-day drinking water exposed rats, substance-related effects were seen at the high and mid dose level (i.e., 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). Specifically, in the mid dose groups, decreased food and water consumption in male rats and reduced water consumption in female rats was noted. Body weight was significantly impaired at the high dose in both sexes. In the mid and high dose, the mean absolute liver weights in male rats were significantly decreased, but not the relative liver weights. No other significant treatment related changes were observed.

Read-Across Exercise

1. Statement target substance(s) and the regulatory endpoint(s) that is to be read across.

There are no *in vivo* repeated-dose data for the vast majority of β-olefinic alcohols. However, there are strong 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 [107-18-6] as a read-across analogue for other β-olefinic alcohols, especially unbranched compounds.

2. Description of the analogues or members of the category.

2.1. Premise

The hypothesis 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.*
- *While 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 metabolized, 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.*

2.2. Justification

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 (Klinger et al., 1986), while the rat oral LD50 for the saturated isomer 1-propanol is 1870 mg/kg (Smyth et al., 1954).

Several 90-day oral repeated-dose toxicity evaluations of 2-propen-1-ol have been conducted. 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 for 13 weeks (females) and 0, 0.13, 0.62, 5.9, 11.6, 25.5, 41.0, and 72.0 mg/kg bw/day (male) (Dunlap et al., 1958). 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) (Dunlap et al., 1958).

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 (Carpanini et al., 1978). 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 (NTP, 2006). 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. The sex difference in 2-propen-1-ol hepatotoxicity in rats was also reported by Rikans and Moore (1987) 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 vacuolization occurred in 12 mg/kg or greater in females; whereas in males, this lesion was first

observed at 25 mg/kg (NTP, 2006). 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.

The second β-olefinic alcohol tested in acute toxicity as well as in 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 (Belsito et al., 2010).

To our knowledge, only one 90-day oral repeated-dose toxicity evaluation of 3-methyl-2-buten-1-ol has been conducted (see ECHA Study report 2002; OECD 2003; Mc Ginty et al., 2010). Following OECD test guideline 408, 3-methyl-2-buten-1-ol was administered to groups of 10 male and 10 female Wistar rats were exposed 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 (ECHA Study report 2002). Substance related effects were seen at the high and mid dose level. In the mid dose groups, decreased food and water consumption in male rats and reduced water consumption in female rats was 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 (Belsito et al., 2010). 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. (ECHA Study report 2003).

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. These ranges of NOAEL values are 10-100 times smaller than those reported for saturated derivatives.

2.3. Applicability domain

The applicability domain for this read-across was confined to subclasses of β-unsaturated 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). A key structural feature was the presence of a free H-atom on the hydroxyl-containing C-atom. Hence, tertiary isomers were not considered as members of this category.

2.4. Analogues or category members

Sixteen β-olefinic alcohols were chosen initially to form a category for the read-across case study and are listed in Table 1. The additional identifiers/information, such as chemical structures and molecular formulas are available in the Table 1 in Annex 1. The additional chemical, 2-propyn-1-ol (i.e., propargyl alcohol, 1-propyn-3-ol) represents the β-acetylenic alcohols. Similar to the potential source substance, 2-propen -1-ol, it is well studied chemical with *in vivo* and *in vitro* data, therefore can serve as "positive control", supporting the evidence for toxicity of β-unsaturated alcohols. Based on extended structural fragments, the β-olefinic alcohol category includes five sub-groups. 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.

Name	CAS No	SMILES	Type of Alcohol	
Straight-chained				
1) 2-Propen-1-ol (1-propen-3-ol; allyl alcohol)	107-18-6	OCC=C	prim. allylic	terminal OH & C=C
5) 2-Buten-1-ol	6117-91-5	OCC=CC	prim. allylic	terminal OH, internal C=C
6) 2-Penten-1-ol	20273-24	OCC=CCC	prim. allylic	terminal OH, internal C=C
7) 2-Hexen-1-ol	2305-21-7	OCC=CCCC	prim. allylic	terminal OH, internal C=C
2) 1-Buten-3-ol (3-buten-2-ol)	598-32-3	C=CC(O)C	sec. allylic	internal OH, terminal C=C
3) 1-Penten-3-ol	616-25-1	C=CC(O)CC	sec. allylic	internal OH, terminal C=C
4) 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)
β-acetylenic				
17) 2-Propyn-1-ol (1-propyn-3-ol)	107-19-7	OCC#C	prim. propargylic	terminal OH & C#CC)

2.5. Purity/impurities

The purity/impurity profile for the analogues listed in 2.4 is unknown. However, since the category is structurally limited, the potential impact of any impurities on the endpoint being considered is very limited. The most likely impurities are other isomers (e.g. *cis* vs. *trans* conformations).

3. Data matrices for assessing similarity

The data supporting the similarity argument for the analogues listed in section 2.4 are reported in Annex 1.

3.1. Structural similarity

As demonstrated in Table 1 of Annex I all the β-olefinic alcohols include 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, another potential category member, 3-methyl-2-buten-1-ol, is dissimilar as it has an alkyl substituent on the olefinic carbon that can inhibit the protein binding site of the vinyl group.

3.2. Chemical property similarity

As demonstrated in Table 2 of Annex I, all the β-olefinic alcohols include in the category have very narrow value ranges 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.

3.3. Chemical constituent similarity

As demonstrated in Table 3 of Annex I, 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.

3.4. Toxicokinetic similarity

As demonstrated in Table 4 of Annex I, 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 (Pietruszko et al., 1973). 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 (Pietruszko et al., 1973). 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) (Pietruszko et al., 1973). 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 (Klesov et al., 1977). The activity of the enzyme appears to be dependent on the lipophilic character of the alcohol.

Fontaine et al. (2002) studied the enzymatically 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 oxidized 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 (2002) 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. The corresponding carboxylic acid may enter the β-oxidation pathway and be subsequently metabolized 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 oxidized to ketones, which cannot be further oxidized. 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.

3.5. Metabolic similarity

As demonstrated in Table 5 of Annex I, all of the β-olefinic alcohols included in the category are predicted by *in silico* tools to be metabolized to the corresponding α, β-unsaturated aldehydes or α, β-unsaturated ketones. These soft electrophiles subsequently react with GSH and protein thiols in hepatocytes (Boyland and Chasseaud, 1967; Fontaine et al., 2002).

From a structural standpoint, only primary and secondary β-olefinic alcohols are able to be activated by ADH to form polarized α, β-unsaturated electrophiles (Bradbury and Christensen, 1991). The availability of H-atoms on the C-atom with hydroxyl OH group is crucial to the metabolic activations 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 metabolized to Michael acceptor electrophilic derivatives by ADH (Veith et al., 1989). It follows that primary β-olefinic alcohols are likely to be more readily converted to reactive metabolites than secondary ones.

Based on metabolic similarity, the read-across category is limited to primary and secondary β-olefinic alcohols. The finding of Moridani et al. (2001) 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 (Moridani et al., 2001).

3.6. Toxicophore similarity

As demonstrated in Tables 6A and 6B of Annex I, based on *in silico* predictions, only the metabolites of β-olefinic alcohols and not the parent compounds triggered the OECD protein binding profiler within the OECD QSAR Toolbox v3.3.5. The all metabolites of β-olefinic alcohols, but 4-methyl-3-penten-2-one are associated with Michael addition or Schiff base formation mechanisms. Moreover, the potency of protein binding varies between the five sub-structure groups what can be accounted for sub-categorisation of β-olefinic alcohols.

3.7. 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 (Schwöbel et al., 2011). As demonstrated in Table 7 of Annex I, the β-olefinic alcohols included in the category are associated via the ADH-induced Michael addition mechanism of action. This mechanism is based on covalent interaction with thiols (Schwöbel et al., 2011).

As noted by Richarz et al. (2013), 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 does-dependent necrosis or mitochondrial-based apoptosis; whereas liver and kidney are the target organs.

Landesmann et al. (2012) 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. (1999) 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 (Strubelt et al., 1999). *In vitro* toxicity profiles for selected β-unsaturated alcohols are reported in Table 2.

Table 2. *In vitro* toxicity profiles for β-olefinic alcohols.

	Name	LDH (U/l)	O2 (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
5)	2-Buten-1-ol	10977 ± 2433	0.47 ± 0.06	0.11 ± 0.01	0.02 ± 0.01
6)	2-Penten-1-ol				
7)	2-Hexen-1-ol				
2)	1-Buten-3-ol	25756 ± 1355	0.19 ± 0.04	0.09 ± 0.00	0.03 ± 0.00
3)	1-Penten-3-ol				
4)	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	3.39 ± 0.45
19)	1-Butanol	8946 ± 2411	0.98 ± 0.40	0.88 ± 0.09	3.76 ± 0.72
20)	1-Pentanol	28959 ± 4142	0.06 ± 0.01	0.22 ± 0.03	2.82 ± 0.36
21)	2-Methyl-1-propanol	11499 ± 2898	0.88 ± 0.10	0.53 ± 0.05	2.38 ± 0.99

22)	3-Methyl-1-butanol	8680 ± 1216	0.22 ± 0.07	0.10 ± 0.01	1.33 ± 0.29
23)	2-Methyl-2-butanol	9353 ± 2582	1.13 ± 0.33	0.62 ± 0.23	1.63 ± 0.25
24)	2-Methyl-3-butyn-2-ol	2078 ± 1524	1.20 ± 0.20	0.68 ± 0.07	1.68 ± 0.33

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 (Strubelt et al., 1999).

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 metabolized and give rise to a metabolite of similar potency to 2-propen-1-ol and thus very likely to cause similar repeated-dose toxicity. The data in Table 2 also support the structural selectivity to the category as tertiary β-unsaturated alcohols, as well as alkanols, do not reduce GSH (i.e., are not metabolized 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 (Hillbom et al., 1974). Food consumption, body weight gain, and liver histopathology were comparable to those of the control group. Additionally, the 90 days 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 (Pilegaard et al., 1993).

3.8. Other endpoint similarity

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

Acrolein is unique among the α, β-unsaturated carbonyl compounds as it alone is the only molecular structure having both a terminal vinyl group and a terminal carbonyl group. These structural feature associated with relative reactivity of polarized α, β-unsaturated molecules, especially where an olefinic moiety conjugated to a carbonyl group, toward the model nucleophile glutathione, have been examined (Schultz et al., 2005). 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 (Schwöbel et al., 2011). 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 (Schultz et al., 2005; Schwöbel et al., 2011). *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.

	Alcohol	Metabolite	Metabolite SMILES	GSH RC_{50}
Straight-chained				
1)	2-Propen-1-ol	2-Propenal (acrolein)	O=CC=C	0.085
5)	2-Buten-1-ol	2-Butenal (crotonaldehyde)	O=CC=CC	0.22
6)	2-Penten-1-ol	trans-2-Pentenal	O=CC=CCC	0.35
7)	2-Hexen-1-ol	trans-2-Hexenal	O=CC=CCC	0.42
2)	1-Buten-3-ol	Methyl vinyl ketone	C=CC(=O)C	0.070
3)	1-Penten-3-ol	Ethyl vinyl ketone	C=CC(=O)CC	0.051
4)	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		not reactive at 1000 mg/l
18)	1-Butanol	1-butanal/1-butyric acid		not reactive at 1000 mg/l
19)	1-Pentanol	1-pentanal/1-pentanoic acid		not reactive at 1000 mg/l
20)	2-Methyl-1-propanol	2-methyl-1-propanal/2-methyl-1-propionic acid		not reactive at 1000 mg/l
21)	3-Methyl-1-butanol	3-methyl-1-butanal/2-methyl-1-butyric acid		not reactive at 1000 mg/l
22)	2-Methyl-2-butanol	2-methyl-2-butanone		not reactive at 1000 mg/l
23)	2-Methyl-3-butyn-2-ol	not metabolized		not reactive at 500 mg/l
24)	2-Methyl-3-buten-2-ol	not metabolized		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: 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 carbon-carbon double bond on 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 thiol reactivity experiments (see Table 3) suggest that the ability of α, β-unsaturated carbonyl compounds other than acrolein (and thus, β-olefinic alcohol other than 2-porpen-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, this mode of toxic action involves metabolism of the parent alcohol to the corresponding α, β-unsaturated aldehyde or ketone via alcohol dehydrogenase (Lipnick, 1985; Veith et al., 1989). The conventional thinking is that, while the parent aliphatic alcohols elicit baseline narcosis toxic action, the metabolites are electrophilic toxicants. Specifically, the metabolites are polarized α, β-unsaturated chemicals which undergo a Michael-type addition to soft nucleophilic sites in proteins (Schwöbel et al., 2011). Bradbury and Christensen (1991) confirmed the role of alcohol dehydrogenase activity in metabolic activation and enhanced toxicity in fish. Specifically, the alcohol dehydrogenase in the gill epithelial cells metabolizes 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 (McKim et al., 1987).

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 (Lipnick, 1985; Lipnick et al., 1987). 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 (Veith et al., 1989).

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.

4. Statement of uncertainty in similarity

Data uncertainty and weight-of-evidence associated with the fundamentals of chemical, transformation/toxicokinetic and toxicological similarity (i.e., toxicokinetic and toxicodynamic similarity of category members) is low-to-moderate. In Annex II, the assessment of uncertainties is presented. In terms of chemistry, the complex extended fragment of the applicability domain of this category leads to moderate similarity of all analogues or category members. Specifically, the key feature, being a primary or secondary β-unsaturated alcohol of short-chain length is common within the category and relevant to the toxicity read across. There are differences among the category members with respect to 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 with alkyl group (e.g., methyl group). Amongst the category members, 2-propen-1-ol is a structurally unique β-olefinic alcohol with both a terminal vinyl group and a terminal hydroxyl. Such 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 questionable if these short-term (i.e., 2-hour) differences are relevant to repeated-dose toxicity.

From a toxicokinetic standpoint, there is low-to-medium uncertainty. The narrow range of carbon atoms of the applicability domain limits the impact of bioavailability. The most likely metabolic pathway of all analogues is considered to be metabolized via ADH oxidation to similar reactive derivatives eliciting the same mechanism of chemical reactivity. This metabolic activation is supported indirectly by the results of the liver profusion studies by Strubelt et al. (1999). However, other metabolic mechanisms, such as ROS formation or P450 activation, cannot be completely ruled out.

All analogues or category members are considered, from a toxicodynamic standpoint, to be only moderately similar. Only two category members: 2-propen-1-ol and 3-methyl-2-buten-1-ol have

been evaluated in mammals for *in vivo* repeated-dose effects. For the *in vivo* acute toxicity, nine of 16 analogues have been tested in mammals.

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 molecular mechanism, soft electrophilic reactivity, is well understood. There is a qualitative adverse outcome pathway available linking electrophilic reactivity via ADH-mediated metabolism to cellular necrosis and/or apoptosis. 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.

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 alcohols is not exhibited *ex vivo* in liver profusion tests.

Uncertainty associated with mechanistic relevance and completeness of the read-across following the traditional exercise is medium. Briefly, uncertainty associated with this read-across stems from the facts that: 1) one source substance, allyl alcohol, is a unique β-olefinic alcohol and is metabolized to a unique electrophile, acrolein, 2) the most likely mode-of-action, liver fibrosis is not 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.

5. Reducing uncertainty by using new methods information

The new methods data reported here are not intended to be inclusive. Rather they are designed to demonstrate how new methods data may aid in reducing uncertainties associated with this read-

across case study. Further studies may be required to validate that the bioactivation in the new methods is part of and similar to what is observed under *in vivo* situations.

The aims were to use this new method data to improve the description and understanding of toxicodynamic similarity and reduce uncertainty associated with *in vivo* read-across from 2-propen-1-ol to other β-olefinic alcohols. Specifically, 2-buten-1-ol, 1-buten-3-ol, 3-methyl-2-buten-1-ol, as well as the β-acetylenic alcohols 2-methyl-3-butyn-2-ol and 2-propyn-1-ol were evaluated. The first three alcohols were expected to give similar results to 2-propen-1-ol, while 2-methyl-3-butyn-2-ol and 2-propyn-1-ol were expected to have dissimilar results. Since 2-methyl-3-butyn-2-ol is a tertiary unsaturated alcohol it was not expected to be metabolically activated via ADH to an electrophilic Michael acceptor. 2-propyn-1-ol which is activated via CYP 2E1 activity (Moridani et al., 2001) is toxicokinetically dissimilar.

An *in vitro* model was used to assess fibrosis of selected β-unsaturated alcohols. The model consists of hepatic organoids (3D co-culture) of human hepatocyte-like cells (HepaRG and primary human hepatic stellate cells (HSC). This culture model has shown to maintain good hepatocyte functionalities and maintain HSCs in a quiescent-like state for 3 weeks (Leite et al., 2015). Furthermore, 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 (Leite et al., 2015). Based on results with 2-propen-1-ol, the 3D HepaRG/HSC co-culture model was used to evaluate five other β-unsaturated alcohols.

Testing was conducted following the protocol of Leite et al. (2015). Briefly, HepaRG/HSC co-cultures and 3D HSC control cultures were generated in 96 round-bottom wells, 1 spheroid/well. Cultures were maintained for 3 weeks with medium exchange every second day. The study was conducted with test compounds in single and repeated fashion. For single dose assays, spheroids were exposed on day 20, while for repeated-dose testing spheroids were exposed on day 8, 10, 12, 14, 16, 18 and 20. In both cases, on day 21 cells were lysed either for ATP measurements (as a toxicity assessment) or mRNA analysis. For dose-response toxicity six different compound concentrations (i.e., 0, 0.064, 0.32, 8, 40 and 200 μM) were tested. Six individual spheroids were exposed and lysed separately, for each compound, concentration and exposure setup. For RNA, the analysis focused on one effective concentration (40μM), determined previously for 2-propen-

1-ol. Additionally, six individual spheroids were pooled for lysis, RNA extraction and analysis. The entire suite of assays was performed in duplicate.

Both single and repeated-dose assays revealed 2-propen-1-ol is toxic (i.e., reduction in % control ATP) at the high concentrations (i.e., 40 and 200 μM) with potency increasing for all tested concentrations upon repeated exposure. However, none of the other tested alcohols showed toxicity for the same concentrations.

The analysis of fibrosis-related gene expression was adopted as an easy and accurate way to screen HSC activation. *In vivo*, upon liver injury HSCs respond by activation which is accompanied by an increased transcription and production/secretion of extracellular matrix; once the injury is repeated the described phenotype will lead to the development of fibrosis. The up-regulation of HSC activation markers such as COL1A1, COL3A1 and LOXL2 at the mRNA level has been established as a way to detect HSC activation in the current 3D model (Leite et al., 2015).

Although it is known that hepatotoxicity leads *in vivo* to HSC activation, this is not a mandatory step and there are hepatocyte-mediated compound effects that also activate HSCs. For this reason the mRNA expression of the main HSC activation markers were checked in the hepatic organoids upon exposure to 40 μM of the different unsaturated alcohols (Table 4).

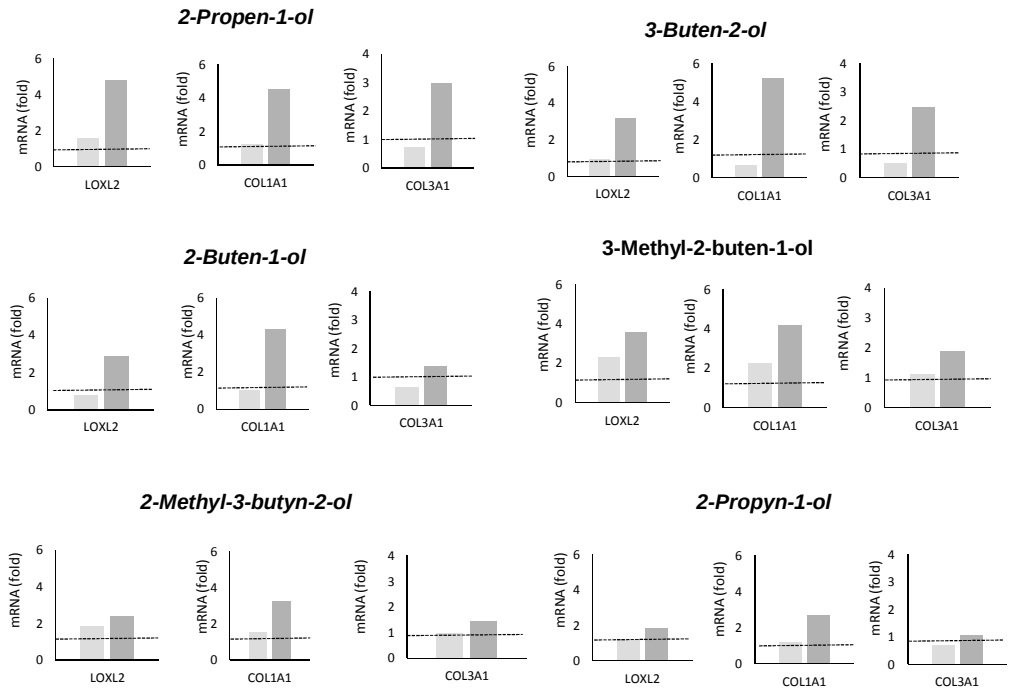
Table 4. Summary of COL1A1, COL3A1 and LOXL2 responses to selected β-unsaturated alcohols. Strong induction indicated by **, while weak induction is indicated by *.

	2-propen-1-ol	**1-buten-3-ol**	**2-buten-1-ol**	**3-methyl-2-buten-1-ol**	**2-propyn-1-ol**	**2-methyl-3-butyn-2-ol**
COL1A1	**	**	**	*	*	*
COL3A1	**	**		*		
LOXL2	**	**	**	*		

Bar graphs demonstrating gene induction are presented in Figure 1. Specifically, 2-propen-1-ol strongly induced (i.e., >3-4 fold up-regulation) the expression of all three tested markers, but only upon repeated exposure. This pattern (strong induction of 3 out of 3 tested markers after repeated exposure), is also observed for 1-buten-3-ol. The up-regulation of the three genes upon repeated exposure, although to a lesser extent, was also observed for 3-methyl-2-buten-1-ol,

however this compound also produces an effect in single exposure. 2-buten-1-ol up-regulates two out of the three tested genes (i.e., COL1A1 and LOXL2) in repeated exposure. The only alcohols that do not strongly induce (i.e., >3-4 fold up-regulation) of at least two out of three markers are 2-methyl-3-butyn-2-ol and 2-propyn-1-ol. Both of the latter alcohols only induce a 2-3 fold up-regulation of COL1A1 without induction of COL3A1 or LOXL2. The triple-bond-containing β- acetylenic alcohols do not exhibit 2-propen-1-ol-like up-regulates of HSC activation markers.

Figure 1. mRNA levels of LOXL2, COL1A1 and COL3A1 in HepaRG/HSC spheroid exposed to 40 μM β-unsaturated alcohols (fold increase with respect to control, GAPDH mRNA was used as a reference gene). Single dose responds in light grey and repeated-dose responds in dark grey.



In another new method study, van de Water and co-workers evaluated 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 (Wink et al., 2014). Briefly, they used the HepG2 BAC-GFP reporter system after induction with 2-propen-1-ol and five structurally-related analogues.

The six β-unsaturated alcohols were screened for a logarithmic dose range: 17.7 µM, 31.6 µM, 56.1 µM, 100 µM, 177 µM, 316 µM, 516 µM and 1000 µMIn addition, all alcohols with the exception of 2-methyl-3-butyn-2-ol were screened at 1770 µM while 2-propen-1-ol was also screened at 3160 µM and 5610 µM. Aflatoxin B1 (AB1)was used as a positive control for p53-mediated DNA damage response.

Stress response activation was evaluated at 24- and 48-hrs after exposure using Nikon confocal microscopy. HepG2 cells were cultured in conventional 2D monolayer and the 3D hydrogel based assay, which shows a more differentiated liver phenotype (Ramaiahgari et al., 2014). Green fluorescent protein (GFP) pixel intensity was measured per single cell for 2D monolayer data. GFP pixel intensity in 3D was measured per spheroid. Error bars were calculated over three replicates.

Since 2-propen-1-ol causes oxidative stress in primary human and rat hepatocytes as well as under *in vivo* circumstances initial work focused on the SRXN1 reporter. After 24-hrs, all tested β-unsaturated alcohols showed a significant and dose dependent activation of SRXN1-GFP expression in both the 2D and 3D assays. Aflatoxin B1 also caused up-regulation of the reporter activity with the point-of-departure concentration starting at 100 µM. 2D cultures were more sensitive to pick of the GFP-SRXN1 reporter activity. After 48-hrs limited up-regulation of SRXN1-GFP was seen both in 2D and 3D compared to 24-hrs. This is likely due to adaptation of the system to the oxidative stress. Nevertheless, a clear dose dependent effect was observed. From the 2D experiments, the fraction of cells where the GFP intensity is higher than two times DMSO GFP was determined. In both experiments: 24- and 48-hrs, more than 50% of the cells showed a response to all tested alcohols. Similarly to the overall SRXN1-GFP intensity, at 48-hrs the percentage of cells that demonstrated a response was less compared to 24-hrs.

The response for p21-GFP induction was also evaluated. Aflatoxin B1 caused an up to 20-fold up-regulation of p21-GFP, which was already clear at 17.7 uM. Significant up-regulation was observed for multiple concentrations for the tested β-unsaturated alcohols. An exception was noted for 3-methyl-2-buten-1-ol, which exhibited high variation between the three replicates. Similarly, for 3D the up-regulation of p21-GFP was strong for AB1; for the tested β-unsaturated alcohols a response was observed for 2-propen-1-ol, 2-propyn-1-ol and 2-methyl-3-butyn-2-ol. At 48-hrs, AB1 caused an even higher induction of p21-GFP reaching over 30-fold induction

under 2D situations; whereas only a mild significant up-regulation of P21-GFP after 48-hrs was observed for the alcohol analogues, with hardly any induction for 2-propen-1-ol and 1-buten-3-ol. The 3D situation showed significant up-regulation for one concentration of 2-propen-1-ol and 3-methyl-2-buten-1-ol at 48-hrs. In general, a limited percentage of cells responded to the tested analogues, all treatments showed maximal up to 20 % of the cells reaching the threshold of two times the negative control (i.e., DMSO) GFP intensity. In contrast, almost all cells responded to AB1.

The response for the BTG2-GFP reporter, which reflects a DNA damage response through activation of p53 was also examined. No significant induction was observed in 2D after 24-hrs of treatment, except for the positive control, AB1, which causes strong DNA damage at 17.7 µM. Also in 3D spheroids AB1 caused a strong activation of BTG2-GFP reporter activity. While exposure to some unsaturated alcohols (i.e., 2-propen-1-ol, 2-propyn-1-ol and 2-methyl-3-butyn-2-ol) resulted in a significant induction of BTG2-GFP; induction was not observed at all concentrations and the induction was not to the extent exhibited by AB1. After 48-hrs exposure, the average GFP intensity in 2D cultures showed some significant up-regulation for 2-propen-1-ol, 2-buten-1-ol, 2-propyn-1-ol, and 2-methyl-3-butyn-2-ol. However, no significant up-regulation is observed in 3D cultures. For all unsaturated alcohols, between 25% and 45% of cells were observed to express GFP intensity higher than two times DMSO GFP intensity.

Table 5. Summary of SRXN1-GFP, the p21-GFP and BTG2-GFP responses to selected β-unsaturated alcohols. Significant up-regulation at any concentration are indicated with an *.

	2-propen-1-ol	**1-buten-3-ol**	**2-buten-1-ol**	**3-methyl-2-buten-1-ol**	**2-propyn-1-ol**	**2-methyl-3-butyn-2-ol**
SRXN1 2D 24h	*	*	*	*	*	*
SRXN1 2D 48h		*	*	*	*	
SRXN1 3D 24h	*	*	*	*	*	*
SRXN1 3D 48h	*				*	
P21 2D	*	*	*		*	*

24h						
P21 2D 48h			*	*	*	*
P21 3D 24h	*				*	*
P21 3D 48h	*			*		
BTG2 2D 24h						
BTG2 2D 48h	*				*	*
BTG2 3D 24h	*				*	*
BTG2 3D 48h						

It can be concluded from these new methods data that:

- Straight chain β-olefinic alcohols induce the main HSC activation markers (i.e., COL1A1, COL3A1 and LOXL); the exception COL3A1 for 2-buten-1-ol is noted.
- Vinylic methyl-substituted β-olefinic alcohols weakly induce the main HSC activation markers tested.
- β-acetylenic alcohols typically do not induce the main HSC activation markers.
- β-unsaturated alcohols primarily activate an oxidative stress response, but not a DNA damage response.
- β-unsaturated alcohols all strongly activate the KEAP1/Nrf2 pathway reporter SRXN1-GFP.
- SRXN1-GFP activation is strongest at 24 hr exposure, likely due to adaptation towards oxidative stress and therefore reduced activity at later time points.
- β-unsaturated alcohols do not effectively activate the p21-GFP and BTG2-GFP reporter and responses are only generally observed >100 µM.

The premise that the 90-day rat oral repeated-dose toxicity of primary and secondary β-olefinic alcohols of short chain length (i.e., C3 to C6), acting as indirect-acting toxicants with the same covalent mechanism of action, is supported by the new methods data reported above. The new

methods data, while limited, also suggest that reduction in reactive potency is associated with HSC activation. While other metabolic mechanisms, such as ROS formation or P450 activation, cannot be completely ruled out, the new methods data are consistent with the ADH pathway of metabolic activation. Taken collectively, these findings demonstrated how new method data reduce the uncertainty associated with mechanistic relevance and completeness of the read-across (i.e., uncertainty in the predictions).

6. Statement of conclusions

The applicability domain for this case study is limited to small (C3 to C6) primary and secondary β-olefinic alcohols. The mechanistic argument is consistent with primary and secondary β-olefinic alcohols being readily metabolized by alcohol dehydrogenase (ADH) to polarized 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 or, as in the case of 3-methyl-2-buten-1-ol, to reductions in food and water uptake. 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.

Results for selected compounds representing each of the five sub-structural groups with the *ex vivo* model, the 2-hour rat isolated perfused liver, are consistent with the mechanistic hypothesis of metabolic activation via ADH to soft electrophiles. Specifically, all tested primary and secondary β-olefinic alcohols 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: 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 new *in vitro* method data reveal that β-unsaturated alcohols primarily activate an oxidative stress response, but not a DNA damage response. While straight chain β-olefinic alcohols induce the main HSC activation markers (i.e., COL1A1, COL3A1 and LOXL), the vinylic methyl-substituted β-olefinic alcohols only weakly induce these markers.

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 *ex vivo*, *in vitro* and *in chemico* data support the premise that 2-propen-1-ol, as the most potent analogue, can be read-across to other primary and secondary β-alkenols.

The *in vitro* and *in chemico* data, but not the *ex vivo* data support the argument for sub-categorization. In the sub-categorization scheme 2-propen-1-ol can be read-across to the 1-alken-3-ols and 2-alken-1-ols and 3-methyl-2-buten-1-ol is read across to 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.

The net result is the uncertainty as described by Schultz et al. (2015) for these β-olefinic alcohols is low-to-medium. Specifically, 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 for all regulatory decisions including risk assessment.

Read-across from 2-propen-1-ol to untested methyl-substituted β-olefinic alcohols is a conservative prediction which may estimate lower than likely repeated-dose potencies. The oral 90-day repeated-dose NOAEL values of 65.4 and 82.1 mg/kg bw/d in male and female rats, respectively, reported for 3-methyl-2-buten-1-ol, may be read across to untested branched β-olefinic alcohols (e.g., 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). However the uncertainty is higher.

Disclaimer: *This case study has been designed to illustrate specific issues associated with read-across and to stimulate discussion on the topic. It is not intended to be related to any currently ongoing regulatory discussions on this group of compounds. The background document has been prepared to facilitate the discussion at the Topical Scientific Workshop and does not necessarily represent ECHA's position. The papers are not final publications and are solely intended for the purposes of the Workshop.*

Acknowledgement: Authors acknowledge funding from the COSMOS Project which was funded by the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement number 266835 and the European Cosmetics Association Cosmetics Europe. This paper was also supported by a consultancy agreement with Cosmetics Europe.

References

Atzori, L., Dore, M. and Congiu, L. 1989. Aspects of allyl alcohol toxicity. Drug Metabol. Drug Interact. 7: 295-319.

Badr, M.Z., Belinsky, S.A., Kauffman, F.C., and Thurman, R.G. 1986. Mechanism of hepatotoxicity to periportal regions of the liver lobule due to allyl alcohol: role of oxygen and lipid peroxidation. J. Pharmacol. Exp. Ther. 238:1138-1142.

Bataller, R. and Brenner, D.A. 2005. Liver fibrosis. J. Clin. Invest. 115: 209-218.

Belsito, D., Bickers, D., Bruze, M., Calow, P., Greim, H., Hanifin, J.M., Rogers, A.E., Saurat, J.H., Sipes, I.G. and Tagami, H. 2010. A safety assessment of non-cyclic alcohols with unsaturated branched chain when used as fragrance ingredients. Food Chem. Toxicol. 48: 1S-S42.

Berggren, E., Amcoff, P., Benigni, R., Blackburn, K. Carney, E. Cronin, M., Deluyker, H., Gautier, F., Judson, R.S., Kass, G.E.N., Keller, D., Knight, D., Lilienblum, W., Mahony, C., Rusyn, I., Schultz, T., Schwarz, M., Schüürman, G., White, A., Burton, J., Lostia, A., Munn, S., and Worth, A. 2015. Chemical safety assessment using read-across: How can novel testing methods strengthen evidence base for decision-making? Environ. Health Perspect. doi: 10.1289/ehp.1409342.

Boyland, E. and Chasseaud, L.F. 1967. Enzyme-catalysed conjugations of glutathione with unsaturated compounds. Biochem. J. 104: 95-102.

Bradbury, S.P. and Christensen, G.M. 1991. Inhibition of alcohol dehydrogenase activity by acetylenic and allylic alcohols: Concordance with *in vivo* electrophilic reactivity in fish. Environ. Toxicol. Chem. 10: 1155-1160.

Castell, J.V., Gómez-Lechón, M.J., Ponsoda, X. and Bort, R. 1997. In vitro investigation of the molecular mechanisms of hepatotoxicity. Arch Toxicol Suppl. 19: 313-321.

Carpanini, F.M.B., Gaunt, I.F., Hardy, J., Gangolli, S.D., Butterworth, K.R., and Lloyd, A.G. 1978. Short-term toxicity of allyl alcohol in rats. Toxicology 9: 29-45.

Dunlap, M.K., Kodama, J.K., Wellington, J.S., Anderson, H.H. and Hine, C.H. 1958. The toxicity of allyl alcohol. A.M.A. Arch. Ind. Health 18: 303-311.

ECHA. Study report 2002. http://apps.echa.europa.eu/registered/data/dossiers/DISS-9d84b546-0f3d-2231-e044-00144f67d249/AGGR-73100920-372f-4a58-910b-f03d8e650766_DISS-9d84b546-0f3d-2231-e044-00144f67d249.html#AGGR-73100920-372f-4a58-910b-f03d8e650766 (accessed on 9 November 2015)

ECHA. Study report 2003. http://apps.echa.europa.eu/registered/data/dossiers/DISS-9d84b546-0f3d-2231-e044-00144f67d249/AGGR-cbeb7b6e-3295-4ae0-b869-c2c6da5edba9_DISS-9d84b546-0f3d-2231-e044-00144f67d249.html#AGGR-cbeb7b6e-3295-4ae0-b869-c2c6da5edba9

ECHA. Study report 2004 http://apps.echa.europa.eu/registered/data/dossiers/DISS-9d9caf30-12fa-6dc6-e044-00144f67d249/AGGR-52470ab1-4563-43a5-a37a-ac370ec78683_DISS-9d9caf30-12fa-6dc6-e044-00144f67d249.html#AGGR-52470ab1-4563-43a5-a37a-ac370ec78683

Fontaine, F.R.; Dunlop, R.A., Petersen, D.R. and Burcham, P.C. 2002. Oxidative bioactivation of crotyl alcohol to the toxic endogenous aldehyde crotonaldehyde: Association of protein carbonylation with toxicity in mouse hepatocytes. Chem. Res. Toxicol. 15: 1051-1058.

Friedman, S.L. 2008. Mechanisms of hepatic fibrogenesis. Gastroenterology 134: 1655-1669.

Hillbom, M.E., Franssila, K. and Forsander, O.A. 1974. Effects of chronic ingestion of some lower aliphatic alcohols in rats. Res. Commun. Chem. Pathol. Pharmacol. 9: 177-180.

Jenner, P.M. 1964. Food flavorings and compounds related structure 1. Acute oral toxicity. Food Cosmet. Toxicol. 2: 327-343.

Jung, S.A., Chung, Y.H., Park, N.H., Lee, S.S., Kim, J.A., Yang, S.H., Song, I.H., Lee, Y.S., Suh, D.J. and Moon, I.H. 2000. Experimental model of hepatic fibrosis following repeated periportal necrosis induced by allyl alcohol. Scand. J. Gastroenterol. 35: 969-975.

Klesov, A.A., Lange, L.G., Sytkowski, A.J. and Vallee, B.L. 1977. Unusual nature of the substrate specificity of alcohol dehydrogenase of different origins. Biorganich. Khim. 3: 1141-1144.

Klinger, W., Devereux, T., Maronpot, R. and Fouts, J. 1986. Functional hepatocellular heterogeneity determined by the hepatotoxins allyl alcohol and bromobenzene in immature and adult Fischer 344 rats. Toxicol. Appl. Pharmacol. 83: 108-111.

Landesmann, B., Goumenou, M., Munn, S., and Whelan, M. 2012. Description of prototype modes-of-action related to repeated-dose toxicity. JRC Scientific and Policy Report 75689.

Lee, U.E. and Friedman, S.L. 2011. Mechanisms of hepatic fibrogenesis. Best Pract. Res. Clin. Gastroentrol. 25: 195-206.

Leite, S.B., Roosens, T., El Taghdouini, A., Mannaerts, I., Smout, A.J., Najimi, M., Sokal, E., Noor, F., Chesne, C. and van Grunsven, L.A. 2015. Novel human hepatic organoid model enables testing of drug-induced liver fibrosis *in vitro.* Biomaterials 78: 1-10.

Lipnick, R.L. 1985. Validation and extension of fish toxicity QSARs and interspecies comparisons for certain classes of organic chemicals. In: Tichy M, editor. QSAR in toxicology and xenobiochemistry. Amsterdam: Elsevier. p. 39-52.

Lipnick, R.L., Watson, K.R. and Strausz, A.K. 1987. A QSAR study of the acute toxicity of some industrial organic chemicals to goldfish. Narcosis, electrophiles and proelectrophile mechanisms. Xenobiotica 17: 1011-1025.

Mc Ginty, D., Jones, L., Letizia, C.S. and Api, A.M. 2010. Fragrance material review on 3-methyl-2-buten-1-ol. Food Chem.Toxicol. 48: S64-S69.

McKim, J.M., Bradbury, S.P. and Niemi, G.J. 1987. Fish acute toxicity syndromes and their use in the QSAR approach to hazard assessment. Environ. Health Perspect. 71: 171-186.

Mekenyan, O.G., Veith, G.D., Bradbury, S.P. and Russom, C.L. 1993. Structure–toxicity relationships for alpha,beta-unsaturated alcohols in fish. Quant. Struct.-Act. Relat. 12: 132-136.

Moridani, M.Y., Khan, S., Chan, T., Teng, S., Beard, K. and O'Brien, P.J. 2001. Cytochrome P450 2E1 metabolically activates propargyl alcohol: propiolaldehyde-induced hepatocyte cytotoxicity. Chem.-Biol. Interact. 130-132: 931-942.

National Toxicology Program (NTP) 2006. Technical Report Number 48 on the Comparative Toxicity Studies of Allyl Acetate, Allyl Alcohol, and Acrolein. 178p.

Organization for Economic Co-Operation and Development (OECD) and Screening Information Datasets (SIDS) 2003. High Production Volume Chemicals 3-Methyl-2-butanol (Cas No.: 556-82-1). Processed by United Nations Environmental Program (UNEP). Available Online: http://www.chem.unep.ch/irptc/sids/OECDSIDS/.

Ohno, Y., Ormstad, K., Ross, D., and Orrenius, S. 1985. Mechanism of allyl alcohol toxicity and protective effects of low-molecular-weight thiols studied with isolated rat hepatocytes. Toxicol. Appl. Pharmacol. l78: 169-179.

Patel, J.M., Gordon, W.P., Nelson, S.D. and Leibman, K.C. 1983. Comparison of hepatic biotransformation and toxicity of allyl alcohol and [1,1-2H2]allyl alcohol in rats. Drug. Metab. Dispos. 11:164-166.

Pietruszko, R., Crawford, K. and Lester, D. 1973. Comparison of substrate specificity of alcohol dehydrogenases from human liver, horse liver and yeast towards saturated and 2-enoic alcohols and aldehydes. Arch. Biochem. Biophys. 159: 50-60.

Pilegaard, K. and Ladefoged, O. 1993. Toxic effects in rats of twelve weeks dosing of 2-propanol, and neurotoxicity measured by densitometric measurements of glial fibrillary acidic protein in the dorsal hippocampus. In Vivo 7: 325-330.

Ramaiahgari, S.C., den Braver, M.W., Herpers, B., Terpstra, V., Commandeur, J.N., van de Water, B. and Price, L.S. 2014. A 3D in vitro model of differentiated HepG2 cell spheroids with improved liver-like properties for repeated dose high-throughput toxicity studies. Arch. Toxicol. 88: 1083-1095.

Reid, W.D. 1972. Mechanism of allyl alcohol-induced hepatic necrosis. Experimentia 28: 1058-1061.

Research Institute for Fragrance Materials, Inc. (RIFM) 2002. 3-Methyl-2-buten-1-ol (Prenol): subchronic toxicity study in Wistar rats administered in drinking water for 3 months. Unpublished report from BASF. Report number 55401, 11 April RIFM, Woodcliff Lake, NJ, USA.

Richarz A.-N., Enoch, S.J., Hewitt, M., Madden, J.C., Przybylak, K., Yang, C., Berthold, M.R., Meinl, T., Ohl, P. and Cronin, M.T.D. 2013. Flexible and transparent computational workflows for the prediction of target organ toxicity. The Toxicologist – A Supplement to Toxicological Sciences 132: 183.

Rikans, L.E. and Moore, D.R. 1987. Effect of age and sex on allyl alcohol hepatotoxicity in rats: role of liver alcohol and aldehyde dehydrogenase activities. J. Pharmacol. Exp. Ther. 243: 20-26.

963 Schultz, T.W., Amcoff, P., Berggren, E., Gautier, F., Klaric, M., Knight, D. J., Mahony, C.,
964 Schwarz, M., White, A. and Cronin, M.T.D. 2015. A strategy for structuring and
965 reporting a read-across prediction of toxicity. Reg. Toxicol. Pharmacol. 72: 586-601.

966 Schultz, T.W., Yarbrough, J.W. and Johnson, E.L. 2005. Structure-activity relationships for
967 reactivity of carbonyl-containing compounds with glutathione. SAR QSAR Environ. Res.
968 16: 313-322.

969 Schwöbel, J.A.H., Koleva, Y.K., Enoch, S.J., Bajot, F., Hewitt, M., Madden, J.C., Roberts, D.W.,
970 Schultz, T.W. and Cronin, M.T.D. 2011. Measurement and estimation of electrophilic
971 reactivity for predictive toxicology. Chem. Rev. 111: 2562-2596.

972 Serafini-Cessi, F. 1972. Conversion of allyl alcohol into acrolein by rat liver. Biochem. J. 128:
973 1103-1107.

974 Smyth, H.F., Carpenter, C.P., Weil, C.S., and Pozzani, U.C. 1954. Range-finding toxicity data.
975 Arch. ind. Hyg. occup. Med. 10: 61-68.

976 Strubelt, O., Deters, M., Pentz, R., Siegers, C.-P. and Younes, M. 1999. The toxic and metabolic
977 effects of 23 aliphatic alcohols in the isolated perfumed rat liver. Toxicol. Sci. 49: 133-
978 142.

979 Trautwein, C., Friedman, S.L., Schuppan, D., and Pinzani, M. 2015. Hepatic fibrosis: Concept to
980 treatment. J. Hepatol. 62*:* S15-24.

981 Veith, G.D., Lipnick, R.L. and Russom, C.L. 1989. The toxicity of acetylenic alcohols to the
982 fathead minnow, *Pimephales promelas*-narcosis and proelectrophile activation.
983 Xenobiotica 19:555-565.

984 Vinken, M. 2013. The adverse outcome pathway concept: A pragmatic tool in toxicology.
985 Toxicology 314: 158-165.

986 Wink, S., Hiemstra, S., Huppelschoten, S., Danen, E., Niemeijer, M., Hendriks, G., Vrieling, H.,
987 Herpers, B. and van de Water, B. 2014. Quantitative high content imaging of cellular
988 adaptive stress response pathways in toxicity for chemical safety assessment. Chem. Res.
989 Toxicol. 27: 338-355.

990 Yarbrough, J.W. and Schultz, T.W. 2007. Abiotic sulfhydryl reactivity: A predictor of aquatic
991 toxicity for carbonyl-containing α,β-unsaturated compounds. Chem. Res. Toxicol. 20:
992 558-562.

993 **Annex II: Template for Assessing Uncertainty for Read-Across**

994 **Table 1. Data Uncertainty and Weight-of-Evidence Associated with the Fundamentals of Chemical,**
995 **Transformation/Toxicokinetic and Toxicological Similarity**

Similarity Parameter	Data Uncertainty [a] (empirical, modelled) (low, medium, high)	Strength of Evidence [b] (low, medium, high)	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.
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 moderate	High	Substituents and functional groups are consistent across all category members. There is a complex extended structural fragments (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 (see Table 2). 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 (see Table 3).
Toxicophores /Mechanistic alerts	Medium	High	Based on *in silico* profilers, category member contains any established toxicophores for protein 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.
Mechanistic	Medium	High	The available AOP hypothesized the mode of toxic action, of

Similarity Parameter	Data Uncertainty [a] (empirical, modelled) (low, medium, high)	Strength of Evidence [b] (low, medium, high)	Comment
plausibility and AOP-Related Events			all category members is related to oxidative metabolism to corresponding α, β-unsaturated electrophilic aldehydes or α, β-unsaturated ketones (see Table 3).
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.
Over all chemical similarity is limited by the complexity of the extended fragment but enhanced by sub-categorisation. Similarity of chemistry within the category (i.e., five structural sub-groups) is low to moderate. Within the category data similarity and weight-of-evidence associated with the fundamentals of chemical transformation/toxicokinetic is moderate to high with uncertainty, mainly related to metabolite reactivity. Within the category data similarity and weight-of-evidence associated with toxicodynamics is low to moderate. Uncertainties associated with mechanistic relevance and completeness of the read-across (i.e., uncertainty in the predictions) are reduced with sub-categorization and the addition of "new methods" data Summary: Key features of chemistry are similar within the category. Key features of transformation toxicokinetics and metabolism are common within the category. Category members exhibit a Michael addition electrophilic reactivity profile with respect to *in chemico* reactivity. Category members are considered mechanistically similar; category members exhibit a Michael addition electrophilic reactivity profile with respect to *in vivo*, *ex vivo* and *in vitro* toxicity.			

[a] Uncertainty associated with underlying information/data used in the exercise

[b] Consistency within the information/data used to support the similarity rational and prediction

999 **Table 2. Template for Assessing Uncertainty Associated with Mechanistic Relevance**
1000 **and Completeness of the Read-Across**

Factor	Uncertainty (low, medium, high)	Comment
The problem and premise of the read-across	Low to Medium	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; 2 of 16	There are only two suitable category member (2-propen-1-ol and 3-methyl-2-buten-1-ol) with *in vivo* apical endpoint data. These source substances represent one each for the two sub-categories (straight-chained and branched.)
Quality of the *in vivo* apical endpoint data read across	Medium	High quality empirical data for the stated regulatory endpoint exists from multiple studies for 2-propen-1-ol and a single study 3-methyl-2-buten-1-ol. These data are inconsistent in regards to qualitative and quantitative descriptions of effects. These in consistencies are eliminated by sub-categorisation.
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. The 90-day oral repeated dose NOAELs for 3-methyl 2-buten-1-ol in rat were reported based on the decreased food and water consumption: 65.4 mg/kg bw/d in males and 82.1 mg/kg bw/d in females.
Evidence to the biological argument for RA		
Robustness of analogue data set	Low; *ex vivo*, *in vitro* and *in chemico* endpoints reveal the same structure-activity relationships.	The available data from *ex vivo* studies of category members is of high quality but limited to one representative compound of the five structural sub-groups. The available data from *in vitro* studies of category members is of high quality but limited to one representative compound of the five structural sub-structural groups. The available data from *in chemico* studies for the category members is 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; limited by lack of mechanistic plausibility.	While data is 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* and *in vitro* data as well as the structural domains of the category. There is general agreement among the dose-response relationships of the tested category members for relevant *in vitro* and *in chemico* events.
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 is limited, the high quality *ex vivo* data (i.e., profused liver) supports metabolism being a key factor to the category and adds to the WoE. The high quality *in vitro* data (i.e., 3D hepatic organiod) supports the metabolic-mediated fibrosis premise and adds to the WoE. The consistency within the *in chemico* data and its consistency with the *ex vivo* and *in vitro* data adds to the WoE. The fact that there is consistent relevant *in chemico* data for most if not all the category members strengthens the WoE. Sub-categorisation (straight-chained and branched) adds markedly to the WoE.
Uncertainty associated with mechanistic relevance and completeness of the read-across (i.e., uncertainty in the predictions) is reduced with the addition of *ex vivo, and in chemico* reactivity data and sub-categorisation. Uncertainty associated with mechanistic relevance and completeness of the read-across (i.e., uncertainty in the predictions) is further reduced with the addition of "new methods" *in vitro* data. The overall uncertainty associated with the read-across predictions is judged to be low to medium.		

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