TCE Meeting Presentation Supporting Material for W. Dekant
Paul Dugard
to:
Marc Rigas
05/03/2010 09:58 PM
Please respond to Paul Dugard
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Dear Dr Rigas:

Please find attached the material on which Dr Dekant's presentation will be based. This has been abstracted from the comments originally submitted
by HSIA during the public review of the IRIS draft and is provided for the convenience of the panel members.

Thank you.

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Toxicological Review of Trichloroethylene
In Support of the IRIS Database (Draft of October 2009)

Comments of Prof. W. Dekant

I have been asked to comment on the IRIS Document on trichloroethylene (TCE) by the Halogenated Solvents Industry Alliance. My laboratory has published extensively on the biotransformation of TCE and was among the first to report formation of glutathione-*S*-conjugates from TCE. My area of expertise is biotransformation of xenobiotics, mechanisms of toxicity, and genotoxicity testing and I have published more than 180 manuscripts in these areas. Moreover, I am, or have been, a member of several advisory panels charged with health risk assessment of chemicals including the European Union Scientific advisory committee on Health and Environment (SCHER). As a member of this committee, I was the lead author of the review of the European Chemicals Bureau risks assessment report on TCE. I also have followed the many controversies in the risk assessment of TCE over the last 30 years.

General comments

The toxicity database on TCE is very large, with a number of controversial areas relevant to health risk assessment. EPA has generated a large document and attempted to comprehensively cover the available toxicology information on TCE and its metabolites. Most of the available studies are covered by the assessment. However, the document fails to provide a detailed evaluation of the strengths and weaknesses of the individual studies and a selection of key studies based on a weight of evidence approach. In several places in the document, study results are just reiterated and some of the conclusions relevant for deriving RfDs and RfCs have apparently been taken from reviews. EPA should develop comprehensive detailed justifications based on evaluation of the individual studies and consideration of data not supporting conclusions by EPA. Identical criteria should be applied to the level of evidence required to support or discount a mode of action (MoA).

Specific comments:

1. Extent of glutathione *S*-conjugate formation from TCE

EPA concludes that the extent of formation of *S*-(1,2-dichlorovinyl)glutathione (DCVG) from TCE in humans is much higher than in rodents. Since this conclusion has a major impact on the derivation of RfCs and RfDs for TCE, it should be fully justified and based on consideration of all available data. Apparently, EPA supports this conclusion based on high blood concentrations of DCVG reported in humans after inhalation of TCE (Lash *et al.*, 1999b). This observation is in contrast to the very low concentrations of the isomers of *N*-acetyl-*S*-(1,2-dichlorovinly)-*L*-cysteine (N-acetyl-DCVC) in urine. If the overall wealth of information is disregarded, it is possible to conclude that urinary metabolite content cannot be used as a quantitative marker for metabolic flux through the glutathione conjugation pathway (Lash *et al.*, 2000) and that most of the DCVG may undergo bioactivation by ß-lyase and the products retained in the kidney. However, a number of observations refute these conclusions:

- In the human study with TCE inhalation, high concentrations of DCVG in blood were indicated using a complex analytical procedure, often called the “Reed-Method” (Reed *et al.*, 1980). This method was developed to determine low concentrations of glutathione and glutathione disulfide and may be used to quantify DCVG formation in biological samples. The method involves reaction of the thiol with iodoacetamide and the amino group with chlorodinitrobenzene, followed by ion exchange chromatography and UV-detection of the dinitrophenyl chromophore. Due to the ion-exchange chromatography with a high salt concentration in the eluate, retention time shifts are common due to column deterioration (Lash *et al.*, 1999b). Since the method is not selective for DCVG and analysis of biological samples produces many peaks, retention time shifts may create problems for locating the DCVG peak.

A number of inconsistent datasets questions the reliability of the “Reed-method” to determine DCVG and DCVC:

- In a study assessing DCVG and DCVC formation in rodents after high oral doses of TCE, DCVG-concentrations reported in blood were high, but did not show dose or time-dependence (Lash *et al.*, 2006). In addition, the study reports high concentrations of DCVC excreted in urine. EPA calls the results of this study “aberrant”, but apparently did not further assess reliability. Others have reported a very low rate of DCVC-formation in vivo (Dekant *et al.*, 1990; Kim *et al.*, 2009) and DCVC has not been reported as urinary metabolite of TCE using either mass spectrometry or HPLC which radiochemical detection after administration of ^{14}C-TCE (Dekant *et al.*, 1986a).
- The “Reed-method” has also been used to determine DCVG-formation from TCE in subcellular fractions from liver and kidney of rats, mice, and humans. Again, high rates of formation of DCVG were reported (table 1). In contrast, using ^{14}C-TCE and radioactivity detection, much lower reaction rates were observed in other studies (table 1). In addition, isolated glutathione *S*-transferases also have a very low capacity to metabolize TCE to DCVG (Hissink *et al.*, 2002) and the application of the “Reed-method” to study formation of *S*-(1,2,2-trichlorovinyl)glutathione (TCVG) from perchloroethylene (PERC) in subcellular fractions also gave much higher rates of

formation (Lash *et al.*, 1998) when compared with methods using ^{14}C-perchloroethylene and HPLC with radioactivity detection (Dekant *et al.*, 1987; Green *et al.*, 1990; Dekant *et al.*, 1998).

Therefore, DCVG concentrations determined by the “Reed-method” may be greatly overestimated. The more reliable and consistent data support a very low extent of DCVG formation in rodents:

- Very low rates of formation of DCVG in rodent liver subcellular fractions are consistent with very low blood levels of DCVG in mice (Kim *et al.*, 2009) and a very low biliary elimination of DCVG in rats after oral administration of doses > 2 000 mg TCE/kg bw (Dekant *et al.*, 1990). In mice, DCVG concentrations were several thousand-fold lower than those of the oxidative metabolite trichloroacetic acid (TCA) (Kim *et al.*, 2009). In rats, biliary elimination of DCVG within seven hours after oral administration was 2 microg and therefore accounted for << 0.01 % of administered dose (Dekant *et al.*, 1990). Due to its molecular weight (> 350 D) and the presence of effective transport systems for glutathione *S*-conjugates in the canalicular membrane, most of the DCVG formed in rat liver is expected to be excreted in bile. Therefore, the low concentrations of DCVG in blood of mice and the low recovery of DCVG in bile of rats after TCE-administration well support very low rates of DCVG formation.
- Even when considering the high rates of DCVG formation reported in subcellular fractions and the only 3-fold difference in reaction rates between mouse, rat and humans (table 1), it is difficult to explain why DCVG-blood levels in mice after a very high oral dose are orders of magnitude lower than those reported in humans after inhalation exposures giving a much lower internal TCE-dose.
- High blood concentrations of DCVG and a high flux through ß-lyase bioactivation are not consistent with the human toxicity data on TCE. Despite high occupational exposures to TCE between the 1950s and 1970s (occupational exposure limits for TCE were 200 ppm in Germany and were often exceeded for prolonged times), overt nephrotoxicity was rarely observed even after many years of exposures (MAK, 1996). Using the blood concentrations reported and extrapolating to a daily exposure to 200 ppm TCE for 8 h, daily doses of DCVC of approx. 5-7 mg/kg bw should have been received by workers. A significant flux through ß-lyase bioactivation should have resulted in renal effects considering the alleged potency of DCVC.
- Kinetic studies on acetylation, and ß-lyase-mediated metabolism of DCVC support a low flux through ß-lyase activation since the relative flux through the *N*-acetylation pathway (detoxication) is one to two orders of magnitude higher than through ß-lyase activation (Green *et al.*, 1997a). In addition, a low flux through ß-lyase is indicated by the recovery of most of a low intravenous dose of DCVC isomers in urine as mercapturic acids in rats (Birner *et al.*, 1997), the weak nephrotoxicity of DCVC (Green *et al.*, 1997a) and observations with PERC, which is also metabolized by glutathione *S*-conjugate formation and ß-lyase. The PERC metabolite *S*-(1,2,2-trichlorovinyl)-*L*-cysteine is cleaved by ß-lyase to dichloroacetic acid (DCA) which, when formed in the kidney, is excreted with urine. While DCA is a metabolite of PERC in rats, this compound is not excreted as a PERC metabolite in humans (Völkel *et al.*, 1998). In addition, dichloroacetylated proteins were detected both in rat kidney proteins and rat blood proteins after PERC inhalation. Such protein

modifications were not detected in blood proteins from humans after identical exposures (Pähler *et al.*, 1999). These observations indicate that flux through ß-lyase in humans is even lower than in rodents.

- Chloracetic acid is formed by ß-lyase from DCVC (Dekant *et al.*, 1988). In rodents, chloroacetic acid and its metabolites (Green and Hathway, 1975; Green and Hathway, 1977) are not significant metabolites of TCE (< 0.1 % of radioactivity in urine) (Dekant *et al.*, 1984; Dekant *et al.*, 1986a). If the ß-lyase pathway is more relevant, such metabolites should be present in urine in higher concentrations. Other metabolites indicative of alternative processing of DCVC have also not been detected in humans exposed to TCE (Bloemen *et al.,* 2001).

Table 1: [SEE FOLLOWING PAGE] Reported rates of formation of DCVC from Trichloroethene (TCE) in rat, mouse and human subcellular fractions. The concentration of TCE in the incubation is based on the amount added. N.d. = not determined

Tissue	Species	TCE Conc (mM)	Rate of DCVC formation (pmol/minxmg)	Analytical method to determine DCVG	Reference
Liver cytosol	Rat	1.4 (^{14}C)	0.54 (non-enzymatic reaction rates substracted)	HPLC with radiochemical detection, peak identity confirmed by LC/MS	(Green *et al.*, 1997b)
	Mouse	1.9 (^{14}C)	0.35		
	Human	1.9 – 2.5 (^{14}C)	0.012 – 0.055		
Liver microsomes	Rat	1.4 (^{14}C)	Not different from non-enzymatic reaction		
	Mouse	1.9 (^{14}C)	n.d.		
	Human	1.9 – 2.5 (^{14}C)	n.d.		
Kidney cytosol	Rat	1.4 (^{14}C)	Not different from non-enzymatic reaction		
	Mouse	n.d.			
	Human	n.d.			
Kidney microsomes	Rat	1.4 (^{14}C)	Not different from non-enzymatic reaction		
	Mouse	n.d.			
	Human	n.d.			
Liver cytosol	Rat	4 (^{14}C)	< 2	HPLC with radioactivity detection, peak identity confirmed by GC/MS after hydrolysis	(Dekant *et al.*, 1990)
Liver microsomes	Rat	4 (^{14}C)	2		
Liver cytosol	Rat	2	121 (males) 81 (females)	Derivatisation and ion exchange HPLC ("Reed-method")	(Lash *et al.*, 1999a)
	Mouse	2	408 (males) 361 (females)		
	Human	1	1 700 – 4 180		
Liver microsomes	Rat	2	171 (males) 120 (females)		
	Mouse	2	666 (males) 426 (females)		
	Human	1	495 – 3 245		
Kidney cytosol	Rat	2	7.5 (males) 5.3 (females)		
	Mouse	2	93 (males) 61 (females)		
	Human	na	810 (vmax)		
Kidney microsomes	Rat	2	Nd (males) 1.0 (females)		
	Mouse	2	91 (males) 278 (females)		
	Human	na	6 290 (vmax)		

In summary, the evidence does not support EPA's conclusions that DCVG is released to the blood from TCE at a high rate in rodents and humans or that the rate is greater in humans than it is in rats and mice. The evidence indicates that the glutathione conjugation pathway is less active in humans than in rodents.

2. The role of glutathione *S*-conjugates in nephrotoxicity and renal tumor formation by TCE

Since *S*-conjugates of TCE are nephrotoxic in rodents and genotoxic in vitro, it is appealing to conclude that *S*-conjugate formation is involved in nephrotoxicity of TCE and that the MoA for kidney tumor formation is genotoxicity. However, a number of contradictory findings are not adequately considered in the IRIS-document:

- Formation rates for DCVC in subcellular fractions from mice and rats are similar (or even higher in mice) suggesting similar doses of DCVC to the kidney in both species (Green *et al.*, 1997a; Kim *et al.*, 2009). Moreover, activation of TCE by the ß-lyase pathway is higher in mice (Eyre *et al.*, 1995), DCVC is more nephrotoxic in mice, and causes higher rates of cell replication and covalent binding in mice as compared to rats (Eyre *et al.*, 1995; Green *et al.*, 1997a). Yet, mice are not sensitive to TCE induced renal tumor formation.
- Based on the nephrotoxicity of DCVC and the low rates of formation of DCVC both in rats and mice in vivo, it is questionable if the very low concentrations of DCVG formed in rodents can explain nephrotoxicity and tumor formation. Extrapolating the DCVG blood concentrations observed after single doses to the doses applied in the carcinogenicity studies with TCE in rats, daily DCVC-doses in the two year studies were less than 0.03 mg/kg bw. This is orders of magnitude below the doses of DCVC required to induce nephrotoxicity during chronic administration (Terracini and Parker, 1965) and further questions an involvement of this pathway in nephrotoxicity of TCE.
- EPA concludes that trichloroethanol and formic acid formation may not be involved in the toxicity of TCE to the kidney due to differences in pathology observed between TCE and trichloroethanol treated rats. In my opinion, such comparisons are difficult since differences in the kinetic profiles of a compound formed as a metabolite or administered per se are likely major confounders. The mode of action for TCE-induced renal tumors due to effects of increased formic acid excretion due to disturbances in intermediary metabolism by trichloroethanol is supported by renal toxicity of trichloroethanol, insufficient rates of DCVC/DCVC-formation to account for renal toxicity and the absence of genotoxic effects of TCE on rat kidney in vivo.
- EPA states that data on VHL gene mutations support a mutagenic MoA in TCE-induced kidney tumors. This is based on studies (Bruning *et al.*, 1997; Brauch *et al.*, 2004) reporting VHL mutations in renal tumors of TCE-exposed individuals. It is concluded that comparison of TCE-exposed and non-exposed patients (Brauch *et al.*, 2004) revealed clear differences with respect to (1) frequency of somatic VHL mutations, (2) incidence of C454T transition, and (3) incidence of multiple mutations. As discussed in Brauch et al. (2004), the mutation frequency in the non-exposed patients (10%) was considerably lower than that commonly observed in sporadic renal tumors, e.g. 82% (Nickerson *et al.*, 2008) or 71% (Banks *et al.*, 2006), and

technical problems using archived tissue samples may be one of the causes. Given that exon 3, which harbors the multiple mutations seen in TCE exposed patients, did not amplify in most of the controls, there is only limited evidence for a difference in the incidence of multiple mutations and frequency of somatic VHL mutations, although the C454T transition appears to be characteristic of tumors in TCE exposed patients. However, the presence of mutations in human tumors does not lead to the conclusion that VHL mutations occur early during carcinogenesis. Hence, they are not evidence for a direct genotoxicity of TCE in the kidney. In contrast, experimental data in rats show that neither TCE nor its active metabolite DCVC induce VHL mutations (Mally *et al.*, 2006), suggesting that VHL mutations in humans may be acquired at later stages of tumor development. While the document argues that the VHL gene may not be a target gene in rodent models of renal carcinogenesis, only few studies have looked at VHL in rats and there is no support for the hypothesis that the role of VHL is different in rats and humans.

- The Eker rat may be a useful rodent model for renal cell carcinoma (RCC), but the molecular basis for chemically induced tumor formation in rats and RCC in humans may be widely different from spontaneous tumor formation in this rat strain, as high-grade RCCs can develop in the absence of mutations in the Tsc2 gene in rats (Toyokuni *et al.*, 1998). Development of high-grade renal cell carcinomas in rats independently of somatic mutations in the Tsc2 and VHL tumor suppressor genes (Toyokuni *et al.*, 1998) demonstrates that mutational inactivation of TSC2 or VHL is not a prerequisite for renal carcinogenesis. The similar pathway activation in Eker rat RCC as that seen in humans with VHL mutations reported (Liu *et al.*, 2003) involves deregulation of HIFalpha and VEGF expression which frequently occur in various cancers and provide little evidence to suggest that Tsc-2 inactivation in rats is “analogous” to inactivation of VHL in human RCC.
- Epidemiological data may support an association between specific VHL mutations and TCE exposure, this does not indicate an early event in RCC and – in the absence of experimental support - should not be taken as support for a mutational MoA.
- EPA uses micronucleus and comet assay data in rat kidney after TCE-administration as support for a genotoxic MoA. However, the positive micronucleus (Robbiano *et al.*, 2004) assay applied a very high dose and used an inappropriate route of administration (ip injection of ½ of the LD_{50}). Due to the high dose applied and the route of administration, the results may be confounded by inflammatory responses and should not be used for conclusions. A comet assay in the kidney using repeated inhalation exposures to TCE was negative (Clay, 2008). The decision to not use this study in the assessment is insufficiently justified. The inhalation study used a higher number of animals (5/group) as compared to the ip study, which states n > 3 with an apparent maximum of 5. The comet assay also shows that administered DCVC is no more than weakly active in the kidney.
- EPA argues that there is no link between nephrotoxicity and renal tumor formation. However, there are a number of compounds that cause renal tumors in rats without being genotoxic. For example, cytotoxicity and regenerative cell proliferation (Swenberg and Lehman-McKeeman, 1999) is accepted as MoA for α_{2u}-globulin

binding agents (TCE does not bind to α_{2u}-globulin, but is most likely to cause renal tumors through nephrotoxicity).

In summary, the data do not support a genotoxic mode of action for kidney carcinogenicity via *S*-conjugates of TCE. The decision of EPA to employ *S*-conjugate-mediated genotoxicity in support of a linear dose response relationship for renal cell carcinoma should be revised to reflect the balance of the data. A non-linear dose response relationship is well supported by the available evidence.

3. Mode of action for liver carcinogenesis

- EPA spends considerable effort to correlate liver tumor induction by TCE in mice with liver tumor induction observed after administration of the TCE metabolites TCA and DCA. Again, such comparisons are inherently complex. Both DCA and TCA were administered with drinking water and TCE studies applied gavage in oil. The different administration regimens will result in different time courses of the administered compounds or metabolites in blood and dose-dependent bioavailability may further complicate the interpretation.
- It is highly questionable whether DCA is involved in liver tumor induction by TCE since it is only formed in very low concentrations from TCE in rodents (Dekant *et al.*, 1986a; Kim *et al.*, 2009). In mice, DCA is formed in concentrations several orders of magnitude below those of TCA. Thus, DCA would be required to be a highly potent liver carcinogen, which it is not. Therefore, the potency data on DCA do not suggest that the high liver tumor incidence induced by TCE in mice is related to DCA formation. In addition, DCA is not a human urinary metabolite of TCE (Bernauer *et al.*, 1996; Bloemen *et al.*, 2001).
- For TCA, EPA derives a dose-dependence from tumor incidence data in drinking water studies. Apparently, EPA assumes a dose-independent high bioavailability of TCA. However, the oral bioavailability of TCA from drinking water is limited, concentration-dependent and significantly reduced at higher concentrations of TCA (Larson and Bull, 1992; Templin *et al.*, 1993; Sweeney *et al.*, 2009). The incidence data therefore need to be corrected to account for the limited bioavailability of TCA at higher concentrations in drinking water.
- The mostly negative data in mutagenicity testing with TCE using liver specific activation and negative in vivo gentoxicity data including a very low DNA-binding in liver of mice (Bergman, 1983; Kautiainen *et al.*, 1997) also do not support a mutagenic MoA for liver tumors. Due to intensive metabolism by oxidation and reduction, chloral hydrate concentrations in the liver are low and chloral hydrate is a very weak mutagen. Therefore, chloral hydrate mutagenicity cannot adequately explain the formation of liver tumors by TCE in mice.

4. Mode of action for lung tumorigenesis.

EPA considers the lung tumors induced by TCE in specific strains of mice as relevant to humans and implies a genotoxic mode-of action. EPA tries to devaluate the hypothesis that chloral may reach high concentrations in mouse lung cells. However, the arguments by EPA are not convincing.

Rat and guinea pig data should not be used to conclude on biotransformation in mouse lung.

- A delivery of TCE from the systemic circulation in mice also causes lung toxicity due to the high metabolic capacity in the target cell. If TCE-metabolites formed in the liver are transported to the lung to cause toxicity there, the species-specificity is difficult to explain since the same metabolites are also present in rats, which do not show lung toxicity.
- A high rate of chloral formation from TCE and limited capacity for further metabolism of chloral (low capacity for reduction of chloral hydrate to trichloroethanol, low capacity for conjugation of trichloroethanol) will result in much higher steady state levels of chloral hydrate in mouse lung Clara cells as compared to rat or human lung (Odum *et al.*, 1992; Green *et al.*, 1997b). The high steady state levels may result in cytotoxicity.
- Cells damaged by the high chloral concentrations formed by TCE-metabolism initiate regeneration and replication to repair and replace the damaged Clara cells (Villaschi *et al.*, 1991) and repeated cycles of damage and regeneration may finally result in lung tumor formation.

Support for a cytotoxic MoA regarding the mouse lung tumors induced by TCE can also be derived from observations with other chemicals. The consequences of Clara cell specific cytotoxicity for tumor induction has been assessed with a number of other chemicals and the very high capacity of the mouse lung Clara cell for biotransformation is also the basis for the mouse-specific lung toxicity. The assessment therefore should integrate this information.

- Styrene, naphthalene, and coumarin induce lung tumors in mice and chronic damage of Clara cells including hyperplasia, often with a time- and dose-related increase in bronchiolar hyperplasia in terminal bronchioles. As with TCE, lung lesions are induced by short term administration, recess after repeated exposures and reappear after continuing exposures. None of these chemical induced lung tumors or histopathologic changes in rat lung (Cruzan *et al.*, 1998; Cruzan *et al.*, 2001).
- Major species differences in lung tumor induction and lung anatomy are one likely basis for the selective tumorigenicity of these chemicals in mice. Lung tumors occur spontaneously in several mouse strains and the incidences of benign lung tumors in control mice are often very high. In general, murine lung tumors are mostly adenomas originating from bronchiolar Clara cells. The adenomas may progress to adenocarcinomas. (Witschi, 1991).
- Clara cells are the major site of xenobiotic metabolism in the mouse lung (Chichester *et al.*, 1991; Buckpitt *et al.*, 1995). In addition to marked species differences in metabolic capacity of Clara cells in different species, species differences in Clara cell abundance and function may contribute to selective pulmonary toxicity in mice. Clara

cell number is significantly higher within the terminal bronchioles of mice relative to rats and humans (Plopper *et al.*, 1980; Lumsden *et al.*, 1984). Clara cells represent approximately 5 % of all cell types and are distributed throughout the airways in mice. In humans, only very few Clara cells are present and are localized in specific regions. Moreover, Clara cells differ morphologically among species, with human cells containing little smooth endoplasmic reticulum.

- TCE and the other chemicals inducing selective lung damage and lung tumors in mice require biotransformation by pulmonary CYP2F and CYP2E1 (Green *et al.*, 1997b; Shultz *et al.*, 1999; Shultz *et al.*, 2001; Born *et al.*, 2002; West *et al.*, 2002; Forkert *et al.*, 2005).
- In mice, both CYP2E1 and CYP2F1 are preferentially localized in Clara cells (Forkert *et al.*, 1989; Buckpitt *et al.*, 1995; Forkert, 1995; Shultz *et al.*, 2001). In rat lung, the expression of CYP2F4, an ortholog of mouse CYP2F2 (Baldwin *et al.*, 2004) is app. 30-fold lower consistent with a much lower turnover of CYP2F substrates in rat. Evidence for the presence of the human ortholog CYP2F1 in human lung is lacking. In rhesus monkeys, CYP2F1 was not detected in the respiratory tract except in the nasal epithelium (Ding and Kaminsky, 2003; Baldwin *et al.*, 2004). CYP2E1 catalytic activity is present in human lung with an activity app. 100-fold lower than in human liver (Bernauer *et al.*, 2006).

In summary, the available information on the presence and catalytic activities of CYP2E1 and CYP2F enzymes in the lung of different species suggest a much higher activity of these enzymes in the mouse, the species susceptible to the pneumotoxicity. Studies directly quantifying relevant metabolite formation from the different pneumotoxic compounds show that mice consistently have a much higher capacity for oxidation as compared to rats and humans. The available data on the mode-of-action for induction of lung tumors share many common features with regard to the induction of Clara cell lesions in the mouse and a number of observations support a non-genotoxic mode-of-action: Glutathione depletion is a major determinant of the toxic responses in the mouse Clara toxicity (West *et al.*, 2000a; West *et al.*, 2000b; Plopper *et al.*, 2001; Phimister *et al.*, 2004; Turner *et al.*, 2005). Glutathione-depletion induced cell death induced by mouse specific Clara cell toxicants initiates extensive cell replication and subsequent hyperplasia which are considered important steps in the multi-step progression to tumor development (Gadberry *et al.*, 1996; Green *et al.*, 1997b; Green *et al.*, 2001).

Additional comments

Page 2-22: Line 36, the exposures in the cardboard workers in Germany likely were much higher, with peaks well above 1,000 ppm and prolonged exposures above the former occupational standard (> 200 ppm TWA).

Page 3-6: The major toxicity of TCE after acute high dose exposure is narcosis. Kidney and liver damage are usually not observed (MAK, 1996).

Page 3-13: Table 3-6, if the data in the table are not considered reliable why are they presented?

Page 3-15: Line 27, TCA reversibly binds to proteins and the reversible protein binding is much more relevant for toxicokinetics of TCE as compared to covalent binding. It should also be noted that the ^{14}C-TCE used in many of the early studies contained a number of reactive impurities.

Page 3-23: Regarding saturation of TCE metabolism in humans, none of the human studies used dose-ranges where saturation of metabolism was seen in rats. Therefore, this conclusion should be removed.

Page 3-24: Lines 9 to 14, the text is not logical. TCE oxide may rearrange to dichloroacetyl chloride and the TCE P450 intermediate may rearrange to give chloral (Miller and Guengerich, 1982; Liebler and Guengerich, 1983; Cai and Guengerich, 2001).

Page 3-25: Lines 20 to 23, TCE oxide does not rearrange to chloral. Therefore, the text is confusing.

Page 3-27, Lines 19 to 25, chloral hydrate has been identified as a circulating TCE metabolite and is also formed as the major product in the microsomal oxidation of TCE (Byington and Leibman, 1965; Cole *et al.*, 1975).

Page 3-35: Metabolite recovery data in male and female human beings are available. In addition, metabolite excretion in humans and rats exposed to TCE by inhalation under identical conditions are available (Bernauer *et al.*, 1996).

Page 3-44: Table 3-23 should include additional data on GSH-conjugation of TCE (Dekant *et al.*, 1990; Green *et al.*, 1997a).

Page 3-46: Information on ß-lyase catalyzed metabolism of DCVC is available (Green *et al.*, 1997a).

Page 3-47: DCVC-sulfoxide; it should be mentioned that sulfoxides and down-stream metabolites have never been identified in rodents after administration of TCE (or PERC) and therefore are, at best, formed in small traces.

Page 4-34: Line 1, conclusion on bacterial mutagenicity. A more detailed weight-of-evidence evaluation of the contradictory database is needed here.

Table 4-18: Robbiano study, the study did not apply DCVG or DCVC and thus should not be included in the table.

Page 4-83: Line 28, DCVC is not a "direct-acting" mutagen since bacteria express ß-lyase (Dekant *et al.*, 1986b). Thus, this is a difference when compared to *S*-(2-chlorethyl)-L-cysteine, which does not require enzymatic transformation.

Page4-443: Lines 6 -7, the reactivity of chloral hydrate and chloroacetaldehyde are highly different and should not be compared. Chloroacetaldehyde is highly reactive with DNA-constituents (Green and Hathway, 1978), whereas chloral hydrate is not.

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