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TOXICITY CRITERION FOR ALPHA-HEXACHLOROCYCLOHEXANE

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ACRONYMS AND ABBREVIATIONS

ALT	alanine aminotransferase
ATSDR	Agency for Toxic Substances and Disease Registry
BaP	benzo(a)pyrene
BMD	benchmark dose
BMDL	confidence limit of the benchmark dose
CYP	cytochrome P450
DEN	diethylnitrosamine
DNA	deoxyribonucleic acid
EPA	U.S. Environmental Protection Agency
GRed	glutathione reductase
GST	glutathione-S-transferase
HCC	hepatocellular carcinoma
HCH	hexachlorocyclohexane
Integral	Integral Consulting Inc.
i.p.	intraperitoneal
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
LOAEL	lowest-observed-adverse-effect level
MF	modifying factor
mg/kg-day	milligram per kilogram per day
MOA	mode of action
MRL	minimal risk level
NADPH	nicotinamide adenine dinucleotide phosphate
NDEP	Nevada Division of Environmental Protection
NOAEL	no-observed-adverse-effect level
OGG1	8-oxoguanine glycosylase
P450	cytochrome P450
PB	Phenobarbital

POD	point of departure
RED	Reregistration Eligibility Decision
RfD	reference dose
RNA	ribonucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substance
TCDD	tetrachlorodibenzo-p-dioxin
UF	uncertainty factor
WOE	weight of evidence
8-OHdG	8-hydroxy deoxyguanosine

EXECUTIVE SUMMARY

Integral Consulting Inc. (Integral) has developed an updated toxicity criterion for alpha-hexachlorocyclohexane (alpha-[HCH]). Alpha-HCH has previously been regulated as a potential human carcinogen by the Nevada Division of Environmental Protection (NDEP) using toxicity criteria housed in the U.S. Environmental Protection Agency's (EPA) Integrated Risk Information System (IRIS) and last updated in1993[1]. This project was initiated by Integral on behalf of Syngenta Crop Protection and Stauffer Management Company to update the NDEP toxicity criterion for alpha-HCH by incorporating 1) recent advances in the approach to carcinogenic risk assessment recommended by USEPA (2005a) and 2) new data on the potential toxicity of alpha-HCH that has been published since the original toxicity criterion was developed.

The collective evidence indicates that alpha-HCH is a hepatocarcinogen in rodents. Alpha-HCH is not mutagenic, and induces a carcinogenic response in rodents via aberrant cell proliferation triggered by absorption in the liver, cytochrome P450 (CYP P450) induction, and oxidative stress. The mode of action (MOA) is sufficiently well described in the experimental literature to conclude with appropriate confidence that alpha-HCH acts via a non-linear MOA, and displays a threshold at which carcinogenicity does not occur. There is insufficient evidence to indicate that alpha-HCH is carcinogenic in humans. However, the physiological process and biochemical pathways observed in animals are present in humans, and thus, the MOA and carcinogenic response in animals is potentially relevant to humans. Following USEPA (2005a) guidance, the weight of evidence (WOE) cancer classification determined for alpha-HCH is: **"suggestive evidence of carcinogenic potential above a specified dose but not likely to be carcinogenic below that dose."**

The liver is determined to be the most sensitive target organ following subchronic and chronic exposure to alpha-HCH. Considering these findings and following USEPA (2005a) guidance, a cancer-based reference dose (RfD) was developed. The recommended cancer-based RfD for alpha-HCH is 0.0003 mg/kg-day. The value is based on a point of departure (POD) of 0.1 mg/kg-day for increased incidence of preneoplastic hepatic foci in rats, and a total uncertainty factor (UF) of 300 (10 each to account for intra- and inter-species extrapolation, and 3 for database uncertainties).

For perspective, the recommended RfD is approximately an order of magnitude lower than the oral chronic non-cancer RfDs and minimal risk level (MRL) established by EPA and the Agency for Toxic Substances Disease Registry (ATSDR) respectively. In their 2006 *Assessment of Lindane and Other Hexachlorocyclohexane Isomers* (USEPA 2006), completed as part of the Reregistration

[1] EPA's IRIS currently classifies alpha-HCH as a class B2, probable human carcinogen (USEPA 2011). The current classification was last reviewed in 1993.

Eligibility Decision (RED) for Lindane, EPA established chronic oral RfDs for alpha-HCH of 0.001 mg/kg-day and 0.008 mg/kg-day. ATSDR (2005) established a chronic oral MRL for alpha-HCH of 0.008 mg/kg-day. The non-cancer RfDs and MRL are all based on hepatoxicity.

1 INTRODUCTION

Integral Consulting Inc. (Integral) has developed an updated toxicity criterion for alpha-hexachlorocyclohexane (alpha-[HCH]). Alpha-HCH has previously been regulated as a potential human carcinogen by the Nevada Division of Environmental Protection (NDEP) using toxicity criteria housed in the U.S. Environmental Protection Agency's (EPA) Integrated Risk Information System (IRIS) and last updated in1993. This project was initiated by Integral on behalf of Syngenta Crop Protection and Stauffer Management Company to update the NDEP toxicity criterion for alpha-HCH by incorporating 1) recent advances in the approach to carcinogenic risk assessment recommended by the USEPA (2005a) and 2) new data on the potential toxicity of alpha-HCH that has been published since the original toxicity criterion was developed. This report presents a summary of the methods and results of the toxicological review and presents a recommended toxicity criterion for adoption by NDEP into its regulatory programs.

2 METHODS

The available toxicological data were compiled and reviewed to assess the potential carcinogenicity and non-cancer effects of alpha-HCH. USEPA's *Guidelines for Carcinogen Risk Assessment* (2005a) provided the over-arching framework for the evaluation and assessment of potential carcinogenic effects, supplemented by recent peer-reviewed literature related to the evaluation of carcinogenic mode of action (MOA) and human relevance (Boobis et al. 2006, 2009; Butterworth 2006; Meek 2008; Meek et al. 2003). Approaches and principles outlined in EPA guidance for dose-response modeling (USEPA 2000) and EPA's review of the reference dose (RfD) process also were applied (USEPA 2002).

Key steps in the assessment were: literature summary and quality assessment; hazard assessment; and dose-response assessment and criterion derivation. The methods utilized for each of these steps are discussed briefly below.

2.1 LITERATURE SUMMARY AND QUALITY ASSESSMENT

A comprehensive literature search was conducted to identify relevant literature to support the evaluation. Data related to the assessment of oral exposures were the focus of the review as this is a principal pathway currently for human exposures to ambient alpha-HCH. EPA and Agency for Toxic Substances and Disease Registry (ATSDR) reviews of HCH toxicity (ATSDR 2005; USEPA 1987, 2001, 2006) provided the starting point for identification of literature to be evaluated. Original studies identified in these documents were obtained for review. In addition, literature searches were conducted to identify more recent toxicity literature relevant to cancer and non-cancer endpoints.

All studies were reviewed and basic information characterizing study design, findings, and dose-response was compiled in a Microsoft Access database. In addition, each study was critically reviewed to assess its quality and reliability using criteria developed from Klimisch et al. (1997), USEPA (2005a), and Durda and Preziosi (2000). Evaluation criteria included:

- Study is conducted using standard methods. Test substance purity and origin are described.
- Controls are included.
- Statistical power is appropriately included in the study design.
- Study design controls for potential confounders. Data on secondary effects which may influence the result are described.
- Methods and results are clearly and completely documented.

- Animal mortality and/or viability of the test system are described.

A summary of each paper and the data quality ranking assigned as a result of the critical review was complied in a Microsoft Access database. The database is provided as Attachment A. The database additionally includes, definitions for the criteria used in ranking each study and notes regarding the rank assigned for each study.

Poor quality and/or unreliable data were excluded from further technical evaluation and from use in the derivation of a toxicity criterion. Data of intermediate quality were used to support qualitative evaluations of toxicity (i.e., hazard assessment). Only high quality data were considered appropriate and utilized for quantitative dose-response modeling.

2.2 HAZARD ASSESSSMENT

Studies of acceptable quality were further reviewed collectively to assess overall human carcinogenic potential and non-cancer effects. The outcome of this step was a determination of the potential human carcinogenicity of alpha-HCH and the identification of the most sensitive target organ/system for dose-response assessment.

2.2.1 Cancer Assessment

A weight of evidence (WOE) approach was taken to determine the carcinogenic potential of alpha-HCH, following USEPA's *Guidelines for Carcinogen Risk Assessment* (2005a). Under the WOE approach, the available data on carcinogenicity, including epidemiological studies, animal bioassays, and *in vitro* assays were critically reviewed. Generally accepted causation criteria (Bradford Hill 1965), including strength, specificity, and consistency of the association, evidence for a dose-response relationship, temporal association between exposure and effect, and biological plausibility, were considered as part of the overall WOE evaluation.

The carcinogenic potential in humans was summarized into a WOE narrative following USEPA (2005a) guidance. EPA classifies potential human carcinogens using the following hazard classification categories:

1. Carcinogenic to humans
2. Likely to be carcinogenic to humans
3. Suggestive evidence of carcinogenic potential
4. Inadequate information to assess carcinogenic potential
5. Not likely to be carcinogenic to humans.

USEPA (2005a) guidance provides for application of more than one hazard classification for a chemical if carcinogenic response is variable depending upon exposure conditions (e.g., route or dose-specific differences).

For potential human carcinogens, the appropriate approach for quantifying dose-response depends on the MOA for the cancer. Historically, EPA has most often assumed a no-threshold model for cancer dose-response assessment and has used a linear, low-dose model to quantify cancer potency. More recently, however, USEPA (2005a) has acknowledged that carcinogenic response of some chemicals occurs via a MOA that has a defined toxicological threshold. In these instances, USEPA (2005a) states that non-linear modeling can be used to derive a cancer toxicity criterion.

To support the development of toxicity criterion for alpha-HCH, therefore, the MOA for carcinogenic response was evaluated along with the human relevance of the MOA. The evaluation of MOA and human relevance utilized a framework consisting of three central questions:

1. Is the WOE sufficient to establish a MOA in animals?
2. Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between animals and humans?
3. Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between animals and humans?

This framework was adopted from Meek et al. (2003) and other publications (e.g., Boobis et al. 2009; Butterworth 2006; Meek 2008) and was based on guidance issued by EPA and the International Programme on Chemical Safety (IPCS) for the assessment of the relevance of animal-derived MOA data to inform human health risk (Boobis et al. 2006; USEPA 2005a).

The assessment of MOA and human relevance provided additional lines of evidence to support the selection of the most sensitive endpoint for derivation of the toxicity criterion.

2.2.2 Non-Cancer Assessment

For non-cancer effects, studies exploring toxic response for non-cancer endpoints in all organ systems were reviewed. Relative potency to target organs based on animal data and the potential for increased susceptibility in human subpopulations were evaluated. The evaluation of relative potency focused on animal studies that considered effects associated with low doses[2] delivered during subchronic or chronic exposure durations because these types of exposure scenarios are most relevant for human health risk assessment (USEPA 1992). Low-dose animal

[2] Based on the experimental literature, these were defined as studies with one or more oral dose less than or equal to 10 mg/kg-day.

studies of reproductive and developmental endpoints were also included, regardless of the exposure duration, as recommended by USEPA (2005b). The potential for increased susceptibility of human subpopulations was evaluated considering lifestage (e.g., age, pregnancy), gender, underlying disease, genetic polymorphisms, and lifestyle factors (e.g., nutrition, smoking).

2.3 DOSE-RESPONSE ASSESSMENT AND CRITERION DEVELOPMENT

The toxicity criterion was derived consistent with the general principles and procedures outlined in USEPA's *Benchmark Dose Technical Guidance Document* (2000) and *A Review of the Reference Dose and Reference Concentration Processes* (2002). First, a point of departure (POD) for the critical effect[3] was selected. The POD is the dose-response point that marks the beginning of a low-dose extrapolation. The point can be the lower bound on dose for an estimated incidence or a change in response level from a dose-response model, a no-observed-adverse-effect level (NOAEL) or lowest-observed-adverse-effect level (LOAEL) for an observed incidence, or change in level of response (USEPA 2011).

The POD was determined by first identifying the endpoints that appropriately reflect, or are closely related to, the critical effect and then selecting the most sensitive. The modeling approach was selected on the basis of the nature of the critical effect and existing toxicity data. For cancer effects, a linear, non-threshold, dose-response model is applied if there is not sufficient evidence to indicate that the MOA is threshold based (USEPA 2005a). If sufficient evidence exists to support a non-linear MOA, the most sensitive endpoint for toxicity is used to determine the response data that are selected for deriving a RfD (USEPA 2005a). In the latter case, the most sensitive endpoint could be either a cancer or non-cancer effect. For threshold-based responses, both a traditional RfD approach, and benchmark dose (BMD) modeling were explored for developing the appropriate toxicity criterion. Uncertainty factors (UFs) and/or modifying factors (MFs) were applied to the POD to account for uncertainties associated with the available data and variability between the test species and sensitive human populations.

[3] For the purposes of developing toxicity criteria, EPA defines a critical effect as the first adverse effect, or its known precursor, that occurs to the most sensitive species as the dose rate of an agent increases (USEPA 2011). EPA defines an adverse effect as a biochemical change, functional impairment, or pathological lesion that affects the performance of the whole organism, or reduces an organism's ability to respond to an additional environmental challenge (USEPA 2011). It is recognized that the distinction between adverse effects and non-adverse effects is not always clear cut, and best professional judgment is required in making that distinction (Bogdanffy et al. 2001; HERA 2004).

3 FINDINGS – HAZARD ASSESSMENT

The collective evidence indicates that alpha-HCH is a hepatocarcinogen in rodents. Alpha-HCH is not mutagenic, and induces a carcinogenic response in rodents via aberrant cell proliferation triggered by absorption in the liver, cytochrome P450 (CYP P450) induction, and oxidative stress. The MOA is sufficiently well described in the experimental literature to conclude with appropriate confidence that alpha-HCH acts via a non-linear MOA, and displays a threshold at which carcinogenicity does not occur. There is insufficient evidence to indicate that alpha-HCH is carcinogenic in humans. However, the physiological process and biochemical pathways observed in animals are present in humans, and thus, the MOA and carcinogenic response in animals is potentially relevant to humans. Following USEPA (2005a) guidance, the following WOE cancer classification was determined for alpha-HCH: **"suggestive evidence of carcinogenic potential above a specified dose but not likely to be carcinogenic below that dose."**

Across all endpoints, the liver was determined to be the most sensitive target organ for alpha-HCH toxicity. A summary of the information supporting these findings is presented below.

3.1 CARCINOGENICITY

A summary of the human, animal bioassay, and *in vitro* data reviewed to develop the finding for carcinogenic potential is presented below. The MOA evaluation, which supports the conclusion regarding the compound's threshold response, is also presented.

3.1.1 Human Data

Table 1 summarizes the study designs, findings, and key limitations of the human data reviewed for evaluating the potential carcinogenicity of alpha-HCH. Only a single epidemiologic study (Mathur et al. 2002) was located in the literature that presented results regarding the potential association of alpha-HCH and human cancer. Mathur et al. (2002) reported a positive association between levels of alpha-HCH in blood and breast cancer in a single age category of women. The study did not account for several potential confounders, including the presence of other organochlorine pesticides and body fat levels (a parameter which is associated with both breast cancer risk and the body burden of lipophilic chemicals such as alpha-HCH). Therefore the study cannot be used to make conclusions regarding the compound's carcinogenic potential.

3.1.2 Animal Bioassays

Table 2 summarizes the study designs, findings, and major limitations of the animal bioassays reviewed for the evaluation of alpha-HCH's carcinogenic potential. The available data clearly show that alpha-HCH exposure is associated with benign and malignant liver tumor formation in multiple mouse strains as well as in rats. Differences in susceptibility to alpha-HCH-mediated liver tumor formation are apparent, with rats being less susceptible than mice. Although the majority of studies using mice were positive for tumor formation (Hanada et al. 1973; Ito et al.1973a,b, 1976; Nagasaki 1975; Tryphonas and Iverson 1983), some negative results have been reported (Siglin et al. 1991, 1995). The negative effect may be strain-specific.

Demonstration of dose- and time-dependency of these effects strengthens the conclusion that alpha-HCH causes tumors in rats and mice. Consistent dose-dependent increases in tumor formation were observed in each study in which multiple dose levels were evaluated (Ito et al. 1973a,b, 1975; Hanada et al. 1973, Puatanachokchai et al. 2006). Additionally, temporal relationships between exposure and increased tumor formation were consistently seen across studies (Ito et al. 1975, 1976, Schulte-Hermann and Parzefall 1981; Tryphonas and Iverson 1983).

Results obtained from initiation-promotion studies for alpha-HCH support the role of alpha-HCH as a tumor promoter. In initiation-promotion studies of alpha-HCH, formation of preneoplastic hepatic foci has been used as a marker for carcinogenic potential. Increases in hepatic foci and area have been consistently observed in laboratory animals dosed with alpha-HCH following a known initiator (Masuda et al. 2001; Puatanachokchai et al. 2006; Schroter et al. 1987; Ito et al. 1983; Luebeck et al. 1995). Importantly, alpha-HCH is not itself a tumor initiator; no hepatic foci were observed in partially hepatectomized rats given a single dose of alpha-HCH followed by 15 weeks of dietary phenobarbital (PB) (Schroter et al. 1987).

Patterns of tumor formation observed in animals strongly suggest that alpha-HCH acts as a tumor promoter. First, multiple studies show that there are doses of alpha-HCH that do not cause malignant tumor formation after long-term or even lifetime exposure (Ito et al. 1973a,b, 1975, 1976). Consistent observations of certain patterns including long time-to-tumor, progression of lesions and tumors from benign to malignant over time (Ito et al. 1976), tumor formation at only one target organ, and reversibility of tumor formation upon cessation of exposure (Ito et al. 1976), additionally support that alpha-HCH is a tumor promoter in animals. Detailed notes on these patterns, by study, are presented in Table 2.

3.1.3 Mutagenicity and Genotoxicity Assays

Table 3 summarizes the short-term mutagenicity and genotoxicity assays reviewed for the evaluation of alpha-HCH mutagenic potential.

Assays measuring various categories of mutagenic /genotoxic endpoints (i.e., gene mutation; deoxyribonucleic acid (DNA) binding; DNA damage or fragmentation, or repair of such damage; and chromosomal abnormalities) reported mixed results. Among those that reported a positive result, however, several compared the mutagenic potential of alpha-HCH to that of compounds that are known to be direct acting mutagens. These studies reported lower levels of direct mutagenic potential for alpha-HCH compared to those compounds established in the literature as having strong mutagenic activity (Iverson et al. 1984; Sagelsdorff et al. 1983; Venkat et al. 1995).

Although some evidence of genotoxicity has been observed, the lack of a consistent positive response in the short-term bioassays conducted in a variety of *in vitro* and *in vivo* systems and evaluating a variety of endpoints associated with DNA damage does not support that alpha-HCH is mutagenic. Additionally, several other scientific and regulatory entities have concluded that alpha-HCH does not act as a direct mutagen. IPCS (1992) concluded that the tumorgenic response observed with alpha-HCH in mice results from a non-genotoxic mechanism. RIVM[4] (2001) state that there are no indications for alpha-HCH being mutagenic, and that alpha-HCH induced tumorgenicity has a non-genetic mechanism.

3.1.4 Mode of Action for Carcinogenicity

As discussed above, the results from animal bioassays provide evidence that alpha-HCH displays a threshold dose below which a carcinogenic response is not exhibited. The key events that are postulated to lead to cancer development in animals, along with a discussion of their potential relevance to humans, are presented below. Tables 2 and 3 summarize the data obtained from the peer-reviewed literature evaluated to support the MOA review.

3.1.4.1 Key Events of Animal Mode of Action

The collective literature indicates that the MOA for alpha-HCH-mediated liver tumor development is increased promotion of cell growth, or mitogenesis. The putative key events underlying its carcinogenic action are absorption in the liver, P450 induction, oxidative stress, and increased cell proliferation, ultimately resulting in benign and malignant tumor formation. A similar MOA has been demonstrated for PB (Fukushima et al. 2005), which is a well accepted tumor promoter in rodents (Klaassen 2001; Klaunig et al. 1990; Kitchin et al. 1994; Aydinlik et al. 2001). With the exception of the process of alpha-HCH absorption, the multiple biochemical and physiological processes underlying these key events are receptor-mediated (e.g., P450 induction) and threshold-based (e.g., oxidative stress), which suggests that the ultimate outcome, tumor formation, would also be threshold-based. The evidence supporting this conclusion is described below.

[4] Rijks Instituut Voor Volksgezondheid en Milieu, National Institute of Public Health and the Environmental, Bilthoven, The Netherlands.

Absorption in the Liver

Alpha-HCH has been detected in liver and other tissues after subchronic or chronic dietary exposure (Fitzhugh et al. 1950; Schroter et al. 1987). Alpha-HCH has also been isolated from mouse liver DNA, ribonucleic acid (RNA), and/or protein following a single oral bolus or intraperitoneal (i.p.) dose (Iverson et al. 1984; Sagelsdorff et al. 1983). The detection of alpha-HCH in the liver supports an association between exposure and the development of liver tumors.

Cytochrome P450 Induction

Exposure to xenobiotics often results in increased P450 enzymatic activity in the liver. Increased P450 activity is, in some cases, an essential component of disease pathogenesis through, for example, formation of a reactive metabolite or reactive oxygen species (ROS; Klassen 2001).

Increased P450 protein and isozyme activity are consistently demonstrated in rats exposed to alpha-HCH in studies of varying experimental design. For example, increased CYP2B, 2C, 2E, and 3A protein or activity have been observed, and these increases were found to be dose- and time-dependent (Masuda et al. 2001; Puatanachokchai et al. 2006; Schroter et al. 1987; Schulte-Hermann and Parzefall 1980, 1981). Importantly, CYP isoform activity increases have been shown to diminish following cessation of exposure, supporting the role of alpha-HCH as a tumor promoter, for which an ultimate tumorigenic effect is due to sustained cellular change mediated by sustained exposure (Schulte-Hermann and Parzefall 1981). The changes in P450 isoform protein and activity are supported by microarray data, which show that P450 isozyme expression increases after HCH exposure (Sumida et al. 2007; Werle-Schneider et al. 2006).

The effect of alpha-HCH on P450 is further demonstrated by increased total P450 levels and increased nicotinamide adenine dinucleotide phosphate (NADPH): P450 reductase activity following alpha-HCH exposure in rats (Barros et al. 1991; Puatanachokchai et al. 2006). A hormetic dose-response curve has been observed for HCH-mediated total P450 and P450 reductase levels, with decreases at low doses and increases at high doses (Puatanachokchai et al. 2006). Such a response strengthens the conclusion that the response is threshold-based, and clearly non-linear at low doses. Moreover, as has been observed for other P450 inducers such as PB (Klassen 2001), alpha-HCH exposure results in proliferation of smooth endoplasmic reticulum (Ito et al. 1973a, 1976; Tsukada et al. 1979). This is likely the direct result of the observed P450 protein increase.

P450 induction is a threshold-based, receptor-mediated process that is regulated largely on the level of transcription. As such, gene expression must be increased before increases in protein and enzyme activity can occur (Klaassen 2001). In general, the effectiveness of receptor-mediated transcription induction depends on affinity of the xenobiotic for the intracellular receptor and affinity of the receptor-ligand complex for the relevant regulatory sequences in

each gene as well as the presence of co-activators or co-repressors (Kohn and Melnick 2002). In such a multi-factorial process there are doses of inducer at which no measurable response would occur, as has been demonstrated for alpha-HCH.

In the case of alpha-HCH, the increases in total P450, P450 isoform protein/activity, and P450 reductase activity have been observed in parallel with increased oxidative stress, increased proliferation or increased liver weight, and hepatic foci formation (Barros et al. 1991; Masuda et al. 2001; Puatanachokchai et al. 2006; Sumida et al. 2007). Additional discussion of the potential role of P450 induction in tumor formation is provided below within the discussion of oxidative stress.

Oxidative Stress

Xenobiotic-mediated production of ROS and the subsequent overwhelming of cellular antioxidant defenses can damage cellular macromolecules, leading to a variety of cellular outcomes including lipid peroxidation and DNA damage (Klaunig and Kamendulis 2004; Klaunig et al. 1998). For alpha-HCH, the data clearly show that exposures induce an oxidative stress response that is, at least to some degree, linked to the response of increased microsomal enzyme activity in the liver. Specifically, in alpha-HCH-treated rats, superoxide anion is detected in the hepatic microsomal fraction, which implicates P450 as the source (Barros et al. 1991). Increased superoxide formation and lipid peroxidation, together with increases in P450 levels, are seen prior to development of microscopic liver lesions (Barros et al. 1991), illustrating a temporal sequence of events. Oxidative DNA damage has also been seen following intermediate alpha-HCH exposure, the development of which follows a hormetic dose-response pattern, with statistically significant decreases in liver 8-hydroxy deoxyguanosine (8-OHdG) at low doses and increases at high doses (Puatanachokchai et al. 2006). However, concomitant increases in DNA repair (i.e., 8-oxoguanine glycosylase [OGG1] message induction) were not observed, which may be a temporal phenomenon or may reflect the limitations of the assay. Whether the observed oxidative lesions result in actual mutations has not been evaluated for alpha-HCH, although the database in general does not support a mutagenic MOA (ATSDR 2005 and references therein). Moreover, it is important to note that the study in which 8-OHdG was observed used animals that had been initiated with a known DNA damaging agent, diethylnitrosamine (DEN). The formation of 8-OHdG in response to alpha-HCH only was not evaluated, so the relevance of this finding to alpha-HCH-mediated liver tumor formation is unclear.

In response to alpha-HCH-mediated production of ROS, cellular antioxidant defenses are upregulated. This is observed after acute or intermediate alpha-HCH exposure. Specifically, increased superoxide dismutase (SOD) activity, glutathione reductase (GRed) activity, catalase activity, and glutathione-S-transferase (GST) activity have been observed in rats (Barros et al. 1991; Puatanachokchai et al. 2006; Schulte-Hermann and Parzefall 1981; Sumida et al. 2007). Alpha-HCH-mediated production of ROS and its potential sequelae – antioxidant enzyme

upregulation, lipid peroxidation, and/or oxidative DNA adduct formation - have not been confirmed in additional studies, nor have they been evaluated in mice, which are more susceptible to liver tumor formation than are rats.

GST activity increases are dose-dependent (Kraus et al. 1981; Puatanachokchai et al. 2006). Importantly, transient increases in GST activity were observed after single intraperitoneal (i.p.) and oral doses (Kraus et al. 1981), demonstrating a similar effect with different routes of exposure. These data further support the characterization of HCH as a tumor promoter whose tumorigenic effects depend upon the sustained cellular changes that are the result of continuous exposure. The observed changes in GST activity are supported by microarray data (Sumida et al. 2007; Werle-Schneider et al. 2006).

The literature on hepatotocarcinogenicity, generally, suggests a role for P450 in superoxide anion production because superoxide anions have been observed in the microsomal fraction of hepatic cells. P450-mediated superoxide production may occur by two mechanisms (Klaunig and Kamendulis 2004). If, for example, alpha-HCH is a low-affinity substrate for a P450 isoform, futile cycling may result in the release of ROS, including superoxide anion. Alternatively, P450 can catalyze the formation of a reactive metabolite that attacks cellular lipids and/or DNA indirectly through redox cycling. The latter hypothesis assumes a rate of alpha-HCH biotransformation that is sufficient for metabolite generation that results in statistically significant production of superoxide anions and lipid peroxidation within a short timeframe. Neither of these hypotheses has been experimentally evaluated for alpha-HCH.

Oxidative stress is a threshold-based process. There are numerous cellular mechanisms for detoxification of ROS; these mechanisms are upregulated in response to increased oxidative stress. Such upregulation has been demonstrated for alpha-HCH. Only when these compensatory systems are overwhelmed do deleterious effects on the cell occur (e.g., lipid peroxidation, DNA damage) (Klaassen 2001). The alpha-HCH data suggest a threshold for P450 induction, oxidative lesions, and cell proliferation (Masuda et al. 2001; Puatanachokchai et al. 2006), although there are very few studies that looked at relevant "key event" endpoints over a range of doses for sufficient duration.

Aberrant Cell Proliferation

The physiological consequences of unmitigated cellular oxidative damage can include necrosis, apoptosis, genetic mutation, and stimulation of cell growth (Klaunig and Kamendulis 2004; Klaunig et al. 1998) which can lead to tumor formation. The data for alpha-HCH support increased growth as a cellular response to oxidative stress, although the precise mechanism by which the growth occurs has not been evaluated. Alpha-HCH exposure consistently results in increased hepatocellular proliferation, as evidenced by increased liver DNA synthesis, hypertrophy, and hyperplasia, leading to increased relative liver weight in both rats and mice. DNA synthesis is increased in hyperplastic and, in some cases, normal liver tissue from exposed mice (Gerlyng et al. 1994; Schulte-Hermann et al. 1981; Siglin et al. 1991, 1995; Tryphonas and

Iverson 1983). Increased DNA synthesis is observed after single oral doses and after long-term dietary exposure (Schroter et al. 1987; Schulte-Hermann et al. 1981, 1983), which demonstrates that the proliferative effect is immediate and is sustained with continuous exposure. Overall liver DNA and ribonucleic acid (RNA) content is increased after exposure to alpha-HCH, and these increases regress after cessation of exposure (Schulte-Hermann and Parzefall 1981), consistent with its role as a tumor promoter. The observed increase in DNA synthesis occurs in hepatocytes of differing chromosomal content (i.e., diploid, tetraploid, or octaploid). However, the proportion of binuclear (as opposed to mononuclear) hepatocytes decreases during mitogenesis (Gerlyng et al. 1994). Decreased binucleation is indicative of loss of terminal differentiation and entrance into an aberrant pattern of cell growth (Guidotti et al. 2003). Supporting the conclusion that alpha-HCH exposure leads to aberrant cell growth is the observation that hepatic cell division decreases overall while individual initiated clone size increases (Luebeck et al. 1995). Alpha-HCH-mediated increases in centrilobular hyperplasia or hypertrophy and liver weight regress after cessation of exposure (Angsubhakorn et al. 1981; Ito et al. 1976; Kraus et al. 1981; Schulte-Hermann and Parzefall 1981), which also demonstrates the tumor promoting effect of the chemical. Dose-dependent increases in liver weight, hypertrophy, GGT-positive foci formation, and DNA content have been observed with long-term alpha-HCH exposure (Fitzhugh et al. 1950; Goto et al. 1972; Ito et al. 1973a,b; Luebeck et al. 1995; Masuda et al. 2001; Schroter et al. 1987). Among mouse strains, there is some evidence for strain- and gender-dependent differences in liver weight increase, severity of microscopic changes (e.g., hyperplasia), and overall tumor incidence (Nagasaki et al. 1975).

3.1.4.2 Weight of Evidence: Evaluation of Causation Criteria

The evidence supporting the postulated key events underlying the mitogenic MOA were evaluated against well accepted causation criteria (Bradford Hill 1965). The collective WOE indicates that this MOA is operational for alpha-HCH induced hepatocarcinogenicity, as discussed below.

Strength, Specificity, and Consistency of the Association

P450-related parameters such as isozyme protein and activity, total P450, and P450 reductase were consistently increased in independent studies of varying experimental design. Statistically significant increases in total P450 and P450 reductase activity and/or protein were seen in 2/2 studies which examined these effects. Statistically significant increases in P450 isozyme activity were seen in 3/4 studies which examined this effect; in the fourth study, increased P450 was also seen but these data were not statistically evaluated. This trend is supported by two microarray studies which found increased P450 isozyme expression; however, the microarray data themselves were not conclusive (i.e., because the microarray results were inconsistent over the multiple time points measured). While the association between alpha-HCH and P450 induction is supported by existing data; the association is not considered definitive because of the small number of studies looking at relevant endpoints and also because limitations in the

study design such as small sample size and use of non-specific P450 isozyme substrates. HCH-mediated P450 increases have not been evaluated in mice, which are more sensitive to tumor formation than rats.

Six independent studies evaluated parameters related to oxidative stress; however, most of the parameters were not measured across multiple studies. Lipid peroxidation, oxidative DNA damage, superoxide anion production, and antioxidant enzyme activities (catalase, SOD, and GRed) were only evaluated in one study each. Significant increases in these four endpoints were observed. In addition, increased GST activity was seen in 2/2 studies which examined this endpoint; the increase was statistically significant in one study and was not statistically evaluated in the second study. The magnitude of the GST effect was different depending on the substrate used and the route of HCH administration. Potential increases in GST expression were also seen in two microarray studies; however, the microarray data were not conclusive. As was the case with P450 induction, the observed association between alpha-HCH and oxidative stress is not considered definitive because of the small number of studies looking at relevant endpoints and because of the inconsistent GST data.

Increased cell growth and proliferation have been consistently observed in numerous independent studies of varying experimental design. Markers of cell growth (e.g., relative liver weight, hypertrophy, foci formation, DNA labeling) were increased in almost every study in which these endpoints were examined. These effects occurred at high incidence (e.g., hypertrophy) or were statistically significant (e.g., relative liver weight) in most cases. Only two studies did not show a significant increase in markers of cell proliferation; the lack of an effect may be due to short study duration or species/strain differences in response.

The association between exposure and any of these endpoints is not specific for alpha-HCH; other chemicals have been observed to exert these individual effects or a combination of these effects. However, the lack of specificity of these effects does not decrease their relevance for alpha-HCH-mediated tumor formation.

Dose-Response

Consistent dose-dependent increases in markers of cell proliferation, P450 parameters, and oxidative stress markers were observed in each study in which multiple dose levels/ concentrations were evaluated, with the number of studies different for each endpoint and therefore providing different degrees of evidence for each effect. No increased response at low doses was consistently observed, suggesting a threshold, and, in some cases, hormesis. A lack of dose-response was observed in one study where the incidence of response was 100% at all doses, which illustrates the potency of the doses used. The overall dose-dependency of the effects, particularly cell proliferation, strengthens the causal association between alpha-HCH exposure and liver tumor formation.

Temporal Association

Temporal relationships between exposure and increased cell proliferation, P450 parameters, and oxidative stress parameters were consistently seen across studies. Specifically, two types of temporal patterns were observed: increased response over time and regression of response following cessation of exposure. However, the number of studies that looked at a temporal response was small (n=7 for markers of cell proliferation, and n=1 each for P450 and oxidative stress endpoints). None of the studies evaluated was inconsistent with a temporal pattern. Regression of increases in P450 activity, liver DNA/RNA content, liver weight, and hypertrophy were seen in several studies following cessation of exposure, which further supports the role of alpha-HCH as a tumor promoter.

3.1.4.3 Human Relevance of Animal Mode of Action

The biologic plausibility of the key events in humans has been well established in general; the qualitative and quantitative plausibility are discussed below.

Qualitative Plausibility of Key Events in Humans

Each of the key events identified for alpha-HCH as occurring in animals (e.g., P450 induction, oxidative stress, and increased cell growth) can also occur in humans. For example, xenobiotics that upregulate CYP isoforms in animals are also known to upregulate the appropriate human homologue. The isoforms induced in animals by HCH appear not to be species-specific, as substrates that elicited increases in the isoforms in animals (e.g., testosterone) are also capable of eliciting a response in humans (Klaassen 2001).

Elevated CYP isoform protein has been detected in samples of human hepatocellular carcinoma (HCC) and peri-tumor tissue. Specifically, CYP2E1 protein was significantly increased in tumor and peri-tumor tissues relative to normal hepatocytes. Peri-tumor tissue exhibited the greatest degree of CYP2E1 staining. Moreover, CYP2E1 staining was proportional to the degree of HCC differentiation, with well-differentiated tumors showing the most staining. CYP2E1 protein was also elevated in cirrhosis samples and samples of adenomatous hyperplasia. The degree of CYP2E1 protein expression or staining was not dependent on the severity of fibrosis or hepatitis, however (Hirose et al. 2002). These data support the relevance of CYP induction as a key event in human liver cancer development.

Some agents that induce liver tumors also induce P450 isoforms (e.g., 2,3,7,8-tetrachlorodibenzodioxin [TCDD], benzo(a)pyrene [BaP], PB), although P450 induction is not always causal for the observed carcinogenic effect (Klaassen 2001).

Generation of ROS such as superoxide anion is known to occur in humans as well. The mechanisms underlying ROS generation in humans are similar to those in animals, namely P450 activity or leakage from the mitochondrial electron transport chain. Moreover, the cellular targets for ROS are the same for animals and humans: macromolecules such as proteins, lipids,

and DNA. Antioxidant defenses such as glutathione, catalase, and SOD are similar between animals and humans. Inflammation and oxidative stress are known risk factors for development of HCC in humans (Feo et al. 2009; Kohle et al. 2008). Moreover, many causal factors are known to exert their tumor promoting effects in humans by an oxidative stress MOA, such as hepatitis, ethanol consumption, arsenic, and carbon tetrachloride (James et al. 2003; Robertson et al. 2001).

Increased cell growth is a hallmark of the carcinogenic process. The biochemical pathways and physiologic processes underlying cell growth are highly conserved among mammalian species. However, the factors that drive a growth response to neoplasia are less well understood. It is well-known that the disease of cancer is characterized by loss of homeostatic growth control. The cellular and molecular events underlying this growth abnormality are complex and are likely tissue- and agent-specific.

Although key events observed in animals have not been directly evaluated in humans exposed to alpha-HCH, the concordance of biochemical pathways and physiological processes between animals and humans provides strong evidence of the relevance of these effects for human health.

Quantitative Plausibility of Key Events in Humans

For some xenobiotics, quantitative differences in toxicokinetic or toxicodynamic factors may exist between humans and animals that drive differences in susceptibility.

The lipophilic nature of HCH isomers in general is likely the primary determinant of the degree of systemic absorption. Uptake into the portal circulation or into different tissues has not been shown to be receptor-mediated. Although small quantitative differences in the exact degree of absorption or exact tissue concentrations may occur between animals and humans, there is no evidence of differences in toxicokinetic factors that are anticipated to drive species susceptibility.

The key toxicodynamic response to alpha-HCH exposure is an increase in cell growth. Increased cell growth is a general and highly conserved process; therefore, it is unlikely that species differences in molecular targets or pathways (e.g., alpha 2microglobulin) would account for any possible differential susceptibility between humans and animals.

In general, the toxicokinetic or toxicodynamic factors that may drive species-specific differences in toxic response appear not to be relevant for alpha-HCH carcinogenicity.

3.1.4.4 Uncertainties for the Weight of Evidence for Carcinogenicity and Mode of Action

Although some data gaps and limitations in the individual studies for alpha-HCH exist, these do not undermine the conclusions for the carcinogenicity classification assigned to the

compound. Uncertainties introduced by the data gaps and study limitations are discussed below.

The lack of reliable human data and the need to rely solely on the results of animal bioassays present an uncertainty for determining the carcinogenic potential of alpha-HCH in humans. The conclusions regarding the carcinogenic potential are based on *in vivo* bioassays in animals, and *in vitro* assays in animal and human cell lines. Limitations of the individual animal studies are shown in Table 2.

Generally, the ability to establish dose-response and temporal patterns is limited by the small number of studies that examined either multiple doses or multiple time points for each relevant endpoint.

The potential association between alpha-HCH exposure and P450 induction or oxidative stress is suggestive but not definitive because few studies evaluated these endpoints. The findings in the few available studies were, however, generally statistically significant and consistent.

Although a general link between oxidative stress and cell proliferation has been established, such a link has not been experimentally evaluated in the case of alpha-HCH. Oxidative stress can affect signal transduction, induce or inhibit apoptosis, activate an inflammatory response, and can attack membrane lipids or DNA (Klaunig and Kamendulis 2004; Klaunig et al. 1998). Any one or combination of these events may underlie the tumorigenic effect of alpha-HCH. Moreover, increased proliferation by itself does not necessarily lead to tumor formation (Melnick et al. 1996).

Additional potential MOAs underlying alpha-HCH mediated tumor formation, either related or unrelated to oxidative stress, have received little experimental evaluation. Key events mediating such MOAs may include suppression of apoptosis, inhibition of intercellular communication, and receptor activation/signal transduction, all of which are likely to be threshold-based. These events can be directly or indirectly mediated by oxidative stress, although this has not been experimentally demonstrated for alpha-HCH.

The exception to this generalization is peroxisome proliferation. Electron microscopic examination shows that alpha-HCH does not result in increased peroxisome area or number (i.e., peroxisome proliferation) (Ito et al. 1973a, 1976; Tsukada et al. 1979). However, electron microscopy has only been conducted in mice. The overall lack of necrotic and fibrotic changes adjacent to hyperplastic areas observed in the livers of alpha-HCH-treated animals suggests that cytotoxicity with regenerative hyperplasia is unlikely to be a relevant MOA for alpha-HCH.

3.2 NON-CANCER ENDPOINTS

Renal, neurological, and hepatic toxicity has been observed following subchronic and chronic exposure to alpha-HCH in animals (ATSDR 2005, independent literature search). Human data for reproductive effects following exposure to alpha-HCH do not indicate positive responses; however, in general, epidemiological studies for alpha-HCH are of limited utility for identifying non-cancer health effects. Table 4 presents a list of the primary references considered in the evaluation of the most sensitive target organ. As shown in Table 4, only low-dose effects (effects that were observed at a LOAEL or NOAEL of 10 mg/kg-day or less in at least one study) were brought forward to the evaluation of the most sensitive target organ and endpoint.

Renal and hepatic toxicity were the only two categories for which effects were observed in subchronic or chronic low-dose studies (defined for the purposes of this evaluation as a study with at least one dose <10 mg/kg-day). Renal effects seen at low doses in rodents were found to result from mechanistic pathways not existing in humans (USEPA 1991), and therefore, were not considered further for this evaluation. The liver was determined by USEPA (2006) to be the most sensitive target organ system for effects following alpha-HCH exposure.

Table 5 presents a comprehensive summary of the effect levels reported for liver toxicity. The table includes effects that are precursors to hepatocarcinogenicity. Because the progression to cancer is recognized to be a continuum of processes and events (which theoretically may or may not progress to cancer) their inclusion here is appropriate. LOAELs ranged from 0.2 mg/kg-day (for increases in the number of hepatic foci in male rats exposed to alpha-HCH for six weeks following a known initiator [Masuda et al. 2001]) to 63 mg/kg-day (for histological changes to the liver in rats chronically exposed to alpha-HCH [Fitzugh et al. 1950]).

3.3 MOST SENSITIVE TARGET ORGAN

The available data indicate that the liver is the most sensitive target organ following subchronic or chronic exposure to alpha-HCH.

4 TOXICITY CRITERION

A final cancer-based RfD of 0.0003 mg/kg-day was established for alpha-HCH. The toxicity criterion is based on the NOAEL of 0.1 mg/kg-day from Masuda et al. (2001) for an increased number of hepatic foci (associated with the LOAEL of 0.2 mg/kg-day) and the combined uncertainty factor of 300 (10 each to account for intra- and inter-species extrapolation, and 3 for database uncertainties).

The process for selecting the study and endpoint for the critical effect, and for determining the POD are documented below. Following, the UFs and/or MFs selected for application to the POD are described.

4.1 SELECTION OF ENDPOINTS AND DATASETS

The available evidence indicates that alpha-HCH acts as a tumor promoter and through a MOA that displays a threshold dose-response. In the case that a non-linear, threshold RfD is determined to be appropriate for modeling cancer risk, USEPA (2005a) specifies that the most sensitive endpoint for toxicity be used to determine the specific response data that are selected for deriving the RfD. The critical organ determined for alpha-HCH is the liver. Therefore, the appropriate toxicity criterion for alpha-HCH is a RfD that assumes non-linearity for hepatocarcinogenic response.

Table 6 presents a comprehensive listing of the endpoints for liver effects evaluated in low-dose animal bioassays conducted via oral administration for subchronic or chronic duration, considered for toxicity criterion development. It shows the endpoints that were determined appropriate for the POD determination. It additionally shows the studies/endpoints for which appropriate data amenable to BMD analysis was available. The specific reasoning for data excluded from the BMD analysis is provided.

Data indicative of, or related to, all of the key events determined for the MOA, with the exception of absorption to the liver, were available. A subset of these data, which are indicative of early precursor events including CYP P450 concentrations and activity; oxidative stress (thiobarbituric acid reactive substance [TBARS], superoxide anion production, SOD, 8-OHdG levels, and GST activity), were not considered for the determination of the POD. These endpoints are related to processes that occur in the MOA for hepatocarcinogenicity of alpha-HCH; however, their occurrence is not tightly linked with the outcome (i.e., their occurrence is not necessarily predictive of a toxic outcome). Endpoints that indicate aberrant cell proliferation (or adaptive changes potentially linked with the key event), including liver weight (relative and absolute), microscopic changes to the liver, hypertrophy, number and area of hepatic foci, were brought forward for the POD evaluation. Additionally, indicators of liver

injury, including alanine aminotransferase (ALT), and gross macroscopic changes to the liver were brought forward for the POD determination.

4.2 DETERMINATION OF POINT OF DEPARTURE

Two approaches for deriving the POD were utilized: a traditional RfD approach and BMD modeling. Although BMD modeling has recognized advantages over the traditional RfD approach (USEPA 2000; Castorina and Woodruff 2003), all data sets are not amenable to BMD modeling[5]. Exploring results via both approaches allowed for a comprehensive evaluation of the available data.

Table 7 presents the results of the traditional RfD approach. The table summarizes the lowest LOAEL and its associated NOAEL for the studies and endpoints considered for the POD. Table 8 presents the results of the BMD modeling for all studies and endpoints considered for which modeling was amenable. In addition to the effect levels provided for the low-dose studies, described in Section 4.1 results from the studies which showed the lowest effect levels for tumor incidence following chronic exposure are additionally presented (Tables 7 and 8). These provide perspective on the relationship between the doses at which the early indicators of hepatocarcinogenicity and tumors occur.

As shown in Table 7, the lowest NOAEL from the studies and endpoints considered for the POD was 0.1 mg/kg-day (associated with a LOAEL of 0.2 mg/kg-day for an increase in the number of hepatic foci in male rats exposed to alpha-HCH following a known initiator [Masuda et al. 2001]). The data for the incidence of hepatic foci from this study was amenable to BMD modeling. The foci data were only shown in a graphical format. According to USEPA guidance (2000) data in this format should not be modeled. Of the studies that modeled successfully, the lowest confidence limit on the BMD (BMDL) was 0.39 mg/kg-day. The BMDL was for data reported by Schroter et al. (1987) for an increase in area of hepatic foci in female rats. For perspective, the LOAEL from the studies which showed the lowest effect levels for tumor incidence was 45 mg/kg-day (Ito et al. 1973a,b). The BMDLs for increased tumor incidence reported by Ito et al. (1973a,b) were 26.7 and 26.3 mg/kg-day.

The NOAEL of 0.1 mg/kg-day associated with increased number of hepatic foci from Masuda et al. (2001) was selected as the POD for alpha-HCH. This value was conservatively selected because it was the lowest of the PODs derived.

[5] All BMD modeling was completed using EPA's Benchmark Dose Software version 2.1, and following EPA guidance on benchmark dose modeling (USEPA 2000).

4.3 APPLICATION OF UNCERTAINTY AND MODIFYING FACTORS TO THE POINT OF DEPARTURE

UFs and MFs to be used for the derivation of a toxicity criterion for alpha-HCH based on the POD for hepatic foci formation are presented below.

- **Intraspecies Extrapolation Factor** – A factor of 10 was selected for this factor to account for the variation in sensitivity among the members of the human population.

- **Interspecies Extrapolation Factor** – A value of 10 was selected for this factor to account for the uncertainty involved in extrapolating from animal data to humans.

- **Subchronic-to-Chronic Duration Factor** – A value of 1 was selected for this factor. Masuda et al. (2001) is a subchronic duration study; there are, however, available chronic studies for alpha-HCH that did not show liver effects (including the same effect measured by Masuda as the most sensitive – e.g., increased preneoplastic foci formation) at lower doses than observed by Masuda et al. (see Table 7; Fitzhugh et al. 1950; Puatanachokchai et al. 2006; Schroter et al. 1987)[6].

- **LOAEL-to-NOAEL Factor** – A value of 1 was selected for this factor. The POD selected was a NOAEL.

- **Database UF** – A value of 3 was selected to account for some gaps in the available data. Animal studies of reproductive and developmental endpoints are not available.

- **Additional MF** – No additional MFs were determined necessary for the derivation of the toxicity criterion.

4.4 SUMMARY OF RECOMMENDED TOXICITY CRITERION

The recommended RfD for alpha-HCH is 0.0003 mg/kg-day. The value is based on a POD of 0.1 mg/kg-day for increased incidence of preneoplastic hepatic foci in rats, and a total UF of 300 (10 each to account for intra- and inter-species extrapolation, and 3 for database uncertainties).

[6] USEPA (2002) describes that a subchronic-to-chronic UF is applied only when a chronic study is not available, and is based on the assumption that effects from a given compound in a subchronic study occur at 10X higher concentration than in the corresponding (but absent) chronic study. This is not the case for alpha-HCH.

5 SUMMARY

Integral has developed an updated toxicity criterion for the chemical alpha-HCH.

The collective evidence indicates that alpha-HCH is a hepatocarcinogen in rodents. Alpha-HCH is not mutagenic, and induces a carcinogenic response in rodents via aberrant cell proliferation triggered by absorption in the liver, P450 induction, and oxidative stress. The MOA is sufficiently well described in the experimental literature to conclude with appropriate confidence that alpha-HCH acts via a non-linear MOA, and displays a threshold at which carcinogenicity does not occur. There is insufficient evidence to indicate that alpha-HCH is carcinogenic in humans. However, the physiological process and biochemical pathways observed in animals are present in humans, and thus, the MOA and carcinogenic response in animals is potentially relevant to humans. Following USEPA (2005a) guidance, the following WOE cancer classification was determined for alpha-HCH: **"suggestive evidence of carcinogenic potential above a specified dose but not likely to be carcinogenic below that dose."**

The liver is determined to be the most sensitive target organ following subchronic and chronic exposure to alpha-HCH. Considering these findings and following USEPA (2005a) guidance the development of a cancer-based RfD is appropriate for the protection of human health.

The recommended cancer-based RfD for alpha-HCH is 0.0003 mg/kg-day. The value is based on a POD of 0.1 mg/kg-day for increased incidence of preneoplastic hepatic foci in rats, and a total UF of 300 (10 each to account for intra- and inter-species extrapolation, and 3 for database uncertainties).

For perspective the recommended cancer-based RfD is approximately an order of magnitude lower than the oral chronic non-cancer RfDs and minimal risk levels (MRL) established by EPA and the ATSDR respectively. In their 2006 *Assessment of Lindane and Other Hexachlorocyclohexane Isomers* (USEPA 2006), completed as part of the Reregistration Eligibility Decision (RED) for Lindane, EPA established chronic oral RfDs for alpha-HCH of 0.001 mg/kg-day and 0.008 mg/kg-day. ATSDR (2005) established a chronic oral MRL for alpha-HCH of 0.008 mg/kg-day. The non-cancer RfDs and MRL are all based on hepatoxicity.

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TABLES

Table 1. Epidemiological Evidence: Alpha-HCH and Cancer.

Endpoint	Study	Summary of Findings	Study Limitations
Breast Cancer			
456	Mathur et al. (2002)	Case-control study of women from India. Found higher levels of alpha-HCH in blood of women (age 41-50) with breast cancer compared to controls. Relationship was not significant for other age groups.	Potential confounders including the presence of other organochlorine pesticides were not controlled for. Lipids in blood were not measured. Method for selecting control group was not discussed fully. Potential for retrospective questionnaire bias was not discussed.

Notes: HCH = hexachlorocyclohexane

Studies in which pesticides were measured, but not detected with adequate frequency for statistical analysis are not included in this table. For alpha-HCH these studies were Cocco et al. (2008); Quintana et al. (2004).

Table 2. Alpha-HCH Carcinogenicity and Mode of Action for Hepatocarcinogenicity.

	Reference	Species, Sex	Study Design	Summary of Findings	Major Study Limitations	Relevance of Findings for Carcinogenicity and MOA
380	Angsubhakorn et al. (1981)	Rat (Buffalo), male	**Duration:** up to 35 weeks exposure; interim sacrifices and 30 week recovery **Sample Size:** 3-8/group **Route:** dietary, ad libitum **Dose Levels:** 0, 500 ppm	Centrilobular hypertrophy observed in treated animals beginning after week 5 (incidence of 4/4) and continuing throughout treatment period (incidence of 5/5 after 35 weeks). Treatment-related centrilobular hypertrophy regressed: incidence of 0/7 after the recovery period. Incidence of foci of cellular alterations was 1/5 after 35 weeks and 1/7 after 35 weeks plus 30 week recovery. No nodules or HCC observed.	Small sample size. Only males evaluated. Only one dose level evaluated. Mortality/general toxicity not reported. No statistical evaluation.	Regression of treatment-related effects suggests non-mutagenic MOA. Supports increased proliferation as key event. Temporal trend observed.
381	Barros et al. (1991)	Rat (Wistar), male	**Duration:** 15 or 30 days **Sample Size:** 6-22/group **Route:** dietary, ad libitum **Dose Levels:** 0, 20 ppm	Increased total P450 levels at 15 days; further increase at 30 days (both significant). Increased P450 reductase at 30 days. Increased TBARS formation in liver homogenates and microsomes after 15 and 30 days. Increased microsomal superoxide production at 15 days; further increase at 30 days. Increased SOD activity at 15 days; decreased at 30 days relative to 15 days (but higher than control). Increased glutathione reductase at 30 days; increased catalase at 15 and 30 days. All changes were statistically significant and were generally time-dependent. No microscopic changes.	Small sample size. Only one dose level evaluated. Only males evaluated. Unclear mortality.	Findings support P450 induction and oxidative stress as key events.
382	Fitzhugh et al. (1950)	Rat (Wistar), male/female	**Duration:** approximately 107 weeks **Sample Size:** 10/sex/group; 20/sex/group controls **Route:** dietary, ad libitum **Dose Levels:** 0, 10, 50, 100, 800 ppm	No gross tumors reported. Necrotic foci (<1 mm diameter) and other degenerative changes observed in highest dose (800 ppm) group. Relative liver weight significantly increased in the 50, 100, and 800 ppm groups (dose-dependent). Rats exposed to 800 ppm had decreased body weight gain and decreased survival compared to controls; MTD exceeded.	Small sample size. Minimal details on histopathology. High overall mortality in the study; evaluations were based either on moribund or found dead animals. Inadequate discussion of mortality/general toxicity. Data were not stratified by sex.	Suggests increased proliferation as key event. Dose-response observed. Inconclusive for carcinogenicity due to inadequate description of liver changes.
577	Gerlyng et al. (1994)	Rat (Wistar), male	**Duration:** 50 hours **Sample Size:** 2-19/group **Route:** oral gavage **Dose Levels:** 0, 150 mg/kg	A single dose of HCH did not alter hepatocyte ploidy. DNA labeling index (BrdU incorporation) was maximal ~30 hours after a single HCH dose and was increased in both mononuclear and binuclear hepatocytes. DNA labeling was significantly increased in diploid, tetraploid, and octaploid hepatocytes following daily 150 mg/kg oral doses of HCH; the proportion of binuclear cells decreased, suggesting aberrant proliferation.	Only males tested. Small sample size. Only one dose level evaluated. The number of repeated doses was not specified for the binucleation experiment.	Supports increased proliferation as a key event.
383	Goto et al. (1972)	Mouse (ICR-JCL), male	**Duration:** 26 weeks **Sample Size:** 10/group **Route:** dietary (unknown if ad libitum) **Dose Levels:** 600 ppm	Hepatoma (10/10) consisting of areas of atypical proliferation, nodules, and tumors. Hepatoma incidence in control animals not reported. No fibrosis. No metastases. Relative liver weight was increased.	Only one dose level evaluated. Small sample size. Only males tested. No statistical analysis. Inadequate characterization of histopathological changes. Mortality not reported. Incidence of benign and malignant tumors not reported. Inadequate translation from German did not allow for comprehensive review.	Inconclusive for carcinogenicity due to lack of tumor/histology data for control animals.
385	Hanada et al. (1973)	Mouse (dd), male/female	**Duration:** 32 weeks plus 5-6 weeks recovery **Sample Size:** 10-11/sex/group; 20-21/sex/group controls **Route:** dietary, ad libitum **Dose Levels:** 0, 100, 300, 600 ppm	1/5 300 ppm male, 1/4 300 ppm female, and 2/4 600 ppm males had liver tumors at the week 26 laparotomy. Hepatoma observed after exposure plus recovery: males 0/14, 1/8, 7/7, 7/7 and females 0/15, 0/8, 2/3, 6/8. Average tumor size increased with increasing dose in exposure plus recovery group. No microscopic peritoneal invasions or metastases seen. Atypical proliferation in liver (hypertrophic foci; associated with liver cell damage) noted in all treated exposure plus recovery mice except females at 100 ppm. Incidence of 8/8 100 ppm males, 7/7 300 ppm males, 3/3 300 ppm females, 7/7 600 ppm males, and 8/8 600 ppm females. One 600 ppm female had mammary carcinoma.	Small sample size. No statistical analysis. Apparent increase in mortality in treated animals that was not dose-dependent. General toxicity data were not reported. No evaluation done at the end of the 32 week exposure period; regression of changes could not be evaluated.	Dose-dependent increase in tumor number and size supports role as tumor promoter. Supports increased proliferation as key event.
363	Ito et al. (1973a)	Mouse (dd), male	**Duration:** 24 weeks **Sample Size:** 20-40/group **Route:** dietary, ad libitum **Dose Levels:** 0, 100, 250, 500 ppm	Dose-dependent increase in HCC (0/20, 0/20, 10/38, 17/20) Dose-dependent increase in liver nodular hyperplasia in treated mice (0/20, 30/38, and 20/20). No metastatic changes or tumors in other organs were noted upon gross examination. Dose-dependent increase in relative liver weight. Severe liver cell hypertrophy observed in 250 and 500 ppm groups; less severe at 100 ppm. Necrotic or fatty change rarely noted. Increased smooth endoplasmic reticulum in carcinomas and non-cancerous tissue. Body weight not affected.	Only males evaluated. No statistical evaluation. Only examined liver histologically. Mortality not reported.	Dose-dependent increase in tumors supports role as tumor promoter. Supports increased proliferation as key event.
364	Ito et al. (1973b)	Mouse (dd), male	**Duration:** 24 weeks **Sample Size:** 26-30/group; 20/group controls **Route:** dietary, ad libitum **Dose Levels:** 0, 50, 100, 250 ppm	Nodule incidence of 23/30 and carcinoma incidence of 8/30 in the 250 ppm group. No nodules or carcinoma in 50 or 100 ppm groups. Centrilobular hypertrophy observed in the 100 and 250 ppm groups (dose-dependent increase in severity). No cirrhosis or metastases. Relative liver weight was increased (dose-dependent). Body weight not affected.	No statistical evaluation. Only males evaluated. Unclear if extra-hepatic tumors/metastases were evaluated microscopically. Mortality not reported.	Dose-dependent increase in tumors supports role as tumor promoter. Supports increased proliferation as key event.
386	Ito et al. (1975)	Rat (Wistar), male	**Duration:** 72 weeks; interim sacrifices **Sample Size:** 5-16/group **Route:** dietary, ad libitum **Dose Levels:** 0, 500, 1000, 1500 ppm	HCC observed only in 1000 and 1500 ppm groups at 72 weeks (incidence of 1/16 and 3/13, respectively). Incidence of nodular hyperplasia as follows: 1000 ppm - 5/12 (48 weeks), 12/16 (72 weeks); 1500 ppm - 10/13 (72 weeks). None in control or 500 ppm groups. Increased relative liver weight in all dose groups at all time points (dose-dependent). Hepatic hypertrophy observed; dose- and time-dependent increase in severity. Bile duct proliferation and oval cells observed at 1000 and 1500 ppm (48 and 72 weeks only).	Control animals sacrificed at different time than treated animals. Mortality not reported. Unclear if metastases were evaluated grossly or microscopically. Insufficient description of general toxicity. Only males evaluated. Small sample size. No statistical evaluation.	Threshold response and long time-to-tumor supports role as tumor promoter. Supports increased proliferation as key event. Dose-response and temporal trend observed.
387	Ito et al. (1976)	Mouse (DDY), male	**Duration:** 72 weeks; interim sacrifices and recovery. **Sample Size:** 12-20/group **Route:** dietary, ad libitum **Dose Levels:** 0, 500 ppm	Centrilobular hypertrophy observed after 16 weeks; regressed following cessation of treatment. Incidence of liver tumors increased progressively with continuous exposure (25% after 16 weeks, 70% after 20 weeks, 100% after 24 weeks); some tumors regressed following exposure cessation. Increased relative liver weight over time; increases regressed following exposure cessation. Metastases to regional lymph nodes, lungs, or kidneys were not observed microscopically. After 24 weeks, most tumors were nodular hyperplasia. At 60-72 weeks, most tumors were HCC. Increased amount of smooth endoplasmic reticulum was observed on electron microscopy in the hyperplastic cells.	Only one dose level evaluated. Only males evaluated. No statistical analysis. Apparent increase in mortality over time and with longer exposure.	Regression of some tumors following exposure cessation supports role as a tumor promoter. Temporal trend exhibited. Supports increased proliferation as key event. Electron microscopy data argue against peroxisome proliferation as a key event.
643	Ito et al. (1983)	Rat (Fisher 344), sex not reported	**Duration:** Initiation with DEN and partial hepatectomy followed by 6 weeks of alpha-HCH exposure. Some rats sacrificed at the end of the 6 weeks; other groups were periodically sacrificed over a 50 week total duration. **Sample Size:** 8-34/group for some endpoints; not reported for other endpoints. **Route:** dietary **Dose Levels:** 0, 1000 ppm	No increase in number or area of hepatic hyperplastic nodules, in number of degenerated hyperplastic nodules, or in number of HCCs was observed compared to control over the 50 week experimental period. Hyperplastic nodule number and area were significantly increased in DEN-initiated, partially hepatectomized rats who received 6 weeks of dietary alpha-HCH and were immediately sacrificed.	Lack of methodological details, including animal sex and group size. No mortality or toxicity data.	Supports classification as a tumor promoter whose effects depend upon continuous exposure. Supports increased proliferation as a key event.
389	Kraus et al. (1981)	Rat (Wistar), male/female	**Duration:** 16 days; interim sacrifices **Sample Size:** 5-10/group **Route:** intraperitoneal or gavage **Dose Levels:** 0, 3, 10, 30, 50, 100, 200 mg/kg	GST activity was significantly increased 2, 4, and 6 days after a single intraperitoneal dose (multiple GST substrates); GST was generally not increased after a single oral dose, except for 6 days post-dose when an HCH metabolite was used as the substrate. GST increases were transient. GST activity increases were dose-dependent and significant at doses at or above 30 mg/kg (multiple substrates). Relative liver weights were significantly increased 2, 6, and 10 days after a single oral dose and 4 and 6 days after a single intraperitoneal dose; increases were transient in the i.p. group. GST activity and relative liver weights were significantly increased 6 days after a single i.p. dose of 200 mg/kg in animals 14, 21, and 42 days old but not in 3 day old rats.	Only males evaluated. Small sample size. Potentially irrelevant route of exposure (intraperitoneal).	Findings support oxidative stress as a key event.

Table 2. (continued)

	Reference	Species, Sex	Study Design	Summary of Findings	Major Study Limitations	Relevance of Findings for Carcinogenicity and MOA
496	Lee and Edwards (2001)	Rat (Wistar), male hepatocytes	**Duration:** 6 hours **Sample Size:** 2 cultures **Route:** *in vitro* **Dose Levels:** 0, 30 microM	Prostaglandin E2 release was not increased in treated hepatocytes.	Small sample size. Only one concentration evaluated. Very little data presented on HCH, including DNA synthesis data. Cell viability and treatment cytotoxicity were not reported.	Inconclusive for MOA due to limitations of the study.
455	Luebeck et al. (1995)	Rat (Wistar) female	**Duration:** NNM initiation, 8 week recovery, then 10 or 28 weeks alpha-HCH followed by 2, 6, or 21 week recovery **Sample Size:** 3-7/group/time point **Route:** dietary **Dose Levels:** 0, 20 mg/kg bw	Volume fraction and mean number of hepatic foci increased over an 18 or 36 week exposure and decreased upon cessation of exposure.	No mortality or toxicity data. Only female rats evaluated.	Supports classification as a tumor promoter whose effects depend upon continuous exposure. Supports increased proliferation as a key event.
339	Masuda et al. (2001)	Rat (F344), male	**Duration:** 6 weeks after initiation by DEN and partial hepatectomy **Sample Size:** 15/group **Route:** dietary, ad libitum **Dose Levels:** 0, 0.01, 0.1, 0.5, 1, 2, 4, 7.5, 15, 30, 60, 125, and 500 ppm	GST-P-positive foci increased in dose-related manner in groups receiving 0.5 ppm or more. Numbers of GST-P positive foci significantly increased in groups treated with 2 ppm and higher, with the exception of 4 ppm. Areas of GST-P positive foci significantly increased in groups treated with 7.5 ppm and higher, with the exception of 15 ppm. Dose-dependent, significant increases in CYP2B protein from 60 ppm were seen. Testosterone 16B-hydroxylation activity was significantly increased in a dose-related manner from 30 ppm. CYP3A protein significantly increased (dose-dependent) from 4 ppm and testosterone hydroxylation significantly increased (dose-dependent) from 15 ppm. Relative liver weight significantly increased in the 7.5, 60, 125, and 500 ppm groups. Body weight significantly decreased in 15, 30, 60, 125, and 500 ppm groups.	Non-isoform-specific P450 substrate. The effect of HCH alone on foci formation was not evaluated. Potential confounding effect of partial hepatectomy. Only males evaluated. Small sample size.	Findings support P450 induction and cell proliferation as key events. Evidence of threshold effects for P450 induction and foci formation. Dose-response observed.
463	Nagasaki et al. (1975)	Mouse (DDY), male; Rat (Wistar), male; Hamster (Golden Syrian), male	**Duration:** 24 weeks **Sample Size:** 20 rats, 16 hamsters, 36 mice; 48 mice in the second experiment **Route:** dietary, ad libitum **Dose Level:** 0, 500 ppm	Two experiments conducted (One for species comparison, and one for comparison of alpha-HCH +/- various other compounds). Nodular hyperplasia (20/20 mice and 13/19 mice in first and second experiments, respectively) and HCC observed (6/20 mice and 8/19 mice in first and second experiments, respectively). Centrilobular hypertrophy seen in all three species; most pronounced in mice. No cirrhosis. No tumors in rats or hamsters. Increased relative liver weight in all three species; most pronounced in mice. Reduced body weight gain in rats and hamsters. Co-treatment with 3-MC but not other enzyme inducers reduced the incidence of mouse liver tumors.	Only one dose level evaluated. Only males evaluated. Small sample size. No statistical analysis; standard deviation for body weights not reported. Only livers examined. Mortality not reported. No evaluation of metastases.	Demonstrates species-dependent differences in liver tumorigenesis, which suggests non-mutagenic MOA. Supports increased proliferation as key event.
463	Nagasaki et al. (1975)	Mouse (DDY); Mouse (CH3/He); Mouse (DBA/2); Mouse (ICR); Mouse (C57BL/6), male/female	**Duration:** 24 weeks **Sample Size:** 13-29/sex/strain **Route:** dietary, ad libitum **Dose Level:** 0, 500 ppm	Strain comparison: Increased relative liver weight in treated males and females of multiple mouse strains; strain differences in the degree of the increase. Centrilobular hypertrophy and oval cells observed in males and females of multiple mouse strains. Strain- and gender-dependent differences in incidence of nodular hyperplasia (16.7-100%) and HCC (0-65%). In general, males were more susceptible than females.	Only livers examined histologically. Only one dose level evaluated. Mortality not reported. No statistical analysis.	Strain- and gender-dependent differences in liver tumorigenesis support non-mutagenic MOA. Supports increased proliferation as key event.
307	Puatanachokchai et al. (2006)	Rat (F344), male	**Duration:** 10 weeks following initiation with DEN **Sample Size:** 12/group **Route:** dietary (unknown if ad libitum) **Dose Levels:** 0, 0.01, 0.05, 0.1, 1, 50, 500 ppm	Dose-dependent increase in number and area of GST-P positive foci (significant at high doses); foci number and area at 0.05 ppm were significantly decreased. The proportion of proliferating cells (i.e., PCNA positive) within GST-P positive foci decreased and then increased (dose-dependent; significant at highest dose). Foci were observed in all treated rats. Total P450 content and P450 reductase activity were significantly decreased at 0.05 ppm but significantly increased at 500 ppm. P450 reductase protein level significantly increased at 50 and 500 ppm. Some significant increases in P450 activity at 50 and 500 ppm. Dose-dependent increases in CYP2B, 2C, 2E, and 3A protein levels; increases were significant at 50 and 500 ppm. Liver 8-OHdG levels significantly decreased at 0.1 and 1 ppm but significantly increased at 500 ppm. GST activity significantly increased at 500 ppm. Decreased body weight gain, significantly increased relative liver weight at 500 ppm. Adenomas and HCCs observed only at 500 ppm (mean of 2.8 tumors per rat).	Small sample size. Only males evaluated. Mortality not reported. Oxidative DNA damage repair data difficult to interpret due to high variation in OGG1 message. P450 activity analyses were not isoform-specific; testosterone was the only substrate used for all isoforms. The effect of HCH alone, without initiation, was not evaluated.	Carcinomas seen only at highest doses tested. Threshold response supports role as tumor promoter. Findings support P450 induction, oxidative stress, and cell proliferation as key events. Dose-response trend observed.
390	Schroter et al. (1987)	Rat (Wistar), female	**Duration:** 17 weeks (initiation); 15-20 weeks following initiation by NNM (promotion) **Sample Size:** 3-8/group (initiation study); 4/group (promotion study) **Route:** gavage or dietary, ad libitum **Dose Level:** single oral bolus dose of 200 mg/kg (initiation) or 0-20 mg/kg-day in the feed (promotion)	**Initiation Study:** No increase in GGT-positive foci in partially hepatectomized rats given a single oral bolus dose followed by 15 weeks of phenobarbital in the diet. **Promotion Study:** Dose- and time-dependent increases in foci number and area were observed after 15 and 20 weeks. Foci area was significantly increased relative to control at mid- to high-doses. Dose-dependent increases in liver mass, liver DNA (both significant at highest dose tested), and P450 activities (not significant) were observed. P450 induction and liver weight increases were not predictive of foci formation. NOELs calculated.	Small sample size. Only females evaluated. Not all data were statistically evaluated. Mortality not reported. Only liver evaluated. The effect of HCH alone, without initiation, was not evaluated in the promotion study.	Alpha-HCH is not an initiator in rats. Findings support P450 induction and increased proliferation as key events. Dose-response observed.
430	Schulte-Hermann and Parzefall (1980)	Rat (Wistar), female	**Duration:** 6 days **Sample Size:** 5-6/group **Route:** oral gavage **Dose Levels:** 0, 200 mg/kg	CYP1A, CYP2B, CYP2A, and CYP3A enzyme activities were increased following a single oral dose.	Only females evaluated. Small sample size. No statistical analysis. Unclear if reaction conditions were optimized.	Findings support P450 induction as key event.
391	Schulte-Hermann and Parzefall (1981)	Rat (Wistar), female	**Duration:** 24.5 months; interim sacrifices and recovery. **Sample Size:** 2-5/group **Route:** oral gavage and/or dietary (unknown if ad libitum) **Dose Levels:** 0 and initial oral dose of 100 mg/kg followed by 18.4 mg/kg-day in the diet; 420 mg/kg in 3 week intervals; or 200 mg/kg in 2 week intervals.	Significantly decreased body weight after 4.5 and 23.5 months of continuous exposure; also decreased after 21.5 months of interval exposure (n=2). Significantly increased relative liver weight after 4.5, 13.5, or 23.5 months of dietary exposure and after 11.5 months of interval exposure; no clear temporal trend. After 11.5 months of interval exposure followed by recovery period, relative liver weights and body weights were similar to control. Significantly increased RNA and DNA in liver after continuous or interval treatment up to 23.5 months; no clear temporal trend. The increases regressed in the 11.5 month interval exposure plus recovery group. Significantly increased cytochrome P450 activity following 4.5, 13.5, and 23.5 months of continuous or 11.5 months interval treatment; no clear temporal trend. The increases regressed in the 11.5 month interval exposure plus recovery group. Pronounced increase in GST activity after 11.5 months of interval treatment or 13.5 months of continuous treatment. Time-dependent increase in incidence of macroscopic and microscopic liver lesions (foci, nodules/tumors, and HCC). Low incidence of HCC (1/6 and 1/8 continuous and interval-treated rats after at least 20 months). High incidence of liver nodules (5/6 and 6/8 continuous and interval-treated rats after at least 20 months; 2/4 and 1/4 continuous and interval-treated rats after at least 11.5 months; 3/8 interval-treated rats after 11 months).	Small sample size. Only females tested. Inconsistent dosing regimen. High incidence of microscopic foci in controls (3/9, 1/3, and 5/6). BW decrease was severe (~20%) in the 21.5 month interval treatment and 23.5 month continuous treatment groups. Mortality not reported.	Regression of liver weights and DNA/RNA increases after treatment cessation supports role as tumor promoter. Long time-to-tumor supports role as tumor promoter. Findings support P450 induction, oxidative stress, and increased proliferation as key events of MOA. Temporal trend exhibited.

Table 2. (continued)

	Reference	Species, Sex	Study Design	Summary of Findings	Major Study Limitations	Relevance of Findings for Carcinogenicity and MOA
548	Schulte-Hermann et al. (1981)	Rat (Wistar), female	**Duration:** 8 weeks after DEN; interim sacrifice **Sample Size:** 5-6 rats/group **Route:** oral gavage **Dose Levels:** 0, 150-200 mg/kg each +/- DEN initiation	After 2 HCH doses at 5 and 8 weeks after DEN initiation, the proportion of GGT-positive foci of larger size increased significantly. DNA synthesis in GGT positive and normal cells was significantly increased in HCH-treated animals relative to the same cell type in control animals. Among HCH-treated animals initiated with either DEN or NNM, DNA synthesis was significantly higher in GGT positive cells compared to normal cells. For one experiment, DEN was given for 40 days followed 25 days later by a single 200 mg/kg oral bolus dose of HCH. DNA synthesis in GGT positive cells was greater than in normal hepatocytes. DNA labeling was not different in GGT positive islands of different sizes (40 days of DEN followed 25 days later by 200 mg/kg HCH). Mitotic index was increased in DEN initiated rats given a single 200 mg/kg dose of HCH 3 or 11 months later. No GGT-positive islands were found in rats treated with HCH alone.	Small sample size. Only females evaluated. Inconsistent dosing regimen. Potential confounding effect of initiator administration.	Supports increased proliferation as a key event. Lack of island formation after HCH alone supports tumor promoter.
550	Schulte-Hermann et al. (1983)	Rat (Wistar), female	**Duration:** unclear (gavage study); 28 weeks (dietary study) **Sample Size:** not specified **Route:** oral gavage or dietary, ad libitum **Dose Level:** 0, 200 mg/kg (gavage); 0, 20 mg/kg (dietary)	DNA synthesis and mitotic index were increased in GGT-positive liver cells in initiated rats after a single oral dose of HCH. Mean GGT-positive island size increased after 28 weeks of dietary HCH exposure in NNM-initiated rats relative to NNM treatment alone.	Sample size not reported. Unclear duration. No statistical analysis. Only females evaluated. The effect of HCH alone on island formation was not evaluated.	Supports increased proliferation as key event.
392	Siglin et al. (1991)	Mouse (B6C3F1), male/female	**Duration:** 14 days or 28 weeks (separate experiments) **Sample Size:** 15/sex/group **Route:** dietary, ad libitum **Dose Levels:** 0, 250 ppm each +/- DEN initiation	No foci or adenomas observed in HCH-only or control mice after 28 weeks. Very high number of adenomas in DEN-initiated male mice (no HCH); number significantly decreased after 24 weeks of HCH exposure. In females, adenoma number in DEN-only mice was very low and significantly increased after 24 weeks of HCH. Adenoma number overall was higher in male mice. Progressively increasing DNA labeling was seen over 14 days of dietary exposure (significant at 7 and 14 days) in foci and surrounding tissue in non-initiated males and females and in DEN-initiated females. DNA labeling was significantly decreased after 14 days in DEN-initiated males.	Only one dose level evaluated. Small sample size. Only liver examined. No bw or mortality data reported. DNA labeling data were not differentiated according to cell type (i.e., foci vs. normal hepatocytes). Questionable relevance of initiation due to differentially high adenoma incidence in DEN-only males.	Gender-dependent difference in alpha-HCH-mediated hepatic tumorigenesis supports a tumor promotion MOA. Supports increased proliferation as key event.
393	Siglin et al. (1995)	Mouse (B6C3F1), male/female	**Duration:** 14 days or 28 weeks (separate experiments) **Sample Size:** 15/sex (promotion study); 10/sex (DNA synthesis study) **Route:** dietary, ad libitum **Dose Levels:** 0, 250 ppm each +/- DEN initiation	Significantly increased relative liver weight in males and females initiated with DEN and exposed to HCH for 24 weeks; no increase after HCH-only treatment. Significantly decreased bw in HCH-only and DEN+HCH males. Foci incidence of 4/15 males and 1/15 females receiving HCH only for 24 weeks (no initiation). Foci incidence 100% in males and females treated with DEN only. Hepatocellular adenoma incidence of 4/15 HCH-only males, 15/15 DEN-only and DEN+HCH males, 0/15 HCH-only females, 1/15 DEN-only females, and 11/15 DEN+HCH females (significant increase) after 28 weeks. DNA labeling after 14 days was significantly increased in normal liver from DEN+HCH males and females compared to DEN only, but not in foci. DNA labeling in general was higher in foci.	Only liver examined. Only one dose level evaluated. Small sample size. Only liver examined. Differential susceptibility of infant male mice to DEN-mediated adenoma formation. Questionable relevance of initiation due to high incidence of foci in DEN-only animals and high incidence of adenoma in DEN-only males.	Gender-dependent difference in alpha-HCH-mediated hepatic tumorigenesis supports a tumor promotion MOA. Supports increased proliferation as key event.
319	Sumida et al. (2007)	Rat (F344), male	**Duration:** 28 days; interim sacrifices **Sample Size:** 4/group **Route:** oral gavage **Dose Levels:** 0, 2, 20 mg/kg-day	Hepatocellular hypertrophy seen in high-dose animals (0/4, 0/4, 4/4) . Significantly increased relative liver weight after 3 days at 20 mg/kg-day; no consistent significant increases in the 2 mg/kg-day group were seen. Dose-response evident but no clear temporal trend. Progressive and significant (except day 3) time-dependent decrease in ALP in 20 mg/kg-day animals beginning 1 day post-dose. Increased GST and P450 isoform expression was seen at the 28 day time point. Some increases also noted at 1 and 3 days, but there was no clear temporal trend.	Only males evaluated. Small dose groups. Results not confirmed with PCR. Inconsistency in most changes over time.	Findings support P450 induction, oxidative stress, and increased proliferation as key events.
378	Thamavit et al. (1974)	Rat (Fisher), male	**Duration:** 6 months; 2 month interim sacrifice and 5 month exposure plus one month recovery **Sample Size:** 3-6/group **Route:** dietary, ad libitum **Dose Level:** 0, 0.06% (600 ppm)	No abnormal histology, nodules, or carcinoma at 2 months or 6 months. Moderate increase in relative liver weight at 2 months; very slight increase at 6 months. Decreased body weight gain after 6 months; no change after 2 months.	Small sample size. Only males evaluated. Only one dose level evaluated. No statistical evaluation. Only evaluated the liver. Mortality not reported. Animals not sacrificed at 5 months to assess the effect of the 1 month recovery period.	Long time-to-tumor, supports tumor promoter MOA.
379	Tryphonas and Iverson (1983)	Mouse (HPB), male	**Duration:** 50 weeks; interim sacrifices **Sample Size:** 75 -treatment group; 48 -control group; 4-9/group interim sacrifices **Route:** dietary, ad libitum **Dose Levels:** 0, 500 ppm	Gross (29% incidence) and microscopic (57% incidence) nodules of the liver observed beginning at 21 weeks of exposure; incidence was 100% at 33 weeks. All nodules were benign (adenoma). No gross evidence of metastases in the lungs. Increased relative liver weight (time-dependent) and megalocytosis observed in exposed mice. Increased mitotic index in megalocytic and nodular hepatocytes. Single cell necrosis, lipid accumulation, and nodules arising from areas of megalocytic cells were observed microscopically. Reduced body weight gain in treated mice after 50 weeks.	Only one dose level evaluated. Small sample size. Only males tested. No statistical analysis. Only examined the liver and lungs. Emaciation noted in mice with severe liver enlargement or large tumors. Initial bw not reported.	Late onset of tumors (benign only) supports role as a tumor promoter. Supports increased proliferation as a key event. Temporal trend exhibited.
396	Tsukada et al. (1979)	Mouse (DD), male	**Duration:** 36 weeks; interim sacrifices **Sample Size:** 6/group **Route:** dietary, ad libitum **Dose Levels:** 0, 500 ppm	Centrilobular hypertrophy observed beginning at 16 weeks. Periportal atrophy observed. Hyperplastic nodules: 1/6 mice at 16 weeks; 5/6 mice at 20 weeks. 2/6 mice had hepatomas at 28 weeks; 3/6 mice had hepatomas at 32-36 weeks. Proliferation of smooth endoplasmic reticulum was noted; peroxisome proliferation was not observed at 16-20 weeks.	Only one dose level evaluated. Small sample size. Only males evaluated. Mortality and bw not reported. Background tumor incidence could not be evaluated in control mice (due to short duration of inclusion in the study).	Time-dependent tumor formation supports role as a tumor promoter. Supports increased proliferation as key event. Data argue against peroxisome proliferation as a key event. Inconclusive for carcinogenicity due to lack of tumor/histology data for control animals.
324	Werle-Schneider et al. (2006)	Rat (Wistar), male liver slices	**Duration:** 24 hours **Sample Size:** 8 liver slices from 4 rats **Route:** *in vitro* **Dose Levels:** 0, 0.1, 1, 10, 100 microM	Some dose-dependent changes in expression are suggested (e.g., ubiquitin, P-glycoprotein, GAPDH, retinoblastoma, p53, UGT isoforms, ERK, GST isoforms, P450 isoforms).	Results not confirmed by PCR. Inconsistency of most changes over time.	Findings support P450 induction and oxidative stress as key events.

Table 2. (continued)

Notes:	8-OHdG	= 8-hydroxy deoxyguanosine
	ALP	= alkaline phosphatase
	BrdU	= bromo-deoxyuridine
	bw	= body weight
	CYP	= cytochrome P450
	DEN	= diethylnitrosamine
	DNA	= deoxyribonucleic acid
	ERK	= extracellular signal-regulated kinase
	GAPDH	= glyceraldehyde phosphate dehydrogenase
	GGT	= gamma-glutamyl transpeptidase
	GST	= glutathione-S-transferase
	GST-P	= glutathione-S-transferase, pi isoform
	HCC	= hepatocellular carcinoma
	HCH	= hexachlorocyclohexane (alpha isomer)
	i.p.	=intraperitoneal
	mg/kg	= milligram per kilogram
	mg/kg-day	= milligram per kilogram per day
	microM	= micromolar
	mm	= millimeter
	MOA	= mode of action
	MTD	= maximum tolerated dose
	NNM	= N-nitrosomorpholine
	NOEL	= no-observed-effect level
	OGG1	= 8-oxoguanine glycosylase
	P450	= cytochrome P450
	PCNA	= proliferating cell nuclear antigen
	PCR	= polymerase chain reaction
	ppm	= part per million
	RNA	= ribonucleic acid
	SOD	= superoxide dismutase
	TBARS	= thiobarbituric acid reactive substance
	UGT	= UDP-glucuronosyl transferase

Table 3. Summary of Mutagenicity and Genotoxicity Assays for Alpha-HCH.

Reference		Test System: *In Vitro/ In Vivo*	Test System: Species/Strain/ Cell Type	Assay/Test	Endpoint	Treatment	Result	Comments
Mutation								
777	Moriya et al. (1983)	*In vitro*	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Ames assay	Mutation	up to 5,000 µg/plate w/ and w/out activation	Negative	
			Escherichia coli WP2	Reverse mutation assay	Mutation	up to 5,000 µg/plate w/ and w/out activation	Negative	
428	Shahin and van Borstel (1977)	*In vitro*	*Saccharomyces cerevisiae* XV185-14C	Reverse mutation assay	Mutation	0.1-200 µg/ml w/ and w/out activation	Negative	
433	Tanooka (1977)	*In vitro*	*Bacillus subtilis* TKJ5211	Spot test	Mutation	5,000 µg/plate	Negative	
DNA Binding								
422	Iverson et al. (1984)	*In vitro*	Calf (thymus DNA)	--	DNA binding	1 µm	Weakly positive	Low levels of DNA binding only, authors state that levels of binding are consistent with a non-genotoxic mechanism for neoplastic response.
		In vivo	Mouse liver	--	DNA binding	25 mg/kg	Weakly positive	Low levels of DNA binding only, authors state that levels of binding are consistent with a non-genotoxic mechanism for neoplastic response.
408	Sagelsdorff et al. (1983)	*In vivo*	NMRI mouse liver	HPLC analysis of nucleosides	DNA binding	6.2-8.5 mg/kg	Weakly positive	Authors characterize results as "minute DNA binding", stating that the level of binding is more than three orders of magnitude lower than would be expected if the mechanism of tumor induction was genotoxicity mediated by DNA binding.
DNA Damage, Fragmentation, and Repair								
290	Kalantzi et al. (2004)	*In vitro*	Human MCF-7 breast carcinoma cells	Comet assay	DNA fragmentation	10^{-4} M	Positive	Authors note that at lower concentrations no comet-forming effects were observed; however, the specific treatment dose or data results are not provided.
			Human PC-3 prostate carcinoma cells	Comet assay	DNA fragmentation	10^{-4} M	Positive	Authors note that at lower concentrations no comet-forming effects were observed; however, the specific treatment dose or data results are not provided.
404	Mattioli et al. (1996)	*In vitro*	Human hepatocytes	Comet assay	DNA fragmentation	0.056-0.32 mM	Positive	Dose-dependent increase in DNA breaks in 4 of 5 donors; however, statistical significance was not evaluated for this cell type.
			Rat hepatocytes	Comet assay	DNA fragmentation	0.056-0.32 mM	Positive	Modest, dose-dependent increase in DNA breaks. Co-treatment with metyrapone (inhibitor of CYP450) resulted in a reduction in the frequency of DNA breaks, and is suggestive of several potential mechanisms: 1) alpha-HCH may be transformed into a reactive species by CYP450 dependent reaction; 2) apha-HCH may interact with CYP450s to generate ROS that cause damage.
			Mouse hepatocytes	Comet assay	DNA fragmentation	0.056-0.32 mM	Negative	
795	Venkat et al. (1995)	*In vitro*	*Escherichia coli PQ37*	SOS microplate assay	Induction of gene cascade involved in DNA repair	NA	See comment	Results provide a relative scale of activity. Alpha-HCH had levels of activity that ranged 1/10 to 1/4 (dependent on dosing vehicle) that of 4-NQO, which is considered to be a direct acting mutagen.
Chromosomal Alterations								
658	Hitachi et al. (1975)	*In vivo*	Liver cells from Donryu rats	Inspection for cell distribution - % by ploidy	Chromosomal abnormalities	600 ppm	Positive	

Notes:

CYP450	= cytochrome P450
DNA	= deoxyribonucleic acid
HCH	= hexachlorocyclohexane
HPLC	= high performance liquid chromatography
mg/kg	= milligram per kilogram
M	= molar mass
mM	= millimole
NA	= not available, dose not specified or unclear
ppm	= part per million
ROS	= reactive oxygen species
µg/ml	= microgram per milliliter
µg/plate	= microgram per plate
µm	= micrometer
--	= specific test name not provided. Only endpoint is provided.
4-NQO	= 4-nitroquinoline oxide

Table 4. Inclusion of Studies Evaluating Alpha-HCH Toxicity for Sensitivity Evaluation, by Endpoint.

	Reference [a]	Included in Sensitivity Evaluation		Reason for Exclusion [b]
Hepatic Endpoints				
380	Angsubhakorn et al. (1981)	No	*	Acute exposure/High dose
381	Barros et al. (1991)	Yes	*	NA
635	Busser and Lutz (1987)	No		Reliability rank
382	Fitzhugh et al. (1950)	Yes	*	NA
577	Gerlyng et al. (1994)	No	*	Acute exposure/High dose
383	Goto et al. (1972)	No	*	Non-primary literature source
385	Hanada et al. (1973)	No	*	Acute exposure/High dose
363	Ito et al. (1973a)	No	*	Acute exposure/High dose
364	Ito et al. (1973b)	No	*	Acute exposure/High dose
386	Ito et al. (1975)	No	*	Acute exposure/High dose
387	Ito et al. (1976)	No	*	Acute exposure/High dose
643	Ito et al. (1983)	No		Acute exposure/High dose
389	Kraus et al. (1981)	No	*	MOA endpoint/*in vitro* /Reliability rank
496	Lee and Edwards (2001)	No	*	Acute exposure/High dose & MOA endpoint/*in vitro*
532	Lee and Edwards (2003)	No	*	Acute exposure/High dose & MOA endpoint/*in vitro*
455	Luebeck et al. (1995)	No	*	Acute exposure/High dose
339	Masuda et al. (2001)	Yes	*	NA
463	Nagasaki et al. (1975)	No	*	Acute exposure/High dose
307	Puatanachokchai et al. (2006)	Yes	*	NA
390	Schroter et al. (1987)	Yes	*	NA
430	Schulte-Hermann and Parzafell (1980)	No	*	Acute exposure/High dose & MOA endpoint/*in vitro*
391	Schulte-Hermann and Parzafell (1981)	No	*	Acute exposure/High dose
548	Schulte-Hermann et al. (1981)	No	*	Acute exposure/High dose & MOA endpoint/*in vitro*
550	Schulte-Hermann et al. (1983)	No	*	MOA endpoint/*in vitro*
392	Siglin et al. (1991)	No	*	Acute exposure/High dose
393	Siglin et al. (1995)	No	*	Acute exposure/High dose
319	Sumida et al. (2007)	Yes	*	NA
378	Thamavit et al. (1974)	No	*	Acute exposure/High dose
379	Tryphonas and Iverson (1983)	No	*	Acute exposure/High dose
396	Tsukada et al. (1979)	No	*	Acute exposure/High dose
324	Werle-Schneider et al. (2006)	No	*	Acute exposure/High dose & MOA endpoint/*in vitro*
Immunological Endpoints				
626	Das et al. (1990)	No		Endpoint not evaluated
660	Sweet et al. (2006)	No		Endpoint not evaluated
323	Wang et al. (2006)	No		Endpoint not evaluated
Mutagenicity/Genotoxicity Endpoints				
422	Iverson et al. (1984)	No	*	MOA endpoint/*in vitro*
290	Kalantzi et al. (2004)	No	*	MOA endpoint/*in vitro*
404	Mattioli et al. (1996)	No	*	MOA endpoint/*in vitro*
777	Moriya et al. (1983)	No	*	MOA endpoint/*in vitro*
408	Sagelsdorff et al. (1983)	No	*	MOA endpoint/*in vitro*
428	Shahin and van Borstel (1977)	No	*	MOA endpoint/*in vitro*
433	Tanooka (1977)	No	*	MOA endpoint/*in vitro*
795	Venkat et al. (1995)	No	*	MOA endpoint/*in vitro*
Neurological Endpoints				
358	Srivastava and Shivanandappa (2005)	No		Endpoint not evaluated
Reproductive/Developmental Endpoints				
642	Hosie et al. (2000)	No		Endpoint not evaluated
456	Mathur et al. (2002)	No	*	Endpoint not evaluated
542	Pathak et al. (2009)	No		Endpoint not evaluated
565	Siddiqui et al. (2003)	No		Endpoint not evaluated

Notes: HCH = hexachlorocyclohexane
MOA = mode of action
NA = not applicable
* = study determined useful for other aspects of the overall HCH re-evaluation (carcinogenicity and/or MOA evaluation) and is presented in Table 1, 2, or 3 of the main text.

[a] Table includes only primary literature, or studies for which a comprehensive review of the study was available. All studies shown are included in the database of literature for the evaluation.

[b] Studies were not selected for the sensitivity evaluation, for a variety of reasons, as presented below:

Reliability rank - animal bioassay was determined to be unreliable for the toxicity evaluation. Due to limited human data, some epidemiological studies for which the reliability was classified as unreliable were presented in the review. In these cases the reliability rank is noted.

Acute exposure/High dose - study was conducted at acute exposure duration and/or at high doses, which were determined not to inform the sensitivity evaluation. For the sensitivity evaluation, studies with a treatment dose of less than 10 mg/kg-day and an exposure duration greater than 2 weeks were included. In a few cases, a low dose study of gestation or early development was also included, even though the exposure duration was less than 2 weeks.

Endpoint not evaluated - endpoint showed no evidence of being a sensitive endpoint based upon data reported in the ATSDR (2005) Toxicological Profile.

MOA endpoint/*In vitro* - study may be useful for determining MOA however does not support dose-response for toxic effects. *In vitro* dose-response data is not comparable to *in vivo* studies.

Table 5. Hazard Identification for Alpha-HCH: Summary of Animal Bioassay Studies at Low Doses, Liver Effects.

		Dose (exposure)					Response			
	Reference [a]	Species, Sex	Study Design	Dose Range (mg/kg-day)	Exposure Duration	Sample Size	Observed Response [b]	LOAEL(s) (mg/kg-day)	NOAEL(s) (mg/kg-day)	Major Study Limitations
381	Barros et al. (1991)	Rat (Wistar), male	Single dose dietary bioassay	0, 20 ppm (0, 2 mg/kg-day)[c]	15 or 30 days	6-22/group	Significant increase in P450 content after both 15 and 30 days.	2		Only 1 dose tested. Insufficient reporting of methods, treatment purity.
							Significant increase in TBARS production after both 15 and 30 days.	2		
							Significant increase in superoxide anion production after both 15 and 30 days.	2		
							Significant increase in SOD activity after both 15 and 30 days.	2		
382	Fitzhugh et al. (1950)	Rat (Wistar), male/ female	Multiple dose dietary bioassay	0, 10, 50, 100, 800 ppm (0, 0.8, 3.9, 7.9, 63.2 mg/kg-day)[d]	Approximately 107 weeks	10/sex/group; 20/sex/group controls	Significant increase in relative liver weight at 50, 100, and 800 ppm.	3.9	0.8	Substantial mortality in both control group and all treatment groups.
							Slight microscopic liver changes seen at 50 and 100 ppm.	3.9	0.8	
							Gross histological liver changes and microscopic liver changes seen at 800 ppm.	63.2	7.9	
339	Masuda et al. (2001)	Rat (F344), male	Multiple dose dietary bioassay	0, 0.01, 0.1, 0.5, 1, 2, 4, 7.5, 15, 30, 60, 125, and 500 ppm (0, 0.001, 0.01, 0.05, 0.1, 0.2, 0.4, 0.75, 1.5, 3, 6, 12.5, 50 mg/kg-day)[e]	6 weeks after initiation by DEN and partial hepatectomy, with sacrifices of surviving animals at week 8	15/group	Relative liver weight significantly increased in the 7.5, 60, 125 and 500 ppm groups, but not in the 15 and 30 ppm groups.	0.75	0.4	Only males were tested. No negative control group.
							Number of GST-P positive foci significantly increased in groups treated with 2 ppm and higher, with the exception of 4 ppm. Dose-dependent increase began at 7.5 ppm.	0.2	0.1	
							Areas of GST-P positive foci significantly increased in groups treated with 7.5 ppm and higher, with the exception of 15 ppm. Dose-dependent increase began at 30 ppm.	0.75	0.4	
							Dose-dependent increase in CYP2B1 protein expression, with significant increase at 60 and 500 ppm.	6	3	
							Testosterone 16B-hydroxylation activities increased in a dose-related manner from 15 ppm, with significant increases at 30 ppm and higher.	3	1.5	
							CYP3A2 protein expression increased in a dose-dependent manner from 4 ppm, with significant increases at 15, 60, and 500 ppm.	1.5	0.4	
							Testosterone 6B-hydroxylation activities increased in a dose-related manner from 7.5 ppm, with significant increases at 30 ppm and higher.	3	1.5	
307	Puatanachokchai et al. (2006)	Rat (F344), male	Multiple dose dietary bioassay	0, 0.01, 0.05, 0.1, 1, 50, 500 ppm (0, 0.00054, 0.0027, 0.0055, 0.055, 2.8, 30 mg/kg-day)[f]	10 weeks following initiation with DEN for 3 weeks	12/group	Significant increase in absolute and relative liver weights at 500 ppm. Significant reduction in both at 1 ppm; however, this was not considered an adverse effect.	30	2.8	Only males were tested. Control group was also initiated, no negative control group.
							Number of GST-P positive foci significantly increased at 50 and 500 ppm. Significant decrease in number of GST-P positive foci at 0.05 ppm; however this was not considered an adverse effect.	2.8	0.055	
							Area of GST-P positive foci significantly increased at 500 ppm. Significant decrease in area of GST-P positive foci at 0.05 ppm; however this was not considered an adverse effect.	30	2.8	
							The proportion of proliferating cells (i.e., PCNA positive) within area of GST-P positive foci decreased and then increased as a function of dose, with a significant increase at 500 ppm.	30	2.8	
							Total P450 levels and P450 reductase levels significantly decreased at 0.05 ppm (not considered an adverse effect), but significantly increased at 500 ppm.	30	2.8	
							Liver 8-OHdG levels significantly decreased at 0.1 and 1 ppm (not considered an adverse effect), but significantly increased at 500 ppm.	30	2.8	
							GST activity increased at 500 ppm.	30	2.8	
							In general, there were dose-dependent increases in CYP2B, 2C, 2E, and 3A protein levels, although the trend was variable, but significantly increased at 50 and 500 ppm. P450 reductase activity significantly increased at 50 and 500 ppm.	2.8	0.055	
							Some increases in P450 activity at 50 and 500 ppm.	2.8	0.055	
390	Schroter et al. (1987)	Rat (Wistar), female	Single dose initiation and multiple dose dietary promotion study	0,200 (initiation); 0, 0.1, 0.5, 2, 7, 20 (promotion)	Single dose (initiation); 15 or 20 weeks (promotion)	3-5/group (initiation)	Significant increase in liver DNA after 15 and 20 weeks at 20 mg/kg.	20	7	Only females were tested. Promotion measured following initiation with known carcinogen.
							Significant increase in liver mass after 15 weeks at 7 mg/kg; and after 15 or 20 weeks at 20 mg/kg.	7	2	
							Significant increase in foci area after 20 weeks at 2 mg/kg; after 4 and 20 weeks at 7 mg/kg; and after 15 and 20 weeks at 20 mg/kg. Dose-dependent increase in monooxygenase activity all doses (not significant).	2	0.5	

Table 5. (continued)

		Dose (exposure)					Response			
	Reference [a]	Species, Sex	Study Design	Dose Range (mg/kg-day)	Exposure Duration	Sample Size	Observed Response [b]	LOAEL(s) (mg/kg-day)	NOAEL(s) (mg/kg-day)	Major Study Limitations
319	Sumida et al. (2007)	Rat (F344), male	Multiple dose oral bioassay	0, 2, 20 mg/kg-day	28 days; interim sacrifices at 1, 3, 7, 14, and 28 days	4/group	Significant increase in absolute liver weight at 3, 7, 14, and 28 days with 20 mg/kg-day, and at 3 days with 20 mg/kg-day.	20	2	Only males were tested.
							Significant increase in relative liver weight at 3, 7, 14, and 28 days with 20 mg/kg-day; and at 3 and 28 days with 2 mg/kg-day.	2		
							Significant decrease in AST levels at 7 days at 20 mg/kg-day; significant increase at 28 days at 2 mg/kg-day.	2		
							Significant increase in ALT levels at 28 days at 2 mg/kg-day.	2		
							Significant decrease in ALP levels at 20 mg/kg-day at 1, 7, 14, and 28 days.	20	2	
							Significant hepatocellular hypertrophy at 20 mg/kg-day.	20	2	

Source: Default dose conversion values obtained from EPA (1988).

Notes:

8-OHdG	= glucose-6-phosphate dehydrogenase
ALP	= alkaline phosphatase
ALT	= alanine aminotransferase
AST	= aspartate aminotransferase
CYP	= cytochrome P450
DEN	= diethylnitrosamine
DNA	= deoxyribonucleic acid
GST	= glutathione-S-transferase
GST-P	= glutathione-S-transferase, pi isoform
HCH	= hexachlorocyclohexane
kg	= kilogram
kg/day	= kilogram per day
LOAEL	= lowest-observed-adverse-effect level
mg/kg	= milligram per kilogram
mg/kg-day	= milligram per kilogram per day
NOAEL	= no-observed-adverse-effect level
PCNA	= proliferating cell nuclear antigen
ppm	= part per million
P450	= cytochrome P450
SOD	= superoxide dismutase
TBARS	= thiobarbituric acid reactive substance

[a] Studies selected for inclusion in this table were limited to those with at least one treatment dose of 10 mg/kg-day or less; and those with subchronic/chronic exposure durations or exposure during early development.

[b] Responses were considered significant only for effects reported to be statistically significant at $p<0.05$.

[c] Dietary concentrations in ppm converted to dose in mg/kg-day using estimated food consumption rate of 0.02 and average body weight of 0.2 taken from the study.

[d] Dietary concentrations in ppm converted to dose in mg/kg-day using estimated average food consumption rate for males and females of 0.03 kg/day and average default body weight for males and females of 0.38 kg.

[e] Dietary concentrations in ppm converted to dose in mg/kg-day using estimated food consumption rate of 0.018 kg/day and a default body weight of 0.18 kg.

[f] Dietary concentrations in ppm converted to dose by study authors.

Table 6. Selection of Endpoints for Critical Effect for Alpha-HCH.

	Reference [a]	Study Design	Observed Response in Liver [b]	Endpoint Selected for Evaluation of POD [c]	Included in BMD Evaluation [d]
381	Barros et al. (1991)	Male rats (Wistar), single dose dietary exposure, exposure of 15 or 30 days	P450 level	No[1c]	--
			Parameters related to oxidative stress (TBARS, superoxide anion production, SOD)	No[1c]	--
382	Fitzhugh et al. (1950)	Male and female rats (Wistar), dietary exposure at multiple doses, exposure of ~107 weeks	Relative liver weight	Yes	No[1d]
			Microscopic liver changes	Yes	No[1d]
			Gross macroscopic liver changes	Yes	No[1d]
339	Masuda et al. (2001)	Male rats (F344), dietary exposure at multiple doses following a known initiator, exposure of 6 weeks	Relative liver weight	Yes	Yes
			Number of GST-P positive foci	Yes	No[2d]
			Area of GST-P positive foci	Yes	No[2d]
			P450 activity and protein levels	No[1c]	--
307	Puatanachokchai et al. (2006)	Male rats(F344), dietary exposure at multiple doses following a known initiator, exposure of 10 weeks	Absolute and relative liver weights	Yes	Yes
			Number of GST-P positive foci	Yes	No[2d]
			Area of GST-P positive foci	Yes	No[2d]
			Proportion of proliferating cells (i.e., PCNA positive) within area of GST-P positive foci	Yes	No[2d]
			P450 expression, protein, and activity and total P450 levels	No[1c]	--
			Liver 8-OHdG levels	No[1c]	--
			GST activity	No[1c]	--
390	Schroter et al. (1987)	Female rats (Wistar), dietary exposure at multiple doses following a known initiator, exposure of 15 or 20 weeks	Liver DNA	Yes	Yes
			Liver mass	Yes	Yes
			P450 activity	No[1c]	--
			Area of hepatic foci	Yes	Yes
			Number of hepatic foci	Yes	Yes
319	Sumida et al. (2007)	Male rats (F344), dietary exposure at multiple doses, exposure of 28 days with interim sacrifices	Absolute and relative liver weight	Yes	Yes
			ALT levels	Yes	No[3d]
			Hypertrophy	Yes	Yes

Table 6. (continued)

Notes:
8-OHdG = glucose-6-phosphate dehydrogenase
ALT = alanine aminotransferase
BMD = benchmark dose
DNA = deoxyribonucleic acid
GST = glutathione-S-transferase
GST-P = glutathione-S-transferase, pi isoform
HCH = hexachlorocyclohexane
mg/kg-day = milligram per kilogram per day
PCNA = proliferating cell nuclear antigen
POD = point of departure
P450 = cytochrome P450
SOD = superoxice dismutase
TBARS = thiobarbituric acid reactive substance
-- = not relevant, endpoint not selected for POD evaluation.

[a] Studies selected for inclusion in this table were limited to those with at least one treatment dose of 10 mg/kg-day or less; and those with subchronic/chronic exposure durations via oral exposure.

[b] Inclusive list of observed effects associated with the liver.

[c] Endpoints were not considered to be appropriate for the POD evaluation for the following reasons.

[1c] Endpoint is determined to be an early precursor that is not closely linked with an adverse effect, and is therefore not necessarily indicative of an adverse effect.

[d] Endpoints that were considered for the POD evaluation were additionally explored using BMD modeling where possible. Data for some endpoints/studies was not amenable to BMD modeling. The following reasons for exclusions are noted:

[1d] Number of animals evaluated was not reported.

[2d] Data presented only in graphical format.

[3d] Only one dose level evaluated or no dose-response trend observed.

Table 7. Study-Specific Critical Effects for Deriving a Toxicity Criterion for Alpha-HCH: Traditional RfD Approach.

	Study	Study Design	Study-Specific Critical Effect [a]	LOAEL(s) (mg/kg-day)	NOAEL(s) (mg/kg-day)	Notes Regarding Other Endpoints Considered for POD
Low-Dose Studies						
382	Fitzhugh et al. (1950)	Male and female rats (Wistar), dietary exposure at multiple doses, exposure of ~107 weeks	Slight microscopic changes and increased liver weight	Males - 3.7 Females - 4.2	Males - 0.74 Females - 0.84	Gross macroscopic liver changes were also identified as an endpoint considered for the POD. Effects to this endpoint were noted at higher doses.
339	Masuda et al. (2001)	Male rats (F344), dietary exposure at multiple doses following a known initiator, exposure of 6 weeks	Increased number of GST-P positive foci	0.2	0.1	Number of GST-P positive foci increased at 0.2 mg/kg-day; however, it is noted that the effect was not dose-dependent in the low-dose range (no increase seen at 0.4 mg/kg-day). Liver weight and area of GST-P positive foci were also identified as endpoints considered for the POD. Dose dependent effects for these endpoints began at higher doses.
307	Puatanachokchai et al. (2006)	Male rats (F344), dietary exposure at multiple doses following a known initiator, exposure of 10 weeks	Increased number of GST-P positive foci	2.8	0.055	It is noted that there was a statistically significant decrease in number and area of GST-P positive foci at 0.0055 mg/kg-day; however, this was not considered an adverse effect, or precursor. Effects including GST-P positive foci area, the proportion of proliferating liver cells within the foci area, and liver weight, were also considered for the POD. Dose-dependent effects for these endpoints began at higher doses.
390	Schroter et al. (1987)	Female rats (Wistar), dietary exposure at multiple doses following a known initiator, exposure of 15 or 20 weeks	Increased area of foci	2	0.5	Effects including increases in foci number and, liver DNA, and liver weight were also considered for the POD. Statistical significance was not reported for foci number, and therefore this effect could not be used with confidence. Increased liver DNA and liver weight were noted at higher doses.
319	Sumida et al. (2007)	Male rats (F344), dietary exposure at multiple doses, exposure of 28 days with interim sacrifices	Increased relative liver weight and increased liver ALT	2	--	Effects including increase in hypertrophy and absolute liver weight were also considered for the POD. Effects to these endpoints were noted at higher doses.
High-Dose Studies						
363	Ito et al. (1973a)	Male mice (DDY). Dietary exposure at multiple doses, exposure of 24 weeks.	HCC	45	18	NA
364	Ito et al. (1973b)	Male mice (DDY). Dietary exposure at multiple doses, exposure of 24 weeks.	HCC	45	18	NA

Notes:
ALT = alanine aminotransferase
DNA = deoxyribonucleic acid
GST-P = glutathione-S-transferase, pi isoform
HCC = hepatocellular carcinoma
HCH = hexachlorocyclohexane
LOAEL = lowest-observed-adverse-effect level
mg/kg-day = milligram per kilogram per day
NA = not applicable
NOAEL = no-observed-adverse-effect level
POD = point of departure
RfD = reference dose
-- = LOAEL was lowest dose tested, no NOAEL

[a] Effect for which the lowest statistically significant effect that was determined to be appropriate for the POD determination was observed. Effects that did not show a dose-related response were additionally not selected for the study-specific critical effect.

Table 8. Results from BMD Analysis for Deriving a Toxicity Criterion for Alpha-HCH.

Reference		Test System	Endpoint	Variable Type	Best-Fit Model [a]	Variation Modeling [b]	BMD [c]	BMDL [c]
Low-Dose Studies								
339	Masuda et al. (2001)	F344 rats (male)	Relative liver weight	C	--	--	--	--
307	Puatanachokchai et al. (2006)	F344 rats (male)	Absolute liver weight	C	Linear	Constant	3.28	2.88
			Relative liver weight	C	--	--	--	--
390	Schroter et al. (1987)	Wistar rat (female)	Foci area	C	Polynomial	Non-constant	0.74	0.39
			Foci number	C	Linear and power	Non-constant	1.58	1.06
			DNA content	C	Linear	Constant	9.63	5.99
			Relative liver weight	C	--	--	--	--
319	Sumida et al. (2007)	Fischer 344 rats (male)	Relative liver weight	C	--	--	--	--
			Absolute liver weight	C	--	--	--	--
High-Dose Studies								
363	Ito et al. (1973a)	DDY mice (male)	Absolute liver weight	C	Power	Non-constant	13.60	9.59
			Nodular hyperplasia incidence	D	Log-logistic	NA	36.97	20.66
			HCC incidence	D	Log-probit	NA	34.72	26.71
364	Ito et al. (1973b)	DDY mice (male)	Nodular hyperplasia incidence	D	Log-logistic	NA	37.24	21.31
			HCC incidence	D	Log-logistic	NA	42.08	26.38
			Absolute liver weight	C	--	--	--	--
			Relative liver weight	C	--	--	--	--

Notes:
BMD = benchmark dose
BMDL = lower 95% confidence interval on BMD
BMR = benchmark response
C = continuous
D = dichotomous
DNA = deoxyribonucleic acid
HCC = hepatocellular carcinoma
HCH = hexachlorocyclohexane
NA = not applicable
SD = standard deviation
-- = modeling was unsuccessful

[a] Criteria used for selection of best-fit model are described in the text.
[b] Applicable only for continuous variables.
[c] BMR for continuous data was 1 SD; BMR for dichotomous data was 10% change.

ATTACHMENT A

LITERATURE REVIEW OF ALPHA-, BETA-, AND GAMMA-HEXACHLOROCYCLOHEXANE [ON ENCLOSED CD]