Beyond Science and Decisions: From Problem Formulation to Dose-Response Assessment:

Case Study:

Criteria Requirements for Data-Driven Carcinogenicity Mode of Action (MoA) Determinations as Exemplified by Chloroform Carcinogenicity

1. Introduction

Risk assessment methods for carcinogens have embraced mode of action (MoA) evaluations for replacing policy-based default assumptions of low dose linearity with biologically-based models. Over the past two decades, experimental data and assessment methods have strengthened non-linear MoA-based approaches for assessing carcinogenesis, and biologically based models continue to refine extrapolations to human cancer risks. Nonetheless, clear criteria have yet to be defined for determining when a MoA has been sufficiently well established in an animal model that it should be used to determine the human relevance of the tumors, and if relevant, replace policy-based linear, non-threshold, default models for extrapolating high dose animal tumor data to human cancer risks with models that use actual chemical-specific data. Lacking such criteria, policy decisions regarding use of MoA-based approaches tend to rely on subjective, case-by-case judgments about what constitutes sufficiency of the data for any particular chemical. A summary of experience using EPA's Cancer Assessment Guidelines (Andersen et al. 2000) asserts:

> *"It is appropriate for expert panels to evaluate the data supporting modes of action on a case-by-case basis. Nonetheless, the determination of the adequacy of the weight of evidence for a mode of action will be pivotal in characterizing the dose-response relationship, predicting its shape in the low-dose region and using this information in the risk assessment for many compounds. Consideration of a general framework for assessing the weight of the evidence for a particular mode of action could serve as an important guide for future efforts, although the panel did not endorse a rigid set of rules for this evaluation."* [Andersen et al. 2000]

Although a rigid set of rules for this evaluation might be too restrictive, clear criteria would not only provide an important weight of evidence framework to guide future efforts, as recommended by Anderson et al., but would enhance scientific integrity and transparency in cancer risk assessments and would facilitate consistency in policy-making. This case study is an initial attempt to define those criteria using a data-rich chemical with a well-defined MoA: chloroform.

Chloroform has been used as a prototype compound for developing MoA-based carcinogenicity extrapolations because of its rich and consistent toxicological data set demonstrating a non-genotoxic, non-linear, cytotoxic mode of tumor induction that occurs only at high doses in rodent models. Chloroform is also the prototype example for demonstrating how the IPCS and Human Relevance Framework (HRF) identifies a cytotoxic MoA for tumor induction in rodents and evaluates its relevance for human cancers and risk assessment (Sonich-Mullin et al. 2001; Meek et al. 2003). Numerous expert panels convened by governmental agencies and multi-stakeholder panels have consistently and unanimously agreed with the non-linear, cytotoxic MoA for chloroform, and have expressed a high degree of confidence in the data and the assessment methods. This high confidence is reflected in the fact that the U.S. EPA and the World Health Organization use a non-linear, cytotoxicity-based dose-response model to assess human cancer risks from exposure to chloroform. Therefore, assessments of chloroform's MoA provide a reliable basis upon which to develop criteria for departing from the default dose-response model for cancer risk assessments.

2. Background

Chloroform ($CHCl_3$) is a drinking water disinfection byproduct that is widely found in drinking water systems. Alternative disinfection processes that generate less chloroform are available, but they generate other byproducts, some of which may be of greater concern than chloroform. Chloroform is carcinogenic in the liver and kidney of mice and in the kidney of rats, exhibiting specificity for species, strain, sex and tissues, and marked dependence on route of administration and dose. The carcinogenicity of chloroform has been investigated extensively over the past three decades in long term animal bioassays, *in vivo* and *in vitro* mechanistic studies, pharmacokinetic evaluations in both animal test species and humans, and epidemiological studies. These investigations have elucidated many key aspects of chloroform carcinogenicity, including the role of genetic and cellular toxicity, hepatic and extra-hepatic oxidative and reductive metabolism, isozyme-specificity of metabolism, dependence of carcinogenicity on cellular damage and proliferation, dose-response characteristics of obligate underlying processes, and the correlation of carcinogenicity with potential MoAs. Several peer-reviewed publications and consensus panel reports have used these findings to evaluate chloroform's neoplastic MoA and derive pharmacokinetic models to predict its dose-response characteristics. For example, Andersen et al. (2000) assert:

> *"The mode of action statement for CHCl3 would be stated in two parts. First, CHCl3 forms oxidized metabolites that cause cell damage in tissues with high concentrations of the relevant metabolizing enzyme. Second, metabolite-mediated cytotoxicity leads to cell death, regenerative hyperplasia, and higher probabilities of cell mutation and cancer. High rates*

of compound metabolism and metabolite-mediated cytotoxicity are thus regarded to be key steps in CHCl3 carcinogenicity."

and:

"For CHCl3, a likely mode of action was the accumulation of reactive, oxidized metabolites, leading to cell toxicity. This mode defines the measure of tissue dose, i.e., concentrations of metabolites produced through the oxidative pathway that will be calculated by the TK model."

Chloroform carcinogenicity is considered to be sufficiently well understood to have served as an example in publications that illustrate use of the IPCS and HRF to evaluate the carcinogenic MoA and its relevance to human risk estimation. The set of key events in chloroform's carcinogenic MoA is described in the most recent of these publications (Boobis et al. 2009; Boobis 2010) are listed in Table 1 below.

TABLE 1. Consensus MoA for Liver and Kidney Tumors Induced by Chloroform

Sequence	Key Event in Chloroform's Carcinogenic MoA
1	*Absorption and distribution to target tissue(s).*
2	*Oxidative metabolism of chloroform by the P450 enzyme CYP2E1 to highly reactive phosgene.*
3	*Sustained cytotoxicity to target cells, hepatocytes and/or renal proximal tubular epithelial cells.*
4	*Regenerative cell proliferation in liver and/or kidney.*
5	*Threshold development of tumors in liver and/or kidney.*

These key events are briefly described below.

1. Absorption and distribution to target tissue(s).

Chloroform is a low molecular weight, lipid soluble compound and as such, it is well absorbed via most routes of exposure, particularly inhalation and ingestion, with rapid and

extensive distribution to all tissues of the body (Boobis et al. 2009). Distribution into fat can influence systemic levels, as can the rate of systemic elimination. The elimination of chloroform appears to be saturable, increasing systemic and target organ exposures disproportionately at higher doses. Accumulation in fat is likely not to be saturable, and although the mechanisms are unknown, elimination may be enhanced by repeated, lower level exposures (Boobis et al. 2009). Given the physical chemical properties of chloroform, its absorption and distribution would be similar by inhalation, ingestion and dermal routes, differing only in kinetics.

A number of physiologically-based pharmacokinetic/phamacodynamic (PB-PK/PD) models have been developed based on the strong biological evidence regarding chloroform's carcinogenic mode of action. These include, among others, Luke et al. (2010), Liao et al. (2007), Tan et al. (2003; 2006), Krishnan & Johanson (2005), Levesque et al. (2002), (Corley et al. 2000), Delic et al. (2000), Reitz et al. (1990). No models were found that were based on a different MoA. One of the earliest and most cited of these studies (Corley et al. 2000) developed a (PB-PK/PD) model based on measured chloroform in the exhaled breath of male and female volunteers exposed via bathwater at two temperatures, 35°C and 40°C. Compared to ingestion of water at the same concentration, dermal exposures were estimated to potentially contribute 1-28% of daily chloroform, dependent on temperature of water and gender of subject (males absorbed more chloroform than females from 35°C water).

The toxicological targets of chloroform - liver and kidney - are identical by dermal, inhalation and oral exposure routes, reflecting several aspects of chloroform's pharmacokinetics and MoA. Chronic bioassays conducted by oral and inhalation routes demonstrate that cytotoxicity and tumor induction depend on the level of tissue CYP2E1 activity and secondarily on levels of anti-oxidants and free radical scavengers such as glutathione (Meek et al. 2002). The requirements for tumorigenesis are a sufficient level of chloroform and consequent metabolism to phosgene to produce chronic cytotoxicity and compensatory regenerative hyperplasia. There is no evidence that these requirements differ by route of exposure. Although skin and lung possess CYP2E, these tissues have lower activity of the enzyme and have not been shown to be sites of tumor induction. CYP enzymes are highly conserved between rodents and humans, and a single gene is the source of CYP2E1 in human and rodent tissues, including liver, lung and skin (Neis et al. 2010; Baron et al. 2008; Du et al. 2004; Ingelman-Sundberg 2004). This limits the potential for large differences in chloroform metabolism between different routes of exposure. Thus, there are no data to suggest a difference in chloroform's MoA for tumor induction between oral, dermal or inhalation exposure, and considerable evidence supporting the single non-linear, threshold, cytotoxicity MoA used in published pharmacokinetic and dose-response modeling studies.

Alternative Hypotheses
None Proffered

2. ***Oxidative metabolism of chloroform by the P450 enzyme CYP2E1 to highly reactive phosgene.***

Boobis et al. (2009) and Boobis (2010) describe several lines of evidence implicating CYP2E1 in chloroform carcinogenicity. Chief among these includes counterfactual experiments conducted in CYP2E1-knockout mice. Counterfactual experiments test whether the effect of interest still occurs when a putative causal step is prevented under conditions that would otherwise produce the effect of interest. In CYP2E1 knock-out mice, chloroform does not cause hepatic or renal necrosis, nor is there found any evidence of regenerative proliferation or increased mitotic indices (Boobis et al., 2009; Boobis 2010; Constan et al. 1999). Because of its highly conserved character and the fact that a single isoform prevails in all species other than rabbit (Boobis et al. 2009; Boobis 2010), this experimental system unequivocally demonstrates the obligate role of CYP2E1 in chloroform carcinogenicity in mice and strongly suggests that its role is generalizable to other species. Corroborative evidence is provided from normal mice and in rats, in which CYP2E1 expression levels within and among tissues correlates well with the extent of chloroform toxicity (Boobis et al. 2009; Boobis 2010). The tissues most affected are kidney and liver.

Metabolic conversion of chloroform to phosgene follows classic Michaelis-Menton kinetics, with no threshold for substrate conversion. The kinetics of conversion are linear up to substrate concentrations that support approximately 70% of the Vmax for CYP2E1, at which point the rate of conversion begins to plateau (Boobis 2010). Although the oxidative metabolism of chloroform to phosgene is not itself a threshold process, it's obligate and predominant role in chloroform metabolism, as demonstrated by counterfactual experimentation, ensures subsequent key events that are thresholded and precludes other pathways that would otherwise not be expected to exhibit a threshold.

Phosgene is a highly reactive electrophile that reacts rapidly to form covalent bonds with intracellular nucleophiles such as glutathione, proteins, lipids and other macromolecules. As a result, phosgene cannot diffuse far from its site of production in mitochondria and the endoplasmic reticulum. This limits its potential molecular targets to those organelles and renders interaction with DNA in the nucleus highly unlikely if not impossible. Conversion to phosgene as an obligate event in chloroform toxicity is thus consistent with the lack of evidence for chloroform-induced DNA damage *in vivo*.

Even though phosgene production occurs from even the lowest doses of chloroform, phosgene reactivity with functional and structural macromolecules appears to be limited - and is likely to be totally prevented at low concentrations - by intracellular pools of glutathione and other free radical scavengers. Thus, these cellular defenses against oxidative damage likely contribute to the apparent threshold for chloroform toxicity and carcinogenesis observed in rodent bioassays. Significant toxicity would not be expected until phosgene production is sufficient to markedly deplete intracellular pools of these protective molecules and exceed the considerable ability of cells to rapidly replenish them (Boobis 2010; Boobis et al. 2009). This is consistent with the fact that chloroform toxicity is observed only in the organelles of cells that express CYP2E1, and the corresponding tissues and organs.

Alternative Hypotheses
Reductive Metabolism

Evidence for reductive metabolism of chloroform suggests that if it occurs at all, it occurs at doses that have already saturated oxidative metabolism in rodents and humans, and above those that produce liver and kidney tumors in rodents (Gemma et al. 2003; Meek et al. 2002; Golden et al. 1997), which are themselves far greater than potential human environmental or industrial exposures. Therefore, reductive metabolism of chloroform is not physiologically relevant to the carcinogenic mechanism, even though it may occur under some experimental conditions.

3. Sustained cytotoxicity to target cells, hepatocytes and ／ or renal proximal tubular epithelial cells.

Phosgene reacts rapidly and covalently with structural and functional macromolecules near its site of production. Although the exact mechanism of cell death is not well understood, these covalent interactions interrupt mitochondrial integrity, reduce cellular energy production, and collapse membrane permeability transition pores (Boobis 2010; Boobis et al., 2009). When this damage reaches a sufficient level, membrane permeability is lost, which is the determining factor in cell death. However, because mitochondria can tolerate some level of insult without any change in membrane permeability, and can repair low-level damage, they would be resilient to low level phosgene production. Mitochondrial resilience and repair is demonstrable in both rodents and humans (Boobis 2010; Boobis et al. 2009) and well explains the observed recovery from low level cytotoxicity following low doses of chloroform. Higher doses of chloroform, sustained for periods of time sufficient to exceed mitochondrial tolerance to damage and repair capacity, result in cell death and tissue necrosis in liver and kidney.

In vitro and *in vivo* evidence indicate that chloroform cytoxicity and cell death exhibit a threshold in both liver and kidney (Boobis 2010; Boobis et al. 2009). Together with the lack of chloroform toxicity in tissues that do not express CYP2E1, these facts strongly indicate that sustained phosgene-induced cytotoxicity is a key event in chloroform-induced carcinogenesis.

Alternative Hypotheses
Genotoxic Mode of Action

Using a published comprehensive, quantitative weight of evidence approach to evaluate large, heterogenous genetic toxicology databases, chloroform's potential mutagenicity was assessed by an expert panel. On a scale of -100 to 100, chloroform scored a -14.3, indicating that the weight of evidence supports a non-genotoxic classification (Andersen et al. 2000). Regarding conflicting data, Andersen et al. (2000) point out *"[T]he fact that a compound causes genotoxicity under some limited set of experimental conditions does not necessarily mean that carcinogenic effects of the compound would be related to mutagenicity."* Boobis (2010) notes that chloroform is generally negative in tests for genotoxicity both *in vitro* and *in vivo*, concluding that *"...the weight of evidence is that genotoxicity is not the mode of action for chloroform."*

4. Regenerative cell proliferation in liver and/or kidney.

When toxicity results in significant cell death, the surviving cells of many tissues may respond with compensatory proliferation. Except when toxicity and cell death are so severe that no viable cells remain, compensatory proliferation regenerates cell numbers, lost tissue, organ function, and functional reserve in both liver and kidney. Compensatory proliferation is counterbalanced by apoptosis so that the overall extent of tissue growth does not exceed the amount necessary to restore functional capacity and reserve. Because both liver and kidney have sufficient functional reserve to tolerate a small amount of damage without the need for compensatory proliferation, there is a level of cell death in these organs below which the response is not observed. Bromodeoxyuridine (BrdU) labeling experiments in both mice and rats have demonstrated doses and durations of chloroform administration below which no increase in proliferation is observable in liver or kidney (Boobis et al. 2009). Induction of the proliferative response is dependent upon sufficiency of both dose and duration of exposure. Moreover, at doses/durations sufficiently cytotoxic to induce the compensatory response, proliferation is reversible and returns to baseline soon after cessation of dosing.

Measurement of cell proliferation is one of the most precise, quantitative and reproducible of cellular measurements, greatly exceeding the precision of tumor dose response assessments

from 2-year bioassays and some other measures of cell toxicity such as histopathology (Butterworth & Bogdanffy, 1999). Precise quantitation makes possible accurate delineation of doses and durations of chloroform exposure that do not induce, versus those that do induce, cell proliferation.

Prolonged cell proliferation is critical for the production and selection of pre-neoplastic cells, which can enter the neoplastic progression to cancer. Under normal circumstances, however, cellular and tissue defense mechanisms, including apoptosis, expunge altered cells, exerting a negative selection pressure that aborts neoplastic progression. Chloroform exposures sufficient to elicit cytotoxicity, cell death, and a compensatory proliferative response in liver and/or kidney have been demonstrated to produce altered cells that can lead to cancer in those organs, but only when the exposure is sufficiently prolonged to allow selection and clonal expansion of altered cells. Otherwise, cells altered during the proliferative response are efficiently eliminated once exposure has ceased. Because proliferation is the event that leads to selection or production of pre-neoplastic cells necessary for the neoplastic progression to cancer, these phenomena provide further mechanistic explanation for the observed threshold in chloroform toxicity and carcinogenicity.

Although the proliferative and damage-repair processes in humans may differ quantitatively from those in rodents (humans generally have more robust repair capacity), they are qualitatively similar. Hence, the same processes that can lead to liver and kidney cancer in rodents are possible in humans, and although humans may exhibit different thresholds of dose and duration, the qualitative similarity of the overall processes are sufficient to expect thresholds for chloroform carcinogenicity in humans as has been demonstrated in rodents (e.g., Boobis 2010; Boobis et al., 2009).

Alternative Hypotheses

Boobis (2010) concluded that *"[T]here is no evidence for direct stimulation of hyperplasia, inhibition of apoptosis or receptor activation by chloroform, making it very unlikely that growth stimulation is the mode of action."* Boobis (2010) ruled out estrogenicity or other hormonal activity at tumorigenic doses, rendering endocrine mechanisms highly unlikely.

5. Development of tumors in liver and/or kidney.

Chloroform doses and dosing regimens that result in cytoxicity and regenerative cell proliferation induce liver cancer in male and female B6C3F_1 mice; kidney cancer in male, but not female, BDF_1 mice; and kidney cancer in male, but not female, Osborne-Mendel rats. No tumors were induced in either male or female Fischer-344 rats (Butterworth & Bogdanffy,

1999). No tumors were induced at doses below those that produce sustained cytotoxicity and regenerative proliferation.

> *"For $CHCl_3$, the panel agreed unanimously that a mode of action involving obligatory cytotoxicity as a precursor to cancer in target tissues was most plausible, i.e., much more strongly supported by the comprehensive data set than any of the other modes of action. To paraphrase the conclusion, there should be no significant carcinogenic risk from $CHCl_3$ at concentrations below those that cause cell damage."* [Andersen et al. 2000]

Chloroform in drinking water fails to induce tumors at daily doses greater than those that produce tumors when given by gavage. Since both methods involve absorption via the gastrointestinal tract, the possible differences would relate only to the vehicle (corn oil versus water) and time-frame of administration. Administration by gavage in corn oil delivers chloroform to the target organs in bolus fashion whereas drinking water distributes the dose over the time period of drinking. Unless a threshold existed for bioactivation of chloroform to a mutagenic metabolite, there would be no reason that a mutagenic mode of action would exhibit this profound difference with time-frame of administration, nor is any mechanism known whereby corn oil would alter metabolism of chloroform by CYP2E1. The most plausible explanation is the pharmacokinetic difference in peak dose, which exceeds the ability of cells to protect against cytotoxicity by bolus gavage but not by divided oral doses (Butterworth et al. 1995a). A pharmacokinetic explanation is consistent with data indicating that the threshold for sustained cytotoxicity and proliferative regeneration matches the observed threshold for tumorigenic response.

Alternative Hypotheses

Butterworth et al. (1995a) compared predictions of the linearized mutli-stage model for hepatocellular carcinoma incidence in female mice administered chloroform by gavage to predictions of the dose-response model based on cell proliferation for tumor incidence in female mice administered chloroform in drinking water. Clearly, the cell proliferation model and not the LMS model agreed with actual data. This indicates that the alternative hypothesis - that chloroform carcinogenesis proceeds via a mode of action that lacks a threshold - fails to account for the observed data as well as the hypothesis that chloroform carcinogenicity proceeds via a cytotoxic mechanism operable only at high doses.

Melnick et al. (1998) and Coffin et al. (2000) demonstrated a lack of consistency between cytotoxicity, regenerative hyperplasia and tumor formation in B6C3F1 mouse liver among trihalomethanes, but those data do not refute other studies showing that cytotoxicity is required but insufficient specifically for chloroform-induced liver tumor formation; the key

event in chloroform's MoA is sustained cytotoxicity that induces regenerative hyperplasia. Low levels of liver toxicity are apparently tolerated and repaired without induction of regenerative hyperplasia and tumor formation (Boobis 2010; Boobis et al. 2009).

3. Methodology for Deriving Criteria from the Case Study on Chloroform

Many critical reviews and consensus panel reports independently conducted thorough analyses of the primary research literature on chloroform. Although these reviews consistently conclude that chloroform's mode of carcinogenic action involves cellular rather than genetic toxicity and occurs only at doses that produce sustained tissue damage and regeneration, the analysts asked different questions of the data and used different rationale to evaluate it. Brief summaries of the published critical reviews and consensus reports is contained in Appendix A.

Because chloroform has been so extensively evaluated, and because the various evaluations have been conducted by different groups using different approaches, these reviews were considered more tractable than primary data for elucidating general criteria applicable to deciding when the data regarding a chemical's carcinogenic MoA are sufficiently well understood to compel its use in cancer risk assessment. Hence, the current case study does not seek to derive criteria from a *de novo* evaluation of the primary data on chloroform carcinogenicity, but relies on these critical reviews and consensus panel reports. To derive criteria based on such reviews and reports, this case study considered whether the various vanalyses of chloroform carcinogenicity addressed certain general, essential elements of data assessment described in an approach for conducting weight of evidence evaluations (Borgert et al. 2011). The use of these essential elements promotes consistency of evaluation for deriving the criteria. Furthermore, since the elements are different from and independent of the approach used in the IPCS and Human Relevance Frameworks, they provide a measure of validation not possible using the same criteria initially used to describe carcinogenic MoAs.

For conducting WoE evaluations, Borgert et al. (2011) recommended several principal components, however, not all would be amenable to evaluating critical reviews as opposed to primary data. Because this case study employs critical reviews rather than primary data, it is not feasible to evaluate literature selection criteria, measurement or reporting validity, or data weighting schemes, as each critical review will have followed its own method. However, it is feasible to consider whether the various critical reviews stated explicit hypotheses, evaluated aspects of data quality and study design, and ruled out alternative hypotheses for the MoA. Thus, for each key event in chloroform's carcinogenic MoA (Table 1), the reviews and consensus reports were evaluated for the following components, listed in Table 2.

TABLE 2. Components for Evaluating Critical Reviews and Consensus Reports on MoA

	Description of Component
a	Support for the particular key event (hypothesis) in the MoA.
b	Evaluation or discussion of data quality supporting (or refuting) the key event.
c	Evaluation or discussion of counterfactual concepts in experimental design and interpretation for data supporting the key event.
d	Evaluation or discussion of alternative hypotheses or data interpretations regarding the key event.

Those principal components were used in this case study to derive the criteria. Among them, particular emphasis was placed on counterfactual lines of evidence as this is one of the most powerful means by which systematic experimentation can demonstrate causal relationships (Borgert et al. 2011). Counterfactuals test whether the effect of interest still occurs when a putative causal step is prevented under conditions that would otherwise produce the effect of interest. Consistent with this emphasis, Sonich-Mullen et al. (2001) note, under the designation of "Strength, Consistency, and Specificity of Association of Tumor Response with Key Events," that

> *"Stop/recovery studies showing absence or reduction of subsequent events or tumor when a key event is blocked or diminished are particularly important tests of the association."*

This alludes to counterfactual tests of the hypothesis, which Borgert et al. (2011) explicitly deem a test of tertiary data validity. Hence, while the IPCS and Human Relevance Frameworks acknowledge the importance of counterfactual hypothesis testing, those publications, perhaps inadvertently, subjugate this objective experimental evidence under a professional judgment about strength, consistency and specificity. Because counterfactual evidence is the essential element of causal reasoning in experimental pharmacology and toxicology, it seems this type of evidence deserves special consideration in evaluating experimental data regarding whether a hypothesized mode of action is causal in producing animal tumors.

4. Results

Table 3 presents the evaluation of results of evaluating components a through d (Table 2, Section *3. Methodology*) for each of the five hypothesized key events in chloroform's carcinogenic MoA (Table 1, Section *2. Background*), listed chronologically by critical review or consensus panel report.

Critical reviews and consensus evaluations have employed different approaches to assess chloroform's carcinogenic MoA. Consequently, none have addressed all four weight of evidence components applied here for all five key events. Taking all reviews collectively, however, reveals that all four components (Table 2) have been addressed for all five key events in chloroform's carcinogenic MoA, including a threshold for tumor development in rodent liver and kidney. Several conclusions are evident from the analysis presented in Table 3 for chloroform, and these assist in developing the criteria to be developed from this case study:

- All four weight of evidence components have been addressed for all five key events in chloroform's carcinogenic MoA, including a threshold for tumor development in rodent liver and kidney. Although some key events were unaddressed in some evaluations, none made countervailing conclusions regarding the five key events.
- Animal tumors have been assessed from predominantly oral exposure, with some data from inhalation routes. Only pharmacokinetic assessments are available from dermal exposure. The physical-chemical properties, pharmacokinetic behavior, role of metabolism in toxicity and tumor development, and experimental data from dermal exposure, albeit limited, would predict no deviation from this MoA irrespective of the route of exposure.
- Three of the five key events in chloroform's carcinogenic MoA appear to exhibit a biological threshold.
- Counterfactual evidence is compelling for three key events, two of which demonstrate a biological threshold.
- Data quality issues were evaluated for most key events, including for principal alternative hypotheses that would predict a non-threshold MoA.
- Alternative hypotheses and interpretations for each key event have been considered.

[SEE TABLE 3. Evaluation of Support for Chloroform Mode of Action]

From these conclusions, criteria were developed that would justify and compel application of non-linear, threshold models for cancer risk assessment so that policy decisions are consistent with the best available science (Table 4).

TABLE 4. Criteria for Sufficiency of Evidence to Support Use of Animal MoA in Determining Human Relevance and Dose-Response Model.

I	Defined key events should be consistently (not necessarily unanimously) supported among objective analyses.
II	Issues regarding data quality should not weaken support for the key events; i.e., data supporting the proposed key event should be of equal or higher quality than contradictory data.
III	At least one of the key events should be counterfactually demonstrated to exhibit a biological threshold.
IV	The MoA should not differ by route of exposure (if a chemical's MoA does differ by route of exposure, the MoA evaluation should reflect those differences).
V	Alternative MoA hypotheses that would dictate a linear biological model of tumor development should be consistently ruled out or considered unlikely among objective analyses.

5. Discussion

Because it addresses fundamental principles of hypothesis testing, the methodology employed here is useful for assessing numerous evaluations regarding chemical MoAs that may employ different approaches and consider evolving hypotheses and data sets. The test case of chloroform enables the defining of strong criteria to justify departure from the linear, no-threshold default assumption and to compel the use of non-linear, threshold models for estimating human cancer risks from the animal tumor data. This is because the data set for

chloroform is comprehensive, thorough, consistent, and exhibits only a few minor uncertainties. More lenient criteria, however, may be appropriate and could be developed based on other case studies.

Within its intended context, the method is believed to be broadly applicable to the question of whether a MoA for any particular chemical has been sufficiently established that it should be used to determine the human relevance of animal tumors and if relevant, to derive dose response models for evaluating human cancer risks as a matter of science and policy. The criteria developed here apply to the first part of the Human Relevance Framework, which is a determination of the MoA in an animal model. They were formulated within the realm of experimental data and may also be appropriate for the second arm of the HRF, which determines whether a MoA in the test species is likely to be relevant for human tumors. Consideration of this second question might also benefit from these criteria, but was not specifically addressed in formulating the current methodology and case study.

Although the criteria do not assume that the MoA is relevant to humans, their use could be helpful in resolving that question because it would help to ensure firm and consistent decisions about when a MoA has been sufficiently well established in an animal model that it should form the basis for the human relevance determination and dose response extrapolation. This is especially true when the animal model forms the basis for assuming potential human carcinogenicity. In that case, without evidence to the contrary, there would be no scientific basis to assume that a different MoA would produce tumors in humans than was demonstrated in animals. This rationale is consistent with the HRF requiring evidence of a distinct difference between human and animal physiology to deem a chemical unlikely to produce cancer in humans based upon its carcinogenic MoA in an animal model.

A similar rationale applies to chloroform carcinogenicity by different routes of exposure. In this case, carcinogenicity is clearly demonstrated to be a function of achieving sufficient chloroform levels and metabolism to phosgene to cause chronic toxicity and regenerative hyperplasia in the target organs where tumors develop, the liver and kidney. Differences in tumor incidence, even by different methods of oral exposure (gavage in corn oil versus drinking water) demonstrate this dose requirement, and are supported by data from inhalation exposures. Furthermore, comparison of the gene for rat, mouse and human CYP2E1 enzyme and pharmacokinetic data in humans indicate no rationale for a deviation from the expected target organs or dose-dependence by the dermal route of exposure. Thus, for chloroform, the data and biological rationale strongly indicate that any tumors would arise via a non-linear, cytotoxic MoA, irrespective of the route of exposure, whether oral, dermal, or inhalation. The use of clear criteria for deciding the sufficiency of the animal MoA should facilitate the decision to adopt a dose-response model for humans based on that particular MoA, and absent

evidence to the contrary, further support the adoption of that MoA for all routes of exposure as illustrated by the case study with chloroform.

Irrespective of whether this method is applied to critical reviews and consensus panel evaluations or to primary data, considerable MoA data are required. The minimum primary data requirements are uncertain, however, as these would vary by individual MoA and perhaps by chemical. This ambiguity does not extend directly to the method itself. For application of the method, a minimum of one comprehensive MoA data set, or MoA evaluation, is required. Conceivably, a single comprehensive data set or evaluation could be sufficiently thorough and rigorous to satisfy all five criteria. In practice, however, application of the method will be strengthened with greater numbers of well-constructed studies, or critical reviews and consensus evaluations, as is evident for the case study example, chloroform.

The strengths of the method derive from the simplicity of the criteria and the fact that they relate to fundamental tenets of the scientific method, listed in Table 2. By design, the criteria are compatible with the considerations used to identify key events in the IPCS and HRFs, yet they are not identical. These are both strengths. That they are compatible lends credibility and ready utility; that they are not identical avoids a potential conundrum similar to that which arises in attempting to validate a model with the same data used to derive the model. Were the HRF or IPCS framework used to derive these criteria, they would not represent a separate and independent synthesis of potentially differing interpretations. Furthermore, because the criteria are not identical to those used in the HRF or IPCS framework to identify key events, they do not bias an evaluation toward the outcomes of either process. In other words, they provide a fair hearing for evaluations conducted by alternative decision structures, which is considered a strength.

The criteria can leverage other scientific processes, such as the HRF and IPCS framework and published critical reviews, rather than demanding a de novo consideration of all primary data. In this regard, the method lends efficiency and strength to the decision-making process. Furthermore, the criteria were developed using chloroform as the test case. The carcinogenic MoA for few, if any other chemical is as well characterized as that of chloroform, and none has been subjected to greater scrutiny or more numerous peer-review evaluations. Therefore, there can be high confidence that the criteria are based on reliable scientific evidence. While using the method to leverage other evaluative processes can lend strength in some regards, as mentioned above, it could also be a weakness in that, if the existing published work were unanimously incorrect regarding one or more of the criteria considerations, the method would not uncover that error.

Because the method was developed based on evaluations of chloroform, which are particularly numerous, thorough, and rigorous, it may be unnecessarily stringent. This potential weakness could be remedied by testing the method against additional case studies that vary in the depth and breadth of published evaluations, and modifying the criteria as necessary. In this regard, the method should reject datasets for chemicals whose MoAs are clearly uncertain, and should accept others with strong datasets, albeit perhaps less strong than for chloroform.

Although the criteria were intentionally developed with broad application in mind, their development solely from the chloroform literature could render them less practical for carcinogenic MoAs other than the cytotoxic, non-linear type. This potential weakness is considered unlikely, but cannot be dismissed until further evaluation resolves the issue.

6. Appendix A

Brief Summary of Reviews and Consensus Reports

IPCS, 1994

The IPCS Environmental Health Criterion #163 reviewed the complete toxicology dossier on chloroform, including the extant animal tumor data and studies addressing MoA. Despite its publication long before the HRF or IPCS MoA framework, this comprehensive review supports the current consensus on key events for the carcinogenic MoA of chloroform. Although the review acknowledged the non-genotoxic, cytotoxicity based mode of action for tumor induction by chloroform, it considered deviation from the default non-linear model premature at that time, noting the lack of data on proliferative regeneration in Osborne-Mendel rats, the rat strain in which kidney tumors had been identified. Reevaluation of the histopathology slides from the study in Osborne-Mendel rats has since resolved this critical issue (Hard et al. in 2000; discussed below).

Butterworth et al. 1995a
Butterworth et al. 1995b
Conolly 1995
Conolly & Butterworth (1995)
Wolf & Butterworth (1997)
Butterworth & Bogdanffy (1999)

This body of work, conducted largely through the Chemical Industry Institute of Toxicology (CIIT) provided the formative basis for MoA assessments of carcinogens, all using chloroform as a model chemical due to the extensive body of literature regarding its MoA in producing liver tumors in male and female mice and kidney tumors in male mice and rats. These reviews were preceded by the IPCS (1994) Environmental Health Criteria for chloroform, which provided substantial support for a cytotoxic carcinogenic MoA for chloroform. These early reviews identify most of the key events now considered established for chloroform's tumorigenic MoA, including the importance of the oxidative metabolite phosgene, the negative body of genotoxicity data for chloroform, the strikingly lower tumorigenic response when equivalent or even higher doses of chloroform are given in drinking water versus corn oil gavage and correlation with cytotoxicity/lethality and induction of compensatory regenerative hyperplasia. As well, these publications began the development of MoA-based models to predict cancer risks for human exposure to chloroform, predicting no carcinogenic response at chloroform doses that fail to produce sustained cytotoxicity. In essence, these publications represent some

of the first examples of threshold models for cytotoxic carcinogenic modes of action, all using chloroform as the prototype example chemical.

Golden et al. (1997)

Golden and co-authors published a critical review of chloroform's carcinogenic MoA and its implications for risk assessment. They identified several of the key events and strengths of evidence for the non-linear, cytotoxic MoA now used to assess chloroform's carcinogenic risks, including the importance of the oxidative versus reductive pathway, the importance of studies with enzyme inhibitors, and why a DNA-damaging MoA via the reductive pathway, if operable, is limited to very high doses. They also explain how the use of oil vehicles is a confounding influence due to known effects on peak blood levels and toxicity of other hydrocarbons, and how this factor lends support to a cytotoxic, thresholded MoA. They discussed questions of data quality in methods suggesting a genotoxic potential and described why the evidence strongly supports a non-genotoxic MoA for chloroform. This review considered the potential for alternative MoAs, noting the inconsistency of the data with a direct effect on cell proliferation, and that proliferation alone is a necessary but not sufficient factor in tumor induction. They concluded the available evidence was strongly consistent with a cytotoxic, non-linear MoA, asserting that the existence of a threshold for these events is beyond dispute. They discussed the implications of this MoA for cancer risk assessment using threshold dose response models.

Hard (1998)
Hard et al. (2000)

Hard specifically addressed mechanisms of renal carcinogenesis in the laboratory rodent. Hard (1998) divided into four categories of tumor induction 1) resulting from direct DNA reactivity, 2) linked to indirect DNA reactivity mediated by oxidative stress, 3) associated with sustained regenerative cell proliferation, and 4) reflecting interaction of chemicals with spontaneous CPN. He discussed in detail the various mechanistic features distinguishing different types of tumor induction in kidney, noting distinctly different patterns of testing results for genotoxic versus cytotoxic chemicals. He discussed chloroform as the prototype example for category 3, where tumor induction occurs as a direct, as opposed to an indirect, response to cyctotoxicity.

Recognizing that the data were less certain regarding a cytotoxic MoA for chloroform in the induction of kidney tumors relative to liver tumors, Hard et al. (2000) reevaluated the 2-year carcinogenicity bioassay in Osborne-Mendal rats in which chloroform was given in drinking water, concluding that all rats treated with the highest dose, 1800 ppm (approximately 160 mg/kg/day) and half the rats treated at the intermediate dose of 900 ppm (approximately 81

mg/kg/day) had mild to moderate changes in proximal convoluted tubules in the mid to deep cortex indicative of chronic cytotoxicity. Tubule alterations noted in those rats were absent from controls and groups treated with lower doses of chloroform (200 ppm and 400 ppm), adding substantial support to the hypothesis that the key events in chloroform-induced carcinogenicity in rat kidney include sustained cellular toxicity and chronic regenerative hyperplasia.

Fawell (2000)

Fawell (2000) reported a risk assessment case study of chloroform and related substances, drawing attention to the differences in tumor incidence observed with different routes of chloroform exposure – oral via different vehicles and inhalation – as supportive of the MoA via cell death from high-dose cytotoxicity leading to regenerative hyperplasia in liver and kidney.

Andersen et al. (2000)

Andersen et al. (2000) reported the evaluation of an expert panel regarding application of the U.S. EPA's Cancer Guidelines to chloroform and dichloroacetic acid. Potential MoAs evaluated were an obligatory role for cell injury with compensatory hyperplasia as a precursor to carcinogenicity against alternatives related to the mutagenic potential of either reduced free radical metabolites or glutathione conjugates. The authors note that cytotoxic and mutagenic MoAs are not mutually exclusive. Both could occur simultaneously, contributing differentially at different doses. Alternatively, each could occur in different dose ranges with a strict dose dependence. However, based on overwhelming data, the panel articulated a cytotoxic MoA statement for chloroform, in two parts. First, chloroform is oxidized to metabolites that cause cell damage in tissues with high concentrations of the relevant metabolizing enzyme. Second, metabolite-mediated cytotoxicity leads to cell death, regenerative hyperplasia, and higher probabilities of cell mutation and cancer. The key events are thus high rates of oxidative metabolism and metabolite-mediated cytotoxicity. The panel agreed unanimously that the comprehensive data set for chloroform most strongly supports this MoA involving obligatory cytotoxicity as a precursor to cancer in target tissues and that there should be no significant carcinogenic risk from chloroform at concentrations below those that cause cell damage.

The authors also reported on a 1998 Workshop held in Hanover, Germany entitled "Issues in Cancer Risk Assessment, noting the proposal of the IPCS framework to assist in making judgments about the sufficiency of available data supporting proposed modes of carcinogenesis. Importantly, the authors briefly describe the numeric weighting scheme for DNA reactivity developed by the ICPEMC (International Commission for Protection against Environmental Mutagens and Carcinogens). This maximum absolute score is 100, with negative

values indicating lack of support and positive values the converse for genotoxicity. The highest score obtained was + 49.7 and lowest –27.7 from the evaluation of more than 100 chemicals. Forty studies on chloroform yielded a quantitative net negative score of –14.3, indicating strong weight of evidence support for a non-genotoxic MoA.

Greim & Reuter (2001)

Greim and Reuter (2001) reported on the 1998 introduction of an extended classification scheme by the German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK Commission). The new classification scheme added two new Categories (4 and 5) to allow classification of substances based on knowledge about carcinogenic modes of action and potency. The essential feature is that substances in these new Categories do not contribute significantly to the risk of cancer in humans provided that an appropriate exposure limit (MAK value) is observed. These authors used chloroform as the prototype example of chemicals to be classified in Category 4, which exhibit a non-genotoxic mode of action.

Meek et al. (2002)
Meek et al. (2003)

Meek et al. (2002) is perhaps the most comprehensive and detailed report published in a scientific journal evaluating the MoA for chloroform carcinogenicity. This publication describes the evaluation of the chloroform as a Priority Substance under the Canadian Environmental Protection Act (CEPA), and focuses largely on the mode of action for liver and kidney tumors. Meek et al. (2003) describe development of the Human Relevance Framework (HRF), a project of the ILSI-RSI (International Life Sciences Institute-Risk Science Institute) that expanded the EPA and IPCS frameworks for MoA evaluation to explicitly address whether tumorigenic MoAs demonstrated in animal test systems should be deemed relevant for human risk assessment. The HRF asks first whether the weight of evidence supports a clear MoA in the animal test model, and second, whether that MoA is also plausible in humans when both toxicokinetic and toxicodynamic factors are considered. Several case studies were used to illustrate various modes of carcinogenesis; chloroform was used to illustrate how the framework is used to demonstrate the MoA comprising sustained cytotoxicity and regenerative cell proliferation.

These publications both conclude with high confidence that the weight of evidence for induction of liver tumors in mice and renal tumors in mice and rats is consistent with a MoA comprising oxidative metabolism principally to phosgene and hydrochloric acid, followed by marked, obligatory cytotoxicity concomitant with a period of sustained cell proliferation and a non-linear dose-response relationship. The authors noted that the evidence for this mode of action is stronger for liver and kidney tumors in mice than for kidney tumors in rats, largely

because of fewer data from long-term metabolism studies in Osborne-Mendel and Fischer 344 rats than from B6C3F1 mice. An alternative MoA involving a combination of oxidative and reductive metabolism, followed by cytotoxic and mutagenic pathways was considered highly unlikely based on several lines of evidence, including striking differences between the carcinogenic action of chloroform and carbon tetrachloride, which produces toxicity secondary to reductive metabolism and is not mutagenic. Nonetheless, Meek et al. (2002) state that weak genotoxicity in the rat cannot be precluded, and that the DNA reactivity of chloroform and its metabolites could be further investigated.

Meek et al. (2003) concluded that the weight of evidence supports that the cytotoxic MoA is relevant for human risk assessment and that non-linear pharmacokinetic models can be appropriately applied to derive large margins of exposure for humans based on tumorigenic doses in animals. Although not stated explicitly in these publications, such models are consistent with a threshold below which tumors would not be predicted in test species or in humans.

Komulainen (2004)

Komulainen (2004) reviewed the data on carcinogenic MoAs for chlorine disinfection by-products, concluding that the data for rodent and kidney tumors induced by chloroform demonstrate metabolic activation to reactive phosgene followed by the determining step of high-dose sustained cytotoxicity and regenerative hyperplasia. The publication primarily cites the consensus reports of expert panels convened by IARC (1991), ILSI (1997), IPCS (1994), and WHO Guidelines for Drinking-Water Quality (1993; 1996; 1998).

Holsapple et al. (2006)

Holsapple et al. (2006) used the IPCS (International Programme on Chemical Safety) and the ILSI-RSI (International Life Sciences Institute-Risk Science Institute) frameworks for conducting MoA analysis and extrapolation to humans to evaluate the plausibility of various modes of rodent carcinogenesis in humans. They used chloroform as the prototype chemical example to consider whether sustained cytotoxicity and regenerative proliferation in rodents is a plausible mechanism for tumor formation in humans. They deemed the data for chloroform sufficient to rule out DNA reactivity as the tumorigenic MoA and to demonstrate parallel dose response characteristics between the key mechanistic events and tumor formation as well as the specificity of those key events in the proposed MoA. They concluded that having satisfied these necessary criteria, the tumorigenic MoA for chloroform in rodent liver, consisting of metabolic activation by CYP2E1 followed by sustained cyctotoxicity and regenerative

hyperplasia, is relevant for humans and is expected to follow non-linear dose response kinetics that entail a threshold.

Boobis (2010)
Boobis et al. (2009)

These most recent reviews generally update application of the Human Relevance Framework developed by ILSI-RSI (see discussion of Meek et al., 2003). These papers summarize and build upon the conclusions of previous consensus panels by adding considerable discussion of absorption kinetics, oxidative metabolism by CYP2E1, the correlation of the key events with tumorigenic incidence, and import of pharmacokinetic/pharmacodynamic modeling. These reviews also mention the lack of data for various potential alternative hypotheses regarding the MoA for chloroform carcinogenicity not mentioned in other reviews, such as direct mitogenesis, inhibition of apoptosis, receptor activation, estrogenicity or other hormonal stimulation. A discussion of the differences between biological and population thresholds completes the discussion with suggestions regarding how remaining uncertainties regarding the human population threshold dose might be addressed.

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