January 4, 2019

Lauren Zeise, Ph. D.
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California Environmental Protection Agency
Post Office Box 4010
Sacramento, CA 95812-4010

Re: Public Health Goals for Trihalomethanes in Drinking Water (First Public Review Draft, October 2018)

Dear Dr. Zeise:

The Chlorine Chemistry Division (CCD) of the American Chemistry Council submits the enclosed comments on the proposed public health goals (PHGs) for the four trihalomethanes. CCD represents major producers and users of chlorine in North America and works to promote and protect the sustainability of chlorine chemistry processes, products and applications in accordance with the principles of Responsible Care®.

Disinfection of drinking water with chlorine is one of the most significant public health achievements of the twentieth century that has saved millions of lives and spared countless illnesses. Because of the inevitable presence of organic matter in source water and distribution systems, disinfection byproducts, such as the trihalomethanes (THMs), are produced in the implementation of this simple, yet vital, public health practice. The potential health effects of the THMs have been well studied and regulations to reduce their concentrations in finished drinking water have been in place since the mid-1980s.

In its current proposal, OEHHA would establish a PHG for each of the THMs that are not supported by the available science or by the conclusions of regulatory bodies in North America and Europe. The attached comments outline our concerns with what OEHHA has proposed, including a detailed analysis of the cancer evidence for chloroform. We look forward to reviewing this information with you and your staff as you consider revisions to the THM draft.

Sincerely,

Judith Nordgren

Judith Nordgren
Managing Director

Enclosure

January 4, 2019

Comments of the Chlorine Chemistry Division
of the American Chemistry Council
On the Draft Public Health Goals for Trihalomethanes in Drinking Water
(First Public Review Draft, October 2018)

Summary

Disinfection of drinking water with chlorine is one of the greatest public health achievements of the twentieth century. While chlorine disinfection can result in the formation of disinfection byproducts (DBPs), such as the trihalomethanes (THMs), it is critical that efforts to control THMs and other DBPs do not compromise the effectiveness of the disinfection process and that a sufficiently protective residual level of disinfectant is maintained throughout the distribution system. Because of the integral connection between disinfecting drinking water and THM formation, it is inappropriate to consider the toxicity of THMs without also considering the public health benefits that are associated with the disinfection process. The proposal to establish individual public health goals (PHGs) for the four substances that are well below the current maximum contaminant level for total THM creates a number of policy issues that should be considered prior to finalization of the goals.

OEHHA's proposed PHGs for chloroform, bromoform, bromodichloromethane (BDCM), and dibromochloromethane (DBCM) should be reconsidered to address several general and compound-specific scientific deficiencies in the agency's Technical Support Document (TSD). Some apply to all four of the proposed PHGs:

- OEHHA's disregard of authoritative conclusions by the US. Environmental Protection Agency (USEPA), the International Agency for Research on Cancer (IARC), the World Health Organization (WHO), and Health Canada regarding the animal evidence for the carcinogenic potential of the substances.

- OEHHA's selection of oral gavage studies as the basis for the PHG calculations when more studies exposing animals through the more relevant routes of diet or drinking water have failed to find carcinogenic effects.

- OEHHA's use of daily water consumption rates that are four times higher than the available exposure data can support.

In addition to these general problems, the substantial body of evidence available for the individual THMs contradicts OEHHA conclusions regarding the mechanism by which the substances cause cancer in animals or their carcinogenic potential. The weight of the evidence (WoE) for chloroform and BDCM establishes that they are not genotoxic carcinogens and supports the conclusions of several authoritative bodies that cancer in laboratory animals only results after sustained exposure to high levels of these substance overwhelms the animal's natural defense mechanisms. Moreover, IARC has determined that the WoE for bromoform

and DBCM do not support classification of these substances as carcinogens. OEHHA previously confirmed IARC's conclusion for DBCM by removing it from the list of chemicals "know to the state to cause cancer" under the California Safe Drinking Water and Toxic Enforcement Act (Proposition 65).

The available scientific evidence, and the consensus of multiple authoritative bodies, do not support the proposed PHGs for the individual THMs. The combined effect of the scientific deficiencies is artificially inflated estimates of human health risk from exposure to individual THMs resulting in individual PHGs much lower than necessary to protect public health.

Chlorination is Critical to Public Health and the Safety of the California Drinking Water Supply

The treatment and distribution of drinking water for safe use is one of the greatest public health and engineering achievements of the twentieth century, and has saved millions of lives and spared countless illnesses.[1] Before U.S. cities began routinely treating water with chlorine, typhoid fever, dysentery, and other waterborne diseases killed thousands annually. Without disinfection consumers are at a significantly higher risk of contracting and spreading waterborne diseases. As more and more U.S. communities began chlorinating and filtering their drinking water, corresponding death rates declined dramatically.

Chlorine is typically added to drinking water as elemental chlorine (chlorine gas), sodium hypochlorite solution (bleach), or dry calcium hypochlorite. Virtually all public water systems use a chlorine-based disinfection method, either for centralized disinfection or as a supplement to other technologies to prevent recontamination of treated water as it moves through the distribution system. Regardless of the type of chlorine applied, primary disinfection is accomplished by free chlorine, which readily penetrate the cell walls, slime coatings, and even resistant shells of most microorganisms to disrupt metabolic processes and rapidly neutralize otherwise harmful biological contaminants. In addition to controlling most disease-causing organisms, chlorination provides residual benefits, including removal of some chemicals that interfere with the disinfection process and neutralizing unpleasant tastes and odors. It is also the ***only*** means of preventing recontamination of finished water between the treatment plant and the tap.

Implementation of the federal and California Safe Drinking Water Act (SDWA) is responsible for significant reductions of waterborne disease risks throughout California and the United States as documented by the Centers for Disease Control and Prevention (CDC).[2] This is in large part

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1 McGuire MJ. *The Chlorine Revolution: Water Disinfection and the Fight to Save Lives.* AWWA: Denver, Colorado (2013).

2 CDC. Surveillance for waterborne disease outbreaks associated with drinking water—United States, 2013–2014. MMWR Surveillance Summaries 66(44):1216–1221 (2017). https://www.cdc.gov/mmwr/volumes/66/wr/mm6644a3.htm?s_cid=mm6644a3_w

due to the federal-state partnership that has evolved since the mid-1970s that has led to improved treatment plant operations and oversight by state regulators, occurrence monitoring and reporting, and health effects research. However, the threat of disease outbreaks due to deficiencies in distribution systems, including microbial growth, leaks, water pressure loss, and main and pipe breaks is increasing as a result of aging drinking water infrastructure. There is no expedient solution to this problem. According to AWWA addressing the adverse impacts of aging drinking water infrastructure in the U.S. will require a $1 trillion investment over the next 25 years.[3] In the interim, maintaining residual disinfection capacity in the distribution system will be a public health protection imperative.

Individual THM Levels Can Vary Based on Source Water Characteristics, Disinfection Method and Other Factors

THM concentrations in treated water depend on several factors, including organic matter concentration, pH, temperature, and bromide ion concentration of the source water, what chlorine-based chemicals are applied, contact time, and the presence of other chemicals that may influence the disinfection reaction. Although several pre- and post-disinfection techniques are available to minimize THMs,[4] care must be taken to avoid the production of other DBPs such as haloacetic acids.[5]

Concentrations of THMs in chlorinated water in treatment plants and distribution systems are approximately twice as high during summer months as during winter months. This is a consequence of the higher concentrations of precursor organic materials in the raw water during warmer periods and because the rate of formation of DBPs increases with rising temperatures.[6] THM levels also can increase as the chlorinated water moves from the water treatment plant through the distribution system, because of the continued presence of a chlorine residual to prevent recontamination of the finished water.

Smaller public water suppliers with less sophisticated treatment systems generally have higher THM levels in their drinking water. For example, in a 1994–2000 national survey of Canadian water suppliers, 274 systems serving populations of less than 1000 people had average THM

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[3] AWWA. Infrastructure Report Card (2017). https://www.infrastructurereportcard.org/cat-item/drinking-water/

[4] Water Resource Federation. Disinfection By-Products: Control Strategies (2017). http://www.waterrf.org/knowledge/dbps/FactSheets/DBP-ControlStrategies-FactSheet.pdf

[5] An important parameter in DBP formation is pH. THM formation increases at high pH and decreases at low pH, whereas the formation of haloacetic acids (the second most common group of disinfection by-products) decreases at high pH and increases at low pH.

[6] LeBel GL *et al.* A one-year survey of halogenated disinfection by-products in the distribution system of treatment plants using three different disinfection processes. *Chemosphere* 34(11):2301–2317 (1997). https://doi.org/10.1016/S0045-6535(97)00042-8

levels greater than 75 microgram per liter (µg/L), and 45 systems had average BDCM levels greater than 10 µg/L.[7] Conversely, where the population was greater than 50 000 (where more sophisticated treatment plants would be expected), there were only four systems whose average THM levels were greater than 75 µg/L, and only one system had an average BDCM level greater than 10 µg/L.

THM concentrations in finished water can be reduced by implementing measures to control the concentration of organic matter upstream of the disinfection process. Modification of chlorination practices, such as optimizing the chlorine dosage and changing the point of contact for chlorine, can also help reduce THM concentrations in finished drinking water as it enters the distribution system. However, alternative pre-treatment measures have no impact on organic matter introduced in the distribution system. The same limitation applies to any post-disinfection THM removal measures applied upstream of the tap.

Alternatives to Chlorine-Based Disinfection Methods Will Not Ensure Safer Drinking Water

Some centralized drinking water treatment plants do employ alternative to chlorine treatment disinfection including chloramines, chlorine dioxide (CLO_2), ozone and ultraviolet (UV) irradiation. Although these alternatives can reduce THM concentrations, none of them can ensure safer drinking water at the without secondary treatment with chlorine.

While the use of chloramines does not form significant levels of THMs, it is a much weaker disinfectant than chlorine and are not recommended as primary disinfectants, especially where virus or parasite cyst contamination may be present.[8] They also are capable of inducing halogen substitution in organic compounds and thus may produce significant quantities of total organic halogen. Little is known about these oxidant residuals. The nature and toxicity of products formed from the organic base precursor fractions, particularly the organic chloramine portion of the chlorine residual, have not been characterized.

CLO_2 is a volatile gas that is generated onsite at drinking water treatment facilities from sodium chlorite. It is a strong primary disinfectant and a selective oxidant also used in conjunction with water pre-treatment and to reduce production of THMs.[9] The main inorganic byproducts of CLO_2 disinfection are chlorite (which is regulated) and chlorate (currently unregulated).

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[7] Health Canada. Guidelines for Canadian Drinking Water Quality: Guideline Technical Document – Trihalomethanes. Ottawa, Ontario (May 2006, with April 2009 addendum). https://www.canada.ca/en/health-canada/services/publications/healthy-living/guidelines-canadian-drinking-water-quality-trihalomethanes.html

[8] National Academy of Sciences. Drinking water and health. Vol. 7. Disinfectants and disinfectant by-products. National Academy of Sciences, National Academy Press, Washington, DC (1987). https://www.nap.edu/catalog/1008/drinking-water-and-health-volume-7-disinfectants-and-disinfectant-by

[9] USEPA. Drinking water treatability data base, chlorine dioxide. https://iaspub.epa.gov/tdb/pages/treatment/treatmentOverview.do?treatmentProcessId=-1277754943

Although CLO_2 can produce an adequate disinfectant residual, it is difficult to maintain, which is why it is rarely used for that purpose.

Ozone has been used as a primary disinfectant in water treatment plants in some parts of Canada and Europe. It is highly effective and does not form THMs, but can form other by-products (*e.g.*, include bromate, acids, and aldehydes) which can be converted to more toxic compounds when the finished water is chlorinated for distribution.

Like ozone, UV can be an effective primary disinfectant and under typical conditions does not produce significant concentrations of THMs either during primary disinfection or secondary chlorination. However, because it is dependent on light transmission to inactivate microbes, this process is more sensitive to variations in water quality characteristics affecting UV transmittance (*e.g.*, turbidity). These issues need to be considered in the design of the system.

None of these alternatives are sufficient in isolation because none of them impart any residual disinfection capacity to the finished water to ensure it will still be safe to drink when it arrives at the tap. Therefore, where they are used as primary disinfection methods they must be supplemented with a chlorine-based secondary treatment to protect the public from biological contaminants that may occur in the distribution system. It bears repeating that the threat of biological recontamination of treated drinking water is increasing with the passage of time, as drinking water infrastructure is operated beyond its design life and investments in system repair and maintenance are deferred in response to new regulatory obligations and conservation-induced revenue declines.

OEHHA Has Not Assessed the Public Health Benefits of the Chlorination of Drinking Water that Can Result in Trihalomethane Formation

The gravity of public health risks addressed by drinking water disinfection necessitate a more thoughtful and balanced approached to THM risk assessment than is reflected in the TSD. OEHHA acknowledges the importance of disinfection relative to incremental cancer risk from exposure to THMs by reference to WHO and IARC findings (page 2), but then proceeds to evaluate THM cancer risks in isolation of the public health risks that could result from actions to reduce THM concentrations. This approach does not satisfy OEHHA's statutory requirements pertaining to the development of PHGs and is potentially harmful to public health.

THMs are present in drinking water as a result of chlorination of organic matter present in raw water supplies. It is therefore critical, in assessing the potential risks associated with the ingestion of THMs in drinking water, to also consider the substantially greater benefits to public health associated with drinking water disinfection. As noted above, the use of chlorine for disinfection has virtually eliminated waterborne microbial diseases because of its ability to kill or inactivate essentially all enteric pathogenic microorganisms, including viruses and bacteria from the human intestinal tract. Chlorine is the most accessible and easily controlled

disinfectant. It is used by the vast majority of public water utilities in California. Chlorine provides a lasting residual disinfection capacity throughout the distribution system to prevent bacterial regrowth. Numerous public health organizations, including the WHO, have consistently described the profound historical and continuing public health benefits that chlorination provides and strongly caution that –

> [I]n attempting to control DBP concentrations, it is of paramount importance that the efficiency of disinfection is not compromised and that a suitable residual level of disinfectant is maintained throughout the distribution system.[10]

In establishing a PHG, Health and Safety Code §116365 directs OEHHA to estimate the level of the contaminant in drinking water "that is not anticipated to cause or contribute to adverse health effects, or that does not pose <u>any</u> significant risk to health" (emphasis added). While §116365 focuses on health effects associated with the drinking water contaminant, OEHHA also must consider "any significant risk to health." In other words, OEHHA must assess all potential health risks that directly relate to the action being contemplated.[11] Due to the inseparable relationship between THM formation and life-saving disinfection of drinking water, and consistent with the WHO's cautionary language about controlling DBPs, any evaluation of THMs must take into account the significant public health benefits associated with chlorine disinfection of drinking water.

The Draft PHGs Are a Significant Departure from the Established Approach to Addressing Trihalomethanes

The California MCL for total THMs of 0.08 mg/L (80 µg/L) is the sum of the concentrations of chloroform, bromoform, BDCM, and DBCM. It was established by the California Department of Public Health in 2006. In 2010 OEHHA proposed a PHG for total THM based on an assessment of "the mean concentrations of each of the four chemicals in California drinking water" but never finalized the PHG. In the current proposal, OEHHA has replaced the single PHG for total THM with separate PHGs for each of the four THMs.

OEHHA's proposal would appear to require that the SWRCB establish separate MCLs for each of the four THMs since there is no "corresponding" PHG for total THM. If, on the other hand, the SWRCB determines that it can maintain the current approach of specifying a single MCL for total THMs, it would need to demonstrate how a single standard for total THM meets the requirement to be "as close as feasible" to the four individual PHGs.

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[10] WHO. Guidelines for Drinking-water Quality. Fourth Edition Incorporating the First Addendum. WHO: Geneva, Switzerland, at 173 (2017). https://www.who.int/water_sanitation_health/water-quality/guidelines/en/

[11] The inclusion of language relating to synergistic and additive effects in (C)(i) and (C)(iv) of §116365 reinforces the legislature's intent that OEHHA not restrict itself solely to consideration of the contaminant in question.

Comments on Health Risk Assessment and PHG Calculation

In addition to the specific comments on OEHHA's evaluation of the toxicity of the individual THMs discussed elsewhere, there are three general comments relating to the assessment of total THMs and the derivations of the four PHGs that require comment: the existing human evidence for an association between THM exposure and bladder cancer, the significant pharmacokinetic differences between oral gavage and drinking water dosing, and the assumptions about drinking water consumption rates that OEHHA employs to calculate the draft PHGs.

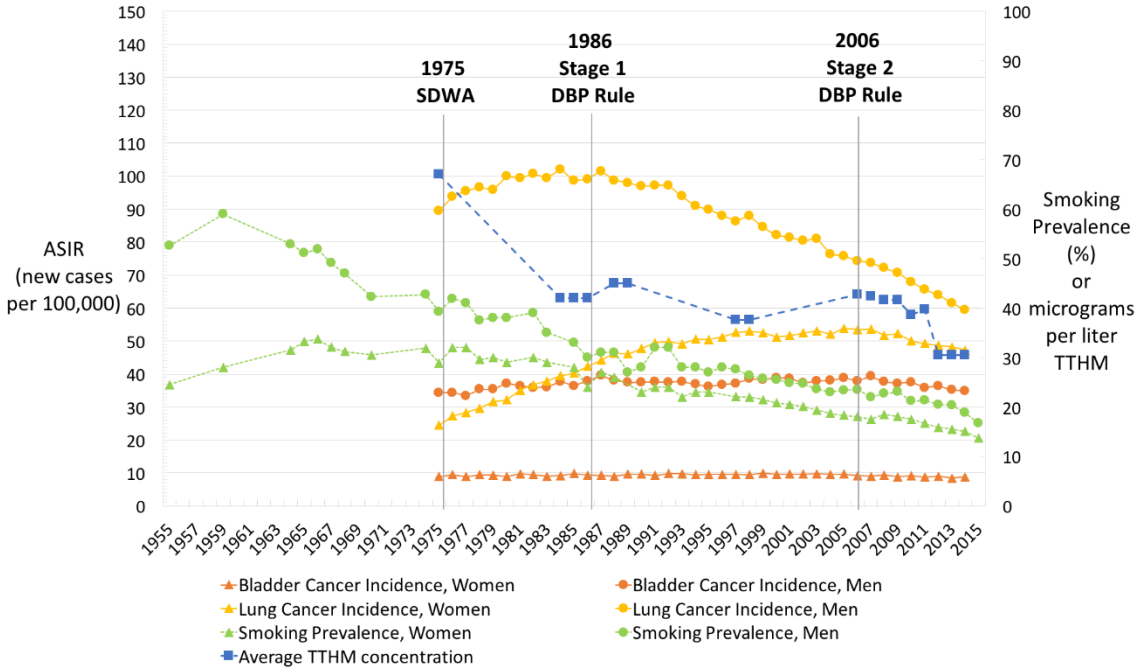

Figure 1. Annual age-adjusted smoking prevalence and bladder and lung cancer incidence, and total THM (TTHM) concentrations in drinking water systems in the United States, 1975-2015.[10]

a. A Recently Completed Analysis Finds no Association between Trihalomethane Exposure and Increased Bladder Cancer Risk

As OEHHA notes in the draft PHG document, USEPA, IARC, WHO, and Health Canada have found the epidemiological evidence for a weak association with bladder cancer to be inconclusive because of the large number of potentially confounding factors. Although bladder cancer incidence rates are a somewhat consistent result in some human studies, it is not possible to assess the potential impact of individual THMs or total THM because of smoking rates and other confounders and the long latency period and high diagnosis age associated with

the disease. However, a recently completed analysis of national trends in bladder cancer incidence provides important new insights into any potential association with THM exposure.

The study found no discernible relationship between total THMs in drinking water and bladder cancer incidence in the U.S. over the more than 40 years since the passage of the federal SDWA.[12] As noted in Figure 1, bladder cancer incidence rates in both males and females have remained relatively stable since the mid-1970s, while THM concentrations have been reduced by more than half over the same time period.

b. There is Strong Evidence of Pharmacokinetic Differences between Exposure in Drinking Water and Dosing by Gavage

The proposed PHGs for the four THMs are based on cancer evidence from gavage studies using corn oil, despite the fact that drinking water and dietary studies have generally produced negative results. In a comparative study of gavage and drinking water exposures, THMs

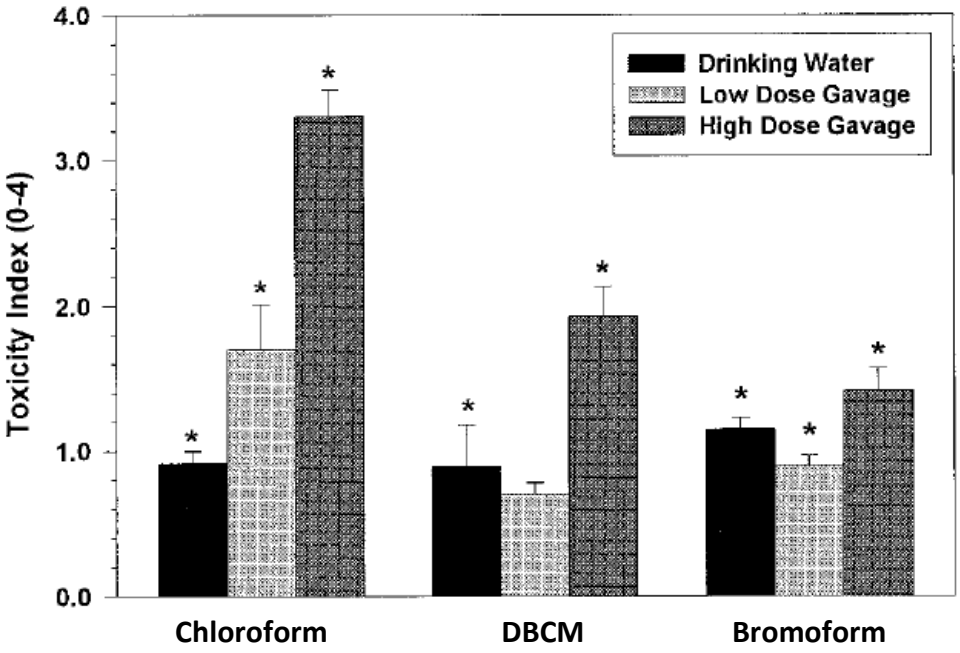

Figure 2. Ability of trihalomethanes to induce liver toxicity; * indicates significant difference from the vehicle control group, p-value <0.05 (Coffin *et al.* 2000)

[12] Cotruvo JA Amato H. National trends in bladder cancer and trihalomethanes in drinking water. *Dose Response* (in press). ASIR – age-specific incidence rate

administered by gavage increased cell proliferation and decreased DNA methylation in mouse livers while dosing in drinking water produced a much smaller effect, particularly for chloroform.[13] These findings are consistent with the dose-response curves observed for the THMs, especially chloroform and BDCM, which suggest that THM levels must be sufficiently high to overcome natural detoxification mechanisms before they can exert a toxic effect. The weaker activity of THMs administered in drinking water likely results from its incremental delivery each time the mouse drinks – in contrast to bolus delivery by oral gavage. The slower rate of delivery by drinking water is expected to result in a lower liver concentration that increases the opportunity for detoxification (Figure 2). Hence, the activity of the THM appears to be dependent on their rate of delivery (*i.e.*, rapidly by oral gavage and more slowly in drinking water).[14]

The slower rate of delivery by drinking water results in lower metabolite concentrations that reduce the likelihood that concentrations will overwhelm reduced glutathione (GSH) and other detoxification mechanisms. This appears to be true even though the doses administered in drinking water produce a greater area under the chloroform blood concentration curve than with bolus delivery (Figure 3).

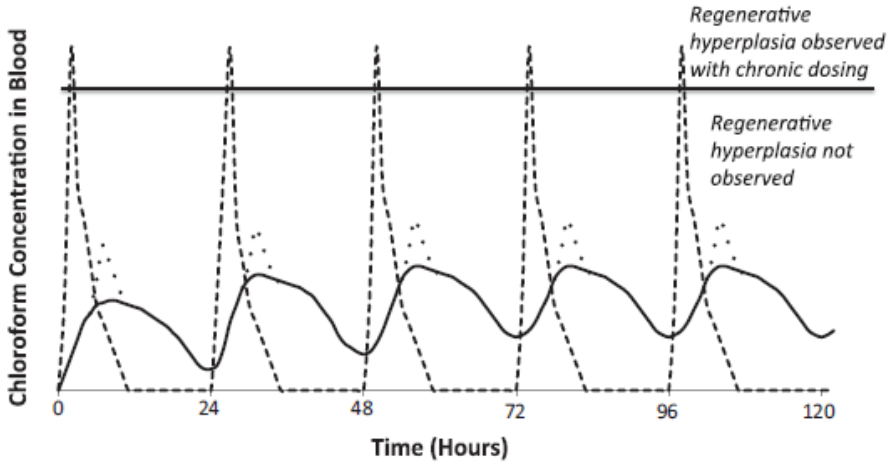

Figure 2. Illustration of pharmacokinetics of chloroform following administration by oral gavage (- - - -), drinking water (——), and inhalation (····) routes. Area under the concentration curve: drinking water > gavage > inhalation[15]

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[13] Coffin JC *et al.* Effect of trihalomethanes on cell proliferation and DNA methylation in female B6C3F1 mouse liver. *Toxicol Sci* 58(2):243-252 (2000). https://doi.org/10.1093/toxsci/58.2.243

[14] Ibid.

[15] Borgert *et al.* Modernizing problem formulation for risk assessment necessitates articulation of mode of action. *Reg Toxicol Pharma* 72:538-551 (2015). http://dx.doi.org/10.1016/j.yrtph.2015.04.018

The pharmacokinetic differences between bolus-gavage and drinking-water (and dietary) dosing appear to play a significant role in explaining the disparity in the observed tumor incidence in the animal studies and need to be taken into consideration in assessing the toxicity of the THMs.

c. OEHHA Has Significantly Overestimated the Drinking Water Consumption Rate in Calculating the Public Health Goals

OEHHA's calculation of the drinking water concentration associated with a cancer risk of 10^{-6} for each of the four THMs is based on a susceptibility-weighted daily water intake (DWI). The weighted DWI, expressed in equivalent liters of water consumed per kilogram body weight per day, or L_{eq}/kg-day, represents the product of the age sensitivity factor (ASF), the time spent in each life stage (expressed as a ratio), and the unweighted DWI for the life stage. Although this approach is consistent with OEHHA's method for accounting for early life-stage exposures, OEHHA proposes to add the weighted DWIs for each life stage to produce a lifetime DWI (DWI_{life}) as opposed to taking an average of the four life stage values.[16] The result is an inappropriately high estimate for lifetime daily water consumption, which also biases the calculated risk value to indicate and improbably high health risk.

Using the DWI_{life} for chloroform of 0.180 L_{eq}/kg-day, for example, a 70-kilogram adult would consume the equivalent of 12.6 liters of water per day – four times the total daily consumption rate of 3 L_{eq}/day assumed in the 2010 draft PHG.[17] While ACC appreciates OEHHA's attempt to account for age sensitivity, an estimate that assumes a four-fold higher consumption rate for three-quarters of an individual's lifetime grossly overstates probable lifetime exposures for water use and consumption, even among outliers in the population. Using the average of the susceptibility weighted DWIs (equal to 0.045 L_{eq}/kg-day), on the other hand, generates a total adult consumption rate of 3.15 L_{eq}/day, which – while still quite high – is consistent with OEHHA's 2010 assumption.

Correcting the approach to calculating the susceptibility weighted DWI reduces the risk associated with a particular concentration level of the THM by a factor of four. As a result, the concentration of THM that results in a 10^{-6} risk will be four times greater than the proposed OEHHA value.

The disparity in assumptions about drinking water consumption rates is even more apparent when the proposed PHGs are compared to the no significant risk levels (NSRLs) developed by OEHHA under Proposition 65. For example, although OEHHA has lowered its cancer potency factor for chloroform by 60 percent (from 0.035 to 0.0137 per mg/kg-day) in the latest

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[16] The lifetime DWI also is discussed in Section 3 of the draft. Although it is not clear how the lifetime value is calculated in this Section, it is significantly less than the sum of the DWIs for the four life stage.

[17] OEHHA. Public health goal for trihalomethanes in drinking water, draft – for public review (September 2010).

assessment, the estimate of drinking water risk for the proposed PHG is dramatically higher. Based on OEHHA's latest estimate, a 70-kg individual would need to consume the equivalent of 50 liters of water daily to achieve the NSRL of 20 micrograms per day (µg/day) for chloroform (Table 1). Similar comparisons between the proposed PHG and the NSRL can be made for

	Cancer Potency Factor (per mg/kg-day)		NSRL (µg/day)	Proposed PHG (µg/L)	Consumption Required to Reach NSRL (L_{eq}/day)
	Prop 65	OEHHA 2018			
Chloroform	0.035	0.0137	20	0.4	50
Bromoform	0.011	0.0109	64	0.5	128
BDCM	0.14	0.087	5	0.06	83
DBCM	--	0.0445	--	0.1	--

Table 1. Comparison of Cancer Potency and Drinking Water Risks Between the Proposition 65 NSRLs and the Proposed PHGs[18]

bromoform and BDCM, requiring the consumption of 128 and 83 liters per day to achieve the respective NSRLs.[19]

Specific Comments on the Draft PHGs

OEHHA's proposal to base its proposed PHGs for each of the THM on cancer risks derived from gavage studies is not supported with the available science and is inconsistent with the conclusions of other authoritative bodies. The evidence for each THM is discussed below.

a. OEHHA Overstates the Potential Cancer Risk from Chloroform Exposure

Experiments have reported that chloroform is carcinogenic in the liver and kidney of mice exposed through oral gavage[20] and in the kidney of rats exposed to high levels in drinking water.[21] Chloroform is listed under Proposition 65 as 'known to the state to cause cancer." It is considered by IARC to be possibly carcinogenic to humans (Group 2B) based on inadequate evidence for carcinogenicity in humans and limited evidence in experimental animals. As noted by USEPA and WHO, however, there is compelling mechanistic evidence that both the hepatic

[18] The NSRL is based on a cancer risk of 1 in 100,000 (10^{-5}), while the PHG is based on a cancer risk of 1 in a million (10^{-6}).

[19] No NSRL exists for DBCM since OEHHA delisted it from Proposition 65 in 1999.

[20] National Cancer Institute. Carcinogenesis bioassay of chloroform. NTIS PB-264018/AS Carcinogen Bioassay and Program Resources Branch, Bethesda, MD, March (1976).

[21] Jorgenson TA *et al.* Carcinogenicity of chloroform in drinking water to male Osborne-Mendel rats and female B6C3F1 mice. *Fundam Appl Toxicol* 5(4):760 (1985). https://doi.org/10.1016/0272-0590(85)90200-3

and renal tumorigenic responses observed in previous carcinogenicity studies of chloroform are mediated by a non-genotoxic mechanism.[22] As a consequence, USEPA concludes that "chloroform is likely to be carcinogenic to humans only under high exposure conditions that lead to cytotoxicity and regenerative hyperplasia."[23]

A state-of-the-science quantitative comparison of the available data for genotoxic and non-genotoxic cancer mechanism is included as Attachment A of these comments, based on a confidence scoring system developed by Becker *et al.* (2017).[24] The results of this comparison indicate that there is strong counter evidence for several of the early diagnostic key events for a mutagenic mode of action (MoA), including three negative in vivo transgenic mouse datasets.

In evaluating the available information on the MoA for chloroform carcinogenicity, the draft PHG rejects the conclusion of USEPA, WHO,[25] and Health Canada[26] that tumors result from tissue regeneration subsequent to toxicity. OEHHA's conclusion is based on the following analysis --

> We found that the evidence is not entirely consistent with tumors in the kidney or in the liver being only secondary to cytotoxicity and tissue regeneration. The relationships among toxicity, tissue regeneration, and tumor formation are not clear. OEHHA considers use of the available cancer data on chloroform and a linear extrapolation in dose-response assessment for development of the health-protective concentration to be appropriate based on our evaluation of the strength of the evidence regarding a potential threshold mechanism.[27]

In support of its conclusion, the draft PHG suggests "a plausible genotoxic mechanism of chloroform carcinogenicity that involves covalent binding of chloroform-derived reactive metabolites to nucleic acid, nuclear protein or phospholipid."

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[22] IPCS. Disinfectants and disinfectant by-products. Environmental Health Criteria 216, International Programme on Chemical Safety, World Health Organization, Geneva (2000). http://www.inchem.org/documents/ehc/ehc/ehc216.htm

[23] USEPA. Toxicological review of chloroform (CAS No. 67-66-3). In support of the summary information on the Integrated Risk Information System (IRIS). Washington, DC (October 2001). https://cfpub.epa.gov/ncea/iris/iris_documents/documents/toxreviews/0025tr.pdf

[24] Becker RA et al. Quantitative weight of evidence to assess confidence in potential modes of action. *Reg Toxicol Pharm* 86:205-220 (2017). https://doi.org/10.1016/j.yrtph.2017.02.017

[25] IPCS 2000.

[26] Health Canada 2006.

[27] OEHHA 2018 Draft PHG, at 265.

Chloroform has produced generally negative results in tests for genotoxicity, however, both *in vitro* and *in vivo*.[28] Using a published, comprehensive, quantitative weight-of-evidence (WoE) approach to evaluate large, heterogeneous genetic toxicology databases, chloroform's potential mutagenicity was assessed by an expert panel convened by the Health and Environmental Sciences Institute of the International Life Sciences Institute. On a scale of negative 100 (-100) to positive 100 (+100), chloroform scored negative 14.3 (-14.3), indicating that the WoE supports a non-genotoxic classification.[29] Regarding conflicting data, the expert panel noted that --

> [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.[30]

As a result, Boobis (2010) concluded that ''the weight of evidence is that genotoxicity is not the MoA for chloroform.''[31] These findings are discussed in greater detail in Attachment A.

OEHHA notes in the draft PHG that oxidative metabolism of chloroform to form phosgene via the P450 pathway is critical to its toxicity. This conclusion is based on several lines of evidence, including the results from studies in CYP2E1 knock-out mice in which exposure to chloroform did not cause hepatic or renal necrosis or evidence of regenerative proliferation or increased mitotic indices. In experiments with normal mice and rats, moreover, CYP2E1 expression levels within and among tissues correlated well with the extent of chloroform toxicity.[32] The tissues most affected are kidney and liver.

Metabolic conversion of chloroform to phosgene follows classic Michaelis–Menten kinetics, with no threshold for substrate conversion.[33] Phosgene is a highly reactive electrophile that reacts rapidly to form covalent bonds with intracellular nucleophiles such as glutathione, proteins, lipids and other macromolecules.[34] As a result, phosgene likely does not diffuse far

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[28] Boobis AR *et al.* IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit Rev Toxicol* 36(10):781–792 (2006). https://doi.org/10.1080/10408440600977677

[29] Andersen ME *et al.* Lessons learned in applying the U.S. EPA proposed cancer guidelines to specific compounds. *Toxicol Sci* 53(2):159–172 (2000). https://doi.org/10.1093/toxsci/53.2.159

[30] Ibid.

[31] Boobis AR. Mode of action considerations in the quantitative assessment of tumour responses in the liver. *Basic Clin Pharmacol Toxicol* 106(3):173–179 (2010). https://doi.org/10.1111/j.1742-7843.2009.00505.x

[32] Ibid.

[33] 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.)

[34] Microsomal studies indicate that about 75 percent of covalent binding following treatment with chloroform is to phospholipids. (OEHHA 2018 draft PHG, at 32)

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.[35] 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*.

Moreover, because mitochondria can tolerate some level of insult without any change in membrane permeability, and can repair low-level damage, they will be resilient to low-level phosgene production. Mitochondrial resilience and repair is demonstrable in both rodents and humans and explains the observed recovery from low level cytotoxicity following low doses of chloroform.[36] 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.

In vitro and *in vivo* evidence indicate that chloroform cytotoxicity and cell death exhibit a threshold in both liver and kidney.[37] Together with the lack of chloroform toxicity in tissues that do not express CYP2E1, these results strongly indicate that sustained phosgene-induced cytotoxicity is a key event in chloroform-induced carcinogenesis.

The characteristics of chloroform-induced tumors are inconsistent with OEHHA's no-threshold hypotheses of carcinogenic action. Mutagenic mechanisms would be expected to produce DNA damage and increase tumor incidence in target organs at any level of chloroform that produces reactive metabolites (*i.e.*, at all doses) -- yet this is clearly not observed. Furthermore, since conversion of chloroform to reactive phosgene increases with increasing chloroform blood concentrations, a mutagenic mechanism cannot be reconciled with the observation that chloroform tumorigenesis occurs following bolus gavage administration, but not with most doses administered in drinking water, which produce a greater area under the chloroform blood concentration curve.

Similarly, cytotoxic mechanisms that lack a threshold would be expected to increase tumor incidence at all chloroform doses at which cytotoxicity is measurable. This is also not observed. Instead, chloroform tumorigenesis requires dosing sufficient to sustain a cytotoxic effect in the kidney and/or liver. These observations argue strongly for a cytotoxic, threshold mechanism of chloroform carcinogenesis and preclude non-threshold mechanisms that might occur by either mutagenesis or cytotoxicity.

Theoretically, genotoxicity could occur and contribute to tumorigenicity at tissue concentrations above those that saturate cellular protections against oxidative damage as well

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[35] Borgert *et al.* 2015.

[36] Boobis AR *et al.* Application of key events analysis to chemical carcinogens and noncarcinogens. *Crit Rev Food Sci Nutr* 49(8):690–707 (2009). https://doi.org/10.1080/10408390903098673

[37] Ibid.

as saturate available lipid and macromolecular targets in cellular organelles. However, this would be an irrelevant speculation because it would still be a threshold process indistinguishable from the cytotoxic pathway that is primarily responsible for chloroform-induced rodent tumors. Thus, even this highly speculative MoA would still support the conclusion that chloroform presents no carcinogenic hazard to humans at doses below the threshold.

Consistent with its conclusion that chloroform may cause cancer via a non-genotoxic (threshold) mechanism, USEPA established a maximum contaminant goal (MCLG)[38] of 70 µg/Liter (µg/L) under the SDWA. Although the biochemical steps by which chloroform induces tumors can occur in humans, the established MoA renders tumor formation unlikely under any foreseeable consumer exposure resulting from its presence in finished drinking water in California.

b. Bromoform Does Not Present a Carcinogenic Risk to Humans

Four cancer bioassays have been conducted with bromoform. The best documented of these are gavage studies conducted by the National Toxicology Program (NTP) that reported a small increase in relatively rare tumors of the large intestine in rats of both sexes. No tumors were reported in mice in the same NTP study.[39] An earlier study by Theiss *et al*. (1977) reported mixed results in male mice administered bromoform by intraperitoneal injection.[40] They reported a significant increase in lung tumor incidence at the intermediate dose (48 mg/kg three times/week), but not at the lower or higher doses (4 and 100 mg/kg). In a feed study with microencapsulated bromoform, there was no evidence of carcinogenicity for male or female rats exposed for 24 months at concentrations of up to 6,500 ppm.[41]

Bromoform was listed as a Proposition 65 carcinogen in 1991. USEPA subsequently classified bromoform as a probable human carcinogen (Group B2) in 1993 based on inadequate human data and sufficient evidence of carcinogenicity in animals. In a more recent review, IARC (1999) classified bromoform as "not classifiable as to its carcinogenicity to humans" (Group 3) based on limited animal data and inadequate human data. The 2008 WHO drinking water guideline for bromoform is based on the IARC Group 3 classification. In its drinking water guidance for

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[38] MCLGs are defined by USEPA as "aspirational" goals and are comparable to the PHG.

[39] NTP. Toxicology and carcinogenesis studies of tribromomethane (bromoform) in F344/N rats and B6C3F1 mice (gavage studies). National Institutes of Health. NTP TR 350 (May 1989). https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr350.pdf

[40] Theiss JC *et al.* 1977. Test for carcinogenicity of organic contaminants of United States drinking waters by pulmonary tumor response in strain A mice. *Cancer Res* 37(8):2717-2720 (1977). http://cancerres.aacrjournals.org/content/37/8_Part_1/2717

[41] Personal communication from Y. Kurokawa, National Institute of Hygienic Sciences, Tokyo, Japan, 1987, to R. Melnick, NTP (cited in NTP 1989).

THMs,[42] Health Canada classifies bromoform as "possibly carcinogenic to humans" (Group IIID), based on limited evidence for carcinogenicity in one species of experimental animals and no data in humans. An expert panel convened in 2002 by Health Canada to assess the toxicological and epidemiological evidence for the THMs for the purpose of drafting an updated Canadian drinking water guideline concluded that there was insufficient information available to calculate a drinking water guideline for bromoform.[43]

The data from a variety of assays on the genotoxicity of bromoform are equivocal. There is some evidence to suggest that bromoform may be weakly mutagenic. Bromoform is largely positive in bacterial assays of mutagenicity conducted in closed systems and was positive in the Ames test in *S. typhimurium* strain, positive in TA98, and negative or equivocal in strains TA1535 or TA1937 (NTP, 1989). Bromoform yielded increased SCE and chromosomal aberrations in mouse and rat bone marrow cells but negative results in other mouse bone marrow tests, the rat liver UDS assay, and in the dominant lethal assay. There is no *in vivo* evidence of genotoxicity with bromoform.

Despite these equivocal results, the draft PHG concludes that the weight of the available evidence indicates that bromoform is mutagenic and genotoxic and that the chemical is a genotoxic carcinogen – with no exposure threshold. This conclusion contradicts those reached by USEPA, IARC, WHO, and Health Canada. These authoritative bodies have rejected a cancer classification for bromoform based on evidence from a gavage study in only one species and equivocal evidence for genotoxicity.

c. The Proposed Public Health Goal Overstates the Potential Cancer Risk for Bromodichloromethane

While tumors have been reported in gavage studies with BDCM, studies in drinking water have been largely negative. NTP reported tumors of the large intestine and kidney in rats exposed to BDCM dissolved by gavage in corn oil.[44] In its gavage study in mice, NTP reported kidney tumors in males and liver tumors in females. In a subsequent drinking water study, NTP did not observe a significant increase in tumors in either rats or mice.[45] Although additional studies in drinking water studies have suggested liver tumors in rats, these studies have reported mixed results or provided only estimates of drinking water consumption. Notably, George *et al.*

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[42] Health Canada 2006.

[43] Health Canada 2006.

[44] NTP. Toxicology and carcinogenesis studies of bromodichloromethane (CAS No. 75-27-4) in F344/N rats and B6C3F1 mice (gavage studies). NTP TR 321. U.S. Department of Health and Human Services. Research Triangle Park, NC (1987). https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr321.pdf

[45] NTP. Toxicology and carcinogenesis studies of bromodichloromethane (CAS No. 75-27-4) in male F344/N rats and female B6C3F1 mice (Drinking Water Studies). NTP Technical Report 532 (2006). https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr532.pdf

(2002) reported liver tumors in rats at the low and mid dose levels, but not at the highest dose, and no evidence of intestinal tumors at any dose level.[46] In a diet study in rats, Aida *et al.* (1992) reported no significant differences in neoplasms between controls and treatment groups dosed for 24 months.[47]

Based on the 1987 NTP gavage study, BDCM was listed as a Proposition 65 carcinogen in 1990 and as a probable human carcinogen (Group B2) by USEPA in 1993. More recently, but prior to the release of the second NTP study results, IARC concluded there was sufficient evidence in experimental animals for BDCM carcinogenicity and classified it as possibly carcinogenic to humans (Group 2B). The WHO drinking water guideline for BDCM cites the IARC Group 2B classification but concludes that, based on the negative results in the 2006 NTP study, "exceedances of its guideline value [of 60 micrograms/Liter] are not likely to result in an increased risk of cancer." Health Canada considered BDCM to be a probable carcinogen in humans, with sufficient evidence in animals and inadequate evidence in humans.[48]

As with the other THMs, OEHHA bases its draft PHG on its conclusion that BDCM is a genotoxic carcinogen with no exposure threshold. *In vitro* mutagenicity testing has produced mixed results, while the results from *in vivo* testing have been negative. Citing positive results in two studies conducted in closed systems to minimize loss of the test substance, OEHHA concludes that BDCM is genotoxic. Despite the evidence for genotoxicity, the disparate outcomes from gavage and drinking water bioassays strongly suggest that application of the default multistage cancer model is inappropriate. This conclusion is supported by an expert panel convened by Health Canada in 2008 to consider new data for BDCM. The panel noted that "the combined data from the two [NTP] studies do not support a linear dose extrapolation."[49]

The draft PHG makes no attempt to reconcile the starkly different outcomes from the two studies by NTP. While it provides a summary of the results of the 2006 NTP bioassay, it appears to dismiss the findings by noting that "[w]ater consumption by the exposed mice was less than that of the controls throughout the study" because of "poor palatability" of the water containing BDCM. OEHHA fails to note that NTP's conclusion that the 2006 study shows no evidence of carcinogenic activity in rats or mice is based on the actual water consumption levels, not on consumption compared to the control animals. OEHHA also does not mention

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[46] George *et al.* Carcinogenicity of bromodichloromethane administered in drinking water to Male F344/N Rats and B6C3F1 mice. *Intl J Toxicol* 21(3):219-30 (2002). https://doi.org/10.1080/10915810290096351

[47] Aida *et al.* Chronic toxicity of microencapsulated bromodichloromethane administered in the diet to Wistar rats. J Toxicol Sci 17(2):51-68 (1992). (Erratum 17(3):167.) https://doi.org/10.2131/jts.17.51

[48] While the results of the 2006 NTP bioassay were available at the time of its review, Health Canada noted that "this report has not yet been peer reviewed and as such is not final, and cannot be used in the risk assessment at this time." Peer review of the bioassay has since been completed.

[49] Health Canada. Findings and recommendations of the BDCM expert panel meeting, September 22nd and 23rd, 2008. A copy of the report can be obtained by contacting Health Canada at water_eau@hc-sc.gc.ca.

NTP's conclusion that "[d]ifferences in organ dosimetry after gavage administration versus drinking water or dietary administration may be important in evaluating the carcinogenic activity of [BDCM]."[50]
The conflicting results from the two NTP studies, combined with equivocal genotoxicity data, suggest a carcinogenic response for BDCM similar to that observed with chloroform. This is the conclusion of NTP, WHO, and Health Canada. Consistent with the conclusion by the WHO, it is unlikely that a carcinogenic risk exists from BDCM exposures from finished drinking water.

d. Dibromochloromethane Does Not Present a Carcinogenic Risk to Humans

Four studies have evaluated the carcinogenic potential of DBCM in laboratory animals. These include NTP gavage studies in rats and mice, a drinking water study in mice, and a chronic dietary study in rats. The NTP gavage studies reported no evidence of carcinogenicity in rats and an increase in liver tumors in male and female mice.[51] Voronin *et al.* (1987) observed no significant tumor increases in mice treated with DBCM in the drinking water, [52] nor were tumors reported in an unpublished 2-year dietary study in rats. [53]

While DBCM was originally listed as a carcinogen under Proposition 65, it was delisted in 1999 following IARC's conclusion that DBCM was not classifiable as to its carcinogenicity to humans (Group 3). USEPA classified DBCM as a possible human carcinogen (Group C) in 1992, based on limited evidence for carcinogenicity in animals and structural similarity to other THMs. The 2008 WHO drinking water guidelines also consider DBCM as not classifiable based on the IARC classification. Health Canada classifies DBCM as possibly carcinogenic to humans (Group IIID) but concluded that there was insufficient information available to calculate a drinking water guideline for the substance.

Despite the clear consensus among other authoritative bodies, the draft PHG concludes that DBCM is carcinogenic noting that it is --

> structurally similar to the other THM species, which are classified as either probable or possible carcinogens; the liver is a common target for THM-related

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[50] NTP 2006, at 46.

[51] NTP. Toxicology and carcinogenesis studies of chlorodibromomethane (CAS No. 124-48-1) in F344/N rats and B6C3F1 mice (gavage studies). NTP Technical Report 282. NTIS PB 86-166675 (1985). https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr282.pdf

[52] Voronin VM *et al.* An experimental study of the carcinogenicity of dichlorobromomethane and dibromochloromethane released during the water chlorination process. *Gig Sanit* 0(1): 19-21 (1987). Cited in USEPA IRIS Chemical Assessment Summary for dibromochloromethane.

[53] Tobe M *et al*. Studies on the chronic oral toxicity of tribromomethane, dibromochloromethane and bromodichloromethane. Unpublished interagency report to the National Institute of Hygienic Sciences, Tokyo Medical and Dental University, Tokyo, Japan (1982). Cited in OEHHA 2018 Draft PHG.

> tumors; DBCM has not been as thoroughly studied as the other THM species, resulting in much less available data to assess; and the data from female mice in the critical study employed for the dose-response analysis show positive association with liver tumors.

In calculating the proposed PHG, OEHHA concludes that DBCM causes cancer via a genotoxic mechanism citing results in two assays conducted in a closed system. Similar to the other brominated THMs, the genotoxicity data for DBCM are equivocal. The cancer evidence is limited with no evidence in the rat studies and only one study in mice reporting a tumor increase. As with the other THMs, moreover, positive cancer results were only noted in the gavage study where pharmacokinetic difference in dosing likely impacted the results. . Given these circumstances, OEHHA's conclusion is at odds with current cancer hazard and risk assessment guidelines which prescribe a weight-of-evidence-based approach. In this case, a risk assessment that employs "the most current principles, practices, and methods used by public health professionals who are experienced practitioners in the fields of epidemiology, risk assessment, and toxicology" as required by Health and Safety Code §116365, would lead to the conclusion that DBCM is not carcinogenic to humans.[54,55] Any cancer risk that may exist, moreover, is likely to exhibit a threshold. This was the conclusion of USEPA when it established an MCLG of 60 µg/L for DBCM.

OEHHA Should Adopt a Single Public Health Goal Based on the Most Relevant Animal Studies

OEHHA's proposal to establish PHGs for each of the THMs based on genotoxic cancer risk is neither procedurally nor scientifically justified. Establishing individual goals for each THM is not consistent with the state's current policy to apply a single MCL for total THM of 80 µg/L and raises a number of issues related to ensuring consistency with the CalEPA's mandate under the state Safe Drinking Water Act. It further fails to consider the critical public health benefit that the disinfection of drinking water with chlorine provides to the state.

In proposing that all four THMs are genotoxic carcinogens, OEHHA ignores the weight of the scientific evidence and the consensus reached by other authoritative bodies and, in the case of DBCM, its own previous conclusions. The available evidence does not support a conclusion that bromoform and DBCM are carcinogenic. Substantial evidence indicates, moreover, that

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[54] USEPA. Guidelines for Carcinogen Risk Assessment (2005), at page 2-1: "Conclusions are drawn from weight-of-evidence evaluations based on the combined strength and coherence of inferences appropriately drawn from all of the available information." https://www.epa.gov/risk/guidelines-carcinogen-risk-assessment

[55] OEHHA. Guidance criteria for identifying chemicals for listing as "known to the state to cause cancer." Science Advisory Board Carcinogen Identification Committee (March, 2001). Subsection 2(A)(ii)(e) describes criteria for establishing causation as part of a weight of evidence analysis supporting cancer hazard identification, including but not limited to biologic credibility (e)(6), existence of multiple, well-conducted studies in different populations (or species) observing the same relationship (e)(7) and the absence of negative studies of comparable quality. https://oehha.ca.gov/media/downloads/crnr/revcriteria.pdf

chloroform and BDCM are threshold carcinogens unlikely to present a cancer risk at levels found in finished drinking water.

ACC urges OEHHA to develop a single PHG for total THM, based on its evaluation of non-cancer effects, that will provide adequate protection for exposure to any of the individual THMs. In establishing such a PHG, OEHHA can also ensure that exposure levels are below the thresholds for potential risks of cancer presented by chloroform and BDCM. Although OEHHA will still need to consider the health effects information for the individual substances, those data should be considered in the context of a single goal rather than individual goals.

Attachment A
Chloroform ($CHCl_3$): Scientific Causality Confidence Score for Potential Mode(s) of Action

Comparing the Weight of Evidence for a Mutagenic Mode of Action to a Threshold Cytotoxicity Mode of Action for Rodent Liver Tumors Caused by Exposure to Chloroform ($CHCL_3$)

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January 4, 2019

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Acronyms and Abbreviations

AHF	Altered Hepatic Foci
AOP	adverse outcome pathway
ASTDR	Agency for Toxic Substances and Disease Registry
BrdU	Bromodeoxyuridine
$CHCl_3$	Chloroform
HCC	Hepatocellular carcinoma
IPCS	International Programme of Chemical Safety
KE	key event
KER	key event relationship
mkd	mg/kg body weight per day
MOA	mode of action
MS	mass spectrometry
OEHHA	Office of Environmental Health and Hazard Assessment
PHG	Public Health Goals
UDS	unscheduled DNA synthesis
WHO	World Health Organization
WOE	weight of evidence

I. Executive Summary

Background: Mode of Action (MOA) is defined as the biologically plausible series of chemical-specific key events that starts with exposure and proceeds through the interaction of an agent within a cell, the subsequent physiological and tissue or organ changes that occur, which then results in an adverse effect or outcome. One of the critical elements of a chemical carcinogenic risk assessment is the determination of the likely operative MOA. Determining the operative MOA by which a chemical can cause cancer is important for characterizing potential human health hazards and for selecting dose-response extrapolation methods for use in risk assessment at environmental levels of exposure. Thus, this case example focused on evaluating hypothesized MOAs involved in the induction of liver tumors in rodents by chloroform ($CHCl_3$) to identify the likely operative MOA.

This case example used the quantitative MOA weight of evidence (WOE) confidence scoring approach described in Becker *et al*., 2017[1]. This method provides a systematic and explicit approach for the following: 1) evaluating a chemical dataset against each hypothesized MOA, using the evolved Bradford Hill causal considerations (biological plausibility, essentiality, dose-response and temporal concordance, consistency, and analogy); and 2) deriving an overall confidence score for each hypothesized MOA. This enables a side-by-side comparison of numerical WOE confidence scores for each hypothesized MOA, and the determination of which MOA is more likely to be operative, based on those confidence scores. Although this analysis addresses liver carcinogenicity specifically, the same approaches are expected to apply to rodent kidney carcinogenicity induced by $CHCl_3$, as similar data exist for kidney.

Analysis and Results: Using the quantitative MOA WOE confidence scoring approach described in Becker *et al*., 2017 and available data for $CHCl_3$, the WOE for a mutagenic MOA (MOA#1) was compared to the WOE for a threshold cytotoxicity/regenerative proliferation MOA (MOA#2). The relevant dose-response and incidence data were summarized, and WOE confidence scores for both a mutagenic MOA and a threshold cytotoxicity/regenerative proliferation MOA were developed.

This analysis indicates the following:

- It is highly unlikely that a mutagenic MOA is plausible for $CHCl_3$-induced rodent liver tumors. Based on its negative MOA confidence score of -34.2, the WOE clearly does not support a mutagenic MOA for $CHCl_3$-induced liver tumors. The negative score indicates there is strong counter-evidence for several of the early, diagnostic, key events (KEs) for a mutagenic MOA. In other words, the available data, including three

[1] Becker RA *et al*., 2017. Quantitative weight of evidence to assess confidence in potential modes of action. Regul Toxicol Pharmacol. 86: 205-220. OPEN ACCESS: http://www.sciencedirect.com/science/article/pii/S0273230017300387?via%3Dihub

negative *in vivo* transgenic mouse datasets, indicate that it is highly unlikely that rodent liver tumors are induced by $CHCl_3$ *via* a mutagenic MOA.

- The MOA causal confidence scoring results indicate that the likely operative MOA is the cytotoxicity/regenerative cellular proliferation MOA, which exhibits a non-linear/threshold dose-response. Therefore, an increase in cancer risk would only occur at doses that exceed a specific threshold exposure. There are significant mechanistic data to support a non-linear, non-genotoxic MOA for the induction of rodent liver tumors by $CHCl_3$, including *in vivo* hepatocellular histopathology and BrdU labelling data from multiple studies and different labs. When all of these lines of evidence were integrated, a positive MOA confidence score of +93.6 was obtained; this WOE clearly supports a cytotoxicity/regenerative proliferation MOA for $CHCl_3$ induction of rodent liver tumors.

- Application of the Human Relevance Assessment to the cytotoxicity/regenerative proliferation MOA demonstrates that this non-genotoxic, threshold MOA would be considered relevant to humans, thus this MOA should serve as a basis for human health risk assessment for exposures to $CHCl_3$.

Conclusions and Recommendations: Based on the WOE (indicated by comparison of the MOA confidence scores), the likely operative MOA for $CHCl_3$ liver carcinogenicity is cytotoxicity/regenerative proliferation, not mutagenicity. The overall pattern of observations is very consistent with a non-linear, threshold mode of carcinogenic action, as evident by the MOA confidence score of +93.6 for the cytotoxicity/regenerative proliferation MOA compared to the mutagenic MOA confidence score of -34.2. Selection of an appropriate dose-response model to identify quantitative risk becomes straight-forward based on the WOE confidence score which shows that the empirical evidence overwhelmingly indicates rodent liver tumors from $CHCl_3$ exposure arise through a non-genotoxic, threshold MOA.

- Given the threshold nature of the likely operative MOA, a threshold dose-response model would be selected, and cancer risk would only be identified at exposures above the identified threshold for hepatic cytotoxicity, similar to the USEPA assessment (2001).

- It would be inappropriate to use a linear or any non-threshold default approach for extrapolating cancer risks. Instead, the causal weight of the scientific evidence analysis supports use of a threshold, non-linear method for determining potential cancer risks (*i.e.,* an extrapolation method based upon cytotoxicity, for which cancer risk would only be operative at doses that exceed the threshold for induction of hepatic cytotoxicity).

- As stated previously, although only liver data were evaluated in this assessment, a

similar evaluation and conclusion is expected for rodent kidney carcinogenicity, with similar data available.

Use of the quantitative confidence scoring method for determining and communicating the more likely operative MOA based on the weight of scientific evidence provides an important opportunity to incorporate MOA more fully into risk assessment. This method provides a scientifically based WOE approach for selecting the most appropriate extrapolation method for determining potential human health risks. In fact, this approach should prove to be useful for many, if not all, of the chemical contaminants that Office of Environmental Health Hazard Assessment (OEHHA) will review/evaluate to develop Public Health Goals (PHGs) that deal with potential carcinogenic risks, especially where alternative (non-mutagenic) MOAs with supporting mechanistic data are credible.

II. Introduction

Understanding a chemical mode of carcinogenic action ensures that the best available science is used for characterizing and quantifying potential cancer risks at environmental levels of exposures.

To improve transparency and objectivity in mode of action (MOA) analysis, the World Health Organization (WHO) International Programme of Chemical Safety (IPCS) MOA framework has recently been extended using an approach that enables quantitative scoring of the confidence in the weight of the evidence (WOE) of alternative hypothesized MOAs. We have attached the abstract and link to the open access, peer-reviewed publication detailing this quantitative method (Appendix A. Becker et al., 2017). As described in the publication, a systematic and explicit approach is used for evaluating a chemical dataset using key events (KEs) in the context of the evolved Bradford Hill causal considerations, in order to integrate evidence and derive confidence scores for potentially relevant MOAs. This enables a side-by-side comparison using numerical scores of scientific confidence in each hypothesized MOA, including a default mutagenic MOA, to better identify the more likely (i.e., best supported) operative MOA. We are in the process of developing several additional case examples on a variety of chemicals to further illustrate the application of the MOA quantitative confidence scoring method and to support the continued refinement of the method.

The quantitative MOA WOE confidence scoring approach detailed in Becker et al., 2017 is a systematic and explicit approach for evaluating the WOE for hypothesized MOA using the evolved Bradford Hill causal considerations (biological plausibility, essentiality, dose-response concordance, consistency, and analogy), to derive confidence scores for potentially relevant MOAs. Biological plausibility is used in developing the alternative hypothesized MOAs that will be evaluated. The WOE confidence scoring components consist of: 1) a set of defining questions for each of the Bradford Hill considerations coupled with a WOE rating and scoring procedure to guide data evaluation and WOE determinations; 2) a procedure to score the evolved Bradford Hill causal consideration for essentiality typically at the MOA pathway level, based on the highest score achieved by any one of the unique key events (KEs) in the pathway (or, if information is available, essentiality may be evaluated for each KE); 3) a technique for including the supporting evidence of later KEs, even though these are disease-specific and not diagnostic of a MOA for a particular chemical, by affording less evidentiary value to later KEs than the earlier, more MOA-specific KEs; 4) hierarchical weighting of the evolved Bradford Hill causality considerations; and 5) a straightforward arithmetic method to characterize the overall confidence score for each hypothesized MOA.

We have developed this case example using the published chloroform ($CHCl_3$) rodent liver tumor MOA data to document the application of the recently peer-reviewed quantitative MOA WOE confidence scoring approach (Becker et al., 2017). The steps of quantitative MOA WOE confidence scoring are discussed in detail in Becker et al., 2017; they are briefly described here to help contextualize the case example tables presented in Sections III and IV.

- Step 1. Identification of postulated MOAs and KEs/KERs for the adverse outcome (AO) of interest (See Section 2.1 of Becker et al., 2017). (Note: Steps 2 through 5 are conducted for each hypothesized MOA.)

- Step 2. Qualitatively evaluate the evidence in support of, or inconsistent with, the KEs/KERs (See Section 2.2 of Becker et al., 2017), using the evolved Bradford Hill causal considerations.

- Step 3. Quantitatively rate each KE/KER using the evolved Bradford Hill causal considerations (See Section 2.3.1 of Becker et al., 2017). In the qualitative and quantitative rating approach (Steps 2 and 3), the individual or series of KEs are evaluated against the defining question for each evolved Bradford Hill causal consideration, using the WOE rating categories to guide the determinations for scoring. The rating categories include strong (3), moderate (2), weak (1), no evidence (0), weak counter evidence (-1), moderate counter evidence (-2), and strong-counter evidence (-3).

- Step 4. Derive the composite score for each KE/KER by multiplying the quantitative rating score by the weight assigned for each of the evolved Bradford Hill causal considerations and adjust based on the MOA evidentiary value of each KE/KER ($\sum$ (weight × rate × evidentiary value) = KE/KER score) (See Section 2.3.2 of Becker et al., 2017). To derive the composite score, each Bradford Hill causal consideration has been given a numerical weight in accordance with their ranked importance, with a summed maximum of 100% (Section 2.3.1 of Becker et al., 2017). Essentiality of the KEs within the MOA is typically considered collectively since the interdependence of KEs is often illustrated through the impact of prevention or augmentation of an earlier KE on later KEs. Furthermore, all KEs/KERs are not necessarily weighted the same. This is because for a given adverse outcome, often the later KEs leading to the adverse outcome are the same for each of the hypothesized MOAs. These later KEs are often indicative of the disease process, whereas the earlier KEs are more chemical-specific and more influential in determination of MOA, so in this method the later KEs that are common across MOAs are assigned an evidentiary weighting value of 10%.

- Step 5. Integrate the evidence of causality for the MOA by calculating the sum of the scores for all KEs/KERs and then dividing by the total maximum score to derive the "MOA confidence score" (See Section 2.4 of Becker et al., 2017). To calculate the overall WOE confidence score for a hypothesized MOA, KE scores are summed and normalized by dividing by the maximum possible score and then multiplying by 100. This simple normalization procedure allows for comparison of quantitative confidence scores in cases where the number of KEs differs between hypothesized MOAs. Total scores may be negative if, for a hypothesized MOA, there is strong counter evidence for several of the early, most diagnostic KEs.

- Step 6. Compare the quantitative confidence scores for the hypothesized MOAs, and select the MOA for which confidence in the supporting data is highest (See Section 2.4 of Becker et al., 2017).

The intent of this case study is to illustrate the quantitative scoring methodology. It is not intended to be a complete discussion of all available and relevant studies. To that end, we did not conduct an in-depth systematic review of the available literature, but we based this evaluation in large part on data and lines of evidence from already published review articles, and those authors' evaluations of the quality of the empirical evidence. The data and lines of evidence used in developing this case example are from scientifically peer reviewed and journal published articles. For the development of this specific case example, the primary author developed the initial evaluation and additional MOA experts provided peer review of the interpretation and quantification of the MOA scores.

III. Evaluating the WOE for a Mutagenic MOA

Chloroform, $CHCl_3$, is a high volume, chlorinated organic that has been shown to induce liver[2] tumors in mice and rats with chronic exposure (NCI, 1976; Reuber, 1979, Yamamoto et al., 1996, 2002). Metabolic activation of $CHCl_3$ is required to form reactive metabolites capable of binding with DNA to form pro-mutagenic DNA adducts. These mis-repaired or unrepaired DNA adducts result in DNA lesions that would be replicated during cell division, leading to fixation of early induced mutations in cancer critical genes in target tissue (liver). The mutated cells proliferate and result in formation of foci of mutated cells, typically with altered growth characteristics (e.g., loss of contact inhibition, unregulated proliferation). These altered hepatic foci (AHF) of mutated cells would undergo progression, with the accumulation of additional mutations and increasing cell proliferation, to become hepatocellular tumors. The sequence of key events (KE) for such a mutagenic MOA is based on the published description of KEs for a mutagenic MOA for induction of hepatocellular tumors by Aflatoxin B1 (AFB1) (Moore et al., 2018).

The following KEs would be expected for a mutagenic MOA for induction of hepatocellular tumors by $CHCl_3$:

- KE1 Oxidative metabolism of $CHCl_3$ by CYP2E1 to highly reactive metabolites in target tissue, *e.g.*, phosgene
- KE2 Formation of pro-mutagenic DNA adducts in target tissue
- KE3 Insufficient repair or mis-repair of pro-mutagenic DNA adducts
- KE4 Early induced mutation in cancer critical genes in target tissue
- KE5 Cell proliferation, clonal expansion of mutant cells, additional mutations, and progression
- AO Development of liver tumors

Data to provide evidence for these KEs would include information on $CHCl_3$ metabolic activation; the DNA adduct profiles, demonstrating recognized pro-mutagenic adducts; data on DNA repair, preferably specific for the pro-mutagenic adducts; evidence of early induction of mutations in cancer critical genes (in liver); hepatic cell proliferation data, including qualitative and quantitative data on liver foci formation and progression; and hepatocellular tumor data, preferably all of this in both rats and mice.

The initial KE and final KE/AO are similar or identical between MOA #1 and MOA #2.

[2] $CHCl_3$ also induces kidney tumors, with similar supporting data. This analysis focuses on liver tumors, with the expectation that the key events and supporting data for the same MOA are similar in kidney.

These proposed key events for a mutagenic MOA for $CHCl_3$ are shown schematically in Figure 1:

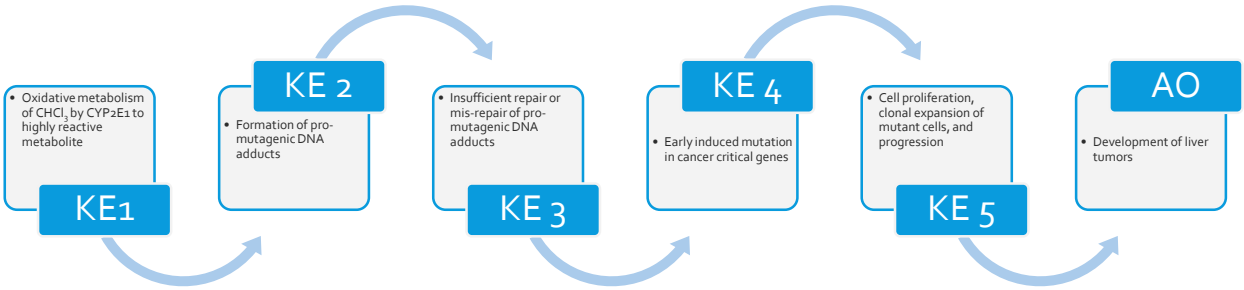

Figure 1. Postulated Mutagenic MOA for $CHCl_3$

$CHCl_3$ also induces kidney tumors, with similar supporting data. This analysis focuses on liver tumors, with the expectation that the key events and supporting data for the same MOA are similar in kidney.

A. Qualitative Evaluation of the WOE for $CHCl_3$ Acting via a Mutagenic MOA

Table 1. Qualitatively Evaluate the Comparative WOE for $CHCl_3$ Acting via The Mutagenic MOA (Borgert et al., 2015; Boobis et al., 2009; Meek et al., 2003; others) – (Step 2)

Key Event	Supporting Data	Potentially Inconsistent	References
KE1: Metabolic Activation of $CHCl_3$ to Reactive Metabolite	Extensive supporting data demonstrate saturable formation (*via* CYP2E1) of phosgene in rodent liver from $CHCl_3$. Key counterfactual evidence demonstrates the lack of liver toxicity with CYP2E1 knock-out (KO) mice, and data demonstrating that pre-treatment with a CYP2E1 inhibitor also blocked liver toxicity in wild type $CHCl_3$-treated mice, further supporting a key role for CYP2E1 (Strong evidence). Quantitative kinetic data are available, including data		KO mice; CYP2E1 inhibition: Constan *et al*., 1999 CYP 2E1 induction: Brady *et al.,* 1989; Kluwe, *et al.,* 1978 Threshold: Ammann *et al.,* 1998; Kluwe and Hook, 1981 Variability: Edwards *et al.,* 1998; Lipscomb *et al.,* 2004;Gemma *et al*., 2003 Conserved gene: Borgert *et al*., 2015; Neis *et al*., 2010;

Key Event	Supporting Data	Potentially Inconsistent	References
	supporting a GSH-dependent threshold for toxicity from phosgene (or other reactive metabolites) generated from $CHCl_3$ (Figure 2). Interindividual variability in human CYP2E1 levels (10- to 12-fold) can affect shape of dose-response curve for hepatotoxicity from $CHCl_3$-generated phosgene; it is likely that variability in CYP2E1 expression has higher impact than variability in GSH levels. Because CYP2E1 is highly conserved between rodents and humans, with a single isoform sourced from a single gene in humans and rodents, including in liver, lung and skin, a common MOA is expected for all exposure routes, resulting from common metabolic products		Baron *et al.,* 2008; Du *et al*., 2004; Ingelman-Sundberg, 2004 PK parameters; PBPK models: Corley *et al*., 1990; Reitz et al., 1980, 1990; Pohl *et al.* 1977; Smith *et al*. 1984
KE2: Formation of pro-mutagenic DNA adduct in target tissue	No data available to support formation of $CHCl_3$-derived pro-mutagenic DNA adducts. There is one report of low levels of binding of $CHCl_3$-derived radiolabel to naked DNA *in vitro*, in the absence of GSH; the bound moiety was not identified. A different, *in vivo*, study reported binding of DNA by $CHCl_3$ but found no differences across tissues, raising questions about validity. Other similar studies did not find DNA binding of radiolabelled $CHCl_3$, *in vivo* or *in vitro*. Although not evidence of pro-mutagenic DNA adducts, there are some data that indicate binding of $CHCl_3$-derived	Despite detection of nuclear protein binding (histone H2B), no DNA binding was detected *in vivo* or *in vitro*, except one report *in vitro* (naked DNA) high dose only, and one report of low level binding equivalent in all tissues evaluated; therefore, formation of pro-mutagenic DNA adducts from $CHCl_3$ is unlikely. UDS data, *in vivo* & *in vitro*, indicate no induction of unscheduled DNA synthesis, which indicates no induction	DNA binding: DiRenzo *et al*., 1982; Diaz Gomez and Castro, 1980a; Colacci *et al*., 1991 Histone adducts: Fabrizi *et al*.., 2003; Diaz Gomez and Castro, 1980b Phospolipid adducts: Vittozzi *et al*., 2000 UDS data: Mirsalis *et al*., 1989; Larson *et al*., 1994b DNA strand breaks: High dose positive: Beddowes *et al*., 2003; Zhang *et al*., 2012

Key Event	Supporting Data	Potentially Inconsistent	References
	radioactivity to nucleophilic macromolecules other than DNA, including nuclear proteins such as histones and phospholipids. These few data are not interpreted as evidence of DNA binding or DNA adduct formation. Although not evidence of pro-mutagenic DNA adducts, there are both positive and negative data on induction of DNA strand breaks by $CHCl_3$ exposure; DNA strand breaks can occur following formation of DNA adducts. In both primary cultures and cell lines, limited data support induction of strand breaks—although only at high doses (8-10 mM), with no response at lower doses, while other data do not show any induction of DNA strand breaks by $CHCl_3$ exposure, some with similar high doses (10 mM). Also not evidence of pro-mutagenic DNA adducts, induction of sister chromatid exchanges (SCEs)—historically classed as genotoxicity-- is no longer considered as evidence of genotoxic effect	of DNA repair & supports no significant DNA damage, *e.g.*, no pro-mutagenic adducts. Also, induction of S-phase DNA synthesis by $CHCl_3$ supports cell proliferation /regeneration MOA. DNA strand breaks generally not induced *in vitro* by $CHCl_3$, for some not even at high doses. NTP's definitive studies of SCEs in CHO cells showed CHCL3 was classified as "questionable" in one study without metabolic activation. This study was then repeated by NTP, and, based upon the results, NTP concluded CHCl3 did not induce SCEs either with or without metabolic activation.	No evidence: Geter *et al*., 2004; Ribeiro *et al*., 2006, 2007; Interpretation of SCEs: Wilson and Thompson, 2007; Bonassi *et al*., 2004
KE3: Insufficient /Mis-repair of pro-mutagenic DNA adducts	There are no data to support occurrence of insufficient / mis-repair of pro-mutagenic DNA adducts following $CHCl_3$ exposure.	UDS data, *in vivo* & *in vitro*, indicate <u>no</u> induction of unscheduled DNA synthesis, which indicates no induction of DNA repair and supports no significant DNA damage, *e.g.*, no pro-mutagenic adducts. Also, induction of S-	UDS data: Mirsalis *et al*., 1989; Larson *et al*., 1994; Reitz *et al*., 1980

Key Event	Supporting Data	Potentially Inconsistent	References
		phase DNA synthesis by $CHCl_3$ supports cell proliferation MOA.	
KE4: Early induction of mutation in cancer critical genes in target tissue	There are no data to support early induction of mutation in cancer critical genes in target tissue. There is a wealth of surrogate mutation data, mostly from *in vitro* assays. The vast majority of those studies are negative or inconclusive, with no increased mutation from $CHCl_3$ exposure. The very few studies that were interpreted to show positive mutation effects from $CHCl_3$ exposure typically present technical flaws, and thus are deficient according to OECD guidance; this affects their validity. For example, positive results for bacteria were obtained but only at 19,200 and 25,600 ppm—exposure levels that are not compatible with life, thus not relevant for humans, and would not be considered valid. For example, one bacterial strain was reported as positive when tested with S9 mix + GSH (not without S9 or with S9 without GSH); however, evaluation of the results does not demonstrate a dose-responsive increase, and only scattered doses showed a 2x increase in mutant colonies over control (not reproducible across experiments). The test concentrations that occasionally showed a 2x increase were 5,000 to 50,000 ppm, not compatible with life.	There are many negative *in vitro* mutation datasets for $CHCl_3$ in bacteria, yeast, and mammalian cells, indicating $CHCl_3$ is not mutagenic *in vitro*. There are three *in vivo* studies on $CHCl_3$ with transgenic mice: one mutation assay and two cancer assays. All are negative: $CHCl_3$ did not induce mutations in *lacI* in liver of transgenic mice after exposures up to 6 months to 90 ppm, a tumorigenic level (DMN did induce mutations). $CHCl_3$ did not induce any tumors in $p53^{+/-}$ transgenic mice or in *ras*H2-Tg mice after 26 weeks of oral gavage dosing. **Lack of induction of mutations or tumors in liver of transgenic mice following repeated exposures to $CHCl_3$ in three separate studies provide strong evidence that $CHCl_3$ is not mutagenic and thus not consistent with a mutagenic MOA.**	Bacterial positive: Pegram *et al*., 1997; Araki *et al.*, 2004; San Agustin and Lim-Sylianco, 1978; Mortelmans & Zeiger, 2000 Yeast positive: Callen *et al*., 1978 Mutagenicity *in vitro* negative results (bacteria & mammalian cells): see OEHHA draft PHG, 2018 for listing of published studies Transgenic studies: Gollapudi *et al*., 1999; Butterworth *et al.,* 1998; Sehata *et al*., 2002 Cytogenetics: Gocke *et al.,* 1981; San Agustin & Lim-Sylianco, 1978; Shelby & Witt, 1995

Key Event	Supporting Data	Potentially Inconsistent	References
	For example, one bacterial strain was positive in an *in vivo/in vitro* host-mediated assay, but the report is missing critical information such as $CHCl_3$ exposure concentration, therefore would be considered as invalid results according to OECD. For example, yeast data for a particular strain (D7) were reported as positive but either did not meet criteria for a valid study (>90% cytotoxicity) or for a positive (2x control), except for a single high dose (41 mM) for some of the assays. Some data on cytogenetic effects (clastogenicity, micronucleus, chromosomal aberrations) are available, but are mostly negative or inconclusive and thus not interpretable/reliable due to technical flaws or lack of details provided. A few positive results were identified but mostly only at very high doses or only as positive trends. **Overall there are not consistent, reliable data to demonstrate a mutagenic effect of $CHCl_3$.**		
KE5: Cell proliferation, clonal expansion, additional mutations (progression)	Increased cell proliferation was shown in liver of $CHCl_3$-treated rats and mice, measured as increased labelling index and/or BrdU incorporation. Quantitative dose-response data on $CHCl_3$-induced cell proliferation demonstrates that sustained, high level $CHCl_3$ is required to induce sufficient	Although providing data on induced liver cell proliferation, the effect in transgenic mouse only followed induction of significant cytotoxicity /degeneration/necrosis from $CHCl_3$ treatment. Stop-exposure studies	Quantitative cell proliferation data: Constan *et al*., 1999; Boobis, 2009, 2010; Larson *et al*., 1994, 1995a,b; Templin *et al.,* 1996, 1998 Transgenic studies with cell proliferation: Gollapudi *et al*., 1999;

Key Event	Supporting Data	Potentially Inconsistent	References
	toxicity to cause cell proliferation. Increased presence of hepatic foci of altered cells is a characteristic of this stage of progression. Quantitative dose-response data are available, based on an initiation-promotion protocol, demonstrating the formation and increased numbers and volume of such hepatic foci following initiation by DEN, identified by altered staining patterns. There is no evidence to support alternative paths to induction of cell proliferation, such as: • $CHCl_3$ directly induces cellular hyperplasia, or • $CHCl_3$ inhibits apoptosis, or • $CHCl_3$ activates nuclear receptors involved in cell proliferation, even at tumorigenic doses.	(6 wks exposed; 7 wks held) did not show any increased hepatic cell proliferation.	Butterworth *et al.,* 1998 Hepatic foci of altered cells: Deml & Osterle, 1985, 1987*;* Osterle & Demrl, 1985; Pereira *et al*., 1982; Sehata et al., 2002 KO mice; CYP2E1 inhibition: Constan *et al*., 1999 Stop-exposure: Larson *et al*., 1996; Templin *et al*., 1996
AO: Liver tumors	Following gavage treatment with $CHCl_3$, both mice and rats showed increases in liver adenomas + carcinomas. Inhalation study showed increased trend only for mouse liver carcinomas + adenomas (rat negative). Route of administration clearly influenced tumor formation as mice treated with similar $CHCl_3$ doses *via* drinking water did not show increases in liver tumors. Additional bioassays exist with generally similar results, typically not showing increases in liver tumors with non-gavage routes (inhalation) or at lower gavage doses (in toothpaste).		Gavage $CHCl_3$: NCI, 1976; Reuber, 1979; Inhalation: Yamamoto *et al.,* 1996, 2002 dH2O: Jorgenson *et al.,* 1985 dH2O single dose level: Tumasonis *et al*., 1985, 1987

Key Event	Supporting Data	Potentially Inconsistent	References
	One drinking water single dose level study reported increased hepatic neoplastic nodules as 'adenofibrosis' in Wistar rats.		

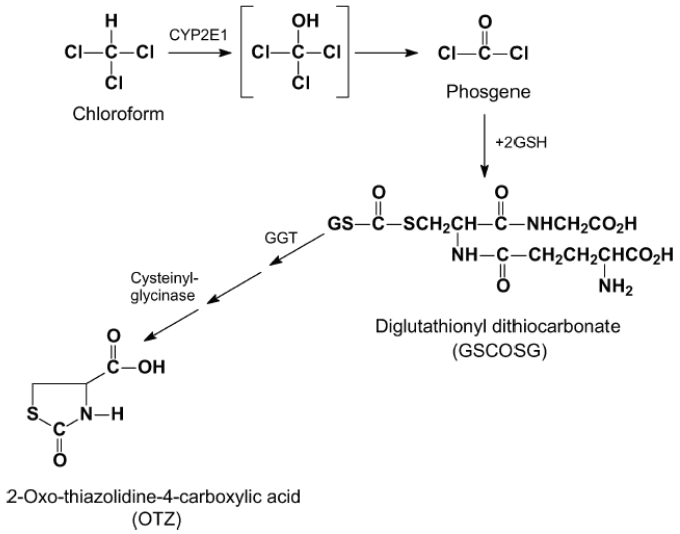

Figure 6 Metabolic activation of chloroform to phosgene and subsequent detoxication by glutathione. Chloroform is oxidized by CYP2E1 to an unstable hydroxylated intermediate, which spontaneously rearranges to the chemically reactive phosgene, the putative toxic species. Phosgene can react spontaneously with glutathione (GSH), the product of which is non-toxic. The glutathione conjugate is further metabolised via γ-glutamyltranspeptidase and cysteinylglycinase to yield 2-oxo-thiazolidine-4-carboxylic acid (OTZ), an unreactive product.

Figure 2. Taken from Boobis et al., 2009

Table 2. Incidence of Liver Tumors and Nodules from Key Studies on $CHCl_3$. (Based on NCI (1976) data re-examined by Reuber (1979): gavage dosing; 5 (rat) or 6 (mouse) d/wk, and on Jorgenson et al. (1985) female mouse drinking water study and Yamamoto et al. (2002) mouse and rat inhalation study.)

Tumor outcome	**Osborne-Mendel Rats***							
	Male				**Female**			
	Colony Control	Vehicle Control	90 mkd	180 mkd	Colony Control	Vehicle Control	100 mkd	200 mkd
Hyperplastic nodules	0/20	1/19	5/50	8/49	1/20^	2/20^	7/39^	12/39^
Hepatocellular carcinoma	0/20	0/19	0/50	2/49	0/20	0/20	2/39	2/39
Adenoma/Carcinoma	0/20[#]	1/19[#]	5/50[#]	10/49[#]	1/20[&]	2/20[&]	9/39[&]	14/39[^^,&]
	B6C3F1 Mice*							
	Male				**Female**			
	Colony Control	Vehicle Control	138 mkd	277 mkd	Colony Control	Vehicle Control	238 mkd	477 mkd
Hyperplastic nodules	1/17	2/17	11/46	0/44	0/20	0/19	1/45	12/40
Hepatocellular carcinoma[a]	1/17	0/17	20/46	44/44	0/20	0/19	40/45	240
Hyperplastic nodules + Carcinoma	3/17[+]	2/17[+]	31/46[@,+]	44/44[@,+]	0/20[k]	0/19[k]	41/45[t,k]	40/40[t,k]
	Female B6C3F1 Mice* **							
	Control	Matched dH_20 Control	34 mkd	65 mkd	130 mkd	263 mkd		
Hepatocellular Adenoma/Carcinoma	21/415	0/47	15/410	9/142	0/47	1/44		
	B6C3F1 Mice** **							
	Male				**Female**			
	Control	5 ppm	30 ppm	90 ppm	Control	5 ppm	30 ppm	90 ppm
Hepatocellular Carcinoma	10/50	0/50	7/50	10/48	1/50	1/49	0/50	3/48
Hepatocellular Carcinoma/Adenoma	14/50↑	7/50↑	12/50↑	17/48↑	2/50↑↑	2/49↑↑	4/50↑↑	6/48↑↑

* Rats: Based on NCI (1976) data as re-examined by Reuber (1979): gavage dosing; 5 d/wk for 78 wk; sacrificed at 111 wk.

** Mice: Based on NCI (1976) data as re-examined by Reuber (1979): gavage dosing; 6 d/wk; 80 wk; sacrificed at 96 wk.

[a] Small + Large carcinomas combined from Reuber (1979).

*** Mice: Based on Jorgenson et al. (1985) drinking water dosing with dH20 matched controls due to high mortality in 2 high dose groups (~25%) during first week from dehydration (refusal to drink treated water); remainder of treatment: 78-90% of control water consumption: 104 wk total.

**** Mice: Inhalation exposures: 6 h/d; 5d/wk; 104 wks; similarly treated rats did not have treatment-related neoplastic liver lesions (hepatocellular adenoma males: 0/50, 0/50, 0/50, 0/50; females: 1/50, 0/50, 2/50, 1/49).

Statistical info: Reuber (1979) Male rats: [#]Trend: p = 0.04502; Female rats ^ Trend: p = 0.03617; ^^ p = 0.03105; [&]Trend: p = 0.01886; Male mice: [@]p = <0.00001; [+]Trend: p = 5.794 x 10-17; Female mice: [t]p = <0.00001; [k]Trend: p = 2.168 x 10-18 ;Jorgensen et al. (1985): no statistically significant differences across Female mouse liver tumors; Yamamoto et al. (2002): Male mice: ↑ = Trend (Peto's): p<0.05; Female mice: ↑↑= Trend (Peto's): p<0.01.

Empirical Support Dose-Response and Temporal Concordance:

KE #1: Good supporting data for the necessity of the metabolism step based on in vitro and in vivo data; extensive information on kinetics of CYP2E1 activity demonstrates $CHCl_3$ is activated to form phosgene, and that this reaction is rapid and is saturable, with a maximum level reached rapidly; phosgene is very reactive thus it is rapidly removed either through further metabolism or through binding with nearby cellular macromolecules, likely highly compartmentalized to the CYP2E1-containing smooth endoplasmic reticulum, further supporting data generally showing no binding of activated $CHCl_3$ metabolites with DNA. There do not seem to be data quantifying a dose-response for formation of phosgene over a range of exposures/doses; however, induction of CYP2E1 in vivo results in an increased metabolism of $CHCl_3$ by liver microsomes from induced rats.

There is strong support for the essentiality of this step provided by the CYP2E1 knock-out mice study demonstrating that $CHCl_3$ treatment of these KO mice does not result in liver toxicity or other subsequent key events. Similar data with chemical inhibitors of CYP2E1 provide additional support for essentiality of this KE. This step initiates the sequence of KEs, thus fits temporally with an early KE.

KE #2: There are no data demonstrating formation of pro-mutagenic DNA adducts from $CHCl_3$ exposure. Reliable supporting data for DNA binding are very sparse, although binding to other macromolecules does occur. Other exposure biomarkers evaluated (e.g., sister chromatid exchanges) are not definitive evidence of DNA damage (no information is lost) and certainly not of pro-mutagenic DNA adduct formation. DNA strand break data are mixed, mostly negative, and again do not provide evidence of pro-mutagenic DNA adduct formation.

This KE would need to occur very early in the sequence of KEs; with no supporting data, temporality cannot really be evaluated.

KE #3: There are no data to demonstrate DNA repair of pro-mutagenic DNA adducts. There are a few datasets, both in vitro & in vivo, measuring UDS following $CHCl_3$ exposure that mostly demonstrate no increase in unscheduled DNA synthesis, a hallmark of DNA repair. Temporality cannot be evaluated due to lack of data.

KE #4: At best, contradictory results exist for mutagenic effects of $CHCl_3$, with most *in vitro* & *in vivo* mutagenicity data negative for mutation induction. The few datasets that have been published as demonstrating mutagenic effects from $CHCl_3$ exposure mostly have significant flaws (*e.g.*, inadequate numbers, no dose-response, not statistically or biologically significant positives) that affect reliability and validity. Perhaps the strongest counterevidence are the three negative transgenic studies; one that did not show any increase in *lacI* gene induced mutations

in any tissue, including liver, and two transgenic mouse studies with no increase in liver tumors after 26 wks exposure to $CHCl_3$; typically, increased tumors would be evident in the *p53*$^{-/-}$ and *ras*H2-Tg models following such an exposure to a mutagenic carcinogen. Temporality cannot be evaluated due to no data.

KE #5: Clear evidence for induction of cell proliferation in liver is provided by several publications from different laboratories. Both gavage and inhalation repeated (13 or 26 wk) exposures to $CHCl_3$ have induced dose-responsive increased labelling indices (LI) in mouse and rat liver, using BrdU labelling to quantitate cell proliferation. Two datasets with transgenic mice included BrdU LI in liver and clearly demonstrated dose-responsive increases in hepatocyte proliferation with $CHCl_3$ exposure (a 26 wk inhalation and a 13 wk gavage).

There is strong support for the essentiality of this step provided by the CYP2E1 knock-out mice study demonstrating that $CHCl_3$ treatment of the KO mice does not result in any increased LI in liver, while the wild-type CYP2E1 mice demonstrated extensive hepatocyte proliferation. Pre-treatment with a chemical inhibitor of CYP2E1 also blocked the (regenerative) cell proliferation in liver of $CHCl_3$-treated mice.

The hepatic cell proliferation is a relatively early event, as some datasets show it has stopped by 13 wks of treatment, while other datasets show its occurrence following 13 or 26 wks of exposure.

The temporal concordance of KE5 is maintained within the sequence of key events, as it occurs later than the expected timeframe for mutation induction

AO: Strong evidence for induction of hepatocellular carcinoma/adenoma by high dose exposure to $CHCl_3$ in mice and rats, with several bioassays (gavage or inhalation) demonstrating increased incidence of these tumors. In addition, there are exposure levels that do not result in hepatic tumors, providing dose-response data. Tumors are identified only following ~50+ weeks of $CHCl_3$ treatment, supporting the temporal concordance of this AO.

Table 3. Dose-Response and Temporal Concordance Table: Mouse

Dose Concordance ↓

Temporal Concordance →						
Temporal	**24 hrs.**	**12 - 48 hrs.**	**48-72 hrs**	**2 d - 2 wks**	**13+ wks**	**2 yrs (Cancer studies)**
Dose/Conc.	KE1: Metabolism to Reactive Metabolites	KE2: Formation of Pro-mutagenic DNA adducts	KE3: Insufficient/Mis-Repair of Pro-mutagenic Adducts	KE4: Early Induced Mutation in Cancer Genes	KE5: Cell Proliferation Progression	AO: Hepato-cellular Adenoma/ Carcinoma
Inhalation (Yamamoto *et al*., 1996, 2002;) Inhalation (Yamamoto et al., 1996, 2002; Templin et al., 1996b, 1998; Larson et al., 1996; Butterworth et al., 1998)						
0 ppm	*[--]*	*[--]*	*[--]*	*[--]*	--	-/+
5 ppm	*[+]*	*[--]*	*[--]*	*[--]*	--	-/+
30 ppm	[++]	*[--]*	*[--]*	*[--]*	+	+
90 ppm	[+++]	*[--]*	*[--]*	*[--]*	+++ *(9- 17x↑)*	++
30 ppm/stop*	*[+++]*	*[--]*	*[--]*	*[--]*	--	*ND*
90 ppm/stop*	*[+++]*	*[--]*	*[--]*	*[--]*	--	*ND*
0 ppm[&]	*[--]*	*[--]*	*[--]*	--	--	ND
90 ppm[&]	*[++]*	*[--]*	*[--]*	--	++	ND
Gavage (NCI, 1976/Reuber, 1979; Larson *et al.,* 1994a,b; Gollapudi *et al.,* 1999; Sehata *et al*., 2002)						
0 mkd	*[--]*	*[--]*	*[--]*	*[--]*	--	-/+
34 mkd**	*[+]*	*[--]*	*[--]*	*[--]*	+	ND
90 mkd**	*[++]*	*[--]*	*[--]*	*[--]*	+	ND
138/238 mkd**	*[++]*	*[--]*	*[--]*	*[--]*	+++ *(30x↑)*	++
277/477 mkd**	*[+++]*	*[--]*	*[--]*	*[--]*	+++	+++
0 mkd^	*[--]*	*[--]*	*[--]*	*[--]*	--	--
24/28 mkd^	*[+]*	*[--]*	*[--]*	*[--]*	+	--
90 mkd^	*[++]*	*[--]*	*[--]*	*[--]*	++	--
140/240 mkd^	*[+++]*	*[--]*	*[--]*	*[--]*	+++	--
0 mkd[%]	*[--]*	*[--]*	*[--]*	*[--]*	--	--
240 mkd WT[%]	*[+]*	*[--]*	*[--]*	*[--]*	+	+
240 mkd *ras*H2-Tg[%]	*[--]*	*[--]*	*[--]*	*[--]*	--	--

+++ : strong response; ++ : moderate response; + : weak response; -/+: background; **--** : no response; [assumed response]; ND : no data; *italicized grey* = data from subchronic or subacute (not chronic) studies; stop exposure: 6 wks exposure, then 7 wks no exposure.

[^] Yanamoto et al., 1996, 2002 source of inhalation tumor data; gavage tumor data from NCI,1976; Reuber, 1979.

* Templin et al., 1996b, 1998 and Larson et al., 1996 (inhalation exposure, with stop-exposure groups);

** Larson et al., 1994a,b (gavage mouse B6C3F1).

[&] Butterworth et al., 1998 lacI transgenic inhalation tumor study: up to 6 mon (180 d) exposure.

^ Gollapudi et al., 1999 *p53-/-* transgenic gavage tumor study: 13 or 26 wks dosing.

[%] Sehata et al., 2002: *ras*H2-Tg transgenic gavage tumor study: 26 wk dosing.

Table 4. Dose-Response and Temporal Concordance Table: Rat

Dose Concordance ↓

Temporal Concordance →

Temporal	**24 hrs.**	**12 - 48 hrs.**	**48-72 hrs**	**2 d - 2 wks**	**13+ wks**	**2 yrs (Cancer studies)**
Dose/Conc.	**KE1: Metabolism to Reactive Metabolites**	**KE2: Formation of Pro-mutagenic DNA adducts**	**KE3: Insufficient/Mis-Repair of Pro-mutagenic Adducts**	**KE4: Early Induced Mutation in Cancer Genes**	**KE5: Cell Proliferation Progression**	**AO: Hepato-cellular Adenoma/ Carcinoma^**
Inhalation (Yamamoto *et al.,* 1996, 2002; Templin *et al.,* 1996c)						
(2*) 10 ppm	*[--]*	*[--]*	*[--]*	*[--]*	--	-/+
30 ppm	*[+]*	*[--]*	*[--]*	*[--]*	--	-/+
90 ppm	[++]	*[--]*	*[--]*	*[--]*	--	-/+
300 ppm *	[+++]	*[--]*	*[--]*	*[--]*	++	-/+
90 ppm/stop*	*[+++]*	*[--]*	*[--]*	*[--]*	+++ *(25x ↑)*	*ND*
300 ppm/stop*	*[+++]*	*[--]*	*[--]*	*[--]*	--	*ND*
Gavage (Tumor data: NCI, 1976/Reuber, 1979; Templin *et al.,* 1996a,c; Larson *et al*., 1995a,b)						
0 mkd	*[--]*	*[--]*	*[--]*	*[--]*	--	-/+
10 mkd**	*[+]*	*[--]*	*[--]*	*[--]*	--	ND
34 mkd**	*[++]*	*[--]*	*[--]*	*[--]*	--	ND
90/100 mkd	*[+++]*	*[--]*	*[--]*	*[--]*	--	++
180/200 mkd	*[+++]*	*[--]*	*[--]*	*[--]*	--	+++
477 mkd**	*[+++]*	*[--]*	*[--]*	*[--]*	++ *(5x ↑)*	ND

+++ : strong response; ++ : moderate response; + : weak response; -/+: background; **--** : no response; [assumed response]; ND : no data; *italicized grey* = data from subchronic or subacute (not chronic) studies; stop exposure was 6 wks exposure followed by 7 wks no exposure.

[A] Yanamoto *et al*., 1996, 2002 source of inhalation tumor data; gavage tumor data from NCI,1976; Reuber, 1979.

* Templin *et al*., 1996c (inhalation exposure); **Larson *et al*., 1995a,b (gavage); Templin *et al*., 1996a (single gavage dosing for intermediate KEs).

B. Evolving Bradford Hill Causal Considerations: Qualitative and Quantitative Data Evaluation

Table 5. Qualitative and Quantitative Rating Categories [See Becker et al., 2017 for details]

Qualitative	Quantitative	Category Description
Strong	3	Multiple studies and/or extensive data provide convincing evidence that the substance causes the KE.
Moderate	2	Some evidence (direct or indirect) indicating the substance causes the KE, but scientific understanding is not yet completely established. There may be some studies that are equivocal.
Weak	1	Very limited evidence (direct or indirect) that the substance causes the KE along this pathway. Scientific understanding of the KE is limited.
No Evidence	0	No data available to support or negate causation of this KE by the substance.
Weak Counter	−1	There is very limited contradictory evidence (direct or indirect) that the substance does not cause this KE.
Moderate Counter	−2	Some evidence (direct or indirect) indicating that the KE is not caused by the substance, but scientific understanding is not completely established. There may be some studies that are equivocal.
Strong Counter	−3	Multiple studies and/or extensive data provide convincing evidence that the substance does not cause this KE.

Table 6. Evolved Bradford Hill Causal Considerations, Defining Questions and Body of Evidence (adapted from Meek et al., 2014 a,b)

Bradford Hill Causal Considerations	Defining Questions	Supporting Evidence	Potentially Inconsistent Evidence
Essentiality	Data that demonstrate a KE is essential, *e.g.*, when a KE is blocked or reduced, downstream KEs and/or the AO do not occur or are not present to the same degree.	Data from CYP2E1-KO mouse, and pre-treatment with CYP2E1 inhibitor, both provide strong supporting evidence for KE1 (CYP2E1 metabolic activation) essentiality, as downstream KEs, including the AO, are blocked or reduced when CYP2E1 is missing.	Three transgenic datasets provide strong counter-evidence, with no data supporting *in vivo* mutation induction (KE4), or increased cell proliferation (KE5) or tumor development (KE6/AO). Stop-exposure data provide strong counter-evidence for essentiality of post-KE1 KEs, as their manifestation through KE4 (early induced mutation) is expected with exposures of 6 wks, yet no AHF/cell proliferation are induced (no liver toxicity) in mice that are exposed to carcinogenic levels of $CHCl_3$ for 6 wks and then held for 7 wks.
Empirical Support – Dose and Incidence Concordance	Are dose-response data available demonstrating monotonic increases in response within KEs?	KE1 occurs first and, based on PK data, it does demonstrate a monotonic dose-response until a plateau is reached due to saturation kinetics. This analysis is not possible for KEs 2 & 3 due to lack of empirical data to support them. KE4 surrogate mutation data generally do not show the typical monotonic dose-responses and mostly show no increases in the induced mutations. KE5 data do show dose-response.	The wealth of induced mutation data is mostly all negative. The three negative transgenic mutation datasets are strong counter evidence for empirical support of KE4.
Empirical Support – Temporal Concordance	Do the KEs demonstrate a temporal relationship across KEs over time to the AO? Do the KEs occur in order?	KE1 occurs first. Analysis of KEs 2-4 is not possible as no adequate empirical data exist. KE5 does occur in temporal order, later in the process, followed by the AO.	Lack of empirical support for KEs 2-4 means that temporal analysis is not possible.
Consistency	Do the data present a consistent pattern, e.g., across organ systems and across species?	KE1 is consistent across species and organs with metabolic capability, further supported by CYP2E1 KO mouse data. The middle KEs (2-4) have no data to support consistency in occurrence. KE5 occurs in both rat and mouse liver, thus demonstrating consistency.	Limited liver tumor response from some datasets.
Analogy	Is this MOA expected given broader chemical-specific knowledge?	Although most cancer QSAR and predictive models such as OncoLogic have a rule that defines haloalkanes with an increased probability of causing liver cancer, without specifying the MOA, typically these chlorinated solvents are not classified as mutagens.	

Table 7. Qualitative Rating of the Key Events for Bradford Hill Causal Considerations (Step 3)

Bradford Hill Causal Considerations	Key Event #1	Key Event #2	Key Event #3	Key Event #4	Key Event #5	AO
	Metabolic Activation	Pro-mutagenic DNA adduct	Insufficient / Mis-repair of DNA adduct	Early Induced Mutation in Cancer Gene	Cell Proliferation / Progression	Hepato-cellular Carcinoma/ Adenoma
Essentiality	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong counter (Quantitative rating = -3)	Qualitative rating: Moderate counter (Quantitative rating = -2)	Qualitative rating: Strong counter (Quantitative rating = -3)	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)
Empirical Support – Dose and Incidence Concordance	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong counter (Quantitative rating = -3	Qualitative rating: Moderate counter (Quantitative rating = -2)	Qualitative rating: Strong counter (Quantitative rating = -3	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)
Empirical Support – Temporal Concordance	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong counter (Quantitative rating = -3	Qualitative rating: Moderate counter (Quantitative rating = -2)	Qualitative rating: Strong counter (Quantitative rating = -3	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)
Consistency	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong counter (Quantitative rating = -3	Qualitative rating: Moderate counter (Quantitative rating = -2)	Qualitative rating: Strong counter (Quantitative rating = -3	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)
Analogy	Qualitative rating: Moderate (Quantitative rating = +2)	Qualitative rating: Moderate counter (Quantitative rating = -2)	Qualitative rating: Moderate counter (Quantitative rating = -2)	Qualitative rating: Moderate counter (Quantitative rating = -2)	Qualitative rating: Moderate (Quantitative rating = +2)	Qualitative rating: Moderate (Quantitative rating = +2)

C. Composite Quantification of the WOE for a Mutagenic MOA

Table 8. Quantification of the WOE for the [postulated] MOA (Step 4 and 5)

Bradford Hill Causal Considerations	Key Event #1	Key Event #2	Key Event #3	Key Event #4	Key Event #5	AO
	Metabolic Activation	Pro-mutagenic DNA adduct	Insufficient / Mis-repair of DNA adduct	Early Induced Mutation in Cancer Gene	Cell Proliferation / Progression	Hepatocellu-lar Carcinoma / Adenoma
Essentiality (40%)	(3) x (0.4) x 1 = 1.2	(-3) x (0.4) x 1 = -1.2	(-2) x (0.4) x 1 = -0.8	(-3) x (0.4) x 1 = -1.2	(3) x (0.4) x 1 = 1.2	(3) x (0.4) x 1 = 1.2
Empirical Support – (20%) Dose and Incidence Concordance	(3) x (0.2) x 1 = 0.6	(-3) x (0.2) x 1 = -0.6	(-2) x (0.2) x 1 = -0.4	(-3) x (0.2) x 1 = -0.6	(3) x (0.2) x 1 = 0.6	(3) x (0.2) x 1 = 0.6
Empirical Support – (20%) Temporal Concordance	(3) x (0.2) x 1 = 0.6	(-3) x (0.2) x 1 = -0.6	(-2) x (0.2) x 1 = -0.4	(-3) x (0.2) x 1 = -0.6	(3) x (0.2) x 1 = 0.6	(3) x (0.2) x 1 = 0.6
Consistency (10%)	(3) x (0.1) x 1 = 0.3	(-3) x (0.1) x 1 = -0.3	(-2) x (0.1) x 1 = -0.2	(-3) x (0.1) x 1 = -0.3	(3) x (0.1) x 1 = 0.3	(3) x (0.1) x 1 = 0.3
Analogy (10%)	(2) x (0.1) x 1 = 0.2	(-2) x (0.1) x 1 = -0.2	(-2) x (0.1) x 1 = -0.2	(-2) x (0.1) x 1 = -0.2	(2) x (0.1) x 1 = 0.2	(2) x (0.1) x 1 = 0.2
TOTAL	+2.9	-2.9	-2.0	-2.9	+0.29 (2.9 X 0.1*)	+0.29 (2.9 X 0.1*)

*Adjustment Factor of 10% (0.1) applied to late key events due to convergence and lack of specificity to a particular MOA (see Becker et al., 2017 for rationale).

Mode of Action Confidence Score = -34.2 = (-4.32) ÷ (12.6) X 100 (Step 5)

D. Key References

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IV. Evaluating the WOE for a Cytotoxicity/Regenerative Proliferation MOA

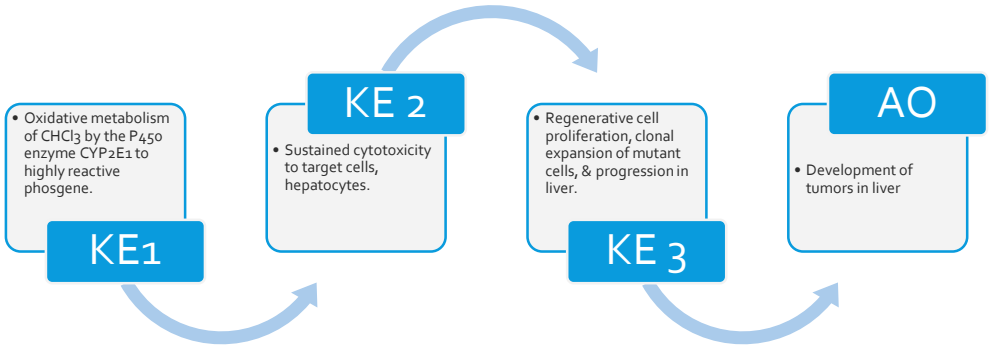
Chloroform, $CHCl_3$, is a high volume, chlorinated organic that has been shown to induce liver[3] tumors in mice and rats (NCI, 1976; Reuber, 1979, Yamamoto et al., 1996, 2002), and it requires metabolic activation to form reactive metabolites capable causing target tissue cytotoxicity. This sustained cytotoxicity induces regenerative cell proliferation, resulting in foci of altered cells that undergo progression which includes induction of mutations and additional cell proliferation. Eventually the altered, mutated foci form hepatocellular tumors. Based on the published description of key events for a cytotoxic, regenerative proliferation threshold MOA for induction of liver tumors by $CHCl_3$ (Borgert et al., 2015; Boobis et al., 2009), the following key events would be expected for a Cytotoxicity/Regenerative Cell Proliferation MOA for induction of liver tumors by $CHCl_3$, which would have a threshold:

- KE1 Oxidative metabolism of $CHCl_3$ by the P450 enzyme CYP2E1 to highly reactive phosgene.
- KE2 Sustained cytotoxicity to target cells, hepatocytes.
- KE3 Regenerative cell proliferation, clonal expansion of mutant cells, & progression in liver.
- AO Development of tumors in liver.

Data to provide evidence for these KEs would include information on $CHCl_3$ metabolic activation; histopathologic details on cytotoxicity/tissue necrosis in liver; (quantitative) hepatic cell proliferation data; data on liver foci formation and progression, and hepatocellular adenoma/carcinoma data, preferably in both rats and mice.

The initial KE and final KEs/AO are similar or identical between MOA #1 and MOA #2.

Provided below is the postulated MOA in Figure 3.



[3] $CHCl_3$ also induces kidney tumors, with similar supporting data. This analysis focuses on liver tumors, with the expectation that the key events and supporting data for the same MOA are similar in kidney.

Figure 3. Postulated Cytotoxic/Regenerative Proliferation Threshold Mode of Action for $CHCl_3$

A. Qualitative Evaluation of the WOE for $CHCl_3$ Acting via a Cytotoxic MOA

Table 9. Qualitatively Evaluate the Comparative Weight of Evidence for $CHCl_3$ Acting via the Cytotoxic/Regeneration Threshold MOA (Borgert et al., 2015; Boobis et al., 2009) – (Step 2)

Key Event	Supporting Data	Potentially Inconsistent	References
KE1: Metabolic Activation of $CHCl_3$ to Reactive Metabolite: Phosgene	Extensive supporting data demonstrate saturable formation (*via* CYP2E1) of phosgene in rodent liver from $CHCl_3$ (Figure 4). Key counterfactual evidence demonstrates the lack of liver toxicity with CYP2E1 knock-out (KO) mice and data demonstrating that pre-treatment with a CYP2E1 inhibitor also blocked liver toxicity in wild type $CHCl_3$-treated mice, further supporting a key role for CYP2E1 (Strong evidence). Quantitative kinetics data are available, including data supporting a GSH-dependent threshold for toxicity from phosgene (or other reactive metabolites) generated from $CHCl_3$. Interindividual variability in human CYP2E1 levels (10- to 12-fold) can affect shape of dose-response curve for hepatotoxicity from $CHCl_3$-generated phosgene; it is likely that variability in CYP2E1 has higher impact than variability in GSH levels. Because CYP2E1 is highly conserved between rodents and humans, with a single isoform		KO mice; CYP2E1 inhibition: Constan *et al*., 1999 CYP 2E1 induction: Brady *et al.,* 1989. Kluwe, *et al.,* 1978 Threshold: Ammann *et al.,* 1998; Kluwe and Hook, 1981 Variability: Edwards *et al.,* 1998; Lipscomb *et al.,* 2004; Gemma *et al*., 2003. Conserved gene: Borgert *et al*., 2015; Neis *et al*., 2010; Baron *et al.,* 2008; Du *et al*., 2004; Ingelman-Sundberg, 2004

Key Event	Supporting Data	Potentially Inconsistent	References
	sourced from a single gene in humans and rodents, including in liver, lung and skin, a common MOA is expected for all exposure routes, resulting from common metabolic products.		
KE2: Sustained cytotoxicity to hepatocytes	Phosgene readily reacts with cellular nucleophiles such as GSH & macromolecules, including proteins, producing covalent adducts, although apparently not with DNA. Modification and/or oxidation of critical macromolecules lead to cytotoxicity and necrosis. Data suggest that mitochondrial dysregulation leading to impairment of the mitochondrial membrane potential (likely *via* effects on mitochondrial permeability transition) is part of cytotoxic mechanisms. Profile of sustained cytotoxicity reaches a peak (typically fairly early in the process) and results in degeneration and necrosis, establishing an adaptive state where the regenerative proliferative stimulus is induced and on-going in KE3 and beyond. Single dose studies are not adequate to initiate the processes involved, and thus not informative for this MOA where repeated exposure is needed. $CHCl_3$ induction of hepatic cytotoxicity /degeneration & necrosis is evident from transgenic datasets (*p53; lacI*), where histopathology clearly shows this occurring (followed by increased cell proliferation). Evidence supports a threshold for induction of cell	Some studies have shown an 'early' peak (a few days or a few weeks into repeated treatment) of toxicity & labelling during chronic exposure; this initial wave can then result in accelerated and continued proliferation of AHF cells, which then continues throughout the chronic exposure period.	Cytotoxic/Proliferation MOA: Cohen, 2010 Covalent adducts: Reynolds and Yee, 1967; Branchlower *et al.,* 1984 Lack of DNA adducts: DNA binding: Diaz Gomez and Castro, 1980a Only Histone adducts: Fabrizi *et al*.., 2003; Diaz Gomez and Castro, 1980b Only Phospolipid adducts: Vittozzi *et al*., 2000 Cytotoxicity/necrosis: Constan *et al*., 1999; Boobis, 2009, 2010; Hartig *et al*., 2005 Mitochondrial dysregulation: Burke *et al*., 2007; Boobis, 2010; Boobis *et al*., 2009 Transgenic studies: Gollapudi *et al*., 1999; Butterworth *et al*., 1998 Threshold for cytotoxicity /necrosis: Ruch *et al.,* 1986; Ammann *et al.,* 1998; Hartig *et al*.,2005; Smith *et al.,* 1985 and Smith *et al*., 1983; Larson *et al*., 1994; Templin *et al*., 1998 Stop exposure: Larson *et al*., 1994, 1995; Liao *et al*., 2007; Templin *et al.,* 1996b

Key Event	Supporting Data	Potentially Inconsistent	References
	death/cytotoxicity (*in vitro* & *in vivo*), which requires a critical degree of phosgene to cause a critical level of cell damage, as low levels of $CHCl_3$ do not cause liver toxicity. This is likely due to mitochondrial resilience and repair in rodent and human liver. Sustained exposure to high levels of $CHCl_3$ is required to exceed cellular repair capacity and cause cell death and tissue necrosis. Additional evidence for a threshold comes from the marked species, strain, sex, and tissue specificity of the toxicity of $CHCl_3$ to the liver. Key supporting counterfactual evidence for the requirement for cytotoxicity includes recovery of tissue when exposure is stopped, where animals were exposed to $CHCl_3$ for 6 wks and then held for 7 wks. The data for animals under the stop-exposure protocol did not demonstrate increases in hepatic toxicity or in regenerative cell proliferation. NB: Although the oxidative metabolism of $CHCl_3$ to phosgene by CYP2E1 is not a threshold event, the necessity to accumulate a certain level of phosgene sufficient to induce significant, sustained cytotoxicity is a threshold event, resulting in a threshold MOA.		
KE3: Regenerative cell proliferation, clonal expansion of mutant cells, &	Increased cell proliferation in liver of $CHCl_3$-treated rats and mice, measured as increased labelling index and/or BrdU incorporation, demonstrated regeneration that follows the $CHCl_3$-induced cytotoxicity.	Some single-dose data do not show a consistent relationship for markers of hyperplasia, including DNA synthesis and	Cytotoxic/Proliferation MOA: Cohen, 2010 Quantitative cell proliferation data: Constan *et al*., 1999; Boobis, 2009, 2010; Larson *et al*., 1994,

Key Event	Supporting Data	Potentially Inconsistent	References
progression in liver	Single dose studies are not adequate to initiate the processes involved, and thus not informative for this MOA where repeated exposure is needed. Quantitative dose-response data on $CHCl_3$-induced cell proliferation demonstrates that sustained, high level $CHCl_3$ is required to induce sufficient toxicity to cause compensatory proliferation. $CHCl_3$ induction of hepatic cytotoxicity /degeneration & necrosis is evident from transgenic datasets (p53; lacI), where histopathology clearly shows this occurring (followed by increased cell proliferation). Increased presence of hepatic foci of altered cells is a characteristic of this stage of progression. Quantitative dose-response data are available, based on an initiation-promotion protocol, demonstrating the formation and increased numbers and volume of such hepatic foci following initiation by DEN, identified by altered staining patterns. There is no evidence to support alternative paths to induction of cell proliferation, such as: • $CHCl_3$ directly induces cellular hyperplasia, or • $CHCl_3$ inhibits apoptosis, or • $CHCl_3$ activates nuclear receptors involved in cell proliferation, even at tumorigenic doses. Thus it is highly unlikely that any direct growth stimulation (rather than compensatory proliferation)	induction of AHF; however, single dose studies are unlikely to adequately initiate these responses.	1995a,b; Templin *et al.,* 1996, 1998 Transgenic studies: Gollapudi *et al*., 1999; Butterworth *et al*., 1998 Hepatic foci of altered cells: Deml & Osterle, 1985, 1987*;* Osterle & Demrl, 1985; Pereira et al., 1982 KO mice; CYP2E1 inhibition: Constan *et al*., 1999 Stop-exposure: Larson, Templin *et al*., 1996; Route/PK comparison: Borgert *et al.*, 2015; Butterworth *et al*., 1995a,b; Conolly and Butterworth, 1995

Key Event	Supporting Data	Potentially Inconsistent	References
	is involved in the MOA for $CHCl_3$. Counterfactual evidence of regenerative cell proliferation is provided by data showing it did not occur in $CHCl_3$-treated CYP 2E1 KO mice or in wild type $CHCl_3$-treated mice pre-treated with a CYP2E1 inhibitor. Additional counterfactual evidence is provided by stop-exposure experiments where animals were exposure to $CHCl_3$ for 6 wks and then held for 7 wks. The data for animals under the stop-exposure protocol did not demonstrate increases in hepatic toxicity or in regenerative cell proliferation. Additional counterfactual evidence is provided by comparisons of PK differences between bolus gavage vs. drinking water administration, as only the gavage route produces cytotoxicity and cell proliferation responses while, despite a similar or higher AUC for $CHCl_3$, the drinking water administration does not.		
AO: Development of tumors in liver	Following gavage treatment with $CHCl_3$, both mice and rats showed increases in liver adenomas + carcinomas. Route of administration clearly influenced tumor formation as mice treated with similar $CHCl_3$ doses *via* drinking water did not show increases in liver tumors. Additional bioassays exist with generally similar results, typically not showing increases in liver tumors with non-gavage routes	One drinking water, single dose level study reported increased hepatic neoplastic nodules as 'adenofibrosis' in Wistar rats.; Inhalation study showed increased trend only for mouse liver carcinomas + adenomas (rat negative)	Gavage $CHCl_3$: NIC, 1976; Reuber, 1979; dH2O: Jorgenson *et al.,* 1985, Inhalation: Yamamoto, 1996, 2002 dH2O single dose level: Tumasonis *et al*., 1985, 1987

Key Event	Supporting Data	Potentially Inconsistent	References
	(inhalation) or at lower gavage doses (in toothpaste).		

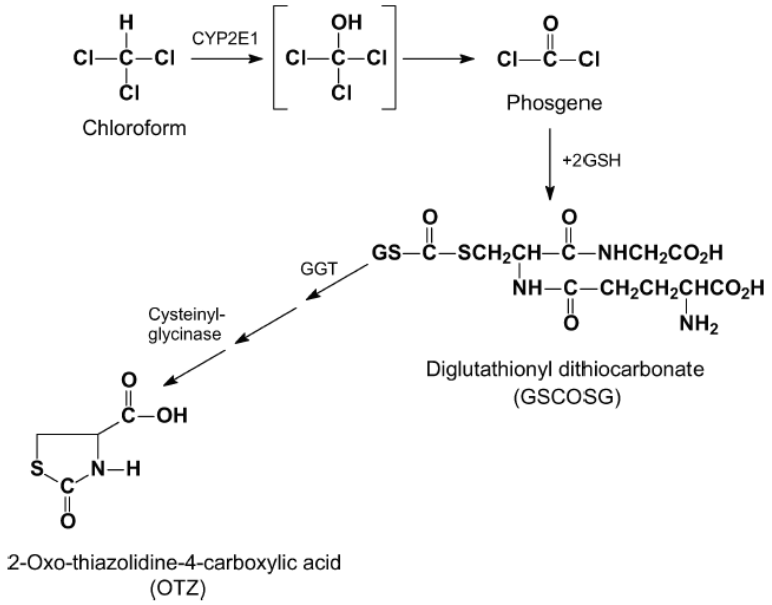


Figure 6 Metabolic activation of chloroform to phosgene and subsequent detoxication by glutathione. Chloroform is oxidized by CYP2E1 to an unstable hydroxylated intermediate, which spontaneously rearranges to the chemically reactive phosgene, the putative toxic species. Phosgene can react spontaneously with glutathione (GSH), the product of which is non-toxic. The glutathione conjugate is further metabolised via γ-glutamyltranspeptidase and cysteinylglycinase to yield 2-oxo-thiazolidine-4-carboxylic acid (OTZ), an unreactive product.

Figure 4. Taken from Boobis et al., 2009

Table 10. Incidence of Liver Tumors and Nodules from Key Studies on CHCl3. (Based on NCI (1976) data re-examined by Reuber (1979): gavage dosing; 5 (rat) or 6 (mouse) d/wk, and on Jorgenson et al. (1985) female mouse drinking water study and Yamamoto et al. (2002) mouse inhalation study.)

Tumor outcome	Osborne-Mendel Rats*							
	Male				Female			
	Colony Control	Vehicle Control	90 mkd	180 mkd	Colony Control	Vehicle Control	100 mkd	200 mkd
Hyperplastic nodules	0/20	1/19	5/50	8/49	1/20^	2/20^	7/39^	12/39^
Hepatocellular carcinoma	0/20	0/19	0/50	2/49	0/20	0/20	2/39	2/39
Adenoma /Carcinoma	0/20#	1/19#	5/50#	10/49#	1/20&	2/20&	9/39&	14/39^^,&
	B6C3F1 Mice**							
	Male				Female			
	Colony Control	Vehicle Control	138 mkd	277 mkd	Colony Control	Vehicle Control	238 mkd	477 mkd
Hyperplastic nodules	1/17	2/17	11/46	0/44	0/20	0/19	1/45	0/40
Hepatocellular carcinoma	1/17	0/17	20/46	44/44	0/20	0/19	40/45	40/40
Hyperplastic nodules + Carcinoma[a]	3/17+	2/17+	31/46@,+	44/44@,+	0/20k	0/19k	41/45 t,k	40/40t,k
	Female B6C3F1 Mice***							
	Control	Matched dH_20 Control	34 mkd	65 mkd	130 mkd	263 mkd		
Hepatocellular Adenoma /Carcinoma	21/415	0/47	15/410	9/142	0/47	1/44		
	B6C3F1 Mice****							
	Male				Female			
	Control	5 ppm	30 ppm	90 ppm	Control	5 ppm	30 ppm	90 ppm
Hepatocellular Carcinoma	10/50	0/50	7/50	10/48	1/50	1/49	0/50	3/48
Hepatocellular Carcinoma /Adenoma	14/50↑	7/50↑	12/50↑	17/48↑	2/50↑↑	2/49↑↑	4/50↑↑	6/48↑↑

* Rats: Based on NCI (1976) data as re-examined by Reuber (1979): gavage dosing; 5 d/wk for 78 wk; sacrificed at 111 wk.

** Mice: Based on NCI (1976) data as re-examined by Reuber (1979): gavage dosing; 6 d/wk; 80 wk; sacrificed at 96 wk.

[a] Small + Large carcinomas combined from Reuber (1979).

*** Mice: Based on Jorgenson et al. (1985) drinking water dosing with dH20 matched controls due to high mortality in 2 high dose groups (~25%) during first week from dehydration (refusal to drink treated water); remainder of treatment: 78-90% of control water consumption: 104 wk total.

**** Mice: Inhalation exposures: 6 h/d; 5d/wk; 104 wks; similarly treated rats did not have treatment-related neoplastic liver lesions (hepatocellular adenoma males: 0/50, 0/50, 0/50, 0/50; females: 1/50, 0/50, 2/50, 1/49).

Statistical info: Reuber (1979) Male rats: #Trend: p = 0.04502; Female rats ^Trend: p = 0.03617; ^^ p = 0.03105; &Trend: p = 0.01886; Male mice: @p = <0.00001; +Trend: p = 5.794 x 10-17; Female mice: tp = <0.00001; kTrend: p = 2.168 x 10-18 ; Jorgensen et al. (1985): no statistically significant differences across Female mouse liver tumors; Yamamoto et al. (2002): Male mice: ↑ = Trend (Peto's): p<0.05; Female mice: ↑↑= Trend (Peto's): p<0.01.

Empirical Support Dose-Response and Temporal Concordance:

KE1: **Metabolic Activation of $CHCl_3$ to Phosgene**:

Good supporting data for the necessity of the metabolism step based on *in vitro* and *in vivo* data; extensive information on kinetics of CYP2E1 activity demonstrates $CHCl_3$ is activated to form phosgene, and that this reaction is rapid and is saturable, therefore a maximum level is reached rapidly; phosgene is very reactive thus it is rapidly removed either through further metabolism or through binding with nearby cellular macromolecules; likely highly compartmentalized to the CYP2E1-containing smooth endoplasmic reticulum, further supporting data generally showing no binding of activated $CHCl_3$ metabolites with DNA. There do not seem to be data quantifying a dose-response for formation of phosgene over a range of exposures/doses; however, induction of CYP2E1 *in vivo* results in an increased metabolism of $CHCl_3$ by liver microsomes from the induced rats.

There is strong support for the essentiality of this step provided by the CYP2E1 knock-out mice study demonstrating that $CHCl_3$ treatment of these KO mice does not result in liver toxicity or other subsequent key events. Similar data with chemical inhibitors of CYP2E1 provide additional support for essentiality of this KE. This step initiates the sequence of KEs, thus fits temporally with an early KE.

KE2: Phosgene induction of Hepatic Cytotoxicity/Necrosis:

Good supporting data from several published studies from different laboratories demonstrate a dose-response in rats and mice for hepatocellular cytotoxicity/necrosis following $CHCl_3$ exposure by inhalation and by gavage. Histopathologic evaluation of liver tissue in mouse and rat following gavage or inhalation exposure to $CHCl_3$ shows that there are doses that do not induce cytotoxicity/necrosis, but that higher exposures do cause liver toxicity. Two datasets with transgenic mice included liver histopathology and clearly demonstrated dose-responsive increases in hepatocyte degeneration and necrosis with increasing $CHCl_3$ exposure (one inhalation and one gavage).

There is strong support for the essentiality of this step provided by the CYP2E1 knock-out mice study demonstrating that $CHCl_3$ treatment of these KO mice does not result in liver toxicity or other subsequent key events. In addition, pre-treatment with a chemical inhibitor of CYP2E1 also blocked the cytotoxicity/necrosis in liver of $CHCl_3$-treated mice, providing additional supporting evidence of essentiality. The stop-exposure experiments also support essentiality of sustained cytotoxicity.

The cytotoxicity/necrosis is an early event but can occur only after the metabolic activation of $CHCl_3$, which is, in itself, relatively unreactive; this supports the temporal concordance of KE2 with the sequence of key events.

KE3: Induction of Regenerative Cell Proliferation in Liver (and hepatic foci of mutant cells, progression)

Strong evidence for induction of regenerative cell proliferation in liver is provided by several publications from different laboratories. Both gavage and inhalation exposure to $CHCl_3$ have induced dose-responsive increased labelling indices (LI) in mouse and rat liver, using BrdU labelling to quantitate cell proliferation. Two datasets with transgenic mice included BrdU LI in liver and clearly demonstrated dose-responsive increases with $CHCl_3$ exposure (one inhalation and one gavage); one transgenic study provides liver AHF.

There is strong support for the essentiality of this step provided by the CYP2E1 knock-out mice study demonstrating that $CHCl_3$ treatment of KO mice does not result in any increased LI in liver, while the wild-type mice demonstrated extensive hepatic regenerative cell proliferation. Pre-treatment with a chemical inhibitor of CYP2E1 also blocked the regenerative cell proliferation in liver of $CHCl_3$-treated WT mice. The stop-exposure experiments also support essentiality of regenerative cell proliferation.

The hepatic regenerative cell proliferation is a relatively early event, as some datasets show it has stopped by 13 wks of treatment; as it is induced by the cytotoxicity/necrosis, the temporal concordance of KE3 is maintained with the sequence of key events.

AO: Hepatocellular Carcinoma/Adenoma

Strong evidence for induction of hepatocellular carcinoma/adenoma by high dose exposure to $CHCl_3$ in mice and rats, with several bioassays (gavage or inhalation) demonstrating increased incidence of these tumors. In addition, there are exposure levels that do not result in hepatic tumors, providing dose-response data. Tumors are identified only following ~50+ weeks of $CHCl_3$ treatment, supporting the temporal concordance of this AO.

Table 11. Dose-Response and Temporal Concordance Table: Mouse

Dose Concordance	Temporal Concordance				
	Temporal	**<24hrs**	**1-2 d to 3 wks**	**3-13 wks**	**2 yrs. Cancer studies**
	Dose/Conc.	**KE 1: Metabolic Activation/Phosgene**	**KE 2: Cytotoxicity/Necrosis**	**KE 3: Regenerative Cell Proliferation**	**AO: Hepatocellular Adenoma/Carcinoma**
	INHALATION ([A]Yanamoto et al., 2002; Templin et al., 1996b, 1998; Larson et al., 1996;) Butterworth et al., 1998)				
	0 ppm[A]	--	--	--	-/+
	(2*) 5 ppm	*[+]*	-/+	--	-/+
	10 ppm	*[+]*	-/+	--	*ND*
	30 ppm	*[++]*	++	+	+
	90 ppm	*[+++]*	+++	*+++ (9- 17x↑)*	++
	30 ppm/stop*	*[++]*	--	--	*ND*
	90 ppm/stop*	*[+++]*	-/+	--	*ND*
	0 ppm[&]	*[--]*	--	--	ND
	90 ppm[&]	*[++]*	++	++	ND
	0 ppm[@]	*[--]*	--	--	ND
	90 ppm WT[@]	*[++]*	++	++	ND
	90 ppm WT + inhibitor[@]	--	--	--	ND
	90 ppm CYP2E1 KO[@]	--	--	--	ND
	GAVAGE (NCI, 1976/Reuber, 1979; Larson *et al.*, 1994a,b; Gollapudi *et al.*, 1999)				
	0 mkd	--	--	--	-/+
	34 mkd**	*[+]*	--	+	ND
	90 mkd**	*[++]*	-/+	+	ND
	138/238 mkd**	*[++]*	++	*+++ (30x↑)*	++
	277/477 mkd**	*[+++]*	+++	+++	+++
	0 mkd^	*[--]*	--	--	--
	24/28 mkd^	*[+]*	-/+	+	--
	90 mkd^	*[++]*	++	+	--
	140/240 mkd^	*[+++]*	+++	+++	--

+++ : strong response; ++ : moderate response; + : weak response; -/+: background; **--** : no response; [assumed response]; ND : no data; *italicized grey* = data from subchronic or subacute (not chronic) studies; stop exposure was 6 wks exposure followed by 7 wks no exposure.

[A] Yanamoto *et al*., 2002 source of inhalation tumor data (trends); gavage tumor data from NCI,1976; Reuber, 1979: males: 138 & 277 mkd; females: 238 & 477 mkd;

* Templin *et al*., 1996b, 1998 and Larson *et al.,* 1996 (inhalation exposure, with stop-exposure groups);

[&] Butterworth et al , 1998 l*acI* transgenic inhalation tumor study: up to 6 mon (180 d) exposure (tumors not measured).

[@]Constan et al., 1999: inhalation (0 or 90 ppm $CHCl_3$ for 6 h/d; 4 d); wild type (WT) and CYP2E1 KO B6C3F1 mouse; BrdU for LI

* Larson et al., 1996 (inhalation exposure, with stop-exposure groups);

**Larson et al., 1994a,b (gavage B6C3F1).

^ Gollapudi *et al*., 1999: oral gavage $p53^{-/-}$ transgenic tumor study: dosing for 13 or 26 wks (both negative for tumors).

Table 12. Dose-Response and Temporal Concordance Table: Rat

Dose Concordance ↓	Temporal Concordance →				
	Temporal	**<24hrs**	**1-2 d to 3 wks**	**3-13 wks**	**2 yrs. Cancer studies**
	Dose/Conc.	**KE 1: Metabolic Activation/Phosgene**	**KE 2: Cytotoxicity/Necrosis**	**KE 3: Regenerative Cell Proliferation**	**AO: Hepatocellular Adenoma/Carcinoma**
	INHALATION (Tumor data: Yanamoto et al., 2002; Templin et al., 1996c)				
	0 ppm[A]	--	--	--	-/+
	(2*) 5 ppm	*[+]*	-/+	--	-/+
	10 ppm	*[+]*	-/+	--	*ND*
	30 ppm	*[++]*	++	+	+
	90 ppm	*[+++]*	+++	+++ *(9- 17x↑)*	++
	30 ppm/stop*	*[++]*	--	--	*ND*
	90 ppm/stop*	*[+++]*	-/+	--	*ND*
	GAVAGE (NCI, 1976/Reuber, 1979; Templin et al., 1996a,c; Larson et al., 1995a,b)				
	0 mkd	***[--]***	--	--	-/+
	10 mkd**	***[+]***	--	--	ND
	34 mkd**	***[++]***	--	--	ND
	90/100 mkd	***[+++]***	--	--	++
	180/200 mkd	***[+++]***	--	--	+++
	477 mkd**	***[+++]***	-/+	++ ***(5x ↑)***	ND

++++ : strong response; ++ : moderate response; + : weak response; -/+: background; -- : no response; [assumed response]; ND : no data; *italicized grey* = data from subchronic or subacute (not chronic) studies.

[A]Yanamoto et al., 2002 source of inhalation tumor data; gavage tumor data from NCI,1976; Reuber, 1979.

* Templin et al., 1996c (inhalation exposure); Larson et al., 1995a,b (gavage); **Templin et al., 1996a (single gavage dosing for intermediate KEs).

B. Evolving Bradford Hill Causal Considerations: Qualitative and Quantitative Data Evaluation

Table 13. Qualitative and Quantitative Rating Categories. [See Becker et al. 2017 for details]

Qualitative	Quantitative	Category Description
Strong	3	Multiple studies and/or extensive data provide convincing evidence that the substance causes the KE.
Moderate	2	Some evidence (direct or indirect) indicating the substance causes the KE, but scientific understanding is not yet completely established. There may be some studies that are equivocal.
Weak	1	Very limited evidence (direct or indirect) that the substance causes the KE along this pathway. Scientific understanding of the KE is limited.
No Evidence	0	No data available to support or negate causation of this KE by the substance.
Weak Counter	−1	There is very limited contradictory evidence (direct or indirect) that the substance does not cause this KE.
Moderate Counter	−2	Some evidence (direct or indirect) indicating that the KE is not caused by the substance, but scientific understanding is not completely established. There may be some studies that are equivocal.
Strong Counter	−3	Multiple studies and/or extensive data provide convincing evidence that the substance does not cause this KE.

Table 14. Evolved Bradford Hill Causal Considerations, Defining Questions and Body of Evidence (adapted from Meek et al., 2014 a,b).

Bradford Hill Causal Considerations	Defining Questions	Supporting Evidence	Potentially Inconsistent Evidence
Essentiality	Data that demonstrate a KE is essential, *e.g.*, when a KE is blocked or reduced, downstream KEs and/or the AO do not occur or are not present to the same degree.	Data from CYP2E1-KO mouse, and pre-treatment with CYP2E1 inhibitor, both provide strong supporting evidence for KE1 (CYP2E1 metabolic activation) essentiality, as downstream KEs, including the AO, are blocked or reduced when CYP2E1 is missing. Stop-exposure data provide evidence for essentiality of KE2 & KE3, as their formation is	None identified

Bradford Hill Causal Considerations	Defining Questions	Supporting Evidence	Potentially Inconsistent Evidence
		significantly/completely blocked and no liver toxicity is induced in mice that are exposed to carcinogenic levels of $CHCl_3$ for 6 wks and then held for 7 wks.	
Empirical Support – Dose and Incidence Concordance	Are dose-response data available demonstrating monotonic increases in response within KEs?	Good dose-response data exist for most all KEs, as shown in Tables 3A & 3B; few data on dose-response for KE1. Data come from mouse & rat, and from different labs, and from different exposure routes, which adds to the strength of evidence. NO(A)El values are identified for some KEs (KE2, KE3). Stop-exposure data provides additional support for dose-response, as KEs do not manifest 7 wks later when exposure is stopped following 6 wks of exposure (elapsed time = 13 wks).	None identified; paucity/lack of actual dose-response data for formation of phosgene from $CHCl_3$, either *in vitro* or *in vivo* in tissues
Empirical Support – Temporal Concordance	Do the KEs demonstrate a temporal relationship across KEs over time to the AO? Do the KEs occur in order?	Good dose-response data exist across most all KEs, as shown in Tables 3A & 3B, demonstrating that the KEs occur in temporal order. Little specific temporal *in vivo* data are available for KE1 itself, which must occur in order to see downstream KEs, however, data that demonstrate blocking KE1 blocks subsequent downstream KEs supports temporal concordance. Available data come from mouse & rat, and from different labs, and from different exposure routes, which adds to the strength of evidence. Stop-exposure data provides additional support for temporal concordance, as later KEs do not manifest 7 wks after a 6-wk exposure was stopped (elapsed time = 13 wks).	None identified
Consistency	Do the data present a consistent pattern, e.g., across organ systems and across species?	Available data are consistent within the target organ system (liver) and across species (mouse and rat). Sex differences can be explained by known biological differences (higher CYP2E1 in female mouse vs male	Limited liver tumor response from some datasets.

Bradford Hill Causal Considerations	Defining Questions	Supporting Evidence	Potentially Inconsistent Evidence
		mouse, thus higher exposure to reactive phosgene and consequently more hepatotoxicity, *etc*., with more liver tumors). Strain differences are not evident in the two mouse or in the two rat strains investigated.	
Analogy	Is this MOA expected given broader chemical-specific knowledge?	Other, structurally similar, haloalkanes have demonstrated induction of liver tumors in rodents *via* a non-genotoxic, cytotoxic MOA with regenerative cell proliferation, similar to what is described for $CHCl_3$ above. This MOA typically involves formation of a more reactive metabolite or toxic moiety (phosgene, in this case), that causes cell membrane damage or cytotoxic effects that lead to sustained regenerative cell hyperplasia and formation of pre-neoplastic lesions/foci, leading to late forming, liver tumors at high doses of chronic exposure. As further demonstration of the consistency and analogy criteria being met, most cancer QSAR and predictive models such as OncoLogic have a rule that defined haloalkanes with an increased probability of causing liver cancer without specifying the MOA.	None identified

C. Qualitative and Quantitative Rating of KEs for Bradford Hill Causal Considerations

Table 15. Qualitative Rating of the Key Events for Bradford Hill Causal Considerations (Step 3)

Bradford Hill Causal Considerations	Key Event #1	Key Event #2	Key Event #3	AO
	Metabolic Activation to Phosgene	**Cytotoxicity /Necrosis**	**Regenerative Cell Proliferation**	**Hepatocellular Adenoma /Carcinoma**
Essentiality	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)
Empirical Support – Dose and Incidence Concordance	Qualitative rating: Moderate (Quantitative rating = +2)	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)
Empirical Support – Temporal Concordance	Qualitative rating: Moderate (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)
Consistency	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)	Qualitative rating: Strong (Quantitative rating = +3)
Analogy	Qualitative rating: Moderate (Quantitative rating = +2)	Qualitative rating: Moderate (Quantitative rating = +2)	Qualitative rating: Moderate (Quantitative rating = +2)	Qualitative rating: Moderate (Quantitative rating = +2)

D. Composite Qualification of the WOE for a Cytotoxicity/Regenerative Proliferation MOA

Table 16. Quantification of the WOE for the [Postulated] MOA (Step 4 and 5): (Quantitative rating) x (BH criterion weight) x (any adjustment factor).

Bradford Hill Causal Considerations	Key Event #1	Key Event #2	Key Event #3	AO
	Metabolic Activation to Phosgene	Cytotoxicity /Necrosis	Regenerative Cell Proliferation	Hepatocellular Adenoma /Carcinoma
Essentiality (40%)	(3) x (0.4) x 1 = 1.2	(3) x (0.4) x 1 = 1.2	(3) x (0.4) x 1 = 1.2	(3) x (0.4) x 1 = 1.2
Empirical Support – (20%) Dose and Incidence Concordance	(2) x (0.2) x 1 = 0.4	(3) x (0.2) x 1 = 0.6	(3) x (0.2) x 1 = 0.6	(3) x (0.2) x 1 = 0.6
Empirical Support – (20%) Temporal Concordance	(3) x (0.2) x 1 = 0.6	(3) x (0.2) x 1 = 0.6	(3) x (0.2) x 1 = 0.6	(3) x (0.2) x 1 = 0.6
Consistency (10%)	(3) x (0.1) x 1 = 0.3	(3) x (0.1) x 1 = 0.3	(3) x (0.1) x 1 = 0.3	(3) x (0.1) x 1 = 0.3
Analogy (10%)	(2) x (0.1) x 1 = 0.2	(2) x (0.1) x 1 = 0.2	(2) x (0.1) x 1 = 0.2	(2) x (0.1) x 1 = 0.2
TOTAL	+2.7	+2.9	+0.29 (2.9 X 0.1*)	+0.29 (2.9 X 0.1*)

*Adjustment Factor of 10% (0.1) applied to late key events due to convergence and lack of specificity to a particular MOA.

Mode of Action Confidence Score = 93.6 = (6.18) ÷ (6.6) X 100 (Step 5)

E. Key References

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V. Conclusions

Comparison of the MOA confidence scores for the two hypothesized MOAs is informative. Analysis of MOA#1, the mutagenic MOA, resulted in a confidence score of -34.2; a negative score indicates availability of data that provide counterevidence, thus contradict, a hypothesis. In the case of MOA#1, there are both significant counterevidence (contradictory evidence), such as the three negative *in vivo* transgenic mouse studies demonstrating that the influential KE4 does not occur, and several KEs that do not have reliable supporting data (KEs 2, 3, and 4), while all of these 3 KEs do have at least some negative (non-supporting) data. Altogether, the assessment results in a negative confidence score. Analysis of MOA#2, the cytotoxicity/regenerative proliferation MOA, presents a different picture, with a confidence score of +93.6 (out of a maximum of 100 possible). Such a high MOA confidence score indicates a wealth of strong supporting data, with little or no contradictory data. This is the case for MOA#2, with qualitative and quantitative supporting data available for most of the KEs.

Uncertainties in these analyses are fairly limited as there are data available for almost all the KEs described. Again, the very strong counterevidence against an influential KE for MOA#1 weighs heavily in the strength of that assessment, but additional *in vivo* transgenic mutation data would certainly help further confirm that $CHCl_3$ does not act as an *in vivo* mutagen, therefore $CHCl_3$ does not act *via* a mutagenic MOA to result in liver tumors. In a similar vein, the very strong *in vivo* mechanistic datasets on liver cytotoxicity/necrosis and regenerative cell proliferation provide significant confidence in the applicability of MOA#2. Indeed, the available data support the existence of a threshold for this MOA-driven response, with no increases in cytotoxicity/necrosis or in hepatic cell proliferation at exposures <10 ppm. Although unlikely to be resolved, one possible uncertainty in the $CHCl_3$ database is the low level of induced liver tumors, with some bioassays not demonstrating unequivocally increased incidences, translating to a low potency of $CHCl_3$. Finally, species extrapolation always adds uncertainty to an assessment. For $CHCl_3$, all the KEs in MOA#2 are predicted to occur in humans, but likely with lower frequency for some, *e.g.,* reduced levels of CYP2E1 would result in decreased levels of reactive metabolites, the initiating KE. The availability of PBPK models describing $CHCl_3$ pharmacokinetic and pharmacodynamic behavior across species helps address this uncertainty with increased confidence, although additional data on human variability is always useful.

The MOA confidence scores developed here provide further support for the application of MOA#2 (cytotoxicity/regenerative proliferation MOA) in conducting human health risk assessment on $CHCl_3$. This is particularly important as this MOA (MOA#2) supports a threshold approach to risk assessment, with evidence to indicate that there is an exposure/dose below that threshold where the MOA for cancer would not be triggered. If the KEs are not triggered, then there will be no subsequent $CHCl_3$-related increased tumor incidence. Conduct

of a MOA-based risk assessment on $CHCl_3$ will allow determination of a threshold dose/exposure, below which no additional cancer risk is expected, in effect a Reference Concentration/Dose (RfC/RfD) for cancer. Indeed, USEPA has supported this idea with development of the RfD value as protective against cancer.

Human relevance of an hypothesized MOA is addressed with the following three questions (Meek *et al*., 2002):

1. Is the weight of evidence sufficient to establish the MOA in animals?
2. Are key events in the animal MOA plausible in humans?
3. Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?

If these questions are answered as 'yes', then the MOA is considered relevant to humans and it can be informative in the conduct of human risk assessment for that chemical. Responses are to be supported by data.

Given the MOA confidence score for MOA#2, the sustained cytotoxicity/regenerative cell proliferation MOA, the first question would be answered 'yes'; there is adequate evidence to establish the MOA#2 for $CHCl_3$ induction of liver tumors in mice (and rats). Concordance of both dose-response and temporality have been established for the KEs along with essentiality, consistency, and analogy/coherence. Analysis of the alternative MOA (MOA#1, mutagenic MOA) resulted in a negative score for MOA confidence.

Given the current understanding of human/tissue response, there are great similarities between rodents and humans, including in acute toxicity and in target tissues for $CHCl_3$. Indeed, all the key events from MOA#2 are plausible in humans, with supporting data for several, and similar processes expected in humans for all (see Table 17: Animal:Human Evidence Concordance). The AO, liver cancer, is the most common cancer in humans, although there are not adequate epidemiological data to demonstrate $CHCl_3$-induced liver tumors in humans. CYP2E1, which activates $CHCl_3$ in rodents, is expressed in human liver and human CYP2E1 can metabolize $CHCl_3$ to reactive metabolites. Both necrosis and regenerative proliferation are believed to be precursor steps in human liver cancer, although data are scarce.

Based on the plausibility in humans of all the KEs for MOA#2, this cytotoxicity/regenerative proliferation MOA should be considered plausible and relevant to human risk assessment of chloroform. Indeed, EPA (2006) concluded in its 2006 assessment of Maximum Contaminant Goals for $CHCl_3$ that "based on an analysis of the available scientific data on chloroform, EPA believes that the chloroform dose response is nonlinear and that chloroform is likely to be carcinogenic only under high exposure conditions" and, as a consequence, "chloroform is likely to be carcinogenic to humans only under high exposure conditions that lead to cytotoxicity and regenerative hyperplasia." In fact, USEPA set an RfD value as protective against cancer based on the cytotoxicity/regenerative proliferation MOA.

Table 17. Concordance Table (Animal Evidence Compared to Human Evidence) for Each Key Event in the Postulate MOA (based on Meek *et al*., 2003).

Key Event	Qualitative Animal Evidence	Qualitative Human Evidence	Quantitative Species Concordance
KE#1	Incidence/severity of toxicity correlate with covalent binding of metabolites in rats and mice, more prevalent in necrotic lesions; CYP2E1 Knock-Out mouse, with no capacity for metabolic activation, does not demonstrate further KEs or AO	Irreversible binding to macromolecules in human liver microsomes requires prior metabolism; PBPK model based on human physiological parameters and metabolic parameters from data from 8 human livers *in vitro*	Based on available data, species concordance is good
KE#2	In all cases where examined, sustained cytotoxicity (as measured by histopath-ological effects and release of hepatic enzymes) confirmed in mouse liver at doses that induced tumors	Liver also a target organ in humans, based on reports of effects associated with occupational (over)exposure	Available data, although limited, indicates species concordance
KE#3	In all cases where examined, persistent regenerative proliferation (as measured by labeling indices) in the liver of mice at doses that induce tumors	No Data	No human data available to demonstrate concordance (or not); processes are expected to be similar across species
AO	Mouse (limited data for rat)	Inadequate epidemiological data	Human data are inadequate to demonstrate concordance (or not); processes are expected to be similar across species; liver tumors are very common human tumors

VI. Appendix A. Quantitative WOE to Assess Confidence in Potential MOAs (Becker et al., 2017)

Quantitative WOE to assess confidence in potential MOAs

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ABSTRACT: The evolved World Health Organization/International Programme on Chemical Safety mode of action (MOA) framework provides a structure for evaluating evidence in pathways of causally linked key events (KE) leading to adverse health effects. Although employed globally, variability in use of the MOA framework has led to different interpretations of the sufficiency of evidence in support of hypothesized MOAs. A proof of concept extension of the MOA framework is proposed for scoring confidence in the supporting data to improve scientific justification for MOA use in characterizing hazards and selecting dose-response extrapolation methods for specific chemicals. This involves selecting hypothesized MOAs, and then, for each MOA, scoring the weight of evidence (WOE) in support of causality for each KE using evolved Bradford Hill causal considerations (biological plausibility, essentiality, dose-response concordance, consistency, and analogy). This early

proof of concept method is demonstrated by comparing two potential MOAs (mutagenicity and peroxisome proliferator activated receptor-alpha) for clofibrate, a rodent liver carcinogen. Quantitative confidence scoring of hypothesized MOAs is shown to be useful in characterizing the likely operative MOA. To guide method refinement and future confidence scoring for a spectrum of MOAs, areas warranting further focus and lessons learned, including the need to incorporate a narrative discussion of the weights used in the evaluation and an overall evaluation of the plausibility of the outcome, are presented.

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JOSEPH A. COTRUVO AND HEATHER AMATO

Trihalomethanes: Concentrations, Cancer Risks, and Regulations

WHILE THE ASSOCIATION BETWEEN TOTAL TRIHALOMETHANES AND DRINKING-WATER CANCER RISKS REMAINS DEBATABLE, MANAGING DISINFECTION BYPRODUCTS WITH SURROGATES CONTINUES TO BE AN APPROPRIATE AND PRACTICAL METHOD FOR MAINTAINING DRINKING WATER QUALITY.

Drinking water chlorination remains one of the greatest public health benefits of science and engineering. Chlorination is a simple, low-cost, and broadly effective technique for disinfecting drinking water and reducing waterborne disease risks. When combined with filtration, chlorination systems provide remarkable reductions in waterborne disease such that source water–related gastrointestinal waterborne disease outbreaks have virtually disappeared when these unit processes operate as designed.

Forms of chlorine include gaseous chlorine, sodium hypochlorite, calcium hypochlorite, chlorinated isocyanurates, and chloramines (combined ammonia and chlorine). Chlorine is chemically reactive and an oxidizing and halogenating agent. In the early 1970s, studies indicated that chlorinated water produced halogenated disinfection byproducts (DBPs) as a function of the levels of natural total organic carbon (TOC) and contact time, pH, and temperature (Bellar et al. 1974, Rook 1974). Use of monochloramine, formed by combining chlorine and ammonia, increased as a secondary disinfectant following this discovery. Monochloramine (i.e., combined chlorine) is much less reactive than free chlorine or hypochlorite, producing lower levels of fewer and different DBPs while retaining some biocidal efficacy during water distribution.

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Four trihalomethanes (THMs)—trichloromethane (TCM; chloroform), bromodichloromethane (BDCM), dibromochloromethane (DBCM), and tribromomethane (TBM; bromoform)—have been a concern since they were found to form in drinking water following reactions between chlorine species and TOC in source waters. Brominated DBPs are produced following chlorine oxidation of bromide to HOBr/OBr^-, an effective brominating agent, and the mixed-halogen total trihalomethanes (TTHMs) depend on precursor concentrations and relative reaction rates.

Along with haloacetic acids (HAAs), TTHMs comprise the major portion of the mass of halogenated DBPs, and their concentrations are regulated in numerous countries. TTHMs were originally regulated in the United States (USEPA 1979) by the US Environmental Protection Agency (USEPA) as a readily analyzed indicator of other DBPs that might be present in much greater numbers but at much lower concentrations. The maximum contaminant level (MCL) of 0.10 mg/L (100 µg/L) was set as the limit for TTHMs in drinking water, taken as the sum of the four most common THMs. The TTHM MCL, a distribution system–wide annual average of quarterly samples, was not really risk-based but rather was based on treatment feasibility while most importantly maintaining adequate disinfection of waterborne pathogens. The MCL applied to large systems; extensions to smaller systems came later. The regulation used TTHMs as an indicator of the presence of other DBPs to drive treatment changes to concurrently reduce other DBPs, an approach analogous to requiring measurement and reduction of *Escherichia coli* bacteria as indicators for sanitary pathogenic microorganisms.

Disinfectant chemistry is complex, and different disinfectants produce arrays of different DBPs. Noting the efficacy of chloramines to reduce DBP formation, many water suppliers shifted from chlorine to chloramine residuals in their distribution systems. Some water suppliers also changed their primary disinfectants from free chlorine to ozone or chlorine dioxide.

USEPA's MCL was found to be feasible, although it was later reduced to 0.08 mg/L (80 µg/L), and five HAAs were added (USEPA 1998). HAAs represent a substantial portion of DBPs, have potential health risk issues, and may be indicators for other DBPs. The MCLs for TTHMs and HAAs were reaffirmed (USEPA 2006) but made more restrictive when the compliance method was calculated on a sampling location–specific basis rather than a system-wide average, although the latter had been previously affirmed on appeal.

CARCINOGENICITY AND REPRODUCTIVE AND DEVELOPMENTAL TOXICITY HISTORY OF TTHMs

USEPA's 1979 TTHM regulation was initiated from the National Toxicology Program's (NTP's) whole animal bioassay results that chloroform was carcinogenic in rats and mice tested at high doses by corn oil gavage (NTP 1976). That numerical TTHM MCL was not based on quantitative toxicology but on analytical and water treatment feasibility and with the intent of using it as a surrogate for reducing other unmeasured DBPs concurrently produced. The other three THMs were grouped with chloroform by structural analogy and similar formation chemistry as there were only limited data from then new and basic in vitro mutagenicity tests.

Some other THMs besides chloroform showed some level of carcinogenicity under animal testing conditions. However, TCM (Jorgenson et al. 1985) and BDCM (NTP 2006) were found not to be carcinogenic when retested in water rather than corn oil. USEPA concluded that TCM and DBCM were not likely to be carcinogenic below a dose threshold (USEPA 1998). The World Health Organization (WHO) Guidelines for Drinking-Water Quality (GDWQ) do not treat TCM, DBCM, and TBM as genotoxic nonthreshold carcinogens and also state that "as BDCM was negative for carcinogenicity in a recent NTP bioassay in which it was dosed in drinking-water, exceedances of the guideline value (currently 0.06 mg/L) are not likely to result in an increased risk of cancer" (WHO 2017). The International Agency for Research on Cancer (IARC) rated BDCM as 2B (IARC 1991). Canada withdrew its cancer risk–based guideline for BDCM in April 2009 (Health Canada 2017, 2009, 2008). IARC determined that TCM was rated 2B, possibly carcinogenic to humans, and consistent with a mechanism of action that involved prior cytotoxicity (i.e., a dose threshold; IARC 1999). DBCM and TBM (Group 3) did not have sufficient evidence to be classified as possibly carcinogenic to humans.

Chloroform has been evaluated for inhalation toxicology in male and female mice in 90-day studies. The no-observed-adverse-effect level for liver cell proliferation, the most sensitive endpoint in female mice, was 10 ppm (Larson et al. 1996); the study authors concluded that no increase in liver cancer would occur in female mice at that inhaled dose.

Population studies suggesting possible reproductive and developmental

> Observed associations between TTHMs and bladder cancer have been incorrectly interpreted by some as causal.

effects have been mixed and inconsistent. In the 2006 revision, USEPA concluded that "the current reproductive and developmental health effects data do not support a conclusion at this time as to whether exposure to chlorinated drinking water or disinfection by-products causes developmental or reproductive health effects," although it went on to say that it supports a potential health concern. In the Six-Year Review (2017), USEPA updated the information on reproduction and developmental toxicity of TTHMs. In general, most of the animal and human studies were inconclusive or negative, and effects in animal studies usually occurred at very high doses and often equivalent to the maternal toxicity levels, which may indicate an indirect adverse effect. Nevertheless, USEPA stated that it continues to support a potential health concern (USEPA 2017).

Health Canada had proposed a guideline of 16 µg/L for BDCM on cancer risk; however, this was later withdrawn on the basis of the NTP BDCM results in water (Health Canada 2017). Reproductive and developmental effect studies concluded that animal effects were observed at high maternally toxic doses and concluded that the weight of evidence did not support an association between those effects and exposure to BDCM at drinking water levels (Health Canada 2008).

Maximum contaminant level goals (MCLGs) in the United States are nonregulatory benchmarks set at the level at which no known or anticipated adverse health effects would occur, including a margin of safety. USEPA sets MCLGs for genotoxic carcinogens at zero as an "aspirational" goal; other chemical MCLGs have finite values. WHO establishes GDWQ for genotoxic carcinogens at the hypothetical 1/100,000 70-year lifetime risk benchmark. The current individual US MCLGs are as follows: TCM, 0.07 mg/L; BDCM, zero; DBCM, 0.06 mg/L; TBM, zero. These have not been reassessed since before 2006. WHO's current health-based guideline values are TCM, 0.3 mg/L; BDCM, 0.06 mg/L; DBCM, 0.1 mg/L; and TBM, 0.1 mg/L (WHO 2017). The USEPA threshold calculation for chloroform used a 20% relative source contribution (RSC) from drinking water; WHO used a 75% RSC, which accounts for most of the four-fold differences in the two values.

The potential for a measurable drinking water contribution to bladder cancer risk is not obvious, and causality associated with drinking water has not been established.

THM RISKS

Some epidemiology studies have suggested—but not consistently—that colon, rectal, and especially bladder cancers could be associated with TTHM exposure (e.g., Hrudey 2012, 2008). However, the assumption that TTHMs are indicators of bladder cancer risk in humans has not been confirmed, and existing data suggest that TTHMs are not good surrogates for some other chlorination byproducts that may increase bladder cancer risk (Bull 2012, Bull et al. 2009). Observed associations between TTHMs and bladder cancer have been incorrectly interpreted by some as causal. Hrudey (2008) concluded that "none of the THMs, nor any other concurrently identified DBPs, have both the capability of acting to cause bladder tumors and sufficient potency and exposure concentration to yield bladder cancer predictions that would accord with epidemiological predictions." USEPA (2003) estimated in its Stage 2 Disinfection Byproducts Rule analysis that lower and upper confidence limits of bladder cancer risk for chlorination of drinking water ranged from 2 to 17%. Bull (2012) concluded that the potential effects of THMs on bladder cancer would be about two orders of magnitude lower than the observed cancer rates reported by some epidemiological studies. Thus, if there is some correlation between chlorination of drinking water and bladder cancer, it would likely be due to other factors.

Bull (2012) stated that results from meta-analyses suggested estimates of approximately 1/1,000 lifetime risk of developing bladder cancer from consumption of chlorinated drinking water. Based on their assessments of several epidemiology studies, Regli et al. (2015) estimated an increased lifetime bladder cancer risk of 0.0001 per incremental µg/L of TTHM, assuming increased source water bromide levels of 50 µg/L. However, USEPA (2006) cautioned that the level of confidence in its calculations did not preclude that the actual number of bladder cancer cases related to drinking water could be zero because causation had not been proved. That lack of causality was restated in USEPA's most recent Six-Year Review document (USEPA 2017).

Brominated THMs and other substances are metabolized by glutathione S-transferase theta 1-1 (GST-T1-1), and some may produce a mutagenic product, so the possibility of a genotoxic mechanism may exist (Ross & Pegram 2004). Some studies in Spain reported a higher risk of bladder cancer among a population subset with genetic polymorphisms coding for activation of brominated THMs, oxygenation of some HAAs, and

metabolism of many industrial chemicals and oxidation of THMs (Cantor et al. 2010). Bull (2012) states that genetic polymorphisms provide substantive evidence that chlorinated drinking water contributes to bladder cancer, but for a number of mechanistic reasons, it does not provide strong evidence that THMs are causally related to bladder cancer.

Cellular-level in vitro studies employing cytotoxicity and genotoxicity have evaluated numerous DBPs for their biological activities. Such studies usually suffer from the lack of consideration of whole animal post-ingestion metabolism and in vivo organ dosages at target organs and cells, in addition to DNA repair processes. Nevertheless, they indicate very low in vitro activity for THMs (Huang et al. 2017, Plewa & Wagner 2009).

Woo et al. (2002) provided a structure–activity assessment of 209 DBPs for carcinogenic potential. None received high ratings; high–moderate ratings were attributed to three MX (halofuranone) chemicals; moderate ratings were attributed to one MX, five haloalkanes/haloalkenes, six halonitriles, two haloketones, one haloaldehyde, one halonitroalkane, and one nonhalogenated aldehyde. The MX compounds are mutagenic in *Salmonella* assays but are not considered very carcinogenic because they are likely rapidly detoxified after ingestion. The remaining 189 DBPs were assigned low–moderate (58), low (98), or marginal (33) concern.

Hrudey et al. (2015) reviewed 10 higher-quality case control studies with some study overlaps, eight of which suggested an association with bladder cancer with odds ratios for men between 1.4 and 2.5, along with two meta-analyses. They stated that

> Quantitative risk estimates derived from toxicological risk assessment for CxDBPs (chlorination DBPs) currently cannot be reconciled with those from epidemiologic studies, notwithstanding the complexities involved, making regulatory interpretation difficult. . . . Replication of epidemiologic findings in independent populations with further elaboration of exposure assessment is needed to strengthen the knowledge base needed to better inform effective regulatory approaches.

They also concluded that "no causal agent with sufficient carcinogenic potency has been identified, nor has a mechanistic model been validated." It is possible that imprecise DBP exposure variables and other assumptions and consequences of multiple contributing risk factors may be larger than the magnitude of potential water treatment–related risks being studied, thus making further studies of the same type not necessarily likely to resolve the issue.

BLADDER CANCER

Bladder cancer rates vary substantially by region and country. Europe and North America have the highest incidence rates, followed by North and West Africa. Age-standardized rates for bladder cancer in the European Union in 2008 were 27.4/100,000 males and 5.6/100,000 females. The highest rates were in Spain, Denmark, Czech Republic, and Germany; the lowest were in Slovenia, Finland, and the United Kingdom (Ferlay et al. 2010). Comparable US incidence was 19.8/100,000 for 2014 (CDC 2017). Race and ethnicity appear to be significant risk factors in the United States; the rate per 100,000 was 21.1 for whites, 11.4 for blacks, 10.7 for Hispanics, 8.1 for Asian/Pacific Islanders, and 8.4 for American Indian/Alaska Native (CDC 2017).

Bladder cancer incidence is correlated with age; about 90% of bladder cancers occur in people over 55 years of age, 70% occur over age 65, and median age at diagnosis is 73 (KenResearch 2017). Five-year survival is 77.3% (NCI 2017). Numerous risk factors contribute to age-related incidences of bladder cancer, including predominantly smoking, exposure to aromatic amines, and several occupations (Action Bladder Cancer UK 2017). Some reports suggest that bladder cancer risk may be about 40% in type 2 diabetes patients, and more so in men than women (Diapedia 2014, Zhu et al. 2013, Larsson et al. 2006).

Diabetes, smoking, age, gender, ethnicity, and chemical contributors may interact to affect the risk of bladder cancer. Other small risk factors like arsenic and polycyclic aromatic hydrocarbon exposures add to contributions from certain medical treatments (ACS 2016). Men are about two to four times more likely to contract bladder cancer than women in their lifetimes; smokers are at least three times as likely as nonsmokers to contract bladder cancer; smoking causes about half of all bladder cancers in both men and women (ACS 2016); and there are numerous other contributors to bladder cancer risk. Some mixed-results studies suggest that drinking more fluids, including drinking water, tends to lower risks (ACS 2016, Michaud et al. 2007). Most dietary components have not been

> Bladder cancer risk from drinking water, if any, is likely small, and it is probably overwhelmed by many other larger risk factors such as smoking, diabetes, and others.

associated with bladder cancer (Cancer Research UK 2017).

Arsenic is a risk factor for bladder cancer at high exposures. Mendez et al. (2016) associated bladder cancer with arsenic in drinking water at >150 µg/L but at <150 µg/L with lower confidence. Other studies have not shown increased cancer risk when arsenic occurs at levels of 3–60 µg/L (Lamm et al. 2004) or <100–200 µg/L, especially for nonsmokers (Tsuji et al. 2014). Median US drinking water levels over the period from 2006 to 2010 were 1.5 µg/L (95th percentile was 15.4 µg/L; Mendez et al. 2016). USEPA's MCL and WHO's GDWQ value are 10 µg/L (WHO 2017, USEPA 2016).

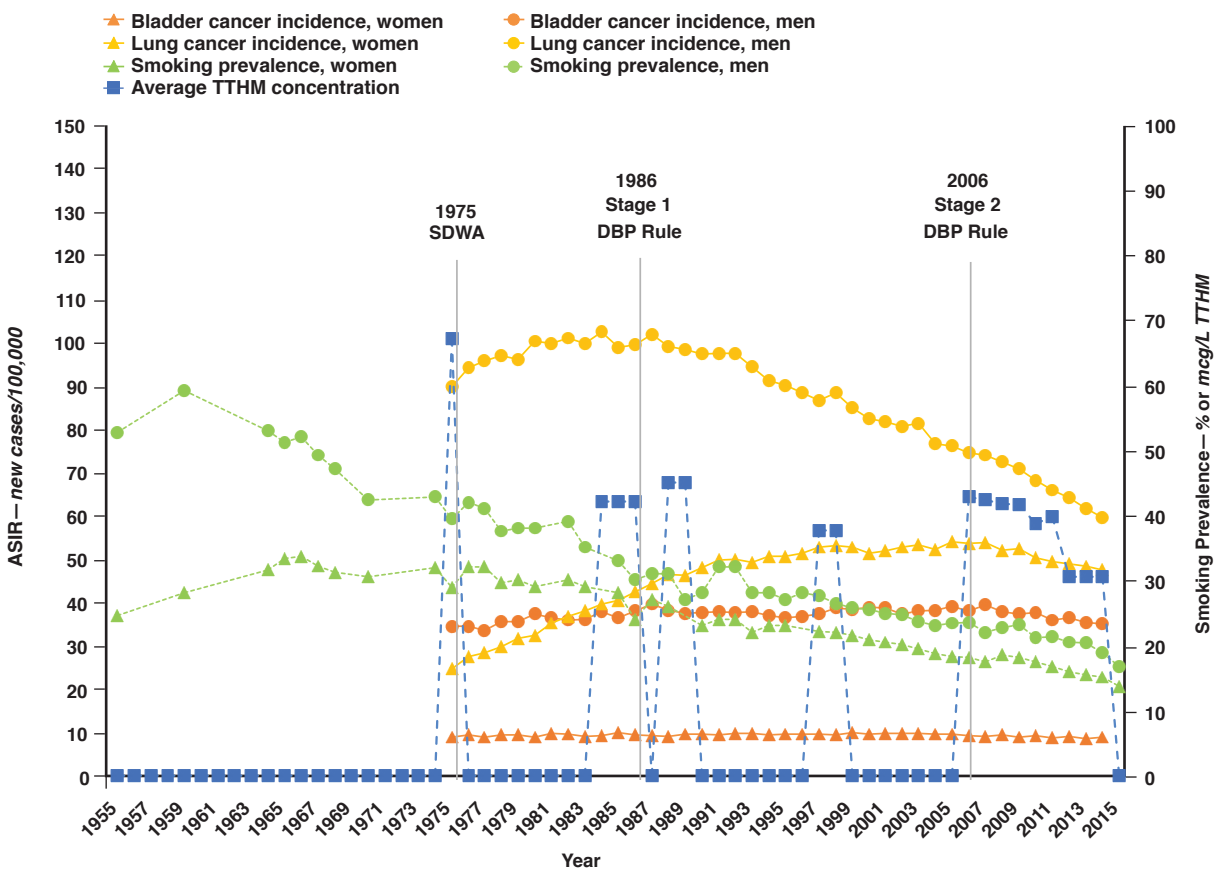
Bladder cancer rates in the United States and Canada have not changed in the more than 40 years since THMs were originally detected in the early 1970s and then regulated in 1979 (Figure 1). US male bladder cancer rates have been consistently 3.5 times female rates; male-to-female rates in Canada have been in the 3.6–4 range (Figure 2). Smoking has declined, and lung cancer rates have also declined, but this has not been manifested in the overall bladder cancer rates. It may be that the latency period for smoking-related bladder cancer is much longer than the latency period for lung cancer.

It remains uncertain whether reduced exposure to TTHMs as a result of drinking water treatment changes has resulted in lower risks to consumers, especially for bladder cancer. Given that THMs are not animal carcinogens at drinking water levels, are there other DBPs that are quantitatively related to TTHM concentrations, such that TTHM reductions might reflect concurrent reductions of those DBPs? It might be hypothesized that reduced drinking water concentrations of TTHMs could concurrently result in reduced exposures to other more potent DBPs and therefore possibly indirectly reduce attributable bladder cancer risks.

TTHMs IN US AND CANADIAN DRINKING WATER SYSTEMS

TTHM data from US locations were extracted from various national or multi-city reports and summaries, primarily from USEPA's national surveys.

FIGURE 1 US annual age-adjusted smoking prevalence, bladder and lung cancer incidence, and TTHM concentrations in drinking water systems, 1955–2015



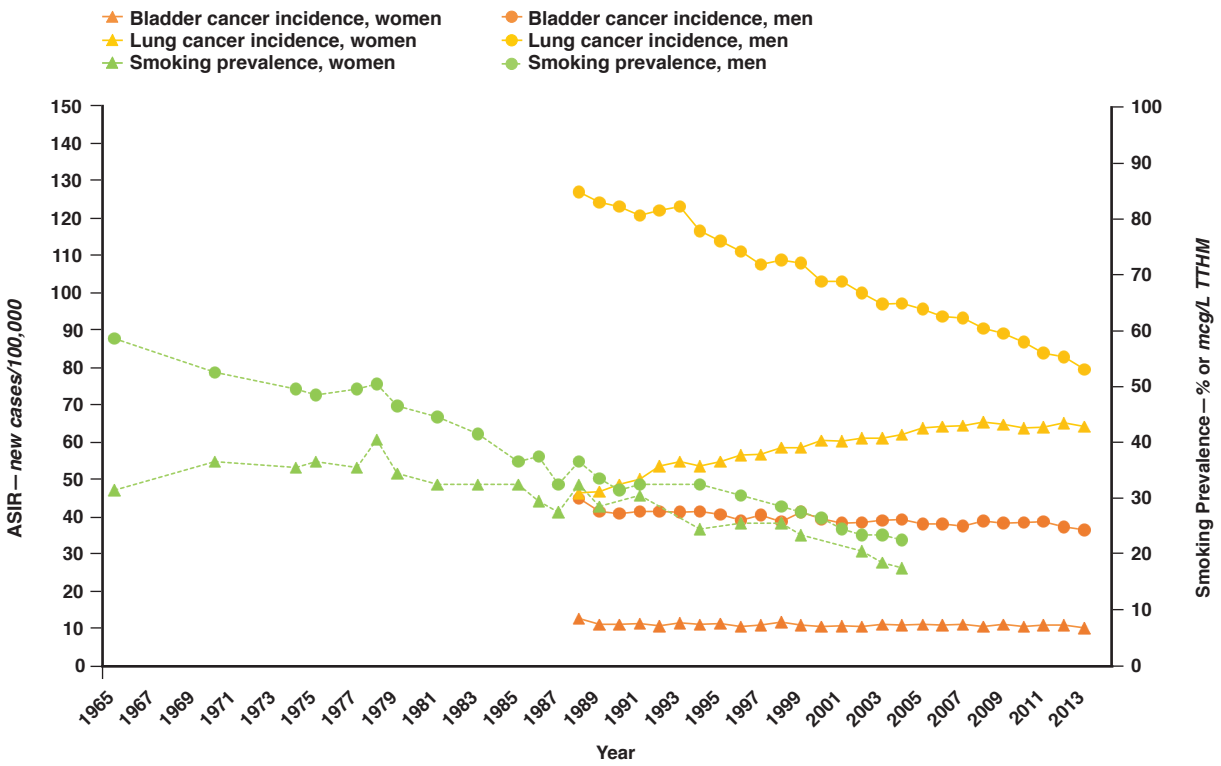
ASIR—age-specific incidence rate, DBP—disinfection byproduct, SDWA—Safe Drinking Water Act, TTHM—total trihalomethane

National annual average TTHM concentrations in micrograms per liter were either directly extracted from published reports or calculated by averaging concentrations across all water systems and all time points with available data in a given year. Table 1 provides a list of TTHM data sources for drinking water systems in the United States by time period.

The United States has multiple databases from its regulatory monitoring requirements and national surveys. The National Organics Reconnaissance Survey (NORS) (Symons et al. 1975) and the National Organics Monitoring Survey in the 1970s related TTHMs to chlorination and water conditions (Table 1). TTHM averages reflect regulatory and treatment technology changes. Average TTHMs in US drinking water supplies were 67 µg/L in 1976, 42–44 µg/L in 1986, and 30 µg/L in 2013–2015. Average TTHM levels were probably at least 67 µg/L before the mid-1970s, when there were no constraints. The highest NORS survey level exceeded 300 µg/L in a water supply in a warm climate with very high TOC water; chlorine was used as a disinfectant and to bleach colored humic substances. TTHM levels have trended downward in part because numerous water suppliers have made treatment changes as previously described.

Similar trends in TTHM reduction technology and concentrations could be expected in Canada. The current Canadian national guideline for chloroform is 100 µg/L (0.1 mg/L) using tolerable daily intake calculations. A summer–winter survey of treated and distributed water from 53 selected water plants in 1993 found that TCM, dichloroacetic acid, and trichloroacetic acid were the major DBPs detected, and HAAs often equaled or exceeded TTHM concentrations (Williams et al. 1997). The population-weighted TTHM average was 30.8 µg/L. Thirty-seven plants used conventional disinfection and alum coagulation, and 15 only disinfected. Most (35) used pre- and post-chlorine dosage; total chlorine doses ranged from 0.1 to 5.75 mg/L (winter) and 1 to 13.6 mg/L (summer). Ammonia followed pre-chlorination in 10 facilities. Facilities (7) using ozone followed by chlorine or chloramine had total chlorine dosages from 0.5 to 3.3 mg/L (winter) and 0.5 to 4 mg/L (summer). TTHM levels in the distribution systems of chlorinating treatment plants ranged from 2.8 to 221.1 µg/L (mean 34.4, winter) and 0.3 to 342.4 µg/L (mean 62.5, summer). TTHM values following chloramine/chloramine or ozone/chloramine ranged from 0.6 to 42.1 µg/L (means 9.9–13.7, winter) and 2.5 to

FIGURE 2 Annual age-adjusted smoking prevalence and bladder and lung cancer incidence in Canada, 1965–2013



ASIR—age-specific incidence rate, TTHM—total trihalomethane

TABLE 1 Data sources for annual average TTHM concentration in drinking water in the United States

Time Period	Data Source	Sample Location	Statistical Summary	Number of Water Systems
1975	NORS	Finished water	Single samples	80
1975–1976	NOMS	Finished water	Single samples	111
1984–1986	AwwaRF	Distribution system	Single samples	727
1988–1989	35-city survey	Finished water	Single samples averaged over four quarters	35
1997–1998	ICR	Distribution system	Average of six quarterly samples	479
2006–2010	Six-Year Review	Distribution system	Single samples	167,000
2012–2015	Seidel et al. 2017	Distribution system	95th percentile quarterly samples	394

Source: McGuire et al. 2003, McGuire & Graziano 2002, McGuire & Meadow 1988

AwwaRF—AWWA Research Foundation, ICR—Information Collection Rule, NOMS—National Organics Monitoring Survey, NORS—National Organics Reconnaissance Survey, TTHM—total trihalomethane

107.8 µg/L (means 32.8–66.7, summer), respectively.

A 2009–2010 survey in 65 selected Canadian facilities indicated a decline in TTHM concentrations and reported a population TTHM average of 20.7 µg/L. Systems employed chlorination (51), chloramination (12), ozonation (8), and ultraviolet light (11). The average TTHM level of surface water facilities was 20.9 µg/L, and the average TTHM concentration in groundwater was 11.6 µg/L (Tugulea 2017).

SUMMARY

THMs have not been determined to be carcinogens under drinking water conditions as indicated by animal bioassays conducted in water rather than corn oil. If THMs correlate with cancer risk, it may be because they reflect the presence of other DBPs potentially present in greater numbers but at much lower concentrations. In the United States and Canada, TTHM concentrations have declined on the basis of published reports, compliance data, and water treatment information from national regulatory authorities. The national time trend bladder cancer data since the TTHMs were discovered and regulated do not reflect a strong linkage between TTHMs and bladder cancer incidence.

Bladder cancer is a disease of older age, and its etiology is complex, with many contributing factors of varying degrees. On the basis of this review, the potential for a measurable drinking water contribution to bladder cancer risk is not obvious, and causality associated with drinking water has not been established. Epidemiological studies using imprecise drinking water TTHM exposure assessments over the long term may include assumptions that have a greater effect on outcomes than the potential risks associated with TTHMs. Bladder cancer risk from drinking water and THMs, if any, is likely small, and it is probably overwhelmed by many other larger risk factors such as smoking, diabetes, and other country-specific factors. Gender and race/ethnicity remain important confounding factors in bladder cancer incidence.

Reproductive and developmental outcomes associated with TTHMs in drinking water were also updated in USEPA's Six-Year Review, and most of the studies it included were negative or inconsistent and/or occurred at maternally toxic doses and doses well above those found in drinking water. However, USEPA has stated continuing concerns. The Six-Year Review concluded that regulatory changes were not indicated at that time for TCM, DBCM, and TBM for toxicity-based MCLGs. With regard to BDCM, the Six-Year Review acknowledged data generated since the 2006 regulation but was not specific as to whether a revised MCLG would be appropriate.

Nevertheless, even though potential TTHM drinking water cancer risks remain questionable and likely small compared with several other factors, DBP management using surrogates continues to be an appropriate and practical strategy for maintaining drinking water quality and avoiding excessive unnecessary exposures. However, as reiterated by WHO, DBP management decisions should never compromise microbial disinfection efficacy, and they should reflect costs and identifiable benefits.

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