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20 February 2013

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Initial Submission for NRC Review of Styrene in NTP's 12th *Report on Carcinogens*

Dear Dr. Reisa and Members of NRC Styrene Review Committee:

SIRC is pleased the NRC Styrene Review Committee (NRC Committee) will review the National Toxicology Program's (NTP) *Report on Carcinogens (RoC), Twelfth Edition* listing of styrene as "reasonably anticipated to be a human carcinogen." By conducting this independent review, the NRC Committee will be able to determine whether NTP has conveyed to the public the best and most informative scientific conclusions regarding styrene and any potential it may have to cause cancer in humans.

Styrene is a well-studied chemical with a robust literature describing investigations of animal toxicology and associated mode of action, cancer epidemiology, and relationships of both genotoxicity and DNA adducts to cancer outcomes. These data provide a breadth of high quality information that can and should be used to inform an evidence based evaluation of the human cancer potential of styrene.

During the past 25 years, the Styrene Information and Research Center (SIRC)* has engaged in an extensive scientific investigation of the potential human health effects of exposure to styrene. Prior to the June 2011 publication of NTP's listing of styrene in the 12th *RoC*, SIRC-sponsored research had been reported in over 55 peer-reviewed publications and in five additional peer-reviewed studies from June 2011 to the present.

* The Styrene Information and Research Center's (SIRC's) mission is to evaluate existing data on potential health effects of styrene, and develop additional data where it is needed. SIRC has gained recognition as a reliable source of information on styrene and helping ensure that regulatory decisions are based on sound science. For more information, visit http://www.styrene.org/.

In its review, the NRC Committee will discover that there are two highly contrasting interpretations of this extensive body of literature concerning styrene.

In SIRC's view, an evidence-based interpretation of the toxicology and human data, including a mode of action evaluation, strongly indicates that styrene is not a human carcinogen.

In distinct contrast, the NTP, using the *RoC's* listing criteria, concluded that the toxicology, mode of action and epidemiology data justified listing styrene as "reasonably anticipated to be a human carcinogen." For reasons that are explained in some detail herein, **SIRC believes that the styrene data do not satisfy any of the NTP's three listing criteria for such a classification, nor is NTP's *RoC* evaluation consistent with an evidence-based evaluation of the data.**

SIRC has closely observed and participated in NTP's review of styrene from its initial proposal to conduct a styrene review in 1994 until the final release of the 12th *RoC* in 2011. On several occasions during the *RoC* review process, SIRC submitted objections to NTP's interpretation of the literature, informed them of key preliminary findings from SIRC's ongoing research program, and presented what SIRC finds to be the most likely interpretation of the data.

Because the *RoC's Styrene Substance Profile* primarily provides only NTP's interpretation of the styrene data, the details of these two contrasting interpretations of the literature may not be readily apparent to the NRC Committee from its initial review of the *Styrene Substance Profile* and the supporting information available from NTP.

SIRC has therefore focused this initial submission on **highlighting these differences in interpretation** in order to aid the committee's independent review of the NTP's decision.

These comments include:

- A summary of the basis for SIRC's integrated evidence-based assessment that styrene is not a human carcinogen.
- A detailed evidence-based critique of NTP's assessment in the *RoC Styrene Substance Profile*, in which NTP posits that the styrene data meet the listing criteria for "reasonably anticipated to be a human carcinogen."

Sincerely,

Jack Snyder, Executive Director
Styrene Information and Research Center

C O N T E N T S

OVERVIEW OF SIRC'S EVIDENCE-BASED INTERPRETATION OF THE SCIENTIFIC LITERATURE

SIRC's interpretation of the scientific literature is summarized below according to the following three bodies of evidence that are important to a consideration of styrene carcinogenicity:

- Data from studies with laboratory animals and consideration of mode of action (MoA);
- Data from epidemiological studies on workers exposed to styrene; and
- Data relevant to interpreting information used by NTP in its review of styrene regarding potential genotoxicity and DNA adduct formation.

Also provided is information regarding all relevant animal, MoA, epidemiological and other data in a comprehensive review of their relevance to human health.

Additional details of SIRC's interpretation of the data are provided in the latter part of this paper where NTP's interpretation of the data is critiqued in detail.

Animal Data

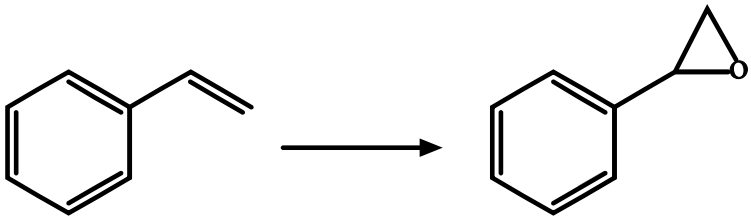
SIRC has attempted to understand the health effects of styrene by hypothesis generation and testing. Given the progression of experimental findings over time, SIRC has refined the styrene cancer hypothesis to align with a consistent, coherent, and scientifically plausible interpretation of the data (see next page).

Animal bioassays

In 1993, SIRC concluded that the existing chronic animal studies of styrene were inadequate for risk assessment purposes. In consultation with the U.S. Environmental Protection Agency and the NTP, SIRC initiated state-of-the-art chronic inhalation exposure studies in both rats and mice.

Upon the completion of these rodent inhalation studies in 1998[1] and 2001,[2] an evaluation of all eight chronic studies of styrene in rats concluded that styrene does not increase tumors of any kind in rats. An inhalation study of styrene in mice resulted in increased lung tumors; two of four gavage studies in mice suggested increased lung tumors. Thus, the animal bioassay data indicate that the only tumor of concern is mouse lung tumor. However, as explained below, the data do not meet the *RoC* listing criteria of "sufficient evidence" of increased tumors by two routes of administration.[3]

Default hypothesis: Styrene causes mouse lung tumors via its main metabolite styrene oxide (SO), and because SO also is a major metabolite of styrene in humans, styrene mouse lung tumors are regarded as relevant to potential human cancer risk (this is also the hypothesis of the NTP *RoC*)



Styrene **Styrene-7,8-Oxide**

Based on finding tumors in the mouse lung, SIRC initially investigated the hypothesis that the MoA for mouse lung tumors was associated with the major metabolite of styrene, styrene-7,8-oxide (SO), produced primarily by the actions of the enzyme CYP2E1 on styrene's vinyl side-chain. However, the data did not support that hypothesis even though styrene is metabolized to SO via the vinyl side-chain. There is much more SO in blood and lung in rats at non-tumorigenic exposures than there is at tumorigenic exposures in mice.[1,2,4] Furthermore, pharmacologic inhibition of CYP2E1 or removal of CYP2E1 (*i.e.*, through knockout mice) did not reduce styrene lung toxicity.[5]

SIRC also considered whether or not R-SO was the toxic metabolite. Research showed organs in rats and mice that were subject to styrene-induced toxicity produced much more R-SO than S-SO.[6] However, single-exposure and 5-day exposure studies using different assay methods demonstrated no difference in lung toxicity between R-SO and S-SO.[5] Therefore, enantiomeric-specific toxicity of SO was also not supported.

Data relevant to the genotoxic-via-SO hypothesis are summarized in Table1

Table 1. Evaluation of hypothesis that styrene induces mouse lung tumors via a genotoxic MoA through SO

Key Findings	*In vitro*	Mouse	Rat	Human	Reference
Styrene negative in Ames assays	Not Supporting				64
Genotoxicity studies of SO	Supporting	+/-	+/-	No data	30
No CA in lungs of mice exposed to styrene		Not supporting			66
No lung tumor initiation in Strain A mice		Not supporting	No data	No data	65
No increased lung tumors from SO		Not supporting	Not supporting	No data	63
Lung toxicity from SO		Supporting	Not supporting	No data	5,6
No decreased lung toxicity when SO decreased (2E1-KO mice)		Not supporting	No data		5
Blood SO rats > mice		Not supporting	Not supporting		4
Lung SO *ex vivo* rats> mice		Not supporting	Not supporting		5
Urinary SO-derived metabolites rats > mice		Not supporting	Not supporting		6
Lack of SO lung toxicity in 2F2-KO mice		Not supporting			13,14
DNA adducts in rats > mice		Not supporting	Not supporting		6
Forestomach tumor incidence in rats			Not supporting		62

Current hypothesis: Styrene causes mouse lung tumors via ring-oxidized cytotoxic metabolites* generated by mouse lung CYP2F2

As the data challenging the SO-associated hypothesis were discovered, a growing body of evidence indicated various chemicals** that are structurally similar to styrene caused lung tumors in mice but not in rats. Several of these substances were also metabolized by CYP2F2 in mouse lung.[2] Importantly, Clara cells, which are the primary target cell for styrene mouse lung toxicity, also are the cell type in which styrene is metabolized by CYP2F2.[7]

Starting in 2001, as an alternative to an SO-mediated MoA, SIRC hypothesized that the generation of ring-oxidized metabolite(s) catalyzed by CYP2F2 rather than the vinyl side chain metabolite SO (via CYP2E1) were responsible for lung toxicity. Several observations prompted this change in direction, including:

- Styrene analogs with a methyl group on the 3 or 4 position*** of the benzene ring do not cause lung tumors;[8,9]
- 4-hydroxystyrene (4HS), one of the ring-oxidized metabolites, was much more toxic to mouse lung than styrene or SO;[10,11] and,
- Ethylbenzene, which does not contain a vinyl moiety, is also a mouse lung carcinogen.[12]

Consistent with this hypothesis, pharmacologic inhibition of CYP2F2 was found to reduce the toxicity of styrene in mouse lung.[5] To avoid confounding influences of pharmacologic inhibitors, SIRC next commissioned research to develop a CYP2F2-knockout mouse to test this hypothesis more definitively. No lung toxicity from either styrene or SO was seen in these knockout mice. Importantly, the absence of SO toxicity in knockout mice further confirmed that SO is not the proximate lung toxic metabolite of styrene.[13,14]

Data relevant to the cytotoxic-via-ring-oxidation hypothesis are summarized in Table 2.

* The metabolites include 4-hydroxystyrene, 3.4-dihydroxystyrene and possibly 4-hydroxystyrene oxide.

** Substances that cause lung tumors in mice but not in rats include styrene, naphthalene, ethylbenzene, cumene (isopropylbenzene), alpha-methylstyrene, divinylbenzene, benzofuran and coumarin.

*** Side chains in these positions on the benzene ring block formation of hypothesized catechol and/or quinone metabolites.

Table 2. Evaluation of hypothesis that styrene induces mouse lung tumors via a cytotoxic MoA through CYP2F2 metabolism

Key Data	*In vitro*	Mouse	Rat	Human	Reference
Styrene negative in Ames assays	Supporting				64
Lung tumors in mice, not rats		Supporting	Supporting		1,2
Lung toxicity in mice, not rats		Supporting	Supporting		6
Toxicity and metabolism in Clara cells		Supporting			6
Lung toxicity from 4HS in mice, not rats		Supporting	Supporting		11
Elimination of lung toxicity from styrene and SO in 2F2-KO mice		Supporting			18
Elimination of ring-oxidation of styrene and 4HS in 2F2-KO mice		Supporting			19
Greater lung toxicity from 4HS than SO		Supporting			10
More ring-oxidized urinary metabolites in mice than in rats		Supporting			6
Limited toxicity from 4HS in 2F2-KO mice		Supporting			18
Styrene analogs (3- or 4-methyl) do not induce lung tumors in mice		Supporting			8,9
Ethylbenzene (no vinyl epoxide) induces mouse lung tumors		Supporting			12
Clara cell toxicity in mice from EB, not rats		Supporting	Supporting		6

Hypothesis: Styrene-induced mouse lung tumors are not quantitatively, and likely qualitatively, relevant for human risk

SIRC hypothesizes that the finding of lung tumors in mice, but not in rats, is based on a cytotoxic and non-genotoxic MoA that occurs to a biologically meaningful degree in mice (tumor responders), but not in rats (tumor non-responders). The mouse-specific mode of action is focused on the formation of ring-oxidized metabolites (catechol and/or quinones) caused by oxidative metabolism catalyzed by CYP2F2. The cytotoxic activity of these metabolite(s) results in reparative cell replication of the terminal bronchioles, the site of origin of mouse lung toxicity and tumors, and eventually progresses to a low incidence of late-developing tumors.

The differential sensitivity of mouse relative to rat lungs is consistent with the observation that rat lungs contain much less CYP2F4, produce much less ring-oxidized metabolites[15] and do not develop lung toxicity or tumors despite substantially higher styrene exposures.[1] Importantly, human lungs have far less amount/activity of the orthologous CYP2F enzyme, CYP2F1. Although one paper reports that bacterially expressed CYP2F1 readily metabolizes styrene, others report very little if any metabolism of styrene by microsomes from human lungs, or from human cell lines that over-express CYP2F1.[5] These findings parallel those obtained with ethylbenzene, in which microsomal metabolism to reactive ring-oxidized metabolites was higher in mice than in rats and absent in humans.[16,17]

The potential quantitative (or qualitative) relevance of CYP2F metabolism of styrene in humans was further examined more recently in a "humanized" transgenic (TG) CYP2F mouse. This mouse model lacks CYP2F2 and expresses CYP2F1 (CYP2F2(-/-)/CYP2F1,2A13,2B6-transgenic). Neither styrene nor SO caused lung toxicity in these TG mice, despite confirmation that CYP2F1 was functionally active.[18]

Therefore, SIRC concludes that mouse lung tumors are not quantitatively (or likely qualitatively) relevant to human cancer outcomes.

Data relevant to the hypotheses that styrene induced mouse lung tumors are not relevant to humans are summarized in Table 3.

Table 3. Evaluation of hypothesis that styrene-induced mouse lung tumors are not relevant to human risk

Key Data	*In vitro*	Mouse	Rat	Human	Reference
CYP2F1 metabolism limited *in vitro*				2 studies supporting 1 not supporting	5
Lung microsomes make ring-oxidized metabolites mouse > rat > human		Supporting	Supporting	Supporting	5
Lack of styrene toxicity in 2F1-TG mice		Supporting		Supporting	18
Lack of SO toxicity in 2F1-TG mice		Supporting		Supporting	18
No styrene excess lung tumors in human studies				Supporting	25

An evidence-based assessment of the animal toxicology and epidemiology data strongly indicates mouse lung tumors are not relevant to humans

Although the majority of styrene is metabolized through SO in liver and lung in both rodents and humans, SO is not the cause of mouse lung toxicity from styrene.

- Reduction of liver and lung SO production by eliminating CYP2E1 did not reduce lung toxicity from styrene.[18]
- Eliminating CP2F2 metabolism completely eliminated lung toxicity from styrene even at near lethal doses.[14,19]
- SO does not produce lung toxicity unless it is ring-oxidized by CYP2F2 (no SO toxicity observed in CYP2F2 knockout mice).[18]
- Thus, SIRC finds compelling evidence that the data support the hypothesis that ring-oxidized metabolites, not SO, cause cytotoxicity to Clara cells and that the data do not provide evidence of genotoxicity in mouse lung.

Furthermore, absence of styrene or SO toxicity following insertion of the human CYP2F1 into the CYP2F2-knockout mice indicates that humans are not likely to produce sufficient ring-oxidized metabolites from either styrene or SO to cause toxicity.[18]

Expert reviews sponsored by the European Union,[20] Denmark[21] and the Texas Commission on Environmental Quality[22] concluded that the potential for styrene to cause cancer in humans was low enough that public warnings are not needed even though these authorities assumed SO played a key role in mouse lung tumors (these reviews were conducted *before* the recent MoA work on styrene was published).

Human Epidemiology Studies

In addition to the mode of action data just discussed, SIRC's hypothesis that styrene-related mouse lung tumors are not relevant to human health is supported by an extensive and robust body of epidemiology data:

- A cohort analysis of about 15,000 styrene-butadiene rubber (SBR) workers suggested an increase in non-Hodgkin lymphoma (NHL) based on cumulative exposure. However, the study findings were confounded by a very low incidence of NHL among unexposed workers in the cohort (36% of expected),[23] and relative risks did not display a consistent exposure-response relationship. No other cancer type was increased in the SBR cohort; specifically, there was no increase in pancreatic or esophageal cancer (see next bullets).
- Three cohorts of reinforced plastics (RPC) workers involving about 50,000 individuals exhibited no increase in leukemia or lymphomas relative to cumulative exposure to styrene,[24,25] and only a borderline increase in malignant lymphomas in one of the three studies relative to average exposure.[26] NHL was not increased in these cohorts.
- The three RPC studies had average follow-up times of 13, 19 and 30 years, with the latter two long enough for a reasonable evaluation of risk. Importantly, exposures of RPC workers were at least ten times higher than SBR workers and without confounding exposure to butadiene. Two of the three RPC studies suggested increased pancreatic or esophageal cancer.
- In contrast to the SBR cohort, NHL was not increased in the RPC cohort and may have been less than expected. A follow-up analysis of the U.S. RPC cohort generated since the *RoC* was published has further supported these conclusions.[27] No increase in NHL was detected based on either cumulative or average exposure, despite more than 30 years of follow-up for at least 85% of the members. Although two of the three earlier RPC cohort studies suggested an increased incidence of pancreatic and esophageal cancer, an increase in esophageal or pancreatic cancer was not detected in the follow-up study.
- When all three RPC studies are combined, there is no evidence of an increase in any type of cancer. (Table 4)

Table 4. Updated epidemiology results based on total cohort mortality analysis in RPC industry

	Ruder		Collins (update of Wong)		Kogevinas		Total	
Endpoint	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp
Total LH	16	21.6	106	126.2	60	64.4	182	212.2
NHL	9	9.1	36	50.0	15	19.5	61	78.6
Esophagus	12	5.3	36	34.0	17	20.7	65	62.0
Pancreas	14	9.8	63	65.6	37	36.9	114	112.3

The robust epidemiology dataset supports SIRC's hypothesis that styrene-related mouse lung tumors are not relevant to human health. Our reasoning is as follows: if styrene acts as a genotoxic, non-threshold carcinogen, as NTP concluded, then a higher dose (seen in the RPC cohort) should lead to a greater association between exposure and the observed effect. It did not. Additionally, the risk should be equally increased in short- and long-term workers as a function of their cumulative exposure.

Furthermore, the theory behind a genotoxic MoA is that the agent causes DNA damage and the risk of developing cancer is proportional to the number of "hits" of the agent on the DNA. This MoA clearly indicates that cumulative exposure is more important than average exposure or duration of exposure as a risk factor for genotoxic carcinogens. Because the results of the RPC studies had much higher cumulative exposures and less confounding from other carcinogens, they should be given the most weight. Also, cumulative exposure is a more meaningful metric than is average exposure (further discussed beginning on page 15).

Based on the styrene epidemiology data existing at the time of the *RoC* review[28] and further strengthened by additional data published since that time, the data do not support any association between styrene exposure and cancer of any type. Therefore, the human data provide neither sufficient nor limited evidence of human carcinogenicity.

Genotoxicity

Styrene and similar chemicals are largely negative in standard genotoxicity assays. Styrene is negative in *in vitro* Ames assays. Styrene is positive in *in vitro* chromosomal aberrations (CA) studies, but negative in a large number of *in vivo* CA and micronucleus studies in laboratory rats and mice; 11 of 12 and 5 of 7 studies were negative for CAs or MN respectively. In addition, two *in vivo* genotoxicity (CA and lung tumor initiation) assays in mouse lung were negative.[29,30] Findings of CA in workers are inconsistent, with about half of the 30 studies of CAs in workers reported increased CAs from styrene exposure.

Over the past 40 years, occupational styrene exposure has decreased by at least two-fold, but the percentage of CA studies in workers reported as positive did not decrease with decreasing styrene exposure, raising a question of whether styrene is the cause. While fewer genotoxicity studies have been conducted on styrene analogs than on styrene, the genotoxicity data on these analogs is even more clearly negative than the generally negative data for styrene.

Overall, the genotoxicty data for styrene do not provide additional support that a causal association with cancer is credible.

DNA Adducts

Low levels of SO-DNA adducts (< 1 per 10^7 nucleotides) found equally in rats and mice[30] suggest that DNA adducts cannot explain why mice get lung tumors and rats do not. Further, DNA adducts were not greater in mouse lung than in mouse liver,[30] thus lacking target organ specificity. Likewise, the finding of very low levels of DNA adducts in styrene workers, when evaluated in the context of similar levels of SO-DNA adducts found in both mouse and rat studies, does not suggest that mouse lung tumors are related to a genotoxic MoA via DNA adduct formation.[6]

NTP provides no data or compelling analysis supporting a potential role of similar low levels of DNA adducts found in humans cause of any type cancer.

Summary of SIRC's Interpretation of the Literature

An increase in mouse lung tumors is the only evidence of styrene-related tumorigenicity in chronic rat and mouse studies. A comprehensive series of MoA investigations is entirely consistent with the hypothesis that CYP2F2 metabolism of styrene in mouse lung causes cytotoxicity via ring-oxidized metabolites, and that chronic injury is the source of the low-incidence of late developing lung tumors.

In contrast, the overall evidence is not consistent with a genotoxic mode of action mediated through either styrene or SO. Styrene and similar compounds

are primarily negative in genotoxicity assays and, specifically, styrene was negative in *in vivo* genotoxicity assays in mouse lung.

Likewise, the finding of very low levels of DNA adducts in styrene workers, when evaluated in the context of similar levels of SO-DNA adducts found in both mouse and rat studies, does not suggest that mouse lung tumors are related to a genotoxic MoA via DNA adduct formation.

SIRC's interpretation of the animal data and MoA derived from the animal data is also consistent with an absence of increased cancer risk observed in large cohorts of workers with relatively high and specific exposures to styrene.

SIRC believes that the animal and epidemiology data do not meet the *RoC* listing criteria, as detailed on beginning on page 13 of this paper, but even if they did, styrene should not be listed in the *RoC* based on the mode of action data for mouse lung tumors, which clearly demonstrates a non-genotoxic MoA based on CYP2F2-generated ring-oxidized metabolites. Furthermore, mouse lung tumors are not quantitatively (or likely qualitatively) relevant to human cancer outcomes.

NTP's View of Styrene and Carcinogenicity

The NTP *RoC* evaluation of styrene presents a very different interpretation of the styrene data. NTP offers a number of possible supports for a cancer listing for styrene, but relies primarily on, and offers the greatest amount of support for, the SO-mediated mode of action hypothesis.[31,32]

The balance of this document provides a more detailed evidence-based, point-by-point analysis of NTP's hypothesis outlined in the *RoC Styrene Substance Profile*; evaluates whether the styrene data satisfy the NTP's three criteria for such a listing of styrene; and considers if NTP has properly applied the best and most informative scientific conclusions regarding styrene and any potential it may have to cause cancer in humans.

REVIEW OF THE STYRENE ASSESSMENT IN THE NATIONAL TOXICOLOGY PROGRAM 12TH *REPORT ON CARCINOGENS* — AN EVIDENCE-BASED OVERVIEW

Introduction

In the 12th *Report on Carcinogens (RoC)*, NTP found "Styrene is *reasonably anticipated to be a human carcinogen* based on limited evidence of carcinogenicity from studies in humans, sufficient evidence of carcinogenicity from studies in experimental animals, and supporting data on mechanisms of carcinogenesis" (emphasis in original).[33]

SIRC believes NTP's conclusion is unwarranted for several reasons, including:

- The data that were available to NTP do not fulfill NTP's listing criteria;[34]
- A detailed evidence-based review of the data does not support a cancer concern for styrene; and
- Peer-reviewed scientific information which has become available since the release of the 12th *RoC* adds additional weight to the conclusion styrene is not a human carcinogen.

SIRC welcomes the NRC Committee's independent determination of whether or not NTP's listing of styrene – and any potential exposure to styrene may have to cause cancer in humans – comports with an evidence-based interpretation of the science. SIRC also urges the NRC Committee to evaluate NTP's procedures and listing criteria as they were applied to styrene and to recommend any changes that may be appropriate.

Note: For ease of comparison, this discussion follows the human, animal and other data sequence used by NTP in its Styrene Substance Profile.

Cancer Studies in Humans

NTP first justifies its styrene classification in the 12th *RoC* by stating styrene meets its listing criterion of "… limited evidence of carcinogenicity from studies in humans…" This criterion calls for the data to indicate that "a causal interpretation is credible" where "alternative explanations … could not be adequately excluded."[3]

The science does not support NTP's finding of "limited evidence" based on human studies

NTP's Styrene Substance Profile states:

Discussion of DNA adducts and genetic studies begins on page 19.

- "Studies on workers…showed (1) increased mortality from or incidence of cancer of the lymphohematopoietic system and (2) increased levels of DNA adducts and genetic damage in lymphocytes from exposed workers."[33]
- "The multi-plant cohort of male styrene-butadiene rubber (SBR) workers found significantly increased risks (SMRs [standardized mortality ratios]) of non-Hodgkin's lymphoma (NHL) … among subgroups of workers…"[35]

NTP spells out its logic for its human data finding in the first paragraph of the *RoC*, and it can be summarized as follows:

- Evidence of increased mortality from or incidence of lymphohematopoietic system cancer such as NHL, based on various measures of exposure, in some (but not all) groups of workers; plus
- Some evidence of increased risks of esophageal and pancreatic cancer among workers (but causality is not established) … therefore "…a causal relationship between styrene exposure and cancer in humans is credible;" and
- NTP's conclusion is supported by findings of DNA adducts and chromosomal aberrations in lymphocytes from styrene-exposed workers.

SIRC believes this reasoning has several substantial weaknesses, if not actual flaws. The following analysis addresses these weaknesses by key topics used by NTP, namely lymphohematopoietic effects, other cancer types, genetic damage and DNA adducts.

Lymphohematopoietic cancers

The "significantly increased" NHL levels among the SBR cohort that NTP cited is highly questionable because (1) NTP's use of the term "increased" is not justified by the data; (2) the finding of an increase in NHL in the SBR cohort was confounded by a very low incidence of NHL among unexposed subjects in that study, and importantly was not corroborated by studies of RPC workers with much higher styrene exposures, no cofounding exposures to known carcinogens and an equivalent number of long-term workers; (3) the flawed application of average exposure in these particular studies as a key metric; (4) NTP's dismissal of two additional studies of RPC cohorts as "less informative;" and (5) disagreement with the *RoC*'s interpretation of the epidemiology data by multiple authors.

1) **NTP's use of the term "increased" for NHL is not justified**. In the *RoC*, NTP appears to refer to any numerical SMR greater than 1.0, regardless of how small, as an "increase." The *RoC* determination relies heavily on such "increases" that were not significantly different than expected (the 95% confidence interval included values less than 1.0).

- Table 3-8 of the *Styrene Background Document*[36] used by NTP to develop its *Styrene Substance Profile* includes an analysis of "Relative occurrence of cancer in 12 cohort studies of populations exposed to styrene" which illustrates NTP's unsupported definition of "significant increase."

  As shown in Table 5, of the 184 statistical comparisons made across all of the cohorts, 103 reported decreased SMRs, 79 reported increased SMRs and only two were neither increased nor decreased. Based on NTP's assessment of NHL in Table 3-8 of NTP's *Styrene Background Document*, four studies reported an increased SMR, three reported a decreased SMR and one had an SMR of 1.0 (none of the eight studies was statistically significant).[36]

Table 5. Summary of epidemiology studies characterized in Table 3-8 of NTP's *Styrene Background Document*, which covered 12cohorts from all three industries

	+	(+)	+/-	(-)	-	Total
All endpoints	11	68	2	97	6	184
All LH	1	5	0	5	0	11
NHL	0	4	1	3	0	8
Esophagus	2	1	0	4	0	7
Pancreas	0	5	1	3	0	8

RoC definitions:

+ SMR >1 and statistically significant
- SMR<1 and statistically significant
+/- SMR = 1
(+) SMR>1, but not statistically significant
(-) SMR<1, but not statistically significant

2) **NTP mischaracterized a 2006 study**[23] that reported statistically-elevated internal rates of NHL and NHL/chronic lymphocytic leukemia (CLL) combined among SBR workers with "highest" styrene cumulative exposure (61.1 or more ppm years). In its discussion of the study, NTP failed to describe that the report itself qualified this result by pointing out that the incidence of NHL was much lower (about 64%

The author of this study disagreed with NTP's interpretation of her study. See page 17.

lower than expected) "… among unexposed subjects compared with external referent populations. For CLL-NHL, results were similar to those for NHL."[37]

Importantly, the increase in NHL based on cumulative exposure in the SBR cohort is not supported by a similar finding in the RPC cohorts, even though the three independent RPC cohorts experienced a ten-fold higher cumulative exposure to styrene that was largely unconfounded by exposures to known or suspected carcinogens.

As the authors of the Delzell SBR study stated:

"Notably, studies of occupational groups exposed to concentrations of styrene higher than those found in the synthetic rubber industry have not reported any consistent increase in NHL deaths or incident cases."[37]

3) **NTP's analysis based on average exposures is flawed**. In its discussion of the Kogevinas RPC study, the *RoC* states:

"No significant relationship with cumulative exposure was observed, although statistically non-significant elevated risks for lymphoma were found for all groups with cumulative exposure greater than 75 ppm. The proportion of short-term workers was higher among the workers with the highest exposure levels (laminators); therefore, measures of exposure intensity (such as average exposure level) may be more informative than measures of exposure duration for evaluating risks."[35]

NTP does not provide a more detailed supporting discussion of why average exposure "may be more informative than measures of exposure duration."

In support of its "limited evidence" conclusions, NTP seems to assume that average exposure was estimated in Kogevinas and multiplied by duration to reach a cumulative exposure and that uncertainties related to duration of exposure for some workers would therefore only affect cumulative exposure findings.

This is mistaken. In fact, both a cumulative exposure and duration of exposure were estimated for each member of the cohort; the average exposure was calculated by dividing cumulative exposure by duration.

Thus, any uncertainties in duration of exposure would affect cumulative and average exposure analyses equally.[26]

In other words, the *RoC* implies that average exposure is a superior exposure metric for cohorts with many short-term workers. However, this view is based on a misunderstanding of how average exposure was determined in this study; in addition, SIRC has not been able to locate a citation in the literature that would support NTP's preference for average exposure over cumulative exposure. Therefore, a borderline increase in lymphoma in one of three RPC studies based on average exposure, where cumulative exposure did not show an effect, is not evidence of an association of styrene with lymphoma.

Additionally, the SMR for the highest exposed group of the Kogevinas RPC study was less than for the two lower exposed groups, the lower 95% confidence

intervals were between 0.34 and 0.82, and the p-value for the trend was 0.52; these do not represent a styrene effect on lymphomas. The other two major RPC cohorts did not find an elevated rate of lymphoma but also did not employ average exposures as a metric.

4) NTP incorrectly dismissed as "less informative" studies of two major RPC cohorts.[33]

NTP dismissed these studies for less than adequate reasons[28] even though NTP admits that "Neither of the two U.S. cohort studies of reinforced plastics workers found a significant association between styrene exposure and lymphohematopoietic cancer…"[35]

- Ruder *et al.*, (2004) is dismissed by NTP as being too small and for lacking an average exposure metric;
- Wong *et al.*, (1994) was criticized by NTP for not reporting results by average *exposure in addition to the exposure duration and cumulative exposure metrics* it did report; and
- While NTP recognized that "workers in the reinforced-plastics industry were exposed to the highest levels of styrene and they had few other potentially carcinogenic exposures," NTP was critical of the RPC studies because "the majority of workers had short periods of employment."[33]

In fact, based on length of follow-up, both the Ruder and Wong RPC cohorts add significantly to the evidence that a causal relationship between styrene exposure and cancer in humans **is not credible**. NTP's mistaken reliance on average exposures is discussed above.

Regarding NTP's criticism of the length of employment, the RPC cohorts have almost the same number of long-term workers as the SBR cohorts (when combined, the Kogevinas and Wong cohorts include about 6,700 workers exposed to styrene for longer than 10 years versus about 8,960 workers in Delzell).

Regarding the relevance of length of employment to the usefulness of the cohort, if one accepts NTP's hypothesis that styrene is acting as a genotoxic, non-threshold carcinogen, it follows that risk should be equally increased in short and long-term workers as a function of their cumulative exposure. A review of cumulative exposure in these studies does not support dismissing the RPC studies:

- Cumulative exposure (SBR)
  - Delzell: 8,962 persons exposed to greater than 13 ppm-years.
- Cumulative exposure (RPC)
  - Kogevinas: 30,516 persons exposed to greater than 75 ppm-years.
  - Wong: 11,870 were exposed to greater than 10 ppm-years and 7,910 of those were exposed to greater than 30 ppm-years.

Further, since the publication of the 12th *RoC*, Collins has updated Wong with an additional 19 years of follow-up, increasing even more the relevance of this cohort incorrectly dismissed by NTP.[27]

The Collins update includes more years at risk than Kogevinas and did not find increased risk for total lymphohematopoietic cancers (although the various leukemias and lymphomas should not be combined), for NHL by either cumulative or *average* exposure metrics, or for esophageal cancer or pancreatic cancer.

When summed across all three RPC studies, if there is any effect, it is a deficit in total LH and NHL cancers and no increase in esophageal or pancreatic cancer. (Table 4)

5) During NTP's styrene assessment process, multiple authors disagreed with NTP's conclusions based on human data:

- In 2009, Dr. Delzell (corresponding author for one of the studies of SBR workers NTP relies on for its determination of "limited evidence" in human studies) submitted a letter to NTP regarding its then draft *Styrene Substance Profile*. Criticizing the NTP draft conclusions and arguing that there is less-than-limited human evidence supporting a cancer concern for styrene, Dr. Delzell stated:

  "The available scientific evidence is not sufficient to conclude that styrene causes lymphoma, leukemia or other cancers. In particular, the lack of consistency, reasonably precise associations between estimated exposure to styrene and NHL or leukemia in the studies of reinforced plastics industry workers is an important shortfall of the evidence for the hypothesis that styrene causes these cancers. This shortfall is not overcome by data from studies of synthetic rubber industry workers, due to the potential for residual confounding by butadiene and the very low levels of styrene experienced by workers in the latter industry."[38]

- Boffetta and his distinguished colleagues (2009) reviewed all of the styrene epidemiological data plus the recommendation of the NTP's styrene Expert Panel regarding the Delzell study, which NTP later incorporated into its S*tyrene Substance Profile,* and did not share NTP's concerns. In fact, this review concluded: "The evidence for human carcinogenicity of styrene is inconsistent and weak. On the basis of the available evidence one cannot conclude that there is a causal association between styrene and any form of cancer."[39]

- Rhomberg *et al.* (2013; presented to NTP during styrene-related *RoC* deliberations in 2009) developed a detailed hypothesis-based review of NTP's classification of styrene in the 12th *RoC*. The authors concluded: "In our view, when all the human evidence is evaluated, and the low numbers of observed cases and the lack of consistent patterns in cancer outcomes within and across cohorts, combined with concerns about co-exposures and confounding are considered, one comes to the conclusion that a causal relationship between styrene exposure and human cancer is not credible, and the standards of 'limited' evidence are not met."[40]

Other cancer types

The *RoC* cites small differences in deaths from pancreatic or esophageal cancer as supporting evidence.[35] The relevant information from the studies of RPC and SBR cohorts includes:

1) **Reinforced plastics and composites (RPC) industry studies**

Both Ruder and Wong reported more pancreatic and esophageal cancer deaths than expected, but the increases were in the second highest exposure groups, not the highest. Over all three RPC cohorts, there were 43 deaths from esophageal cancer (vs. 33.3 expected; SMR = 1.29 (0.95-1.74)) and 70 from pancreatic cancer (vs. 63.5 expected; SMR = 1.10 (0.86-1.40)); the actual observed rates for these cancers are not significantly higher than expected. Table 4 provides these data updated by Collins; in the updated combined data, there is no indication of esophageal (SMR = 1.05 (0.81-1.34)) or pancreatic SMR= 1.02 (0.84-1.22)) cancer.

2) **Styrene-butadiene rubber (SBR) industry studies**

Neither pancreatic nor esophageal cancer was increased in the SBR cohort.

Rhomberg reviewed this information and reported:

> "In certain studies there were some statistically significant associations noted with some styrene exposure metrics for lymphohematopoietic, esophageal, and pancreatic cancers, but the risk estimates were not markedly large (*i.e.*, most were below 2) and were far outnumbered by null associations for each cancer type. In addition, most analyses were based on a small number of observed cases, which resulted in unstable estimates, vis-à-vis wide confidence intervals that either included or were generally close to 1. Furthermore, there were significant and non-significant negative associations reported for certain cancer types that were often as strong as positive associations reported for others. Just as it is unlikely that these negative associations are reflective of a protective mechanism for styrene, the few positive associations are unlikely to reflect a causal association."[41]

In summary, the data available to NTP at the time of the 12th *RoC* do not support a conclusion that a "causal" association between styrene and human cancer is "credible;" furthermore, more recent data casts even greater doubt on any association between styrene and human cancer. The human data available to NTP in 2011, as well as more recently-published data 1) are consistent with SIRC's interpretation of the animal data; 2) specifically counter NTP's proposed interpretation of a genotoxic mode of action; and 3) do not support NTP's conclusion of "limited" human evidence. Thus, a listing of styrene as "reasonably anticipated to cause cancer in humans" based on epidemiological evidence is not supported.

DNA Adducts

NTP's *Styrene Substance Profile* states: "Detection of styrene-7,8-oxide—DNA adducts at base pairing sites and chromosomal aberrations in lymphocytes of styrene-exposed workers supports the potential human cancer hazard from styrene through a genotoxic mode of action."[42]

Styrene mode of action for mouse lung tumors is discussed on pp. 4-7.

NTP's use of data regarding DNA adducts to support "limited evidence" in styrene-exposed workers is misleading because, as discussed earlier, there is no observed increased cancer risk in humans and NTP provides no data or compelling analysis supporting a role of DNA adducts in causing any of the cancers reported in the human studies, or for that matter, in animal tumor findings.

As indicated in the *RoC*, DNA adducts have been reported in some studies of styrene-exposed workers. Boffetta concluded, after reviewing all of the epidemiological studies as well as NTP's conclusion that the Delzell study supports a finding of limited human evidence: "one cannot conclude that there is a causal association between styrene and any form of cancer."[39] In referencing the Boffetta report in the *RoC*, NTP chose not to quote this conclusion. However, the NTP does cite the Boffetta *et al.* review with regard to its observations regarding genotoxicity, but here too fails to include the caveats of the Boffetta report that make it clear that Boffetta *et al.* do not support NTP's view that "a causal interpretation is credible." Specifically, in their report the Boffetta *et al.* reviewers question the plausibility of a genotoxic MoA for styrene in humans:

"Several studies showed low levels of DNA adducts in lymphocytes of workers exposed to styrene. Limited by their small size and lack of control for potential confounders, these studies provide evidence for a genotoxic effect of styrene in humans, probably mediated by the metabolite, styrene 7,8-oxide. Several issues should be considered in the interpretation of DNA adduct data on NHL risk. Following styrene exposure, rats and mice form adducts similar to those found in humans. Although levels are higher in rats, no excess cancer incidence has been detected. Furthermore, agents known or suspected to cause NHL in humans are believed to act through immune disregulation rather than through DNA damage."[43]

Similarly, Rhomberg observed:

"Extensive data show, however, that a genotoxic mode of action for styrene is unlikely. Although SO can adduct to proteins and DNA, low levels of SO-DNA adducts have been observed *in vivo*. Increases in SO-DNA adducts have not been observed in mouse *versus* rat lung or mouse lung *versus* mouse liver after inhalation exposure to styrene; thus, the increased incidence of lung tumors in mice is not accompanied by an increase in DNA adducts."[44]

Although DNA adducts formed in both liver and lung tissues are derived from styrene oxide, mode of action investigations strongly indicate that mouse lung toxicity and tumors derive from a different metabolite.

Genetic Studies: Chromosomal Aberrations (CA) and Micronucleus (MN)

In the 12th *RoC*, NTP states: "Both styrene and styrene-7,8-oxide caused cytogenetic effects (sister chromatid exchange, chromosomal aberrations, and micronucleus formation) in human lymphocytes or other mammalian cells *in vitro*."[42] This information is not compelling when placed in the context of the full genotoxicity database for styrene and similar chemicals.

An analysis of the literature cited in the 1994[45] and 2002[46] assessments of styrene carried out by the International Agency for Research on Cancer (IARC) indicates that NTP's consideration of CA is misleading:

- Increased CA were found in several *in vitro* studies using human lymphocytes, but styrene did not cause increased CA or MN in *in vitro* studies using animal tissues despite the fact that SO is common styrene metabolite in both humans and animals.[47]
- An increased incidence of CAs or MN are generally not found in *in vivo* animal studies with controlled styrene exposures even at very high doses; 11 of 12 and 5 of 7 studies were negative for CAs or MN respectively.[47] The *in vivo* CA and MN studies of SO also do not present a clear picture of genotoxicity, as only two of the seven studies reported increases.
- About half of the 30 human studies of RPC workers have found increases in CAs,[47] but the percentage of positive studies has not similarly decreased as general exposures in the industry have decreased significantly, raising a question on whether styrene is the cause.
- Most of the *in vitro* ("comet") assays have been conducted using very alkaline conditions; under alkaline conditions, DNA strands break at abasic sites (undergoing DNA adduct repair), and thus increased comet formation under alkaline conditions may represent broken DNA strands or breaks caused during the assay.[48]
- DNA damage is not evidence of cancer.[49]

In summary, data related to DNA adducts and CAs do not support a credible association between styrene exposure and human cancer.

Studies in Animals

In addition to finding "limited evidence" from human studies, one of NTP's justifications for its styrene classification in the 12th *RoC* is based on "… sufficient evidence of carcinogenicity from studies in experimental animals …" NTP's listing criteria for "sufficient evidence" from studies in animals call for the data to indicate "… there is an increased incidence of malignant and/or

combination of malignant and benign tumors (1) in multiple species or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site, or type of tumor, or age at onset." NTP also states "…there may be substances for which there is evidence of carcinogenicity in laboratory animals, but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.[3]

Therefore, in order to meet its own criteria for "sufficient evidence of carcinogenicity" from animal studies, NTP needs to find that at least one of the following criteria has been met:

1) Positive in more than one species or more than one tissue site; or
2) Positive by multiple routes of exposure; or
3) Exhibits an unusual degree with regard to one of several factors.

NTP's criteria allow animal data to be disregarded if compelling data exists indicating a substance acts by a mode of action that is not relevant to humans.

NTP appears to accept that styrene does not fulfill criterion #1, *i.e.*, it produced tumors only in one species (mouse) and in one tissue site (lung). NTP also does not employ the "unusual degree" criterion (#3) in its discussion of styrene in the *RoC*, which is consistent with observations that the mouse lung is a common tumor site in cancer bioassays, and styrene exhibited only a modest increase in late developing tumors. Thus, NTP argues in the *RoC* that styrene meets criterion #2 based on results in *one species* (mice) by *two routes of exposure* (inhalation and oral/gavage).

NTP's finding of "sufficient evidence" based on animal studies is incorrect because only *one study* in *one species* (mice) by *one route of exposure* (inhalation) provides clear evidence of increased tumors and these lung tumors are likely caused by a cytotoxic mode of action that is not quantitatively, or even more likely qualitiatively, relevant to humans.

NTP's *Styrene Substance Profile* states:[50]

- "Styrene caused lung tumors in several strains of mice by two different routes of exposure. The most robust studies are two-year studies of inhalation exposure in CD-1 mice (Cruzan *et al.* 2001) and oral exposure (by stomach tube) in B6C3F$_1$ mice (NCI 1979)."
- "These findings are supported by findings of lung tumors in both sexes of O20 mice exposed to styrene (Ponomarkov and Tomatis 1978)."
- The evidence from studies in rats is insufficient for reaching a conclusion concerning the carcinogenicity of styrene."

Studies in mice

NTP is correct that Cruzan found benign and malignant lung tumors in CD-1 mice following inhalation exposure to styrene.[2]

This study provides support for one of the required two or more findings in multiple species/tissue sites and/or routes of exposure which are necessary for a finding of "sufficient evidence." However, for mode of action reasons (see pages 2-7), SIRC does not believe the findings of mouse lung tumors are relevant to humans.

To find a second route of exposure, NTP turned to a 1979 National Cancer Institute (NCI) study.[51] For the reasons indicated below, NTP erred in finding that the NCI study supports a finding of "sufficient evidence," and so NTP does not demonstrate tumors in more than one species, more than one tissue site, or by more than one route of exposure.

NTP's interpretation of the NCI study as representing a positive finding of mouse lung tumors is not only clearly at odds with the conclusions of the NCI study authors, but also relies on an inappropriate reconsideration of historical control data that is contrary to NTP's published recommendations regarding consideration of historical controls. In addition, NTP's use of "two-routes of exposure" to justify its listing of styrene in the *RoC* ignores the robust body of mode of action evidence indicating a lack of relevance of mouse lung tumors, by whatever route of exposure, to an assessment of human cancer risk.

The *RoC* states: "In male B6C3F$_1$ mice, oral/gavage exposure to styrene increased the combined incidence of benign and malignant tumors (alveolar/bronchiolar adenoma and carcinoma), and a positive dose-response trend was observed (NCI 1979)."[50]

But, contrary to NTP's statement, the NCI study concluded there was no more than "suggestive evidence of carcinogenicity…and that, under the conditions of this bioassay, no convincing evidence for the carcinogenicity of the compound was obtained …"[52]

1) According to the NCI report's summary, the study found: "In male mice, there was a significant positive association between styrene dosage and the incidences of a combination of adenomas and carcinomas of the lung … However, the variation of the incidence of these neoplasms in historical control male mice at this laboratory does not permit a firm conclusion of carcinogenicity."[53]

   In other words, per standard practice in toxicology research, since the incidence of tumors in the exposed animals was not statistically different than the incidence observed in the past among the same laboratory's non-exposed control population, the finding may have been due to chance and was not considered to be definitive.

Before it was finalized, the NCI study was peer reviewed by a panel of several toxicologists and pathologists, two of whom served as primary reviewers. The panel discussed and approved the wording of the study report, which included the following:[54]

"The primary reviewer for the report on the bioassay of Styrene said that the conclusion in the report was that, under the conditions of test, there was an increased incidence of lung tumors in treated male mice. The finding provided suggestive evidence for the carcinogenicity of the compound. Although the lung tumors were statistically significant, they were not given more weight because the incidence in the matched vehicle controls was lower than expected based on historical data."

"The secondary reviewer of the bioassay of Styrene said that the evidence was inadequate to suggest that the compound was carcinogenic in mice or rats, under the conditions of test. He opined that the increased incidence of lung tumors in male mice was an experimental vagary and that Styrene should be retested in a more susceptible strain … He indicated that the statistical aspects of the study were overemphasized and insufficient attention was given to the tumor biology. In conclusion, the secondary reviewer said that the study was negative in both mice and rats, under the conditions of test …"

2) While the *Styrene Substance Profile* does not disclose exactly how NTP reinterpreted the results of the NCI study to conclude "Styrene caused lung tumors in several strains of mice and by two different routes of exposure,"[50] the *Styrene Background Document* used to prepare the *Styrene Substance Profile* is more forthcoming:

"NCI questioned the significance of these lung tumors because the incidence in the control group was unusually low compared with historical untreated controls, and only small numbers of vehicle historical controls were available from the same testing laboratory. [However, a larger number of vehicle (corn oil)-treated historical controls from this same time period (prior to 1979), with similar study duration, and from the same source as the styrene study were available from a different testing laboratory. Results from these historical vehicle controls indicated that the concurrent vehicle controls in the NCI study were not unusually low and the lung tumor incidence in the high-dose group was significantly increased compared with those historical controls.]"[55] (Square brackets in original)

In other words, NTP developed a new historic control sample based on the experience of a different research facility (Hazleton) to compare with the NCI styrene study (conducted at Litton), and on this basis elevated the findings of the NCI study beyond its own conclusions in order to cite "two routes of exposure" and thus support a finding of "sufficient evidence" in animals to justify listing styrene in the *RoC*.

NTP itself had previously published analyses as to whether it was appropriate to use animals from one laboratory as historic controls for another. NTP's analysis recommended *against* such substitution, specifically noting that the incidence

of lung tumors varied considerably among laboratories.[56] NTP's new historical control was generated to compare studies with corn oil gavage control. However, analysis of the NTP historical control showed no difference in lung tumor frequency between gavage and dietary studies. Therefore, the original historical control used by NCI involving corn oil and diet studies from the same laboratory as the basis for the historical control analysis is more appropriate. Table 6 shows why NTP's disregard of its own recommendations in using historical control data from another laboratory resulted in a re-interpretation of the NCI study that appeared to inappropriately meet the NTP listing criteria.

Based on the above, the 1979 NCI oral/gavage study falls short of supporting a finding of "sufficient evidence" under NTP's listing criteria for the *RoC* and NTP was incorrect to substitute a new historical control sample in order to try to fulfill its listing criteria.

3) NTP also indicates the findings of Cruzan and NCI "… are supported by findings of lung tumors in both sexes of O20 mice exposed to styrene (Ponomarkov and Tomatis)."[57]

This is a misuse of the findings of this study:

- In this study of very high oral doses of styrene (1,350 mg/kg for 16 weeks), more than half of the exposed animals died with severe lung and liver congestion by week 20.
- There was an increase in lung tumors in the surviving animals, but the severe toxicity and mortality in the O20 mice limits the value of this study as providing evidence of tumors in the absence of a lethal dose.

Therefore, like the NCI study, Ponomarkov and Tomatis does not support a finding of "sufficient evidence" by NTP.

Studies in rats

NTP found: "The evidence from studies in rats is insufficient for reaching a conclusion concerning the carcinogenicity of styrene."[50] This is an understatement. The eight chronic studies clearly support a conclusion that inhalation of styrene does not cause cancer in rats.

New research since the *RoC*

No new animal bioassays regarding styrene have been published since the *RoC* was released.

Table 6. Lung tumor incidence in male control mice at Hazleton and Litton from 1st 200 NCI studies demonstrate a much higher incidence at Litton than at Hazelton

Study	No. of Studies	Ave. % Lung tumors	Individual study results
Litton: 91 week studies			
Corn Oil	2	0	0, 0
Diet	14	11	20, 6, 0, 16, 20, 5, 16, 11, 11, 17, 5, 10, 11, 5
Hazleton: 91 week studies			
Corn oil	12	4	0, 0, 11, 0, 0, 0, 18, 0, 0, 11, 5
Diet	14	2	5, 0, 0, 0, 0, 11, 0, 6, 0, 0, 0, 6, 5, 0
Litton: 104 week studies			
Corn oil	1	5	
Diet	35	20	0, 25, 33, 11, 20, 10, 18, 15, 15, 35, 45, 0, 26, 10, 15, 21, 5, 20, 35, 20, 15, 30, 35, 20, 25, 20, 32, 35, 20, 13, 17, 8, 15, 20, 27
Water gavage	1	5	
Hazleton: 104 week studies			
Diet	7	11	2, 13, 4, 18, 18, 12, 10

NTP's Mode of Action Analysis is Not Supported

In addition to finding "limited evidence" from human studies and "sufficient evidence" from animal studies, NTP's justifications for its styrene classification in the 12th *RoC* include "… supporting data on mechanisms of carcinogenesis." NTP's listing criteria include the following:

"Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to, dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub-populations, genetic effects, or **other data relating to mechanism of action** or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals, but **there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans."**[3] (**emphasis added**)

NTP's Styrene Substance Profile states:

"The primary metabolite of styrene, styrene-7,8-oxide, is listed in the *Report on Carcinogens* as *reasonably anticipated to be a human carcinogen*[*] based on sufficient evidence in experimental animals …"[50]

- "The mechanisms of styrene carcinogenicity are not fully understood … The proposed mechanisms for the carcinogenicity of styrene include both genotoxic and non-genotoxic pathways, which are not necessarily mutually exclusive."[50]
- "Although styrene disposition differs quantitatively among species, no qualitative differences between humans and experimental animals have been demonstrated that contradict the relevance of cancer studies in rodents for evaluation of human hazard. Detection of styrene-7,8-oxide-DNA adducts at base-pairing sites and chromosomal aberrations in lymphocytes of styrene-exposed workers supports the potential human cancer hazard from styrene through a genotoxic mode of action."[42]

In summary:

1) NTP cites the 2002 listing of styrene-7,8-oxide (SO) in the 10th *RoC*[58] as supporting evidence of its decision to list styrene in the 12th *RoC*.
2) Even though NTP proposes that more than one MoA is possible for styrene, NTP concludes that a genotoxic MoA, based on metabolism of styrene to SO, is plausible and "supports the potential human cancer hazard from styrene."[59]

[*] This quotation refers to an additional criterion by which NTP can list a substance in the *RoC, i.e.*, a substance shares a metabolite that is already listed in the *RoC*.

Reliance on the Listing of SO in the 10th *RoC* As a Rationale for the Listing of Styrene Is Not Justified

SO's listing is based on studies that reported forestomach tumors in both sexes of rats and mice where SO was administered orally (gavage), and increased liver tumors in low-dose male mice only:

- Forestomach tumors developed only following extensive toxicity, corrosion and cell replication which paralleled the degree of tumors.[60]

  Thus, the reported tumors are likely due to extensive cellular damage at the site of administration and not due to a genotoxic MoA.[61,62]

- The reporting of liver tumors only in male mice at the lowest dose tested is not a strong indicator of liver carcinogenicity from SO and may reflect a chance difference.[63]

  Thus, while it is true that SO is listed in the *RoC*, a brief review of the data does not appear to support a genotoxic MoA for styrene-induced lung tumors in mice through SO; this raises questions about the validity of NTP's listing of SO in the *RoC*. This paper, however, will not further address this topic, as it is outside its scope.

  In contrast to the *RoC's* reliance on a genotoxic MoA for styrene via SO, SIRC concludes, as discussed earlier, that an overall **evaluation of the data that was available to NTP when the *RoC* was published supports a non-genotoxic (cytotoxic) MoA** for mouse lung tumors rather than a genotoxic MoA based on SO metabolism.

The genotoxicity data do not support a listing of styrene in the *RoC*

The key data which support this conclusion include:

- Styrene, in general was not mutagenic in 14 standard Ames tests.[64]
- Styrene did not cause increased MN in five of seven *in vivo* assays.[64]

  - Styrene did not initiate lung tumors in Strain A mice in an initiation/promotion assay, a response expected of genotoxic carcinogens.[65]

- Styrene did not induce CA in the lungs of mice exposed to 125, 250 or 500 ppm styrene for two weeks.[66]
- *In vitro* studies report increased chromosomal aberrations (CA), micronuclei (MN) and sister chromatid exchange (SCE).[67]
- Mixed results reported for CA and MN in workers in industries where there is styrene exposure.
- "Positive" results are found as often in recent studies when workplace exposures have been significantly reduced compared to older studies. An inherent limitation of these studies is their lack of control for confounding exposures.

- Studies have reported low levels of protein and DNA adducts in mice, rats and humans exposed to styrene.[68] These are unrelated to tumors in animals as rats develop higher adduct levels than mice, but do not get tumors.
- Cross-species data do not support a genotoxic MoA of styrene metabolites.
- Oral administration of SO to mice did not result in increased lung tumors even though PBPK modeling indicates the level of SO in the lung would be the same as or higher than that produced by metabolism of 40 ppm inhaled styrene (a clearly tumorigenic dose).[69]
- SO is produced extensively in mouse lung at 40 ppm (tumors found), but
  - Blood level of SO in rats exposed to 1,000 ppm styrene (no increase in tumors) is about 100 times higher than SO levels in mice at 40 ppm.[1,2]
  - Eight-times higher amounts of SO occur *ex vivo* in rat lung at 1,000 ppm than at 40 ppm in mice; author of paper concluded SO not responsible for cytotoxicity from styrene exposure.[4]

Despite NTP's assertions to the contrary, a review of the evidence in favor of a genotoxic MoA for styrene is weak at best and is insufficient to support a listing of styrene due to "supporting data on mechanisms of carcinogenesis."

Evidence of a Cytotoxic, Non-Genotoxic Mode of Action Is Compelling and Is Contrary to NTP's Listing Decision

In the *RoC*, NTP stated:

- "Styrene is metabolized primarily in the liver and the lung. In mice, the Clara cell is regarded as the major lung-cell type in which styrene is activated …"[50]
- "Cytotoxicity can cause regenerative hyperplasia, leading to the promotion of spontaneous or styrene-induced mutations and tumor formation. Styrene caused lung tumors and pulmonary toxicity in mice but did not cause lung tumors in rats."[42]
- "Indirect data supporting the role of Cyp2f in styrene-induced lung toxicity comes from short-term intraperitoneal injections studies with wild-type and Cyp2e1 knock-out mice."[42]
- "Interspecies differences in lung toxicity are proposed to result from differences in the extent of metabolism of styrene to ring-oxidized metabolites by Cyp2f in the Clara cells."[42]

NTP does not explain why it found a genotoxic MoA to be credible, or why the support for a cytotoxic MoA was insufficient to "contradict the relevance of cancer studies in rodents for evaluation of human hazard." As previously discussed, the data do not support a conclusion that mouse lung tumors from styrene are caused by an SO or genotoxic MoA (Table 1).

The available data provide compelling evidence that a cytotoxic MoA best explains why lung tumors are observed in tests of laboratory mice, but not in rats exposed to styrene (Table 2). Furthermore, there is convincing evidence that this MoA does not occur to a biologically meaningful extent in humans (Table 3). These data are sufficiently strong to "contradict the relevance of cancer studies in rodents for evaluation of human hazard," and therefore NTP should have determined that the mouse lung tumors, when coupled with mode of action data, did not meet the *RoC* criteria for "sufficient evidence" in animals.

The Scientific Soundness of NTP's Listing Criteria

The NRC Committee will also be addressing the extent to which NTP's listing criteria are likely to lead to scientifically sound listing recommendations. The following three references may be of value to the committee in addressing this portion of its charge:

- A report by Dr. R. Belzer, "The *Report on Carcinogens*: What Went Wrong and What Can be Done to Fix it"[70] (includes a discussion specifically on NTP's styrene analysis).
- An article by Rodu, B. *et al.*, "Evaluation of the National Toxicology Program Report on Carcinogens" that compares the listing criteria to those of the International Agency for Research on Cancer.[71]
- Written testimony of Dr. J.S. Bus before a 2012 joint Congressional Committee.[72]

Conclusion

As indicated in the *RoC* and reviewed in these comments, NTP characterized cancer studies in humans as showing "limited evidence," meaning NTP determined that a causal association of styrene with cancer in humans is credible.

However, the epidemiology studies show no consistent increased incidence of, or mortality from, any type of cancer. Thus, the data do not support the NTP's conclusion that a causal association between styrene exposure and human cancer is credible, particularly in light of recently published epidemiology data. Nor is there concordance of tumor incidence and tumor types among animals and humans, showing that no particular cancer has been consistently observed among all available studies. A recent update to one of these cohorts provides even more clarity to this conclusion.

NTP based its finding of "sufficient animal evidence" on lung tumors induced in two mouse studies by two routes of administration. However, NTP's supporting analysis of one of the studies inappropriately went counter to the interpretation of the original authors and the study's peer review panel and also inappropriately used historical control information from another laboratory (this action was counter to NTP's published recommendations for use of such data). Thus, the animal data do not provide sufficient evidence of tumors by two routes of exposure.

Even if the animal research had met the "sufficient animal evidence" criteria, the available data on the MoA for styrene-induced lung tumors in mice should have been found to be sufficient to "contradict the relevance of cancer studies in rodents for evaluation of human hazard."

The mode of action data also demonstrate that the observed mouse lung tumors are not quantitatively, or even more likely qualitatively relevant to humans. Newly-completed studies further reinforce the view that the MoA for styrene-induced lung tumors in mice is related to a cytotoxic MoA involving ring-oxidized metabolites and not a genotoxic MoA involving styrene-7,8-oxide, and that this mode of action is unlikely to function in humans. Likewise, SO-induced rodent forestomach tumors (SO is listed in the *RoC*), are likely related to a mode of action that is unrelated to styrene mouse lung tumors.

An overall assessment of the available body of science at the time the *RoC* was published confirms that, as a whole, the science does not support the characterization of styrene as "reasonably anticipated to be a human carcinogen." Data published since June 2011 further support this conclusion.

Finally, putting aside the listing criteria, there are distinctly contrasting interpretations of the science:

1) NTP's hypothesis is that lung tumors in mice result from a genotoxic mode of action involving styrene-7,8-oxide. However, this hypothesis was clearly inconsistent with almost all aspects of the existing mode of action information available to NTP at the time of the *RoC* review, and is even further misaligned with research that has more recently become available.

2) In contrast, the conclusion is that mouse lung tumors are not quantitatively (or likely qualitatively) relevant to human cancer outcomes to any meaningful extent is strongly consistent with an evidence-based evaluation of the toxicology and epidemiology data. This data-driven conclusion is based on a cytotoxic mode of action that occurs to a biologically meaningful degree in mice, but not in rats, involving CYP2F2-generated ring-oxidized metabolites. The action of these metabolites results in reparative cell replication of the terminal bronchioles, the site of origin of mouse lung toxicity, eventually progresses to late-developing tumors.

   This cytotoxic mode of action was derived from the data, has been tested by experiment, and has been shown to be consistent with direct experimental data as well as with indirect evidence from epidemiology and other available information.

   SIRC welcomes the NRC Committee's independent determination of whether NTP has properly applied the best and most informative scientific conclusions regarding styrene and any potential it may have to cause cancer in humans.

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