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Dr. Linda S. Birnbaum
Director
National Institute of Environmental Health Sciences / National Toxicology Program
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Re: Knock-Out Mice Research Supports Conclusion that Styrene is NOT a Carcinogen

Request to Remove Styrene from 12th Report on Carcinogens Based on Lack of Scientific Validation

Dear Dr. Birnbaum:

The Styrene Information and Research Center (SIRC)[1] has just received the results of research with "knock-out" (KO) mice that provides strong confirmation that our hypothesized mode of action for mouse lung tumors is valid and that, consequently, the animal studies cited by NTP as the basis for listing styrene in the 12th *Report on Carcinogens* (*RoC*) cannot be relied upon to conclude that styrene is "Reasonably Anticipated to be Carcinogenic to Humans."

We have obviated that confounder by evaluating the critical role of CYP2F2 metabolism in mediating mouse lung toxicity in CYP2F2-specific knock-out mice. The CYP2F2-KO mice were developed by Dr. Xinxin Ding, who has a grant from NIEHS to develop CYP-KO mice. The study discussed below demonstrates the value of this type of NIEHS-funded research, and how SIRC was able to practically leverage that research to better inform an understanding of the relevance of the mouse mode of action information to potential human health issues.

We previously proposed that metabolism of styrene by CYP2F2 in mouse lung is responsible for the unique sensitivity of mice to lung toxicity and subsequent lung tumors. Consistent with that hypothesis, rats, which express much less of the corresponding rat isozyme CYP2F4, do not develop lung toxicity or tumors from styrene exposure. Importantly, humans express very little of the human-equivalent isozyme, CYP2F1, which also is not active in metabolizing styrene. Thus, we hypothesized that styrene-induced mouse lung tumors are not relevant for human risk assessment (Cruzan et al. 2009. Regul. Toxicol. Pharmacol. 55(2):205-18.)

[1] 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.

Although a previous study has shown that styrene lung toxicity is attenuated when mice are pretreated with pharmacologic inhibitors of CYP2F2 (summarized in Cruzan et al., 2009), such experiments are potentially confounded by mixed affinity of the inhibitor for multiple P450s. We have obviated that confounder by evaluating the critical role of CYP2F2 metabolism in mediating mouse lung toxicity in CYP2F2-specific knock-out mice. Unaltered, or wildtype (WT) mice treated with 400 mg/kg styrene displayed mild atrophy of the bronchiolar epithelium due to necrosis and exfoliation of Clara cells concurrent with areas of epithelial regeneration. The cumulative BrdU-labeling of S-phase cells (LI) was markedly increased in both proximal (46.8±7.9%) and terminal bronchioles (62.4± 2.7%) compared to WT control mice (2.2±0.5% and 4.4±0.9%, respectively); the LI for terminal bronchioles is shown in the attached table. Clara cells contain the majority of lung P450 metabolism, and also are the locus of the lung tumors in mice.

No evidence of Clara cell toxicity was observed in the pulmonary airways of 400 mg/kg-treated KO mice. The cumulative LI was not increased in proximal (1.1±0.3%) or terminal bronchioles (2.2±0.7%) compared to KO control mice (3.2±0.6% and 2.0±0.7%, respectively). The 15-fold increase in BrdU labeled cells in WT mice and NO toxicity from styrene in CYP2F2-kockout mice further supports our hypothesis that mouse lung tumors in styrene-exposed mice are caused solely by CYP2F2 metabolism, and are not related to generation of styrene-7,8-oxide (SO) as has been previously hypothesized by NTP and others. This conclusion is further consistent with the observation that styrene lung toxicity was not attenuated in CYP2E1 knock-out mice; CYP2E1 metabolism is recognized as the major source of SO formation (summarized in Cruzan et al., 2009). Overall, these data therefore strongly support our hypothesis that the mouse lung tumors from styrene exposure are not relevant for human risk assessment.

We also are attaching a copy of an abstract of this work, which has been submitted for presentation at the 2011 Society of Toxicology meeting.

NTP concluded that styrene should be included in the 12th *RoC* based on increased lung tumors in mice by two routes of exposure and a hypothesized genotoxic mode of action from its metabolism to styrene-7,8-oxide primarily by CYP2E1. As noted above, previous research, not relied upon in NTP's decision, demonstrated that lung effects from styrene are not related to 2E1 metabolism or likely even to SO itself (blood SO levels at non-tumorigenic doses in rats were substantially higher than SO levels at tumorigenic doses in mice). The present research demonstrates that styrene lung toxicity in mice is completely related to CYP2F2 metabolism. Rats and humans are not susceptible to styrene lung effects because they have much lower levels and activity of CYP2F directed to styrene metabolism.

The early findings from this ongoing program of knock-out mouse research surely represent a legitimate scientific basis for excluding styrene from the 12th *RoC*. NTP's consideration of the new CYP2F2 data would help to ensure that NTP does not move forward precipitously to classify styrene, particularly when state-of-art approaches to investigating styrene's mode of action – which directly benefit from NIEHS' investment in the Ding knock-out mouse research – clearly show such action is not scientifically grounded or justified. In the final paragraph of the listing criteria, such a reason is cited as an example of the basis for not listing a substance, i.e., when an animal study is not relevant to humans.[2]

[2] "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."
http://ntp.niehs.nih.gov/index.cfm?objectid=03C9CE38-E5CD-EE56-D21B94351DBC8FC3

In addition, as we have previously brought to your attention, NTP's proposed listing of styrene is diametrically opposed to the recent conclusion of the European Union,[3] which, after a comprehensive evaluation of both the animal toxicology and human epidemiology, concluded styrene should not be listed as a carcinogen of any concern. Furthermore, a review of the epidemiology data by a world-class team of epidemiologists (Boffetta et al [2009][4]) clearly stated the collective human data provide no evidence of an association between styrene exposure and cancer in humans (despite remarkably confusing NTP statements that the Boffetta report actually supports NTP's conclusion).

NTP's assertion in the Draft Substance Profile that there is "limited" evidence in the styrene human data rests solely on a reinterpretation of Delzell et al (2006)[5] that in fact concluded there was "insufficient" evidence of cancer. Likewise, in order to qualify an assertion of "sufficient" evidence in the animal data via two routes of exposure, NTP upgraded the published "suggestive evidence" conclusions of an NCI mouse study[6] by applying new, and inappropriate, historical control data to the tumor data evaluation. Even if the NTP's highly debatable upgrading of the NCI mouse study was judged acceptable, the new knock-out animal data strongly suggest the mouse lung tumor response is irrelevant to human risk and thus the "two routes" basis for using animal data to support an *RoC* classification is rendered moot. To date, neither of the NTP's novel reassessments has been externally peer-reviewed or published in the literature. Indeed, NTP continues to insist that these reassessments did not occur – although we would urge you to review the conclusions of the original published authors accessible via the links provided herein, which clearly show that – as published – the Delzell and NCI studies in no way support "limited" human evidence or "sufficient" animal evidence.

In summary – and certainly given the evidence of the new knock-out mouse data – NTP's proposed styrene listing is not scientifically grounded or justified under any of the *RoC* criteria for listing. Given these new mode of action data, and coupled with the significant reinterpretation of existing animal tumor and human epidemiology evidence in the NTP Draft Substance Profile, we do not believe that NTP should proceed with its proposed listing decision for the 12th *RoC*.

Such a listing has the very real potential of causing serious harm to the styrene-based enterprise, and no qualification on NTP's part that the *RoC* is "only a hazard assessment" will ever reduce the external impacts of an NTP carcinogen listing. Revisiting the science once the industry mode-of-action studies are completed would come too late to temper the serious negative impact a *RoC* listing would have on styrene. The dramatic disagreement with NTP's position reached by other highly reputable review bodies also suggests a premature *RoC* listing would not result in any commensurate public health benefit.

As indicated by the newly generated knock-out mice data, we are making rapid and substantial progress in clarifying the mode of action of styrene-induced mouse lung tumors. Our research plan includes using the knock-out mice to further clarify the penultimate metabolite(s) responsible for styrene lung toxicity. We are also well along in creating a "humanized" mouse in which CYP2F2 is replaced with the human CYP2F1 isozyme. Our hypothesis is that the

[3] http://echa.europa.eu/chem_data/transit_measures/annex_xv_trans_reports_en.asp

[4] http://journals.lww.com/joem/Abstract/2009/11000/Epidemiologic_Studies_of_Styrene_and_Cancer__A.5.aspx

[5] http://www.ncbi.nlm.nih.gov/pubmed/17326338

[6] http://www.ncbi.nlm.nih.gov/pubmed/12778193

humanized mice will be similarly resistant to styrene lung toxicity. We would be very willing to meet with the NTP to provide more details of the timing and nature of our research plans, which we believe would make available a significant and valuable body of new mode of action data for consideration in a subsequent 13th *RoC* listing review.

Therefore, based on the availability of these new data, and the other scientific weaknesses we have cited relative to NTP's justification for listing styrene, SIRC asks that you remove styrene before proceeding to finalize the 12th *Report on Carcinogens*.

As with our earlier communications to you, we ask that this letter be added to the styrene docket for the 12th RoC, as part of SIRC's public communications to NTP on this matter.

Very truly yours,

signature redaction

Marcy Banton, DVM, PhD, DABVT
LyondellBasell Corporate HSE/Product Safety
Chairperson
SIRC Science & Technology Task Group

signature redaction

James Bus, PhD, DABT
The Dow Chemical Company
Vice Chairperson
SIRC Science & Technology Task Group

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George Cruzan, PhD, DABT
Toxworks
Science Consultant to SIRC

cc:
The Honorable Kathleen Sebelius / Secretary, U.S. Department of Health & Human Services
Dr. Ruth Lunn / NTP

ATTACHMENTS:
Initial CYP2F2 Knock-Out Mouse Exposure Study Findings
Abstract Submission to Society of Toxicology

Initial CYP2F2 Knock-Out Mouse Exposure Study Findings

September 30, 2010

Previous SIRC research has demonstrated that oral, intraperitoneal, or inhalation exposure to styrene causes lung toxicity in mice, but not rats. Lifetime inhalation exposure also resulted in lung tumors in mice, but not rats. SIRC has postulated that the lung effects in mice are the result of CYP2F2 metabolism of styrene. Rats are resistant to styrene lung toxicity because there is a lower level of CYP2F4 in rat lungs than CYP2F2 in mouse lung cells. Supporting evidence includes reduction in mouse lung toxicity from styrene if CYP2F2 is inhibited by 5phenyl-1-pentyne, and that there is no reduction in lung toxicity by inhibiting CYP2E1 or in CYP2E1-knockout mice.

To further test this hypothesis, SIRC contracted with Dr. Xinxin Ding, an expert in cytochrome metabolism and cytochrome knockout mice, to develop a CYP2F2-knockout mouse using C57Bl/6 mice. These mice appear completely normal; they are viable and reproduce readily and do not have unusual levels of other cytochromes. SIRC transferred founding 2F2-KO mice to Taconic to establish a specific pathogen free breeding colony. The colony is set up with 22 males and 44 females in breeding cages which each contain 1 male and 2 females. They are currently producing offspring for future experiments.

SIRC used 14 extra males not needed to set up the breeding colony for a preliminary study of styrene. 7 male KO mice were exposed to styrene orally at 400 mg/kg/day for 5 days, and the other 7 were not exposed to styrene for comparison. To provide a reference point, similar groups of 7 normal C57Bl/6 (called wild-type, WT) mice were used as controls or exposed to styrene at 400 mg/kg/day for 5 days. In the WT mice exposed to styrene there was lung toxicity and increased cell replication; cell replication was increased 15-fold compared to the WT controls. In the KO mice exposed to styrene there was no (0%) increase in cell labeling and no pathology.

Future experiments in the KO mice will attempt to determine what role, if any, metabolism of styrene to styrene oxide plays in lung effects and to identify what metabolite(s) from styrene cause the toxicity. Further experiments with a mouse in which the mouse CYP2F2 has been replaced by the humans CYP2F1 will examine whether the human CYP2F is capable of producing the toxic metabolites produced by the mouse CYP2F2. These strains of mice can also be used to determine how much metabolites are produced in mice and humans and provide a quantitative comparison.

This preliminary study clearly demonstrates that styrene toxicity in mouse lung is totally dependent on metabolism by CYP2F2. Since humans have very small amounts of CYP2F, lung toxicity and tumors are not expected from styrene in humans.

BrdU Labeling Index of airway epithelium (% BrdU-labeled epithelial cells) in terminal bronchioles of WT and KO mice

Styrene Dose mg/kg/day	Wildtype (WT) %	Knockout (KO) %
0	4.4 ± 0.9	2.0 ± 0.7
400	62.4 ± 2.7[a]	2.2 ± 0.7[b]

Group means ± standard error of the means; n = 6-7 mice/group
[a] Significantly different from respective control group given 0 mg/kg/day ($p \leq 0.05$)
[b] Significantly different from 400 mg/kg/day WT group ($p \leq 0.05$)

Numeric Density of BrdU-labeled epithelial cells (cells/length of basal lamina) in terminal bronchioles of WT and KO mice

Styrene Dose mg/kg/day	Wildtype (WT) cells/mm	Knockout (KO) cells/mm
0	6.6 ± 0.9	3.3 ± 1.3
400	77.5 ± 6.0 [a]	3.1 ± 1.0 [b]

Group means ± standard error of the means; n = 6-7 mice/group
[a] Significantly different from respective control group given 0 mg/kg/day ($p \leq 0.05$)
[b] Significantly different from 400 mg/kg/day WT group ($p \leq 0.05$)

Abstract of Study Report Submitted to Society of Toxicology

No Lung Toxicity from Styrene in CYP2F2 knockout mice. G. Cruzan[1], J. Bus[2], X. Ding[3], J. Hotchkiss[2], J. Harkema[4], R. Gingell[5], [1]ToxWorks, Bridgeton, NJ; [2]The Dow Chemical Company, Midland, MI; [3]State University of New York at Albany, Albany, NY; [4]Michigan State University, East Lansing, MI; [5]Shell Oil Company, Houston, TX;

Styrene induces lung tumors in mice, but not in rats. We hypothesized that the mouse lung tumors were mediated by mouse specific CYP2F2 generated cytotoxic, ring-oxidized metabolite(s) producing repeated localized injury to Clara cells, the site of CYP2F2 metabolism and the tumorigenic response. This hypothesis was consistent with previous data indicating that styrene lung toxicity was not attenuated in CYP2E1 knockout mice (2E1 produces styrene-7,8-oxide). To test our hypothesis we developed CYP2F2-/- (KO) mice on a C57BL/6 background. The 2F2-KO mouse was produced by replacing the third coding exon with a neomycin resistance gene followed by homologous recombination in C57BL/6-derived Bruce4 mouse embryonic stem cells. Male WT and KO mice received 0 or 400 mg/kg/day styrene in corn oil by gavage for 5 days (n=7/group). WT mice treated with 400 mg/kg styrene displayed mild atrophy of the bronchiolar epithelium due to necrosis and exfoliation of Clara cells concurrent with areas of epithelial regeneration. The cumulative BrdU-labeling of S-phase cells (LI) was markedly increased in both proximal (46.8±7.9%) and terminal bronchioles (62.4± 2.7%) compared to WT control mice (2.2±0.5% and 4.4±0.9%, respectively). No evidence of Clara cell toxicity was observed in the pulmonary airways of 400 mg/kg-treated KO mice. The cumulative LI was not increased in proximal (1.1±0.3%) or terminal bronchioles (2.2±0.7%) compared to KO control mice (3.2±0.6% and 2.0±0.7%, respectively). This study clearly demonstrates that styrene toxicity in the mouse lung is dependent on metabolism by CYP2F2, and that the mouse tumor response may not be relevant to human risk in that human lung expresses CYP2F1, an isoform not suspected of catalyzing significant styrene metabolism.