Hypothesis-Driven MOA Analysis: CYP2F2

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Animal Cancer Studies

- Rats: 8 studies, based on WOE no increased tumors
- Mice: 5 studies, only lung tumors

Progression of Toxicity in Mice

- Single exposure – inc. LDH, Cells and protein in BALF
- Up to 2 weeks – inc. BrdU labeling, dec. CCSP
- 3 months – decreased staining of Clara cells; cellular crowding in terminal bronchioles
- 12 months – hyperplasia in terminal bronchioles
- 18 months – hyperplasia in terminal bronchioles extending into alveolar ducts
- 24 months – lung tumors, mostly benign

Urinary Excretion Patterns

Species	SO-EH (%)	SO-GSH (%)	PAA (%)	Ring(%)
B6C3F1 mouse	49-52	33-25	12-17	4-8
CD1 mouse	51-59	20-27	21-22	4-8
F344 rat	68-72	23-26	3-5	<1
Human (2-4 hr)	95		5	ND
Human (4-9 hr)	100		ND	ND

SO-EH = styrene oxide + epoxide hydrolase
SO-GSH = S) = glutathione conjugates
PAA = phenylacetaldehyde pathway
Ring = ring oxidized or ring opened

EPA 2005 Cancer Guidelines

- Animal tumor MOA Analysis
  - Identify Key Events
  - Determine concordance of Key Events with tumors
  - Identify Specificity and Consistency
  - Examine Other Potential MOAs
  - Determine extent of Key Events in Humans

MOA Hypothesis 1

- 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.

- Hypothesis not supported by the data

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

Key Findings	***In vitro***	**Mouse**	**Rat**	**Human**
Styrene negative in Ames assays	Not supporting			
Genotoxicity studies of SO	Supporting	Mostly not supporting	Mostly not supporting	No data
No CA in lungs of mice exposed to styrene		Not supporting		
No lung tumor initiation in Strain A mice		Not supporting	No data	No data
No increased lung tumors from SO		Not supporting	Not supporting	No data
Lung toxicity from SO		Supporting	Not supporting	No data
No decreased lung toxicity when SO decreased (2E1-KO mice)		Not supporting	No data	

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

Key Findings	***In vitro***	**Mouse**	**Rat**	**Human**
Blood SO rats > mice		Not supporting	Not supporting	
Lung SO *ex vivo* rats> mice		Not supporting	Not supporting	
Urinary SO-derived metabolites rats > mice		Not supporting	Not supporting	
Lack of SO lung toxicity in 2F2-KO mice		Not supporting		
DNA adducts in rats > mice		Not supporting	Not supporting	
Forestomach tumor incidence in rats			Not supporting	

Hypothesis 2

- Lung metabolism of styrene by CYP2F2 produces ring-oxidized metabolites that are toxic to Clara cells. Continued exposure results in continual cell replication, leading to hyperplasia. Newly formed Clara cells lack production of CCSP(CC10), which reduces resistance to lung tumors.

- This hypothesis is supported by the data

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

Key Data	*In vitro*	Mouse	Rat	Human
Styrene negative in Ames assays	Supporting			
Lung tumors in mice, not rats		Supporting	Supporting	
Lung toxicity in mice, not rats		Supporting	Supporting	
Toxicity and metabolism in Clara cells		Supporting		
Lung toxicity from 4HS in mice, not rats		Supporting	Supporting	
Elimination of lung toxicity from styrene and SO in 2F2-KO mice		Supporting		
Elimination of ring-oxidation of styrene and 4HS in 2F2-KO mice		Supporting		

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

Key Data	***In vitro***	**Mouse**	**Rat**	**Human**
Greater lung toxicity from 4HS than SO		Supporting		
More ring-oxidized urinary metabolites in mice than in rats		Supporting		
Limited toxicity from 4HS in 2F2-KO mice		Supporting		
Styrene analogs (3- or 4-methyl) do not induce lung tumors in mice		Supporting		
Ethylbenzene (no vinyl epoxide) induces mouse lung tumors		Supporting		
Clara cell toxicity in mice from EB, not rats		Supporting	Supporting	

Labeling index (% of cells labeled with BrdU) in terminal bronchioles of wild type and CYP2F2(-/-) mice exposed to styrene, or styrene oxide, for 5 days.

Treatment (mg/kg/day)	CYP2F2(+/+) [WT]		CYP2F2(-/-) [KO]	
	Males	Females	Males	Females
Control - 0	5.2	5.7	3.8	4.6
Styrene - 200	47.2	34.9	2.9	4.6
S-SO - 200	37.8	46.6	4.2	4.5
R-SO - 200	39.0	43.9	4.4	4.7

- Cruzan et al., 2012

Role of Ring Oxidation

- 4-8% of styrene urinary metabolites in mice are ring-oxidized; 0.5% in rats
- No lung tumors in mice from 3-methyl-styrene or 4-methylstyrene (no ring oxidation possible)
- 4-Hydroxystyrene (4HS, 4-vinylphenol) more toxic than styrene or SO (NOAEL 2 mg/kg/day over 2 weeks compared to 100 mg/kg/day for styrene)

Role of Ring Oxidation

- WT mice produce 4HS, 3,4-dihydroxystyrene and 4-hydroxystyrene oxide from styrene in lung
- Limited ring oxidation of styrene in 2F2-KO mice
- Limited ring oxidation of 4HS in 2F2-KO mice
- Some toxicity from 4HS in 2F2-KO mice

BrdU Labeling index in terminal bronchioles of WT, and KO mice exposed to 4-hydroxystyrene for 5 days

Treatment	Males	Females
WT Controls	5.1(0.5)	5.8(1.1)
WT (105 mg/kg/day)	**48.0** (1.7)	**51.7** (1.4)
KO Controls	4.5(0.5)	4.2 (0.5)
KO (60 mg/kg/day)	6.3 (0.8)	8.4 (0.6)
KO (105 mg/kg/day)	6.0 (0.5)	13.4 (1.4)

Dose-Response and Temporality for Styrene - Inhalation

Exposure conc	CYP2F2 metabolism in lung	Clara cell cytotoxicity	Hyperplasia in terminal bronchioles	Lung tumors
20 ppm	*	Not tested	12 mo. - - 18 mo. - ++ 24 mo. - ++	12 mo. – 18 mo. – 24 mo. M-, F+
40 ppm		5 days - + BrdU 13 wks. – altered cells (50 ppm) ++	12 mo. - + 18 mo. - ++ 24 mo. - +++	12 mo. – 18 mo. – 24 mo. M+, F+
80 ppm		Not tested –BrdU 13 wks. – altered cells (100 ppm) +++	12 mo. - ++ 18 mo. - +++ 24 mo. - +++	12 mo. – 18 mo. – 24 mo. M+, F-
160 ppm		5 days - +++ BrdU 13 wks. – altered cells (150 ppm) +++	12 mo. - +++ 18 mo. - +++ 24 mo. - +++	12 mo. – 18 mo. – 24 mo. M+, F+

Dose-Response and Temporality for Styrene – Gavage

Dose (mg//kg/day)	CYP2F2 metabolism in lung	Clara cell cytotoxicity	Hyperplasia in terminal bronchioles	Lung tumors
175 (B6C3F1)	*	Not measured	Not recorded	M-, F-
200 (B6)	No increase in BrdU labeling in CYP2F2-KO mice	5 days – +++ increase in BrdU labeling	No long term study	No long term study
375 (B6C3F1)		Not measured	Not recorded	M +/-, F -
400 (B6)		5 days – +++increase in BrdU labeling	No long term study	No long term study

*Metabolism by CYP2F2 has been established using microsomes from whole lung.

Summary of Mouse Lung Tumor MOA

- Mouse lung tumors not related to SO – No lung tumors from SO
- No lung tumors in absence of ring oxidation – 3-,4-MeS
- 4HS toxic at much lower dose than styrene or SO
- Some 4HS toxicity even in absence of CYP2F2 metabolism
- CYP2F2 creates ring-oxidized metabolites that are toxic to Clara cells

Human Relevance of CYP2F2 MOA

- Human isoform is CYP2F1
- 2F2 and 2F1 shown to metabolize 3-methylindole; 2F1 metabolizes naphthalene about 1% of 2F2 rate; preliminary indication styrene not metabolized by 2F1
- Developed transgenic mouse to study 2F1 ability to produce toxic metabolites

CYP2F1 Transgenic Mouse

- Inserted transgene containing DNA for CYP2F1 (lung and nasal), 2A13 (lung and nasal), and 2B6 (liver) into CYP2F2-KO mice
- 2F1 and 2A13 protein expressed in lung and nasal tissue of these mice
- CYP2F1 present in TG mouse lung at 1/40th level of 2F2 in WT mice (greater than amount in human Clara cells)
- Lung microsomes from TG mice metabolize naphthalene (Ding) and 3-methylindole (Yost)

CYP2F1 Transgenic Mouse

- No lung toxicity from styrene or SO in TG mice
- Some toxicity from 4HS in TG mice

Labeling index (% of cells labeled with BrdU) in terminal bronchioles of wild type and CYP2F2(-/-), 2F1-tg mice exposed to styrene for 5 days.

	WT Mice		TG Mice	
	Males	Females	Males	Females
Control	4.7 (2.0)	7.2 (2.7)	5.2 (2.1)	7.5 (3.0)
Styrene - 200	35.4 (9.6)	50.0 (8.9)	2.7 (1.1)	3.5 (0.9)
R-SO - 200	35.6 (2.5)	42.0 (8.5)	6.3 (2.2)	6.0 (2.9)
S-SO - 200	27.5 (17.0)	42.3 (1.1)	4.3 (3.0)	6.8 (2.0)
4HS - 105	48.0 (1.7)	51.7 (1.4)	12.3 (6.6)	22.9 (8.3)

Conclusions

- Mouse lung tumors from styrene exposure caused by ring-oxidized metabolites produced by CYP2F2 metabolism
- Styrene oxide is NOT the toxic metabolite
- Human CYP2F1 does not produce sufficient metabolites to cause toxicity or tumors
- Mouse lung tumors from styrene do not indicate human risk of cancer from styrene