Naphthalene Carcinogenicity: Hypothesis-Based Weight-of-Evidence Evaluation

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Beyond Science & Decisions:
From Problem Formulation to Dose-Response Assessment
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Key WoE Questions

- Based on observed positives, what hypothesized causal processes are necessary? Sufficient?
- How do they generalize? What other manifestations should they have?
- If hypothesis were wrong, how else would one explain the array of outcomes?

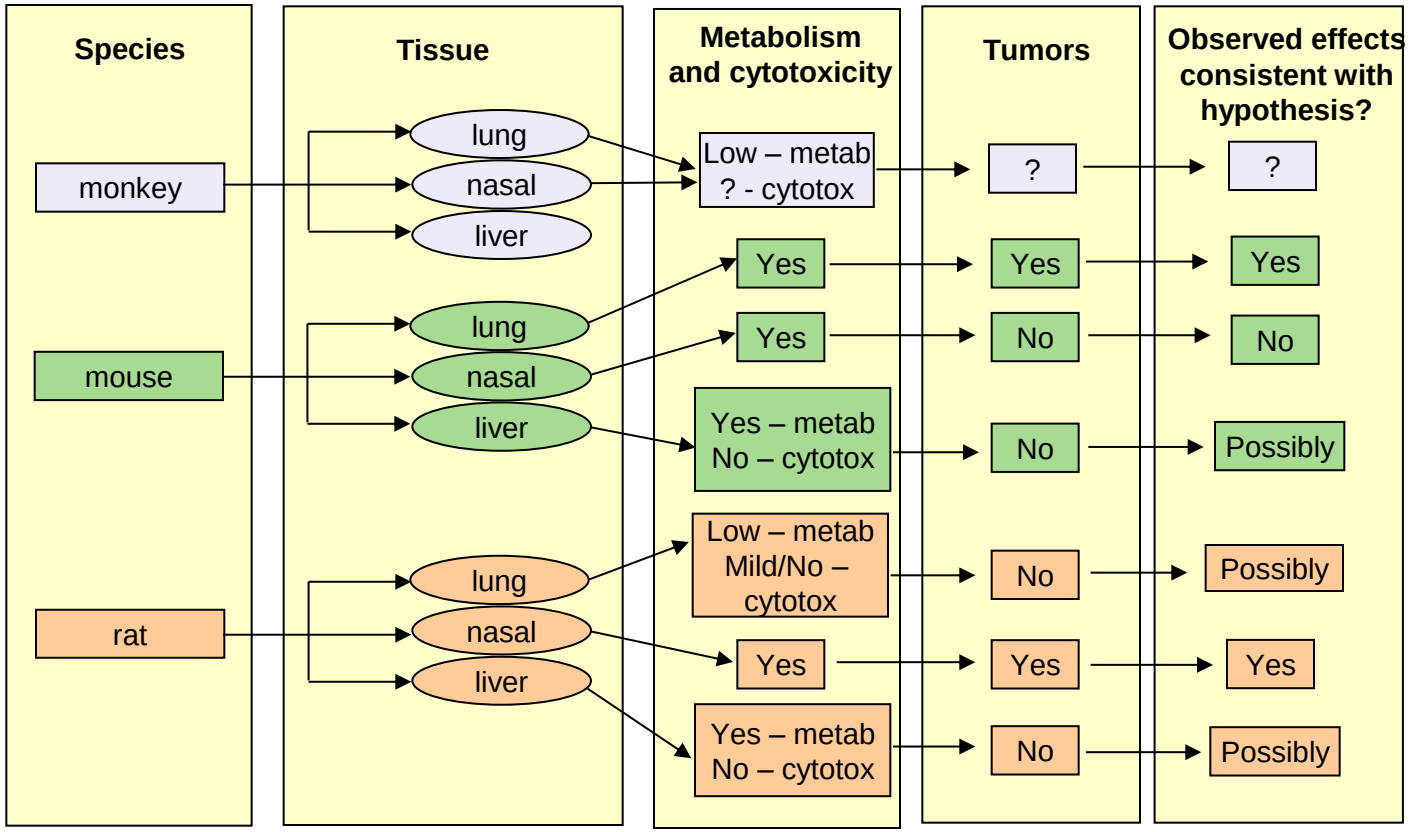
For Observed Outcomes that are Candidates for "Evidence"

- Why we think they happened where they did.
- Why we think they didn’t happen where they didn’t.
- Why we think the "did-happen" factors would also apply to the target population.
  - Might apply? Probably apply? Known to apply?
- Are there discrepant observations, and if so, how do we account for them?
- Are our "whys"
  - Observable underlying causes?
  - Reasonable guesses based on wider knowledge, other cases?
  - *Ad hoc* assumptions without evidence, needed to explain otherwise puzzling phenomena?

Experimental Evidence for Naphthalene Carcinogenesis

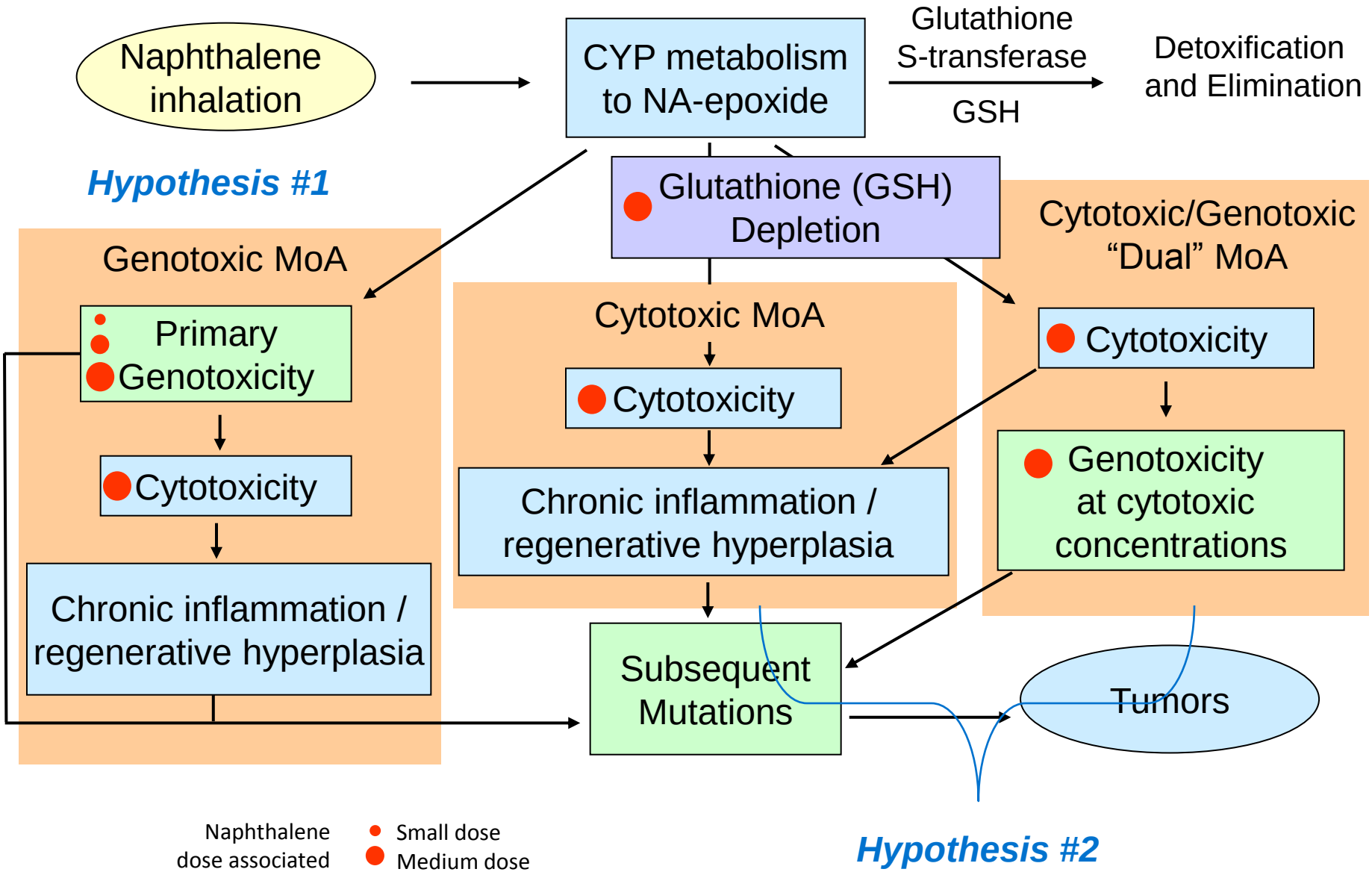
- Inhalation of naphthalene (10-60 ppm) causes olfactory epithelial nasal tumors in rats (but not mice) and benign lung adenomas in mice (but not rats) (NTP, 1992, 2000)
  - Tumors confined to tissues directly exposed to naphthalene
  - Tumors associated with widespread cytotoxicity and inflammation
  - Tissues subject to toxicity are sites of concentrated and localized metabolic activity toward naphthalene
  - IP injection causes similar pattern of cytotoxicity and metabolic activation, suggesting very specific and local MoA
- No positive human evidence for naphthalene's carcinogenicity – nasal tumors are rare

Initial Questions Based on Species Differences



- Given the inconsistency across animal tissues and species, why should we assume human respiratory tissue will respond like the mouse lung or the rat nose?
- How might we account for the observed outcomes? What are possible reasons for lack of tumors in the mouse nose? And how might our understanding of this change our proposed animal MoA?

Hypothesized Modes of Action (MoA)



Hypothesis #1

- Naphthalene metabolites (e.g., naphthalene-1,2-oxide, 1,2-naphthoquinone, and 1,4-naphthoquinone) are generated early in the carcinogenesis process (at subcytotoxic doses), and that one or more of these metabolites reacts with deoxyribonucleic acid (DNA), or generates ROS that can react with DNA, as an early, initiating event leading to tumors in mouse lung tissue and rat nasal tissue.

Hypothesis #2

- High-dose cytotoxicity is hypothesized to be necessary for tumor formation in rat nose and mouse lung – and presumably, in any human target tissue.
- Mice have nasal toxicity without tumors, showing that cytotoxicity, even if necessary, is not sufficient. Some rat/mouse difference must make naphthalene sufficient to cause tumors in rat nose but not in mouse nose.
- A candidate difference is that, in rat but not mouse nose, high doses produce genotoxic metabolites after GSH depletion.
- Sub-cytotoxic exposures in humans is hypothesized to be insufficient to cause tumors owing to lack of necessary cytotoxicity and lack of low-dose genotoxicity sufficient to affect tumor risk.

Seven Steps of the HBWoE Approach

Applied to Naphthalene –

Rhomberg, LR; Bailey, LA; Goodman, JE. 2010. "Hypothesis-based weight of evidence: A tool for evaluating and communicating uncertainties and inconsistencies in the large body of evidence in proposing a carcinogenic mode of action - Naphthalene as an example." Crit. Rev. Toxicol. 40:671-696.

1	Systematically review individual studies potentially relevant to causal question at hand, focusing on evaluation of study quality.
2	Within a realm of investigation (*e.g.*, epidemiology, experimental animal, or mode of action studies), systematically examine the data for particular endpoints across studies, evaluating consistency, specificity, and reproducibility of outcomes.
3	Identify and articulate lines of argument ("hypotheses"), newly proposed or those already put forth, that bear on the available data, and discuss how studies are used for each hypothesis to infer human risk.
4	Evaluate the logic of the proposed hypotheses with respect to each line of evidence.
5	Evaluate the logic of the proposed hypotheses with respect to all lines of evidence holistically so that all of the data are integrated and allowed to inform interpretation of one another.
6	Describe and compare the various alternate accounts of the observations at hand, with a discussion of how well each overarching hypothesis is supported by all of the available data, the uncertainties and inconsistencies in the data set, and any *ad hoc* assumptions required to support each hypothesis.
7	Formulate conclusions and any proposed next steps (*e.g.*, sharpening of proposed hypothesis already put forth; propose additional testing to clarify data gaps).

- Systematic review, evaluate study quality
- Consistency, specificity within Epi, within Tox
- Articulate logic for why data constitute "evidence"
- Evaluate hypotheses: w.r.t Epi; w.r.t Tox
- Evaluate evidence for epi/tox commonality in causes
- Formulate competing "accounts" – sets of explanations of outcomes
- Conclusions, identify studies that can sharpen

Data Integration Phase of the HBWoE Approach

Steps 4-6 of our approach

4	Evaluate the logic of the proposed hypotheses with respect to each realm of evidence, considering plausibility, specificity, and consistency across studies.
5	Evaluate the logic of the proposed hypotheses with respect to all realms and lines of evidence so that all of the data are integrated and allowed to inform interpretation of one another.
6	Describe and compare the various accounts of the observations at hand, with a discussion of how well each overarching hypothesis is supported by all of the available data, the uncertainties and inconsistencies in the data set, and any *ad hoc* assumptions required to support each hypothesis.

- Evaluate hypotheses: w.r.t Epi; w.r.t Tox
- Evaluate evidence for epi/tox commonality in causes
- Formulate competing "accounts" – sets of explanations of outcomes

Intended to be flexible and iterative

Data Integration Phase of the HBWoE Approach

- Integrates all data by asking how each realm of evidence informs interpretation of the others.
- Identifies consistencies and parallels across realms AND lack of consistencies (i.e., across tissues and species, with consideration of toxicity and metabolism).
- Asks how the consistencies and inconsistencies inform interpretation of each other and ultimately how they inform a potential MoA.
- Negative studies and lack of effects are also important.
- Process is iterative and not linear, does not have to follow steps in sequence, and can often lead to more questions.
- Goal is to try to trace the logic of the data (which is often complex and not easily arranged) in context of proposed hypotheses, while identifying key questions that need to be addressed to better understand the MoA.
- Ultimately does not prove or disprove any one hypothesis, but provides a comparison (or accounting) of how the data support each hypothesis and which one is better supported by the available data.

Role of Generalization in HBWoE

- Necessary to try to infer *general* processes from observations of *instances* of that process.
- The generalized process ought to apply to other situations, or at least have reasons why it does not.
- If there are limits to the generalization (*e.g.*, it applies to one species but not another, to males but not females, at this dose but not that dose), then the plausibility of such exceptions, in view of available evidence, becomes part of the evaluation.

In 2010, published HBWoE Evaluation for Naphthalene Carcinogenesis

Critical Reviews in Toxicology, 2010; 40(8): 671–696

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REVIEW ARTICLE

Hypothesis-based weight of evidence: A tool for evaluating and communicating uncertainties and inconsistencies in the large body of evidence in proposing a carcinogenic mode of action—naphthalene as an example

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New Naphthalene Studies Since 2010

- Animal studies
  - 90-day low-exposure rat inhalation study shows minimal to no effect in nasal epithelium at 1 ppm (Dodd *et al*., 2012)
  - Work in progress:
    - Defining cellular patterns of toxicity in the rat nose (Morris and Van Winkle)
    - Defining acute cytotoxicity in rhesus monkey nasal and lung explants (Van Winkle *et al.*)
- Epidemiology reviews
  - Lewis (2011), Magee *et al.* (2010)

New Naphthalene Studies Since 2010

- Metabolism/Toxicokinetic studies
  - DeStefano-Shields *et al.* (2010) – observed protein adducts in rhesus monkey and rat nasal epithelium at similar rates of formation
  - CYP2F2 knockout mouse (Li *et al*., 2011) – CYP2F2 key for lung but not nasal metabolism
  - Mouse nasal uptake (Morris *et al*., 2013) – naphthalene readily taken up and metabolized in mouse upper respiratory tract
  - Metabolism kinetics in rodent and monkey nasal and airway microsomes (Buckpitt *et al.* 2013) – rates much lower in monkey compared to rodent
  - Generation of naphthalene reactive metabolites in rat, mouse, and human nasal and lung cells (Kedderis *et al*., manuscript in preparation)

New Naphthalene Studies Since 2010

- Metabolism/Toxicokinetic studies (cont.) – Work in progress:
  - Evaluating GSH response in rat nasal explants (Morris and Van Winkle)
  - Evaluating naphthalene metabolism mass-balance in rat and monkey nose and airway explants (Buckpitt *et al.*)
  - *CYP2A13/2F1*-Humanized Mouse Model (Li *et al*.)
  - PBPK model (Campbell and Clewell)

New Naphthalene Studies Since 2010

- Mode of Action studies
  - Mutagenesis study (Meng *et al*., 2011) – no increase in p53 mutant fraction in rat nasal respiratory and olfactory epithelia (up to 30 ppm naphthalene, 90-day study)
  - Recio *et al.* (2012) (TK6 human lymphoblast cells) provides strong support for threshold mode of action
    - Significant increase in micronuclei only at concentrations that also induced cytotoxicity, resulting in NOEL for genotoxicity
    - Dependence on GSH depletion
  - Proposed MoA – aryl amidase pathway (Piccirillo *et al*., 2012)
  - (Pham *et al*., 2012a,b) – Naphthalene metabolite protein adduct formation *in vitro* (epoxide, diolepoxide, quinones)
  - Genomics (Clewell *et al.*, work in progress)

Some inconsistencies within the data

- Protein Adducts in Monkey Nose and Lung:
  - Rates of naphthalene metabolite protein binding are similar in rat and rhesus monkey nasal epithelial explants (DeStefano-Shields *et al.* 2010) and in mouse and monkey lung explants (Cho *et al.* 1994; Boland *et al.* 2004)
  - Inconsistent with low rates of naphthalene metabolism in primate nose and lung (i.e., 70- to 100-fold lower compared to rodents)
- Possible reasons we are exploring:
  - Are explant studies predictive of *in vivo* situation?
    - Possible artifact of use of explants due to lack of competing clearance by liver?
  - Are metabolism studies predictive of *in vivo* situation?
  - Do protein adducts cause cytotoxicity in explants?
  - Are protein adducts related to toxicity *in vivo*?

Comparison of Accounts (Example)

Account for Hypothesis #1	*Ad hoc* explanation?	Plausibility that additional data will support explanation	Account for Hypothesis #2	*Ad hoc* explanation?	Plausibility that additional data will support explanation
Animal Data					
explanation and reasoning for key observation		plausible	explanation and reasoning for key observation - may be counter to hypothesis #1	yes	plausible
Epidemiology Data					
explanation and reasoning for key observation		plausibility can reasonably be excluded	explanation and reasoning for key observation - may be counter to hypothesis #1		plausible
Mechanistic Data					
explanation and reasoning for key observation		plausibility can reasonably be excluded	explanation and reasoning for key observation - may be counter to hypothesis #1		plausible
Human Relevance					
explanation and reasoning for key observation	yes	plausibility can reasonably be excluded	explanation and reasoning for key observation - may be counter to hypothesis #1		plausible
Relative weight of evidence for accounts	weaker			stronger	

Notes:

Shaded cells are ad hoc assumptions and/or where additional data are unlikely to support explanation. Accounts with the fewest shaded boxes are considered stronger.

Dose-Response Analysis and Extrapolation to Humans

Challenges in DR Analysis for Naphthalene

- Dose-response data for naphthalene exposure and potential respiratory carcinogenicity in humans is indirect
  - olfactory epithelial nasal tumors in rats (but not mice); benign lung adenomas in mice (but not rats)
- NTP bioassays were only conducted at high doses (10-60 ppm naphthalene) that were also highly cytotoxic
  - providing no information on incidence of non-neoplastic lesions at lower doses
- Evident lack of a tumor effect (nasal and lung) in occupationally exposed people and for people in the general population
- The question of naphthalene's potential respiratory carcinogenicity in humans bears entirely on experimental evidence in rats and mice

Our Approach

- Asks the following questions:
  1. How can we use the results of HBWoE evaluation (i.e., MoA) to inform the dose response?
  2. Since the NTP data are the result of high, cytotoxic doses, how can we better understand what is happening at low doses?
  3. What is likely to be responsible for rat nasal tumors and what is relevance to humans for either nasal tissues or for other places in the respiratory tract?

Our Approach

- To align exposure-response relationships of key precursor non-neoplastic lesions and gene expression responses to exposure-response relationships for tumors
  - GSH depletion
  - Cytotoxicity
  - Respiratory epithelial hyperplasia
  - Tumors
  - Gene expression data
- To develop a sequence of key events for tumor formation in the rat nose that is consistent with the tissue dose-response and the biologically plausible mode of action supported by the HBWoE evaluation.
- To consider how applicable the rat nasal tumors are to serve as a basis for estimation of potential human respiratory-tract cancer risk.

Application of the HBWoE evaluation to inform the dose response

- Preliminary Conclusions of HBWoE Evaluation:
  - The MoA likely involves high-dose cytotoxicity and possibly high-dose genotoxicity from downstream naphthalene metabolites.
  - More strongly support a threshold mode of action (MoA)
  - There is a much larger degree of *ad hoc* argument in the hypothesis that accounts for the data as supporting an initiating genotoxic mode of action.
- The dose-response evaluation needs to attempt to identify this threshold in rodent tissues, and to identify the lowest doses associated with non-neoplastic lesions that are likely precursors to tumors (e.g., cytotoxicity, inflammation, and regenerative hyperplasia).

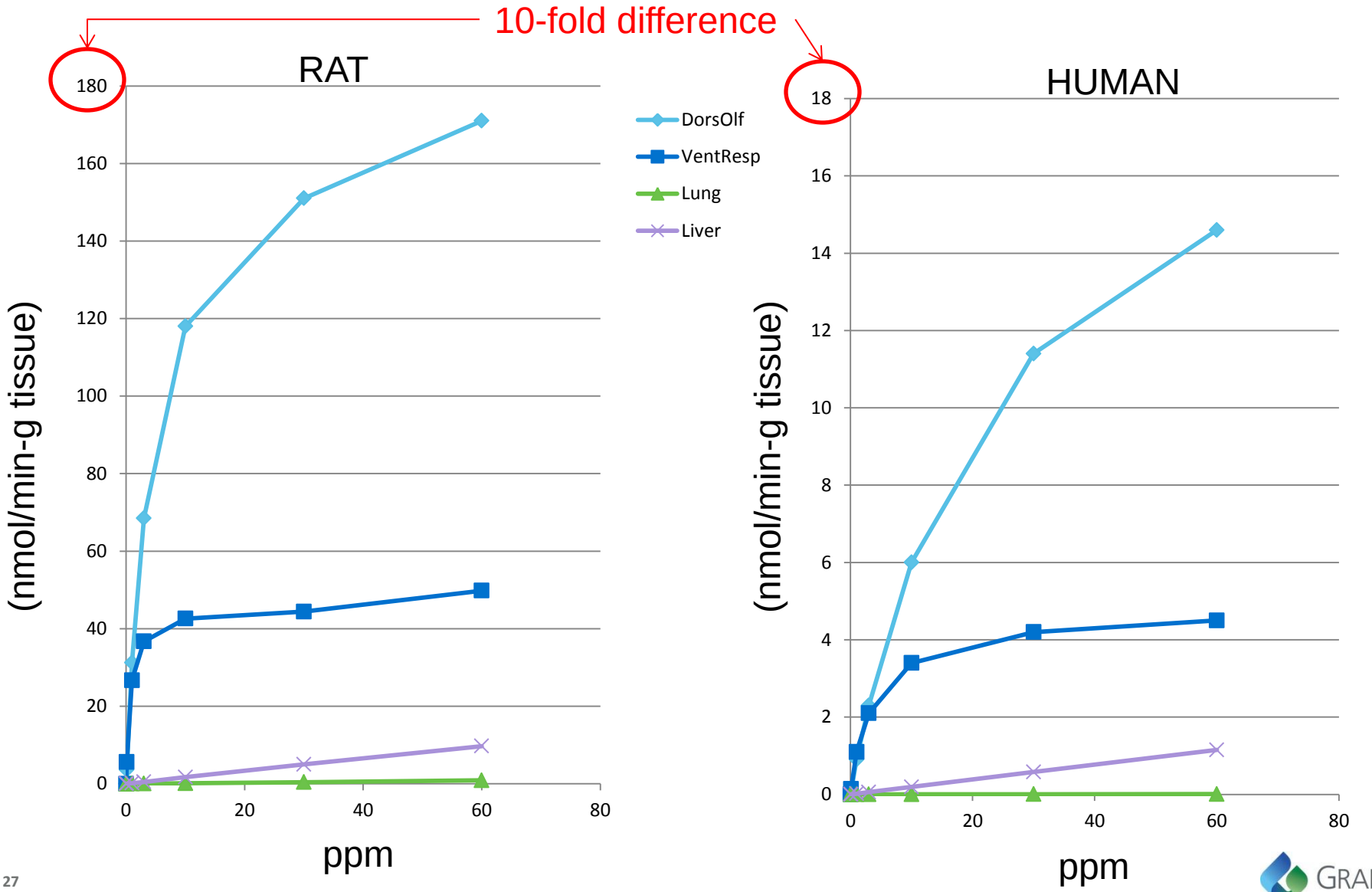
How can we better understand what is happening in rat nasal tissues at low doses?

- Dodd et al. (2012) 90-day inhalation study
  - Identified dose (1 ppm) where minimal to no non-neoplastic nasal epithelial effects observed
  - Suggests threshold
- Evaluate pathology of animals in 90-day study compared to NTP studies (animals with and without tumors)
- Our approach is based on presumption that no effect level for non-neoplastic lesions will also be no effect level for tumors.
- Also will be considering gene expression changes at low doses (Clewell et al., manuscript in preparation).
- PBPK model will provide tissue dose information in locations of nasal lesions, and prediction of doses associated with non-neoplastic effects at low dose.

Dose-Response Modeling of Rodent Data

- Modeling Approaches
  - Benchmark Dose Software Analyses
    - Neoplastic and non-neoplastic lesions
  - Categorical Regression (CatReg) Analyses
    - Non-neoplastic lesions (accounts for severity)
  - Multistage Weibull (MSW) Analyses
    - Neoplastic analyses that account for Time-to-Tumor
- Used PBPK data to model metabolized dose

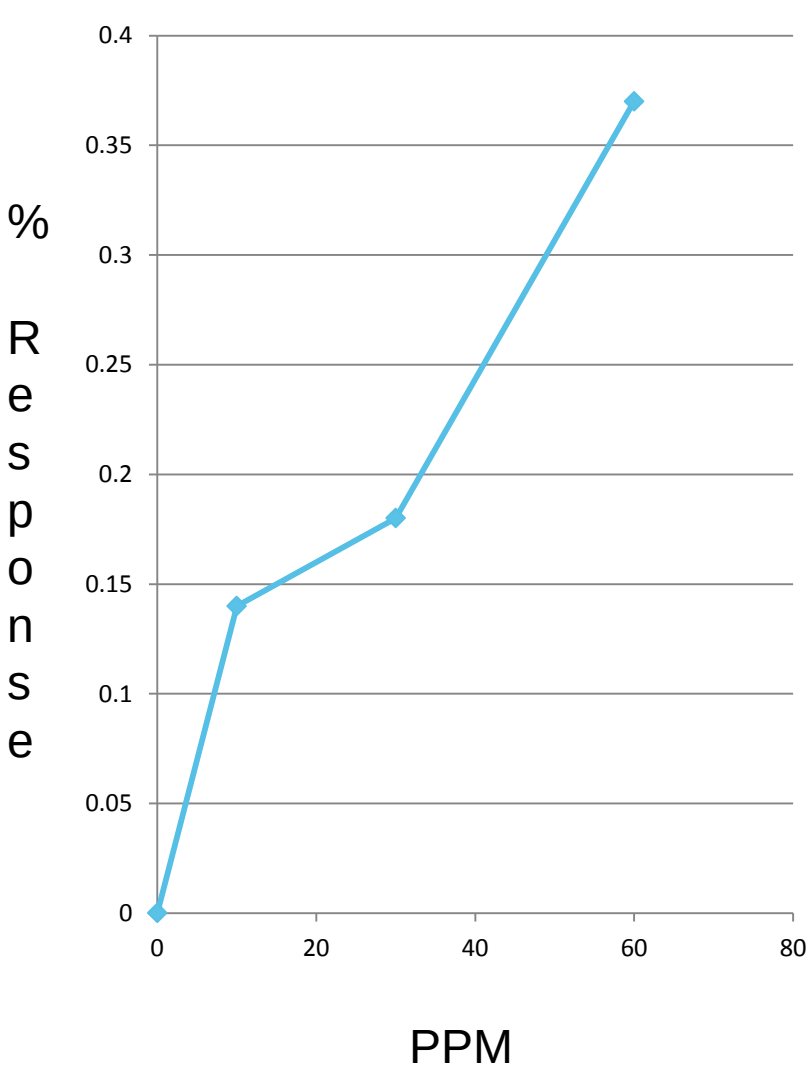
Amount Naphthalene Metabolized – Based on PBPK Model

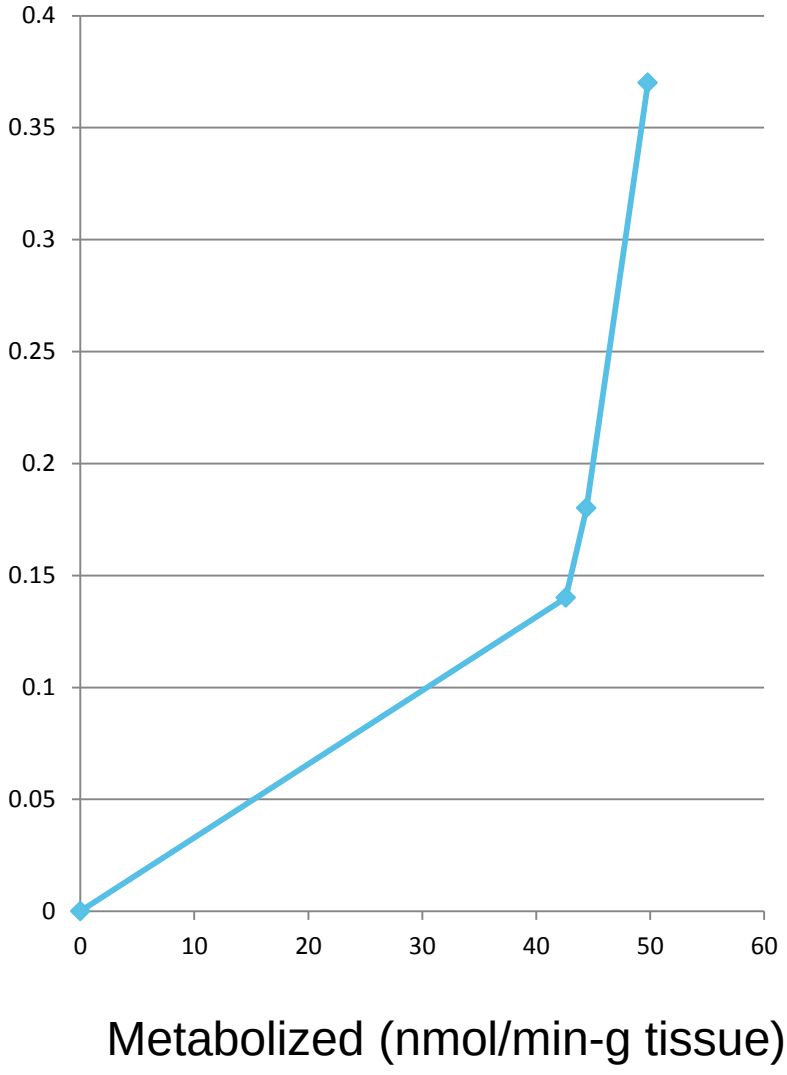


Rat Tumor Dose-Response Based on Administered and Metabolized Dose

- Dose-response based on metabolized dose appears to have a markedly non-linear
  - Male and female respiratory epithelial adenomas (NTP)
  - Male and female olfactory epithelial neuroblastomas (NTP)
  - Non-neoplastic lesions (NTP, 2000; Dodd et al. 2012)
    - Respiratory epithelial hyperplasia
    - Degeneration
    - Squamous metaplasia
    - Goblet cell hyperplasia

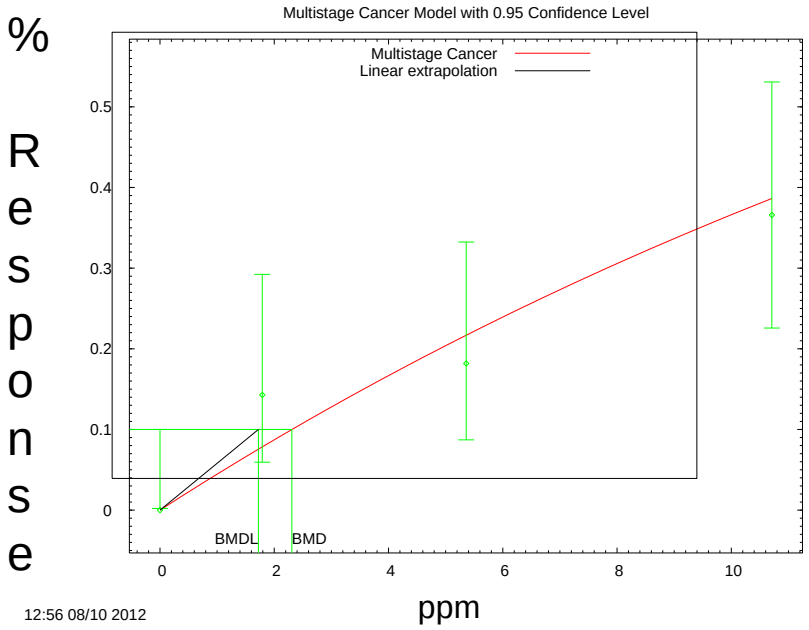
Male Rat Respiratory Epithelial Adenomas (NTP)





Male Rat Respiratory Epithelial Adenomas (NTP) – Multistage Model

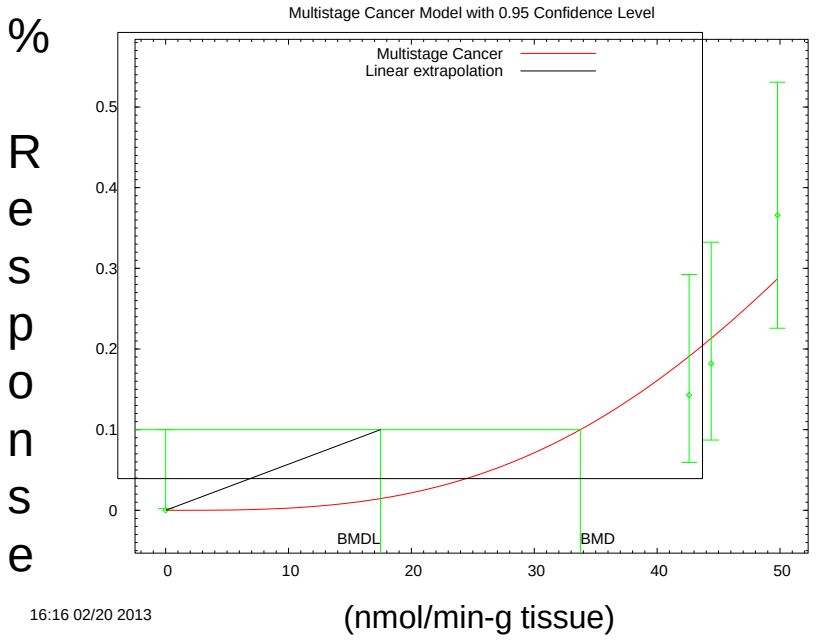
administered dose



m_rea

Conc	Adj Conc	N	Effect
0	0.00	44	0
10	1.79	42	6
30	5.36	44	8
60	10.71	41	15

metabolized dose

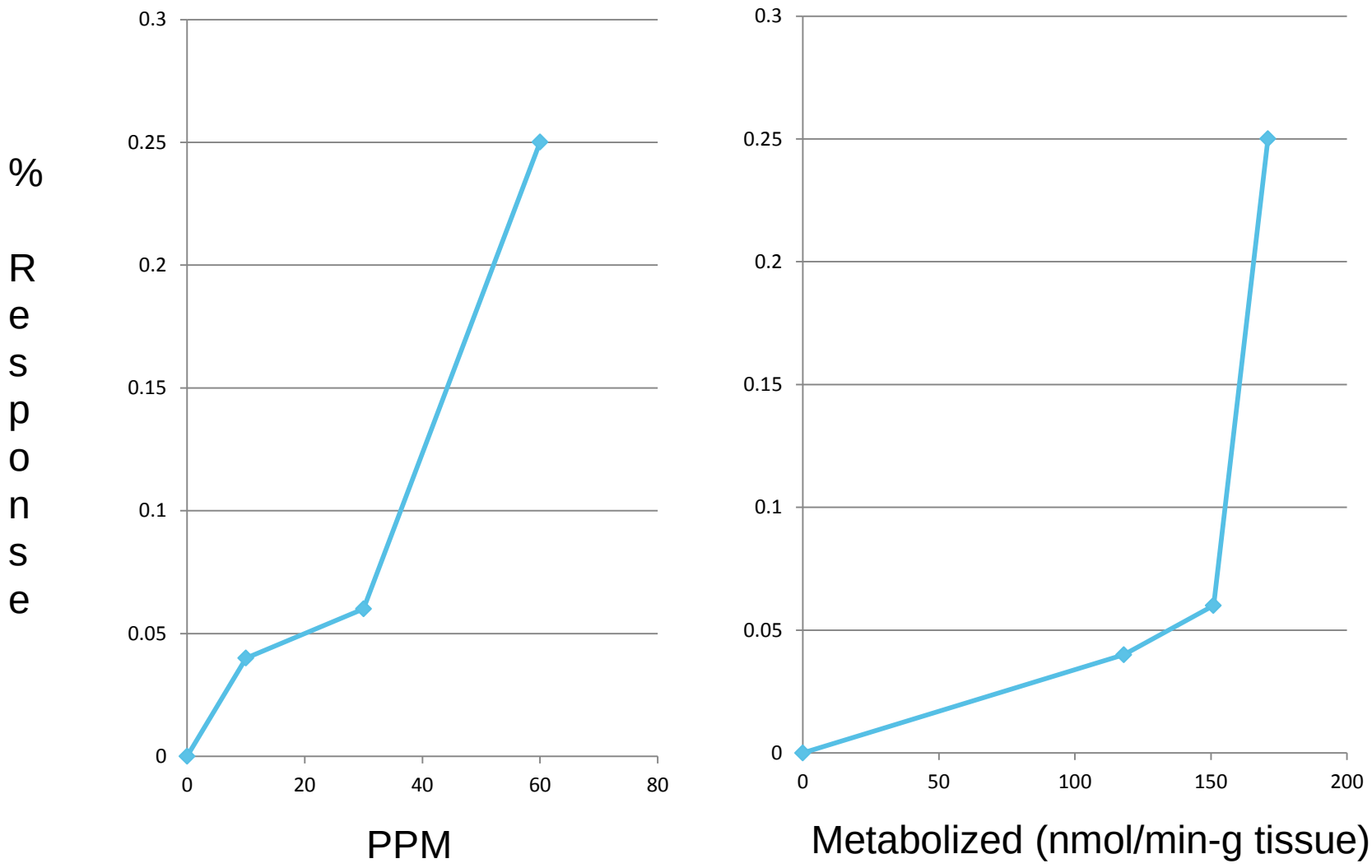


m_rea_md

Conc	Amt Mtb	N	Effect
0	0.0	44	0
10	42.6	42	6
30	44.4	44	8
60	49.8	41	15

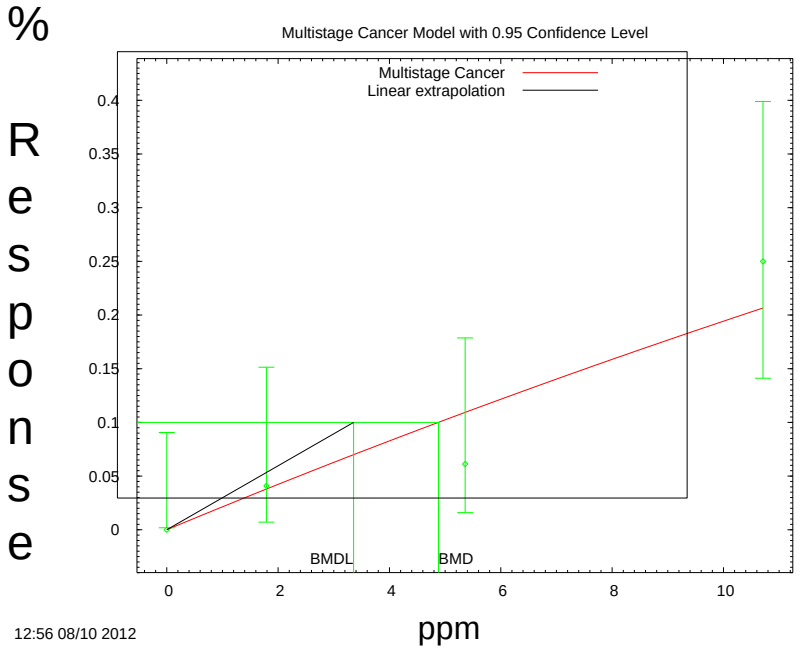
Adj Conc = Conc x 6/24 hr/day x 5/7 day/week = continuous exposure

Female Rat Olfactory Epithelial Neuroblastomas (NTP)



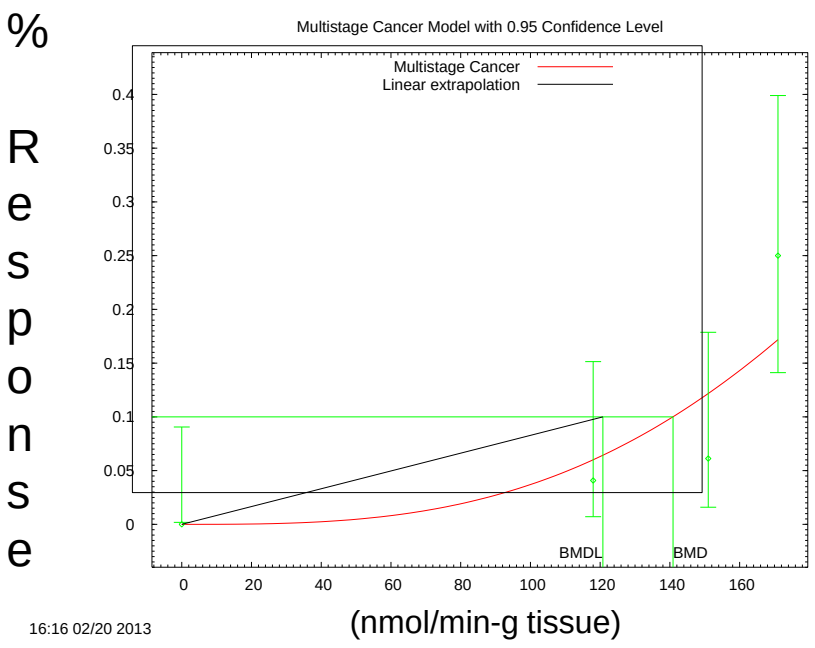
Female Rat Olfactory Epithelial Neuroblastomas (NTP) – Multistage Model

administered dose



f_oen	Conc	Adj Conc	N	Effect
	0	0.00	49	0
	10	1.79	49	2
	30	5.36	49	3
	60	10.71	48	12

metabolized dose



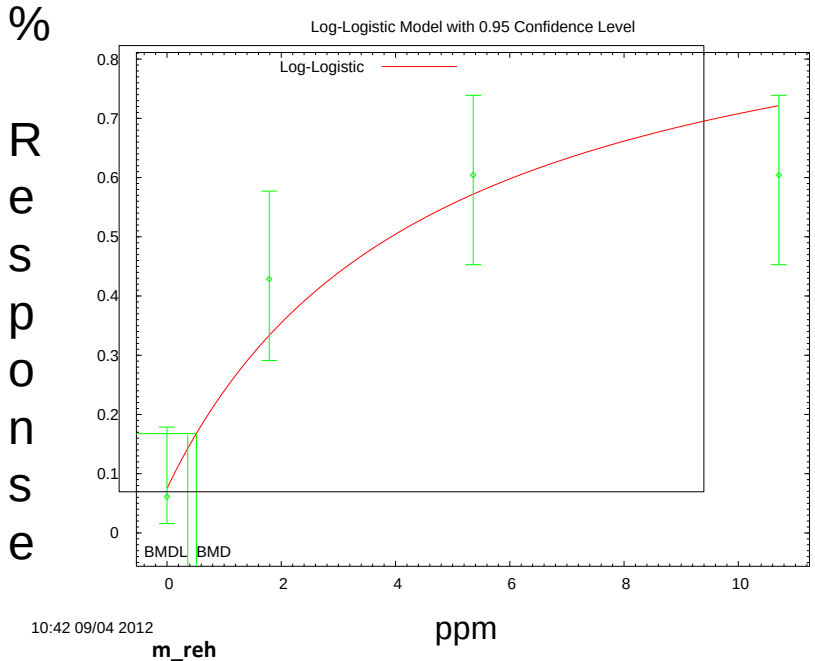
f_oen_md	Conc	Amt Mtb	N	Effect
	0	0.0	49	0
	10	118.0	49	2
	30	151.0	49	3
	60	171.0	48	12

Adj Conc = Conc x 6/24 hr/day x 5/7 day/week = continuous exposure

Non-neoplastic Dose-Response

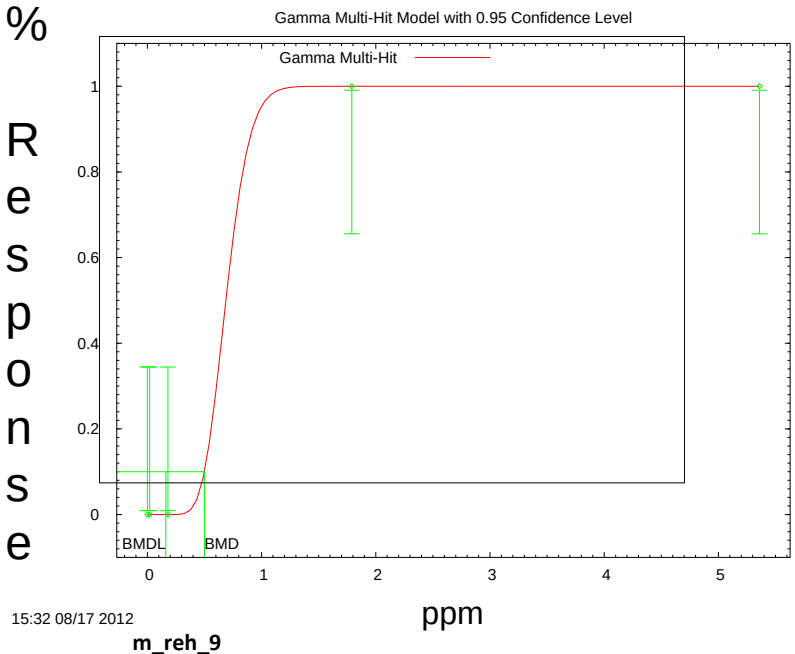
Male rat respiratory epithelial hyperplasia (NTP vs. 90-day administered dose)

NTP (2000) 2 year Study



Conc	Adj Conc	N	Effect
0	0.00	49	3
10	1.79	49	21
30	5.36	48	29
60	10.71	48	29

Dodd et al. (2012) 90 day Study

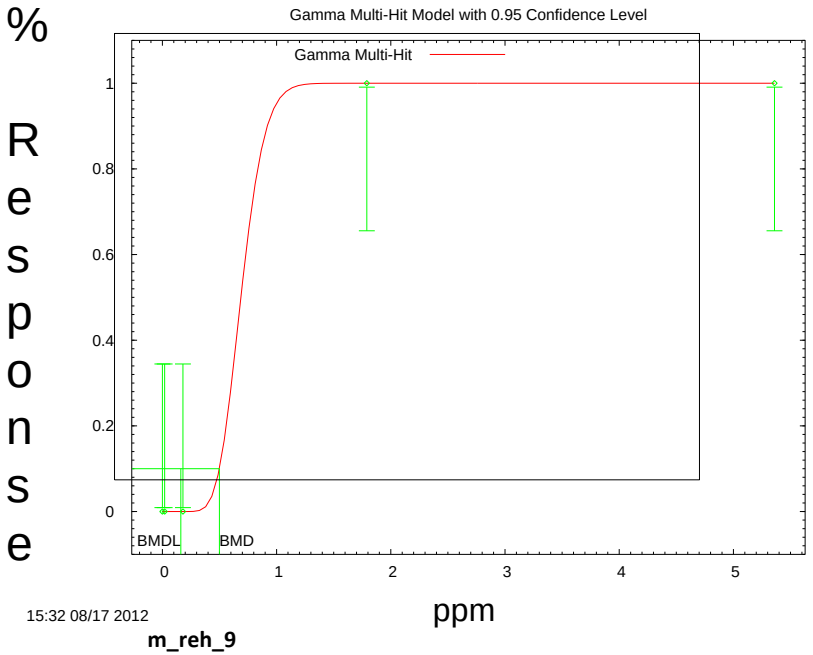


Conc	Adj Conc	N	Effect
0	0.00	10	0
0.1	0.02	10	0
1	0.18	10	10
10	1.79	10	10
30	5.36	10	10

Adj Conc = Conc x 6/24 hr/day x 5/7 day/week = continuous exposure

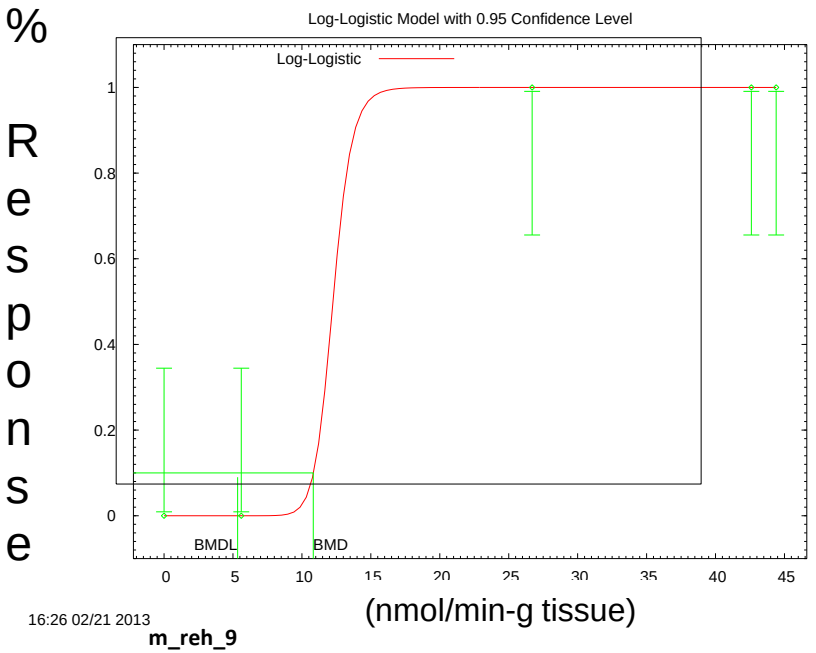
Male rat respiratory epithelial hyperplasia (90-day)

administered dose



Conc	Adj Conc	N	Effect
0	0.00	10	0
0.1	0.02	10	0
1	0.18	10	10
10	1.79	10	10
30	5.36	10	10

metabolized dose



Conc	Amt Mtb	N	Effect
0	0.0	10	0
0.1	5.6	10	0
1	26.7	10	10
10	42.6	10	10
30	44.4	10	10

Adj Conc = Conc x 6/24 hr/day x 5/7 day/week = continuous exposure

Working through inconsistencies in bioassay data to inform dose-response

- Issue:
  - 90-day study suggests respiratory epithelial hyperplasia (REH) is possible key event in rat nasal tumor formation.
  - But, tumor sites in rat NTP bioassay do not correlate well with REH – i.e., animals with tumors did not consistently have REH.
- Questions:
  - Tumor obliteration of non-neoplastic lesion in NTP assay?
  - REH not precursor to tumorigenesis?
- Unpublished work helpful for sorting through issue:
  - Harkema 2001 review of NTP rat assay observed non-neoplastic lesions (REH, inflammation, cytotoxicity) concurrent with neoplastic lesion. Seems inconsistent with NTP.
  - Dodd et al. mapping studies observed REH at 90-day time point in same locations as tumor sites in NTP rat assay.
  - Suggests REH lesions are precursors

What is responsible for rat nasal tumors and what is relevance to humans?

- Based on prediction of tissue doses associated with key non-neoplastic effects at low doses in rats, and a predicted no effect level:
  - Use PBPK model (linked to computational fluid dynamic models of human air flows and metabolic capacities) to predict metabolically activated doses in human nose and other locations in the respiratory tract.
  - Ask whether humans have sufficient metabolic activation in nasal or non-nasal tissues to be near levels needed to produce the key non-neoplastic lesions seen in the rat nose.

Naphthalene Species/Tissue Extrapolation