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Regarding: TOXICOLOGICAL REVIEW OF ACRYLAMIDE (CAS No. 79-06-1): In Support of Summary Information on theIntegrated Risk Information System (IRIS) *December 2007*

Office of Environmental Information (OEI) Docket (Mail Code: 2822T)
U.S. Environmental Protection Agency
1200 Pennsylvania Ave., NW.
Washington, DC 20460.

Dear Colleagues:

We appreciate the opportunity to express our views on this important Agency action and highlight some issues of general and specific interest.

However, our review of your draft document and these comments on it are not comprehensive; we would welcome the opportunity to share additional comments with you in the future. Having conducted numerous noncancer and cancer dose response assessments for chemicals of environmental interest for our federal, state, and industrial colleagues, we appreciate the challenge of addressing complex issues, such as appropriate literature to cite, judgment of critical effect, exploration of mode of action, selection of critical study, choice of point of departure for dose response assessment and investigation of attendant uncertainties. We recently developed a detailed dose response assessment on thyroid tumors for acrylamide (Dourson et al., 2008). This text has been submitted to Regulatory Toxicology and Pharmacology. We have received permission to share it with you and it is attached. We are also continuing our research on mammary tumors and tunica vaginalis mesotheliomas (TVMs) and will notify EPA of progress in these areas as well.

We find ourselves in agreement with EPA's acrylamide text in several places. Specifically:

- We agree with EPA on page 150 that acrylamide shows an increase in both DNA synthesis and DNA damage in mammalian tissues and cells, suggesting that DNA reactivity and cell proliferation, in concert, may contribute to the observed acrylamide-induced carcinogenicity in the rat target tissues. In fact, based on EPA (2005), we judge that it is likely that multiple modes of action (MOA) are occurring with these tumor endpoints and that for at least one of the tumor endpoints, the thyroid, these MOAs can be decoupled to support a more accurate dose response assessment.

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- We agree with EPA on pages 151 & 152 that attempts to quantify mutagenic dose response are clearly in the right direction, and warrants further support and research. Dourson et al. (2008) shows such a quantification in relationship to thyroid tumors.
- We agree with EPA on pages 190 &191 that tumors with statistically significantly elevated incidences in both of the available rat bioassays (thyroid tumors in both sexes, mammary gland tumors in females and tunica vaginalis mesotheliomas in males) should be considered for dose response assessment and to the exclusion of other tumors. We judge that the thyroid tumors provide the best experimental data on which to contemplate possible dose response relationships.
- Moreover, we find EPA's non-cancer dose response assessments to be very nicely done, and substantively agree with EPA's selection of critical effect, benchmark dose (BMD) modeling and selection of point of departure, choice of uncertainty factors and description of confidences for both the Reference Dose (RfD) and Reference Concentration (RfC). We have not reviewed the noncancer assessment at the same level of depth as the cancer assessment, but we do not see the need for any substantive changes in the key decision points for the hazard identification and dose response assessment for noncancer endpoints.

However, the document would be greatly enhanced by a more thorough consideration of alternative cancer MOAs, including consideration of the possibility that multiple MOAs (including a mutagenic MOA) apply. Such an evaluation would identify each of the key events for each of the alternative MOAs, and evaluate the key events against the modified Hill criteria. Although this has been done to a large degree for the mutagenic MOA, and to a somewhat lesser degree for nonmutagenic MOAs, additional consideration of the nonmutagenic MOAs is possible and desirable, based both on the acrylamide data and the underlying biology of the target tissues. Such an analysis has been done by Dourson et al (2008) for the thyroid. As discussed below, and in our general comments, we have also analyzed TVM and mammary tumors. The conclusions of these analyses are consistent with the discussion in EPA's risk characterization recommending additional studies of hormone disruption, as well as with EPA's MOA conclusion that a mixed MOA is possible. These analyses can significantly enhance the overall understanding of MOA in the context of the underlying biology, as well as the understanding of the potential interplay between different MOAs. At a minimum, these additional considerations warrant a more thorough discussion to enhance the document.

We also have several disagreements with EPA. Specifically, with EPA's:

- Presentation of the <u>MOA for tumors endpoints</u>, which we find overly simplistic. For example, many mechanisms exist within and beyond the two broad categories that EPA labels as "genotoxicity" and "hormone-related," for example, growth stimulation in the thyroid as described further in Dourson et al. (2008) and in an attachment to this letter. EPA's minimal discussion of these categories severely impacts the overall confidence in EPA's selected approach.

- Combination of mammary tumors for hazard identification in Table 4-33 (page 141), which we find as inherently inconsistent. Tumors are being combined by EPA authors that other scientists (including EPA scientists, e.g., for the hazard identification for atrazine) do not normally combine. In addition, certain statements on statistical significance (or lack of these statements) are incorrect based on tables presented in the Johnson et al. (1986) and Friedman et al. (1995) papers.
- Combination of thyroid and TVM tumors for dose response assessment. This disagreement is based on two broad observations. First, the MOAs of these tumors are clearly different (as shown in a comparison of Dourson et al., 2008, and comments in the attachment). Second, based on a consideration of MOA data and a screening level comparison of TVM incidence in humans, and acrylamide-related incidence of these tumors in rats, quantitative linear extrapolation of the TVM incidence from F344 rats exposed to acrylamide to humans is clearly inconsistent with the observed human tumor data, and thus not appropriate. Rather we recommend that EPA focus on the thyroid tumors as a basis for quantification, as described in Dourson et al. (2008).
- Mortality adjustment for thyroid tumors in the Friedman et al. (1995) study. A principal reason for conducting such an adjustment is to recognize that when experimental animals die before developing the tumor of interest, the actual lifetime cancer risk maybe higher than that observed in the study. However, our analysis of raw data from this study does not show any consistently lower or higher tumor incidence in rats dying more than one month prior to terminal kill compared with rats dying within one month of terminal kill. Thus, a mortality adjustment does not appear to be needed.
- Combining tumors for dose response assessment. Contrary to a statement on page 205 that, EPA cancer guidelines suggest only two approaches for calculating the risks when there are multiple tumor sites in a data set to assess the total risk, EPA's cancer guidelines provide a number of alterative ways to combine tumor incidences in addition to these two. See page 3-24 and 3-25 of EPA (2005).
- Early life stage risk. Use of its analysis of the Gamboa et al. (2003) study on page 165 to suggest that the cancer slope adjustment factor for early life exposure is needed. This suggestion is fundamentally unsound. For example, Gamboa et al. (2003) show that neonatal mice are less sensitive to adduct formation than adults to AA exposure. The fact that neonatal mice are less sensitive is consistent with EPA (2005) guidelines, page 2-29, which states that carcinogenic metabolites formed through P450 activity will have limited effects in the young. Acrylamide is metabolized to glycidamide by way of P450 activity. This suggests that EPA's cancer slope adjustment factor for early life exposure is specifically not needed.

We also note that significant literature was missed, for example:

- Abramsson-Zetterberg, L. 2003. The dose-response relationship at very low doses of acrylamide is linear in the flow cytometer-based mouse micronucleus assay. Mutation Research 535: 215-222. These authors discuss dose response information for a surrogate genotoxicity endpoint.
- Capen, C.C. 1996. Chapter 3: Anatomy. In Werner and Ingbar's The Thyroid: A fundamental and clinical text. Braverman L.E., and Utiger, R.D. (Eds.). Lippincott-Raven. Philadelphia- New

York. p.19-46. This author discusses information on a possible site of action for thyroid growth stimulation.

- Chico Galdo, V., Massart, C., Jin, L., Vanvooren, V., Caillet-Fauquet, P., Andry, G., Lothaire, P., Dequanter, D., Friedman, M. and J. Van Sande. 2006. Acrylamide, an *in vivo* thyroid carcinogenic agent, induces DNA damage in rat thyroid cell lines and primary cultures. Molecular and Cellular Endocrinology: 257-258:6-14. These authors discuss mechanisms of thyroid tumor formation from acrylamide exposure.
- Dulak, L. H., 1989. A lifetime oncogenicity study in rats with acrylamide. American Cyanamid Company. Performed by: Tegeris Laboratories and Pathology Associates, Inc. Study No. 85033. This author shows the raw data for the Friedman et al. (1995) study
- Imai, T., Cho, Y. M., Hasumura, M., Hirose, M., 2005. Enhancement by acrylamide of N-methyl-N-nitrosourea-induced rat mammary tumor development-possible application for a model to detect co-modifiers of carcinogenesis. Cancer Lett. 230, 25-32. These authors discuss possible promotional effects of acrylamide.
- Hurley, P.M., Hill, R.N., and R.J. Whiting. 1998. Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents. Environmental Health Perspectives. 106: 437-445. These authors discuss pesticides and likely MOAs for thyroid tumorigenesis.
- Lafferty, J. S., Kamendulis, L. M., Kaster, J., Jiang, J., Klaunig, J. E., 2004. Subchronic acrylamide treatment induces a tissue-specific increase in DNA synthesis in the rat. Toxicol. Lett. 154, 95-103. These authors show data consistent with thyroid growth stimulation in Fisher 344 rats
- LoPachin R, Barber D, He D, Das S. Acrylamide inhibits dopamine uptake in rat striatal synaptic vesicles. *Toxicol Sci* 2006; 89: 224-234. These authors present evidence for effects on dopamine signaling (rather than just dopamine levels in the brain).
- Maniere, I., Godard, T., Doerge, D. R., Churchwell, M. I., Guffroy, M., Laurentie, M., Poul, J. M., 2005. DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide. Mutat. Res. 580, 119-29. These authors show data that can be used to quantitatively address mutagenicity and tumor endpoints.
- Marxfeld, H., et al., 2006. Gene expression in fibroadenomas of the rat mammary gland in contrast to spontaneous adenocarcinomas and normal mammary gland. Experiment. Toxicol. Pathol. 58, 145-150. These authors discuss possible MOAs for mammary tumors.
- McConnell, E. E., et al., 1986. Guidelines for combining neoplasms for evaluation of rodent carcinogenesis studies. JNCI. 76, 283-289. This author describes general rules for combining different types of tumors for hazard identification.
- Twaddle, N. C., Churchwell, M. I., McDaniel, L. P., Doerge, D. R., 2004. Autoclave sterilization produces acrylamide in rodent diets: implications for toxicity testing. J. Agricul. Food Chem. 52, 4344-9. These authors describe contamination of rat chow with acrylamide.
- Williams, E., 1995. Mechanisms and pathogenesis of thyroid cancer in animals and man. Mutat. Res. 333, 123-129. This author states that thyroid tumor growth from genotoxicity is not likely in humans without growth stimulation.

A complete list of other relevant literature is on an attachment.

We briefly further describe each of these general disagreements in an attachment, along with other specific comments.

Our mission is to protect public health, as is yours. We encourage EPA to hold fast to this mission, and to adhere to its recently published guidelines (2005), in order to use the available and extensive information on the tumor and genotoxicity information on acrylamide in a more scientific appropriate way. Such use will enable credible risk management decisions that ultimately best protect the public's health.

Sincerely,

Michael L. Dourson, Ph.D., DABT, ATS
Director

Lynne Haber, Ph.D., DABT
Manager, Research Program

Andrew Maier, Ph.D., CIH, DABT
Associate Director

for Richard Hertzberg, Ph.D.
TERA Fellow

The opinions expressed in this commentary reflect those of *TERA* and do not necessarily represent the views the sponsors of Dourson et al. (2008). Staff time to develop these comments was supported by *TERA*, and not any outside party.

Attachment

General Comments

Mode of Action for Tumor Endpoints. EPA's presentation of the MOA is very simplistic and does not adequately discuss differences between genotoxicity effects that would not be expected to be linear and data regarding truly linear effects. The focus on mutagenicity would be enhanced if it addressed the multiple underlying mechanisms for the observed mutagenicity data and other genotoxicity. The concepts of GSH-dependence and oxidative stress seemed absent, and the role of clastogenicity versus mutagenicity seems to have been addressed only in a limited fashion. Thus, many mechanisms that would impact the likely accuracy of a low-dose linear approach were given limited treatment in the text. For example, the statement on Page 224 related to the potential for evidence of other MOAs to support sublinear curvature in the low-dose region is a point that could be enhanced based on our research results (Dourson et al., 2008).

Moreover, while we agree with EPA that the available genotoxicity data indicate temporal concordance, the available genotoxicity data are not dose response concordant, as clearly shown by Allen et al (2005) and in a separate analysis by Dourson et al. (2008) using newly published *in vivo* genotoxicity data. Such dose response concordance is as important as temporal concordance in the evaluation of this MOA as per EPA guidelines (2005).

Lumping of all other MOA possibilities in the "hormone related MOA" seemed minimalistic as well, although the text did identify the key salient data gaps. Increased text would provide a better picture of the current science. For example, growth stimulation and hormone-related MOAs deserves more discussion and analysis. See Dourson et al (2008)

Our analysis leds us to propose that if mutagenicity is operating to cause tumors, it most likely is doing so along with another MOA. In fact, EPA's guidelines (2005) state that when two MOAs operate in the same tissue or organ, the biological data should be used to "decouple" the two MOAs in order to determine the contribution of each MOA to the tumor risk. Thus, at least some of the high dose tumors are likely to be caused by other MOAs.

MOA for Thyroid Tumors. The hormonal support for thyroid tumors is more than just "relatively little." Please see Dourson et al., (2008) for a discussion of this and other support for thyroid growth stimulation.

MOA for Mammary Tumors. Potential key papers related to the mammary tumors were not mentioned by EPA in the tumor mode of action section, and which would impact the discussion. Specifically, key omissions include LoPachin et al. (2006), which shows evidence for effects on dopamine signaling (rather than just dopamine levels in the brain), and the rat mammary tumor promotion study by Imai et al. (2005). In addition key MOA considerations for mammary tumors that should be more fully described in the IRIS draft findings are as follows:

- The assessment should provide in the analyses the biological basis for combining or not combining mammary tumor types. Our MOA analyses found no evidence that

fibroadenomas can progress directly to malignant tumors. This conclusion is based on analyses of the literature on comparative gene expression profiles across mammary tumor types (e.g., Marxfeld et al., 2006), evaluation of evidence for increased malignant breast cancer risk in humans who have had benign mammary tumors (e.g., Hartmann et al., 2005), and the chronic bioassay data itself in which individual rats did not show both fibroadenomas and adenocarcinomas. Thus, according to EPA guidelines, these stromal tumors (fibroadenomas) should not be combined with epithelial tumors (adenomas or adenocarcinomas) in conducting a linear assessment.

- We do identify evidence for linkages between epithelial and stromal cell growth regulation via epigenetic mechanisms that might support consideration of a non-linear analysis using combined mammary tumors.
- Thus, the discussion of mammary tumors should evaluate systematically the variety of plausible MOAs. These include: 1) genotoxicity both direct and secondary to oxidative stress, 2) endocrine effects due to age-related hyperprolactanemia or perturbations secondary to neurotoxicity, and 3) the potential promoting effects of paracrine regulation of mammary epithelial cell growth by stromal cells. Our analysis shows that none of these mechanisms strongly out weigh the others, that the most likely explanation for the tumor observations is a combination of multiple MOAs, and that the overall weight of evidence favors a non-linear modeling of the mammary tumors that captures multiple MOAs.
- As noted above an approach based on application of the Hill Criteria should be shown for each of these possibilities. Such an analysis will highlight the possibility of a role of mutagenicity, it will highlight the likely role of these other possible MOAs, and thus inform the most biologically relevant dose-response approach that is consistent with EPA guidelines.

<u>MOA for TVMs</u>. We have also conducted a detailed evaluation of the TVMs and mesotheliomas seen in the acrylamide exposed F344 rats, considering both the available information on the biology of these tumor types in general, the incidence in humans, and the rarity of this tumor type in species and strains other than the F344 rat. The analysis concluded that only some relatively minor fraction of the TVMs in rats exposed to acrylamide may be relevant to humans. Multiple MOAs may apply for the TVMs, and some contribution from a mutagenic MOA is plausible. Leydig cell tumors (LCTs) may play a role in TVM formation, and this role could be endocrine, paracrine, or the result of a physical interaction, such as pressure from the increased size of testes bearing LCTs. Alternatively, the same hormonal milieu that predisposes F344 rats to LCTs may also predispose them to TVMs, although at a lower level. In light of the available data on MOAs for the TVMs, the incidence of TVMs and total (non-pleural) mesotheliomas in humans, as reported by the SEER registry (Young et al., 2007; Greenberg et al., 2002), and estimated acrylamide intake in food, it is inappropriate to apply a linear slope factor from the TVM data in F344 rats to humans. Specific data supporting this analysis includes:

- TVMs occur almost exclusively in male F344 rats. No increase in mesothelial tumors were reported in the female F344 rats in the acrylamide bioassays, indicating that the acrylamide-related TVMs do not reflect a general tumorigenic influence on mesothelial tissue. The observation that TVMs occur preponderantly in F344 rats means that some

aspect of the biology of this strain makes it particularly susceptible to this tumor type. The specific factor making the F344 rat susceptible is not definitively understood.

- One hypothesis is that TVMs seen after acrylamide exposure are secondary to the enhancement of LCTs in F344 rats. This relationship between TVMs and LCTs could be endocrine, paracrine, or the result of a physical interaction, such as pressure from the increased size of testes bearing LCTs.
    - The background incidence of LCTs is too high to observe an effect of acrylamide on LCT incidence. It may be possible to evaluate the effect of acrylamide on LCT size, but this study has not been conducted
    - Data are available suggesting that acrylamide increases LCT size (Iatropoulos et al., 1998), but the data are weak to moderate.
    - The evidence is stronger for an effect of acrylamide on earlier key events in the development of LCTs. The strongest data support the hypothesis that acrylamide affects LCT development by acting on levels of prolactin and/or testosterone (Ali, 1983; Uphouse et al., 1982; Friedman et al., 1999), but the data are insufficient to definitively determine how these effects occur. Increased dopaminergic activity is consistent with the observed effects on prolactin and testosterone, and interactions of acrylamide with the dopaminergic system have been documented, but there is no clear evidence showing acrylamide to be a dopamine agonist.
    - If acrylamide does affect LCTs via increasing dopaminergic activity, that MOA for LCT development is not relevant to humans (Clegg et al., 1997).
    - The other MOAs for the formation of LCTs described by Clegg et al. (Clegg et al., 1997) are all nonmutagenic, and if acrylamide were to increase LCT incidence or size via any of these MOAs, a nonlinear approach would be used for low-dose extrapolation for an effect on LCTs
    - The data regarding a causal connection between LCTs and TVMs are weaker than the data supporting an effect of acrylamide on LCTs, but an association is observed.
    - The physical proximity of the tumors and substantial concordance between the size of LCTs and progression of tunica vaginalis tumors following acrylamide exposure (Iatropoulos et al., 1998) suggests a relationship between the two tumor types. TVMs are found almost exclusively in the presence of LCTs, although there is not complete concordance between these two tumor types in the overall literature (Ilgren, 1993; Shipp et al., 2006). Concordance appears to be stronger for acrylamide
    - The data reviewed are not sufficient to distinguish between there being a causal relationship between LCTs and TVMs, and the hypothesis that both of these tumor types respond to some other influence (e.g., hormonal milieu of the F344 rat). Both mechanisms may apply. For example, an effect external to the testis may predispose F344 rats to the development of TVMs, and this predisposition being enhanced by increased testis size due to an effect of acrylamide on LCTs.
    - Based on the MOA(s) for LCT formation, the proportion of TVMs that are secondary to LCT formation would either (1) not be considered relevant to humans (if they result from increased dopaminergic activity) or (2) a nonlinear approach would be appropriate for extrapolation to low doses.

  - No evidence of direct hormonal communication between Leydig cells and the tunica vaginalis was located.
- Another hypothesis is that TVMs seen after acrylamide exposure result from mutagenicity or other DNA reactivity of acrylamide or its metabolite glycidamide on the tunica vaginalis. This effect may be enhanced by endocrine or paracrine influences. Although the overall data on mutagenicity do not support mutagenicity being the primary cause of the TVMs, a small contribution of mutagenicity to the development of these tumors is plausible. Also, acrylamide could cause mutations via indirect mechanisms of reaction with DNA, such as resulting from oxidative stress. Linear low-dose extrapolation would not be expected to be appropriate for an oxidative stress MOA.
- Yet another hypothesis is that TVMs seen after acrylamide exposure result from some other (as-yet-unidentified) hormonal signal that may also play a causal role in LCT development, with the TVMs occurring in parallel with the LCTs, rather than being secondary to them.
- Finally, LCTs may be one of multiple pathways for development of TVMs (i e , one of multiple potential precursors), with contributions from mutagenicity and/or endocrine influences.
- While the data are insufficient to definitively show any one MOA occurs, the relevance to humans of the TVMs remains a possibility. However, if the tumors occur in humans, the potency would be expected to be much lower than in F344 rats Overall, these data suggest that a mutagenic MOA cannot be ruled out, but a nonmutagenic MOA is more likely driving the tumor response. Alternatively, a mutagenic MOA may be responsible for a small percentage of the total tumor response, with the majority of the response due to a nonmutagenic MOA.

Combination of Mammary Tumors The combination of mammary tumors in Table 4-33 (page 141) is inherently inconsistent. Tumors are being combined that others (including EPA scientists as mentioned earlier) do not normally combine, and certain statistically significant statements (or lack of these statements) are incorrect based on tables presented in the Johnson and Freidman papers. For example, mammary adenomas did not statistically significantly increase in either study, nor were fibroadenomas statistically significantly increased in Johnson et al (1986). However, adenocarcinomas were statistically significantly increased in Johnson et al. (1986). Moreover, no clear rationale was provided for combining of tumors or discussion of the important issue of ability of tumors to progress to malignancy. This key issue seems to have been missed or disregarded.

An alternative approach consistent with EPA guidelines and more in-line with the weight of evidence would be to develop a linear assessment based on the incidence of adenomas and adenocarcinomas (but not fibromas or fibroadenomas). The dose-response modeling and underlying biology support use of a probit model with a BMR of 0.02 similar to analyses noted in Dourson et al. (2008). Such an approach yields a cancer slope factor of 0.022 per mg/kg-day as compared to a slope factor of 0.055 mg/kg-day based on the multistage model with a BMR of 10%.

Combination of Thyroid and TVM Tumors EPA's combination of thyroid and TVM tumors for dose response assessment is not appropriate. Our disagreement with EPA on this point is based

on two broad observations. First, the MOAs of these tumors are clearly different as shown by detailed analyses of Dourson et al (2008) for thyroid tumors and as shown above for TVMs. For example, thyroid tumors are conservatively assessed by considering a mutagenic MOA in the low-dose region and thyroid growth stimulation MOA at higher doses (Dourson et al., 2008). In contrast, the MOA for TVMs includes physical interaction, a unique hormonal milieu of male F344 rats, and possible mutagenicity. Second, a screening level comparison of TVM incidence in humans, and acrylamide-related incidence of these tumors in rats, shows that a quantitative extrapolation of the TVM incidence from rats to humans is not appropriate, as shown below:

- The available TVM data could be used to develop a very conservative estimate of the risk of TVMs using either a linear or nonlinear extrapolation from an LED10. CalEPA (2005) calculated upper bound human potency estimates for TVM of 0.58 and 0.4 per (mg/kg-day) from the Johnson et al. (1986) and Friedman et al. (1995) studies. JECFA (2005) estimated that average acrylamide intake at the national level ranged from 0.3 to 2.0 μg/kg-day. For high percentile consumers (90th to 97.5th percentiles), intake estimates ranged from 0.6 to 3.5 μg/kg bw per day, and up to 5.1 μg/kg bw per day for the 99th percentile consumer. JECFA stated that children appeared to ingest approximately two to three times the adult intake when expressed on a body weight basis.
- Using the average of the two slope factors calculated by CalEPA (2005) of 0.49 per mg/kg-day and an average intake of 2 μg/kg-day would result in a risk of TVMs in the human population of 0.00098, or a risk of almost 1 in a thousand.
- Of the available epidemiology studies of acrylamide and cancer, studies of the Marsh cohort (Marsh et al., 1999; Marsh et al., 2007) investigated the incidence of cancer of the testis and male genital tract. No effect was seen, although the absolute numbers of observed and expected cancers was very low (1-2 in a cohort of up to 8508), and the statistical power to detect an increase was low, as reflected by the broad confidence limits (Erdreich and Friedman, 2004). However, the very low background incidence of TVMs in humans would make a small increase on the order of 10^{-4} or 10^{-5} easily detectable, and an increase of 1 in a thousand would be quite evident, and likely would be reported in the literature, independent of any association with acrylamide exposure. Human exposure to dietary acrylamide has occurred for long enough that latency would not be an issue in the quantitation.
- Based on these considerations alone, quantitative extrapolation from the rat TVMs to risk in humans does not appear to be appropriate.
- One might consider that the target for acrylamide is mesothelial tissue in general, rather than the tunica vaginalis mesothelium. The human incidence of peritoneal mesotheliomas is higher than that for TVMs, but similarly is below the incidence expected based on linear extrapolation from the F344 rat data and dietary intake of acrylamide.
- Thus, this screening-level evaluation based on cancer risks extrapolated from the TVM data in rats is inconsistent with the human data on TVM and mesotheliomas incidence. This inconsistency could reflect a biological difference between F344 rats and humans. Alternatively, the discrepancy could be because the screening-level quantitation used a linear extrapolation, while a biphasic approach may be more appropriate.

<u>Mortality adjustment for thyroid tumors</u>. EPA conducted a mortality adjustment for thyroid tumors in the Friedman et al. (1995) study. A principal reason for conducting such adjustments is to recognize that when experimental animals die before developing the tumor of interest, the

actual lifetime cancer risk maybe higher than that observed in the study However, our analysis of raw data from the Friedman et al. (1995) study does not show any consistently lower tumor incidence in rats dying more than one month prior to terminal kill compared with rats dying within one month of terminal kill. Thus, a mortality adjustment for the thyroid tumor response does not appear to be needed.

Combining Tumors for Dose Response Assessment EPA (2005) lists multiple ways to combine tumors for dose response assessment (see page 3-24 and 3-25 of EPA, 2005). Based on our analysis that indicated two MOAs appropriately model tumors occurring in the thyroid, we chose to pool the results from both sexes and both studies in order to increase the data set and thus improve the ability to identify where each MOA was most influential. This pooling of tumor responses was done according to EPA (2005) and resulted in a dose response assessment curve with 19 data points, which enabled the MOA "decoupling" concept of EPA to be further investigated The support for this dual MOA and the pooling of thyroid tumor data is shown in more detail by Dourson et al. (2008).

The cancer modeling section states that the linearized multistage model is to be used unless it does not fit well. That is contrary to good modeling practice, in which the list of candidate models is determined first by those that match the understanding of the physical process being modeled. For acrylamide, that process seems to involve both a mutagenic and a nonmutagenic (threshold like) mode of action. The set of candidate models should then be restricted to those that allow a low slope at low doses, where the genotoxicity is operational, and a transition to a higher slope at moderate to high doses. The logistic and probit models allow that behavior, whereas the multistage does not.

Early Life Stage Risk. On page 165, EPA states that

> "Aside from the assumption that early life stages are more susceptible to mutagens, there are limited data on early-life susceptibility to [acrylamide] AA-induced carcinogenicity Gamboa et al. (2003) measured DNA adduct formation in selected tissues of adult and whole body DNA of 3-day-old neonatal mice treated with AA and [glycidamide] GA. In adult mice, DNA adduct formation was observed in liver, lung, and kidney with levels of N7-GA-Gua around 2000 adducts/108 nucleotides and N3- GA-Ade around 20 adducts/108 nucleotides Adduct levels were modestly higher in adult mice dosed with GA as opposed to AA; however, treatment of neonatal mice with GA produced five- to seven-fold higher whole body DNA adduct levels than with AA. The authors suggest that this is due to lower oxidative enzyme activity in newborn mice."

And thus a reasonable conclusion from this text would be that neonatal mice are less sensitive than adults to the carcinogenic effects of acrylamide exposure, because they make less of the genotoxic metabolite glycidamide, correct? In fact, such a conclusion is consistent with several studies in humans that show CYP2E1 levels to be absent or minimal during gestation, followed by a rapid increase immediately following birth, and a gradual increase into adulthood (e.g., Vieira et al., 1996, Hakkola et al., 1998, Johnsrud et al., 2003). In addition, EPA (2005) guidelines, page 2-29 states that "...metabolic capacity with respect to P450 enzymes in newborn children is extremely limited compared to that in adults, so that a carcinogenic metabolite formed through P450 activity will have limited effect in the young, whereas a carcinogenic agent

deactivated through P450 activity will result in increased susceptibility of this lifestage ...". Thus, based on all of these results, an adjustment to the cancer slope factor for early life exposure is specifically not needed.

Specific Comments

- Scientists that reviewed the document outside of EPA's ORD are not listed. Was this an oversight?
- We do not agree with EPA's text on page 81, and elsewhere, that brain tumor incidence rates should have been more fully documented in the Friedman et al. (1995) study. All brains were evaluated in females and in control and high dose males. It is a standard operating procedure to only analyze intermediate doses if the high dose shows an increased incidence (it did not in Friedman et al. (1995). We agree with EPA that Friedman et al. (1995) could have done a better job in the reporting of spinal cord tumors, but do not see a higher incidence of tumors in this latter organ with the available data.
- EPA's text on page 192, and specifically in Table 5-8, is incorrect, in that the raw data of Friedman et al. (1995) DOES report a follicular cell adenoma in the assessment. The number of rats should be 3/102 instead of 2/102.
- EPA's text on page 192 and appendix D - EPA describes the selection of the multi-stage model because the "linear approach is a default option if the MOA of carcinogenicity is not understood." However, our more complete analysis of the MOA for thyroid (Dourson et al., 2008), and analyses for TVM and mammary tumors concluded that at least two MOA were ongoing and likely in different parts of the dose response curve. For thyroid tumors, this allowed us to determine that the probit model, rather than the multistage model, was a better fit of the observed multiple modes of action for acrylamide (Dourson et al., 2008).
- EPA's text in Appendix D shows that EPA did not combine male and females from Friedman study, whereas a statistical analysis of the thyroid tumor responses shows no difference; there is likewise no statistically significant difference in female and male response for thyroid tumors in the Johnson et al. (1986) study (unpublished observations, but available upon request).
- Control doses in both the Johnson et al. (1986) and Friedman et al. (1995) studies actually contained acrylamide. We estimate these "control" doses at 0.002 mg/kg-day (Dourson et al., 2008). EPA's tumor dose response assessments should incorporate this adjustment.
- EPA's text in Appendix D shows that EPA combined the two control groups into one control group in the Friedman et al. (1995) study, whereas these control groups were actually dosed groups.
- EPA (2005) guidelines suggest using the lowest point of departure, or benchmark response (BMR), that is consistent with the data. EPA's acrylamide draft focused on the response of only one sex of one study and chose a 10% BMR. In contrast, pooling the thyroid tumor results from all 4 studies (one of EPA's possible approaches) gives 19 doses and allows a lower point of departure to be determined. Dourson et al. (2008) shows this as a 2% BMR.

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Evidence-Based Dose Response Assessment for Thyroid Tumorigenesis from Acrylamide

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Abstract

Acrylamide is commonly found in various foods. Cancer studies in rats have reported increases in tumors of the thyroid, mammary tissues, tunica vaginalis of the testis, and sometimes tumors at other sites. We review relevant studies on acrylamide's DNA toxicity, tumor formation and the manner of its tumor formation. We find, as do others, that glycidamide (a metabolite of acrylamide) causes mutations, but acrylamide does not. We also find that thyroid tumors are most consistently sensitive in rats, being evoked in each of 4 long-term experiments. We evaluate the common manners of this tumor formation in the thyroid, including both mutagenicity and thyroid growth-stimulation. Consistent with the overall weight of the evidence, we conclude that both of these manners or modes of action may be occurring. We conservatively assume that the mutagenic mode of action determines the low-dose response and we conclude that growth stimulation dominates the response at higher doses. Following the suggestion in U.S. EPA guidelines, we "decouple" these modes of action and determine that the probit model, unlike the multistage model, best reflects the overall data set and is further supported by the likely underlying "decoupled" biology. We use the probit model to identify a health-protective, linear cancer slope factor (SF) of 0.030 $(mg/kg\text{-}day)^{-1}$ for the low area of the dose response curve associated with possible mutagenicity. We also identify a Reference Dose (RfD) in the range of 0.05 to 0.02 mg/kg-day for the high area of the dose response curve associated with the growth stimulation. This latter value can be used to determine the upper range of risk.

Introduction

A number of investigators have conducted dose response assessments for acrylamide due to its widespread occurrence in foods. Some of these investigations have modeled a large spectrum of tumors in a more screening level assessment (e.g., OEHHA, 2005). Such screening level assessments are useful as an initial means of determining whether risks are of sufficient concern to warrant further study. Shipp et al. (2006) developed a more comprehensive assessment. Such comprehensive assessments provide the necessary analysis and data for enhancing the confidence in risk management decisions.

Any review of a chemical's potential cancer causation in humans should first start with human data. Marsh et al. (2007) reported that exposures to acrylamide in an occupational cohort were not associated with elevated cancer mortality risks in brain and other central nervous system, thyroid gland, testis and other male genital organs, esophagus, rectum, pancreas, and kidney. Except for respiratory system cancer, attributed to muriatic acid exposure, none of the mortality

excesses was statistically significant. Swaen et al. (2007) examined the long-term health effects of occupational exposure to acrylamide as well and found no exposure-related, cause-specific SMR for any of the investigated types of cancer. Recent epidemiology studies of acrylamide in food, including prospective data from large cohorts, do not find a higher risk of several cancers in humans (Pelucci et al, 2006; Mucci et al., 2003, 2004, 2005, 2006). A recent prospective study of dietary acrylamide assessed breast, endometrial and ovarian cancer (Hogervorst et al, 2007). As in Mucci et al (2005), Hogervorst et al. (2007) found no evidence for an elevated risk of breast cancer. Increased risk was seen in the highest quintile of acrylamide exposure for both endometrial and ovarian cancer in never smokers, and borderline increase was observed for ovarian cancer in the total group. No statistically significant increased risk was seen for endometrial cancer in never-smokers.

In contrast to these mostly negative human studies, acrylamide carcinogenicity studies in rats have reported increases in both benign and malignant tumors of the thyroid, mammary tissues, and tunica vaginalis of the testis, as well as sporadic findings of other tumors. In particular, thyroid tumors were seen in both sexes of the two available long-term bioassays of acrylamide (Johnson et al., 1986; Friedman et al., 1995). Johnson et al. (1986) conducted several interim kills that showed that the onset of tumors was generally late in the experiment (at nearly 2 years). Latency information was not reported in the Friedman et al. (1995) study.

As discussed more fully by Capen (1992, 2001) and Williams (1992, 1995), the etiology of thyroid tumors has been studied in both humans and experimental animals. Thyroid tumorigenesis in humans has two identified modes of action (MOAs): growth stimulation and mutagenesis associated with radiation---the only known human thyroid carcinogen (Hurley et al., 1998). The growth stimulation MOA is typically caused by a disruption in thyroid hormone synthesis or metabolism leading to a decrease in thyroid hormone levels and a rise in thyroid stimulating hormone (TSH). Tumors arising from growth stimulation in humans are typically benign. The mutagenic MOA for the thyroid involves direct mutation of thyroid DNA or growth stimulation in combination with multiple mutations. Moreover, the increase in cellular growth rate also increases the probability of a mutation. However, tumors in humans do not form in the presence of mutagens if TSH-stimulated growth is prevented (Williams, 1995).

Rat follicular cell thyroid tumor etiology is similar to that of humans, beginning with the follicular cell, progressing to follicular adenoma and ending in follicular carcinoma (Capen, 1992). However, unlike development of follicular cell tumors in humans, rat thyroid carcinogenesis can result from growth stimulation both with and without mutagenesis (McClain, 1992). In addition, other differences as described by Capen (1992, 2001) need to be considered when analyzing tumor incidence in humans and rodents, and in any extrapolation of rat tumor incidence data to humans. The first difference is the lack of thyroid-binding globulin in rodents. This globulin is responsible in humans for binding and transporting thyroid hormones in the blood. This globulin allows more circulating hormone reserves in humans than in rodents. As a result, the rodent thyroid is less able than the human thyroid to maintain thyroid hormone homeostasis. The second difference is that the half-life for the thyroid hormone, thyroxine (T_4), is shorter in rodents than in humans (12 hours versus five to nine days), and TSH levels are 25 times higher in rodents than humans. These factors indicate that the rodent thyroid is much more active (and functions at a higher level) than that of humans. This makes rats more susceptible to

thyroid tumors than humans and care must be taken in any extrapolation from these rodents (Meek et al., 2003). The U.S. Environmental Protection Agency (U.S. EPA, 1998) uses this information to assume that humans and rodents are equally sensitive to thyroid carcinogens, with a corresponding default interspecies extrapolation factor of 1-fold. However, this assumption is readily seen to be conservative based in part on the observation that approximately 10-times the amount of T4 is needed for rats when compared with humans, when both are thyroid deficient (Capen, 2001).

Acrylamide, and its more DNA-reactive metabolite glycidamide, are widely distributed throughout all of the tissues of the body, with the only significant accumulation occurring in red blood cells (DeJongh et al., 1999; Dybing et al., 2005; Kirman et al., 2003). Furthermore, while reaction with DNA has been proposed as a plausible mechanism of tumorigenesis, similar levels of DNA adducts have been observed in the various organs of rats exposed to acrylamide, and maximum levels of DNA adducts in mice are observed in the liver, which is not a target of acrylamide-induced carcinogenesis in the rat (mouse carcinogenesis testing is ongoing) (reviewed by Dybing et al., 2005). In rats, no apparent kinetic explanation exists for the tissues that are targets for acrylamide-induced tumorigenesis. If the target tissue specificity is due to kinetic phenomena, one would expect the target tissues to have higher doses of some likely toxic moiety, or of a surrogate tissue dose metric. These data suggest that the mechanism of acrylamide-induced carcinogenesis is driven by dynamic differences among the tissues, rather than kinetics. The fact that many of the tumor sites in experimental rats are also associated with hormone production is not inconsistent with a hypothesis that dynamic differences among tissues form a key determinant for acrylamide tumorigenesis.

The current U.S. EPA (2005) cancer risk guidelines discuss a low-dose linear, non-threshold dose-response approach for a mutagenic or direct DNA acting MOA, and a so-called non-linear or Reference Dose (or Reference Concentration) approach for MOAs not involving direct DNA interaction. When two MOAs operate in the same tissue or organ, the guidelines recommend that biological data should be used to "decouple" the two MOAs in order to determine the contribution of each MOA to the tumor risk.

The purpose of this manuscript is to briefly review relevant experimental animal studies on acrylamide for thyroid tumorigenicity, genotoxicity, and thyroid growth stimulation. We then follow the U.S. EPA (2005) framework for analyzing the weight of evidence supporting a mutagenic MOA and a thyroid growth stimulation MOA. Based on the results from this MOA analysis, we then determine the most appropriate health conservative approach for low-dose extrapolation of thyroid tumors found in rats to potential tumors in humans.

Methods

We first describe our methods for analyzing mutagenicity and growth stimulation modes of action. Afterwards, we describe our method for modeling tumors that is supported by both mutagenic and thyroid growth stimulation.

Mode of Action. We use the mode of action (MOA) framework within the recent cancer risk assessment guidelines of the U.S. Environmental Protection Agency (U.S. EPA, 2005), which

built on the work of Meek et al. (2003). In accordance with these guidelines, we consider whether each MOA is sufficiently supported by the existing human or experimental animal data, and whether the available evidence suggests these MOAs are relevant to humans. Based on the data describing the key precursor events, we also discuss lifestages that could be more susceptible for dose-response analysis. Furthermore, as per U.S. EPA (2005) guidelines, the model used for extrapolation to low doses is determined based on the most relevant MOA(s) given our current understanding of the science.

U.S. EPA (2005) lists several potential MOAs in general. Specifically, for the thyroid tumors described here we investigate two MOAs:

- A heritable mutation to thyroid follicular cell DNA,
- Thyroid follicular cell growth stimulation.

Both of these MOAs have supporting data and have been discussed by other investigators in dose response assessments for acrylamide (e.g., OEHHA, 2005; Shipp et al., 2006).

We first analyze the mutagenicity data to determine the extent to which a mutagenic mode of action is responsible for the thyroid tumors observed in the acrylamide cancer bioassays. To make that evaluation, one is interested in the consistency of, or concordance between, the pattern of thyroid tumor response, on the one hand, and the pattern of the selected genotoxicity measures. This comparison is ideally done between the tumor data and a marker of mutagenicity in the same species, sex, and tissue. Such mutagenicity data are not available for acrylamide. Therefore, the relevant available *in vivo* genotoxicity data are used as potential surrogates for mutation events possibly leading to tumor development. Concordance of the tumor and genotoxicity patterns is based on comparison of the dose-response curves describing the observed tumor and genotoxicity data sets.

In order to represent the tumor dose-response for this comparison, we follow the standard U.S. EPA practice of fitting a multistage model to the thyroid tumor data (U. S. EPA, 2005). The degree of the polynomial in that model is set to one less than the number of dose groups. The data are represented as proportions (or observed probabilities); the number of animals with thyroid tumors is divided by the total number of animals in each dose group. The multistage model predicts the probability of tumor response as a function of dose. The U.S. EPA software, BMDS (version 1.4.1; U.S. EPA, 2003), is used to obtain and evaluate the model fits.

For any genotoxicity endpoint (MF, % Tail DNA, Olive Tail Moment, all of which are continuous or pseudocontinuous endpoints), a power model is used to relate the mean response to the dose level, i.e.,

$$m(d) = a + b*d^c,$$

where d is dose, m(d) is the mean response at dose d, and the parameters a, b and c are to be estimated. It is assumed that the genotoxicity observations are normally distributed around those means with a dose-dependent variance related to the mean response:

$v(d) = \alpha * m(d)^{\rho}$

where v(d) is the variance around the mean for dose d, and α and ρ are parameters to be estimated. When appropriate (i.e., when suggested by likelihood ratio test results), ρ is set to 0 to yield a constant-variance model. BMDS (version 1.4.1) is used to fit this model to the data.

The model fits to the genotoxicity data predict mean response as a function of dose. But, because our goal is to compare the genotoxicity and tumor dose-response patterns, a common response "scale" is needed. Thus, genotoxicity modeling results are converted to a probability of response scale, where the response in this case is some "adversely" high level of genotoxic insult. Considering that the original-scale genotoxic responses are assumed to vary normally around dose-dependent means, the probability of an "adversely" high insult is just the likelihood, given the normal distribution of responses, that the level of insult will be greater than a specified cut point. Conversely, given a probability of response, and the model-estimated mean and variance, one can determine what cut point gives that probability. This idea of converting between a continuous measurement and a probability of an "adverse" response is familiar from the so-called hybrid approach used in the definition of benchmark doses for continuous endpoints (Kodell and West, 1993).

Given a background probability of adverse response, β, the definition of which is discussed below, the equation for the probability of response as a function of dose is:

$$P(d) = 1 - N(m(d), v(d), N^{-1}(m(0), v(0), 1-\beta))$$

where N(x, y, z) is the probability that a normal random variable with mean x and variance y will be less than z; $N^{-1}(\mu, \sigma^2, t)$ is the inverse normal function giving the value such that the cumulative probability that a normal random variable with mean μ and variance σ^2 will be equal to t; and β is the assumed background probability of response. The values of m(d) and v(d) are as determined by the fit of the power model to the genotoxicity data, as discussed above. Note that when d=0,

$$\begin{aligned} P(d) = P(0) &= 1 - N(m(0), v(0), N^{-1}(m(0), v(0), 1-\beta)) \\ &= 1 - (1-\beta) = \beta, \end{aligned}$$

which confirms that β is the background (i.e., no-exposure) probability of response.

Thus, a key step of this analysis is the definition of the background response probability. A background response rate of around 0.01 is often implicitly assumed in benchmark dose analyses for continuous endpoints. However, such a default choice is not considered appropriate for the current analysis, since the question being addressed is not what level of the genotoxic response is "adverse" per se, but how the genotoxicity and cancer dose-responses compare. For that comparison, a "normalization" of the genotoxic response probabilities that equates responses in the absence of exposure is desired, for reasons explained in the Discussion section. To achieve that normalization, the background probability of response for all genotoxic endpoints is set equal to 0.024, which is an estimate of the background thyroid tumor response rate obtained by

pooling the control responses from the four cancer bioassays being considered (males and females in both the Johnson et al. and the Friedman et al. studies).

Evaluations of the concordance of the genotoxicity and tumor endpoints are based on the inspection of the probability of response for the respective dose-response curves. The predicted probabilities of response for the genotoxicity endpoints are plotted on the same graph as the predicted probabilities of the tumor response. Determinations are made with respect to the consistency of those predicted curves. Concordance is judged to be stronger when the probabilities of a genotoxic endpoint are generally and consistently close to the probabilities of the thyroid tumor responses across dose levels. Concordance is similarly judged to be stronger when the pattern of increases in the probability of one response is reflected in the pattern of increases observed for the other endpoint (e.g., if a genotoxicity endpoint and the tumor response are concordant, one would expect to see substantial increases in the probability of the genotoxic endpoint in the range of doses where tumor probability increased substantially).

The evaluation of concordance, though based on the quantitative representation of the dose-response relationships for the endpoints considered here, is ultimately a scientific judgment based on the consistency of the overall shape of the dose-response relationships using the criteria listed above, rather than looking for parallelism over the entire dose-response curves. When lack of concordance between thyroid tumors and any given genotoxicity endpoint is suggested by this determination, the inference is that the genotoxic endpoint could not be a surrogate for a presumed one-and-only acrylamide-dose-related mutagenic precursor event for the thyroid tumor response. When the general pattern over *all* genotoxic endpoints evaluated is determined to indicate lack of concordance, the inference is that a mutagenic mode of action (as the single acrylamide-dose-related precursor) is not supported as a reasonable basis for assessing acrylamide risk, based on the assumed concordance between the thyroid tumors in the F344 rat and the model species/tissues that are the source of the genotoxicity data, and taking into account the caveats about doses and duration of dosing noted in the discussion.

We then evaluate whether or not a growth stimulation mode of action is primarily responsible for the thyroid tumors observed in the acrylamide cancer bioassays. For this MOA, we reference Capen (2001) and U.S. EPA (1998) among several resources to evaluate thyroid tumorigenesis.[1]

[1] The role thyroid growth stimulation in the development of thyroid follicular cell tumors has been reviewed many times (e.g., Capen, 1992, 2001; EPA, 1998; Hill et al., 1989). For example, thyroid releasing hormone (TRH) is released by the hypothalamus, and stimulates the pituitary to release thyroid stimulating hormone (TSH). TSH stimulates the thyroid to transport inorganic iodide into thyroid follicular cells, where it is converted to an organic form, and made into thyroid hormones (T3 and T4), which are then released to the body. High thyroid hormone levels result in negative feedback that reduces the levels of TRH and TSH levels. Conversely, decreased thyroid hormone levels result in increased TSH release by the pituitary. A number of different mechanisms exist by which growth stimulation can result in tumors, but the different mechanisms have in common increases in cell division in the thyroid. This increase in thyroid cell size, hypertrophy, and hyperplasia, if sustained over many months, will likely lead to thyroid tumor formation.

For example, U.S. EPA (1998) asks for evidence in the following 5 areas in order to show a thyroid-pituitary MOA:[2]

1. Increases in cellular growth (e.g., increased thyroid weight, hypertrophy or hyperplasia, documentation of proliferation detected by DNA labeling or mitotic indices);
2. Changes in thyroid and pituitary hormones;
3. Location of site of action (e.g., thyroid, liver, or peripheral, and enzyme target within the target organ);
4. Dose correlations among thyroid effects and cancer;
5. Reversibility of effects when chemical dosing stops.

Method For Modeling Tumors Via Mutagenic and Thyroid Growth Stimulation MOAs. The U.S. EPA approach for quantitatively characterizing cancer risk begins by modeling the available data and defining a Point of Departure (POD). The POD is a dose-response point that is estimated for a specified response, the Benchmark response (BMR), near the low end of the dose-response data (U.S. EPA, 2005). The acrylamide analysis focuses on experimental animal data, thus the approaches described here are those most relevant to animal studies. Risk levels of 1-10% are commonly used for the BMR, and the U.S. EPA cancer guidelines (U.S. EPA, 2005) specify that the POD should be the lowest POD that is adequately supported by the data. The dose at the BMR, called the Benchmark Dose (BMD), is usually defined in terms of extra risk. For a specified BMR, the BMD is then the dose that satisfies the following formula for extra risk:

$$BMR = \frac{P(BMD) - P(0)}{1 - P(0)}$$

As per EPA (2005) guidelines, we represent uncertainties in the estimation of risk as the best estimate and its upper bound. A lower bound on risk is often desired to show the full range of uncertainty, but current EPA (2001) BMD software does not provide a way to estimate this risk in many of its models. For policy reasons, the POD is usually defined using the upper bound on the risk, which is associated with the lower 95% confidence bound on the prescribed dose, referred to as the lower bound on the benchmark dose, or BMDL. However, as noted above, U.S. EPA (2005) also recommends presenting the best estimate POD, referred to as the benchmark dose, or BMD, to improve the uncertainty description. Another common term is the Slope Factor (SF). This value is calculated from the BMD or BMDL and represents the slope at the given parameter; the SF can be used to determine risk at lower doses.

[2] Although stated otherwise, not all of these criteria are required. For example, in 3 of 4 examples that U.S. EPA (1998) uses to more fully explain its framework, U.S. EPA does not even discuss evidence for reversibility. Follow up conversations with U.S. EPA staff acknowledged that based on many years of experience, reversibility, while desirable, is not always necessary to demonstrate a plausible growth stimulation mode of action (Burnam, 2007). Recent lectures by EPA staff also indicate that sustained increased thyroid hormone is not required (Haber, 2007), since the thyroid hormones may return to normal but the organ is at a different homeostatic set point.

The U.S. EPA software package, BMDS (v. 1.4.1) is used here for estimating BMD, BMDL and SFs. Adequacy of fit is judged by the model significance level (where p>0.10 indicates acceptable fit). Models with acceptable fit are then compared using 4 additional parameters: the Akaike Information Criterion (AIC)---a measure of the deviance of the model fit adjusted for the degrees of freedom, residuals, the difference in ratios between the BMD and BMDL, and visual fits to the actual data.

The next step depends on the MOA that has been determined to apply to the tumor type of interest. For a mutagenic MOA, the modeling assumption in the absence of more refined data is no threshold dose, and low-dose linearity. A line connects the POD to the origin, corrected for background. The slope of the line is used to estimate a risk per incremental increase in dose. Using a BMD based on extra risk, one calculates the SF directly from the desired BMR level. The stability of the slope estimate is gauged by evaluating it for different BMR and BMD values. For example, if the BMD at 0.02 excess risk equals 0.83, then:

SF = BMR/BMD = 0.02/0.83 = 0.024

When the chemical acts via a non-mutagenic MOA, U.S. EPA describes a nonlinear approach. In this case, the POD (based on either tumors or a precursor endpoint) is used to develop a Reference Dose or Reference Concentration for oral or inhalation exposures, respectively, following the procedures prescribed by U.S. EPA for non-cancer toxicity, with the BMDL commonly divided by one or more uncertainty factors (U.S. EPA, 2002).

However, if supporting data exist, U.S. EPA (2005) guidelines also allow the separate evaluation of MOAs in different parts of the dose response range. In this case, the guidelines are not prescriptive, but an approach consistent with the guidelines would be to select a model within U.S. EPA's array that best fits the most relevant data from a MOA(s) perspective. Afterwards, different approaches may be used at points of the dose response curve associated with the appropriate MOA(s).

Results

We first describe tumor observations in experimental animals, focusing on thyroid tumors because each of 4 experiments showed these tumors to be statistically significantly different than controls. We then show an assessment for a heritable mutation to thyroid follicular cell DNA, focusing on concordance in time and dose response of surrogate mutations. We then discuss an assessment for thyroid follicular cell growth stimulation. Afterwards, we describe the modeling of tumors based on both mutagenic and thyroid growth stimulation and the extrapolation of our findings in rats to humans.

Tumor Observations in Experimental Animals. A 2-year toxicity and cancer study for acrylamide using male and female Fischer 344 rats was conducted by Johnson et al. (1984) and published by Johnson et al. (1986). Interim results were reported by Gorzinski et al. (1984). Groups of 90 rats/sex/dose group (900 total, housed double occupancy) were administered acrylamide in drinking water, to maintain doses of 0, 0.01, 0.1, 0.5, or 2.0 mg/kg-day for both sexes with free access to water. Endpoints evaluated were: mortality; gross pathology; routine

hematology and urinalysis; body weight; organ weight for brain, heart, liver, kidneys, and testes; microscopic pathology for all lesions, tissues and organs with emphasis on the brain (6 sections), spinal cord (3 sections), peripheral nerves (3 separate nerves). Cumulative mortality (found dead plus moribund killed) did not show a dose-related trend until the 21st month of the study; from month 21 to the end of the study, mortality was statistically significant for the high dose group of both sexes. Decreased mean body weight was statistically significant for males in the high dose group, mean body weights for all other dose groups of both sexes were either similar or equivalent to controls. In female rats increased tumor incidences were observed in the thyroid, mammary gland, CNS, oral tissues, uterus and clitoris. Male rats had increases in thyroid and scrotal (tunica vaginalis of the testis) tumors. Subsequent to this study, it was discovered that rat chow routinely contains acrylamide, and thus, the control group of this study is expected to have been exposed. Estimates of baseline levels of acrylamide in rat chow are in a range of 3 to 130 ppb, but generally ~20 ppb or less (Twaddle et al., 2004). From these data, we calculate an average of 27 ppb based on analysis of several unaltered diets. Although the use of this average is reasonable, we conservatively use a value of 20 ppb to estimate the dose associated with the control level in this Johnson et al. (1984; 1986) study. This estimated dose is approximately 0.002 mg/kg-day [0.020 mg/kg of diet (i.e., 20 ppb) x 0.086 food feeding factor for a chronic study in F344 rats (U.S. EPA, 1988) = 0.002 mg/kg-day].

A second 2-year cancer study with male and female Fischer 344 rats exposed to acrylamide was conducted by Dulak (1989) and published by Friedman et al. (1995). A total of 585 males and 300 females (housed individually) were dosed with acrylamide in drinking water, to maintain doses of 0, 0, 0.1, 0.5, or 2.0 mg/kg-day for males and 0, 0, 1.0, or 2.0 mg/kg-day for females with free access to water. The study contained two control dose groups to allow statistical comparison between the control groups for background tumor variability. An unbalanced study design was used, with additional animals in the control and low-dose groups, in order to increase the statistical power to detect increases in tumors at low doses. Endpoints evaluated were: mortality; gross pathology; body weight; organ weight for brain, liver, kidneys, and testes; microscopic pathology for thyroid, brain (3 levels), spinal cord (3 levels), uterus and mammary glands (females); testes (males); and gross lesions in all control and high dose animals and any animals killed moribund or found dead in the other dose groups. Survival for the males was good for the first 15 months; mortality increased during month 17 for the high dose group and continued through the end of the study. Survival for the females was good for the first 23 months, mortality increased for the high dose group during the last month of the study. Generally decreased physical condition (decreased food consumption, scant feces, thin animal) was seen in both sexes for the high dose groups in the last months of the study, consistent with the increased mortality toward the end of the study. In female rats, increased tumor incidences were observed in the thyroid and mammary gland. Male rats had increases in thyroid and scrotal tumors. An adjustment for control dose is also made for this study, as above.

The fraction of thyroid tumors as a function of acrylamide dose from these two long-term bioassays is shown in Table 1. These fractions are not statistically significant until the high doses of either sex in either study, but the mid dose in the Friedman et al. (1995) study appears biologically significant in females. In the Johnson et al. (1986) study, serial kills were conducted that identified the onset of these tumors as late in the experiment (at nearly 2 years), and none of the tumors were considered to be the primary cause of death. Information on tumor onset was

not reported in the Friedman et al (1995) study, but it was reported in Dulak (1989). Thyroid tumors were not more frequent in rats at the end of the study when compared with rats dying earlier.

Recent U.S. EPA (2005) guidelines for cancer risk assessment state that the mode of action should be evaluated in determining the approach for dose response assessment from positive human or experimental animal tumor data. This evaluation is accomplished by first proposing a MOA, including identification of key events. Data on these key events, including all available in vivo, in vitro, and mechanistic studies are then evaluated relative to the modified Hill criteria, including strength, consistency, specificity of the association between the key event(s) and tumor outcomes, as well as consideration of the consistency of the dose-response and temporal relationship between the key event and tumors, biological plausibility of the proposed MOA, and coherence of the overall database. A biologically-based dose-response (BBDR) model is the preferred method for low dose extrapolation. Absent such a model, low dose extrapolation usually procedes by either a linear or a non-linear model based on one or more combinations of relevant tumors. The guidance also notes "If there are multiple modes of action at a single tumor site, one linear and another nonlinear, then both approaches are used to decouple and consider the respective contributions of each mode of action in different dose ranges." Afterwards, determination of the human equivalent dose from the experimental animal dose is accomplished by a comparison of human and experimental animal kinetics or a default procedure.

Therefore, we first evaluated the biological data regarding the MOA for thyroid tumorigenesis, paying particular attention to the weight of evidence regarding the two primary MOAs: a heritable mutation to thyroid follicular cell DNA, and thyroid follicular cell growth stimulation. As part of this analysis of MOA, we compared the available genotoxicity data with the tumor data, in order to test the Hill criteria of temporality and dose-response. Based on the evaluation of the MOA, the approach for dose-response modeling and extrapolation to low doses is identified and applied to obtain a point of departure and cancer slope factor.

Assessment for a heritable mutation to thyroid follicular cell DNA. The genotoxicity data on acrylamide have been discussed in a number of reviews (e.g., Dearfield et al., 1988, 1995; EU, 2002; OEHHA, 2005; Shipp et al., 2006), and so this text highlights primarily key and newer studies. Overall, the genotoxicity data indicate that acrylamide is clastogenic, and that its metabolite glycidamide is an active mutagen *in vivo* and *in vitro*.

Acrylamide is consistently negative in bacterial mutation assays in the presence and absence of exogenous metabolic activation, while glycidamide is a direct-acting mutagen in such assays. A positive result was observed with acrylamide in the mouse lymphoma gene mutation assay (Moore et al., 1987), but the increase was limited to small colonies, and so reflected clastogenicity, rather than gene mutation. Other *in vitro* mammalian cell gene mutation assays with acrylamide are positive at cytotoxic doses (Knapp et al., 1988, in mouse lymphoma cells), negative (with Chinese hamster V79H3 cells, Tsuda et al., 1993), or are negative, but tested to insufficiently high doses. Results are available from a few *in vivo* somatic cell gene mutation assays. Neuhauser-Klaus and Schmahl (1989) found that acrylamide is positive in a mouse specific locus assay designed to detect somatic mutations in the developing fetus. However, no analysis was conducted to determine whether the observed effects are due to point mutations, or

to larger DNA lesions, such as whole chromosome loss or somatic recombination. Therefore, these results are not informative as to whether acrylamide (or its metabolite) is interacting directly with DNA.

Transgenic animals with easily retrievable markers for detecting mutations have the potential to improve the detection of somatic cell gene mutations. Small, but consistent and statistically significant increases over controls in mutation frequencies in the bone marrow are observed in validation studies with the *LacZ* transgenic system in mice treated with acrylamide (Hoorn et al., 1993; Myhr, 1991). A recent publication by Manjanatha et al. (2006) also showed that repeated oral doses of acrylamide and glycidamide resulted in dose-related increases in *HPRT* mutation frequency in lymphocytes, and *cII* mutation in liver cells of transgenic mice. Interestingly, similar dose-responses are observed for acrylamide and glycidamide. Molecular analysis of the mutations indicated that acrylamide and glycidamide produced similar mutation spectra, which is consistent with acrylamide exerting its mutagenicity in Big Blue mice via metabolism to glycidamide.

Acrylamide is clearly clastogenic in the presence and absence of exogenous metabolic activation in *in vitro* assays in Chinese hamster V79 or V79H3 cells (Knaap et al., 1988; Tsuda et al., 1993). Studies on spindle disturbances suggest a protein-interactive mode of action (Adler et al. 1993). *In vivo* cytogenetics studies found that acrylamide caused chromosome aberrations in the bone marrow of mice at doses as low as 50 mg/kg i.p., the lowest dose tested (Adler et al., 1988). Increased bone marrow aberrations are also reported by Shiraishi (1978) in mice at 100 mg/kg i.p. Adler et al. (1988) also reported statistically significant dose-related increases in micronuclei in bone marrow cells; positive results in micronucleus assays are also reported in several other studies in the bone marrow, peripheral blood, and spleen (e.g., Abramsson-Zetterberg, 2003; Backer et al., 1989; Cihak and Vontorkova, 1988; Knaap et al., 1988; Russo et al., 1994). EU (2002) noted that a negative result in an unpublished bone marrow micronucleus assay (Sorg et al., 1982) may have been related to the use of inappropriate sampling times. Micronuclei are indicators of chromosomal damage, which may result from such mechanisms as spindle malfunction and inappropriate chromosome segregation, but generally is not a result of direct DNA reactivity.

Direct binding of acrylamide with DNA *in vitro* indicates that acrylamide forms adducts only weakly (Solomon et al. 1985), with adduct yields approximately two orders of magnitude lower than potent direct DNA alkylating agents such as diethylsulfate (Dearfield et al. 1995). *In vivo* studies in rats and mice have found that exposure to acrylamide results in DNA adducts, primarily due to glycidamide binding to the N7 position of guanine or the N3 position of adenine. However, comparable levels of DNA adducts have been found in the liver, lung, kidney, spleen, brain and testis, showing no specificity for the tissues that are targets for tumor development in rats (Segerback et al., 1995, Doerge et al, 2005). *In vivo* studies also showed that acrylamide increased DNA synthesis in the target tissue (thyroid, testicular mesothelium, adrenal medulla), but not in non-target tissue (liver) (Lafferty et al., 2004); and this tissue-specific effect is also accompanied by DNA damage (Klaunig and Kamendulis, 2005). Comet assay results also indicate that DNA damage is evident in multiple organs following in vivo exposure of rats (Maniere et al., 2005). However, the mechanism for these tissue-specific DNA reactivities is not clear. For example, when oxidative metabolism of acrylamide to glycidamide was inhibited,

acrylamide-induced DNA synthesis (a possible measure of DNA repair or cell regeneration) was reduced only in the adrenal medulla, but such effect was not apparent in the testicular mesothelium and was equivocal in the thyroid (Lafferty et al., 2004). These data suggest the involvement of glycidamide in the induction of DNA synthesis and presumably adrenal medullary pheochromocytomas, but the observed DNA synthesis in the thyroid and testicular mesothelium may not be related to glycidamide.

The lack of tissue-specific DNA reactivity might be due to differences among tissues in indirect DNA damage. For example, acrylamide has a well-described affinity for sulfhydryl groups on amino acids (Cavins and Friedman, 1968), and such activity could result indirectly in DNA damage – particularly in rapidly growing cells or cells with lower capacity to maintain proper reduction:oxidation balance. In this case, acrylamide could bind to the sulfhydryl groups of proteins that control chromosome segregation, resulting in chromosome damage. Alternatively, acrylamide binding to the sulfhydryl groups in reduced glutathione could alter glutathione homeostasis, and decreased levels of this protective molecule could result in secondary or oxidative DNA damage. The former mechanism would be consistent with the weight of evidence described above showing that acrylamide is clastogenic in a number of different assay systems and evidence that acrylamide interferes with structural protein function.

The latter mechanism, oxidative stress, has been demonstrated in several studies. Park et al. (2002) showed that acrylamide treatment caused morphological transformation of Syrian hamster embryo cells. This response was not altered by co-treatment with an inhibitor of oxidative metabolism (i.e., by decreasing metabolism of acrylamide to glycidamide), indicating that acrylamide, not glycidamide, was the causative agent. The transformation response was increased in acrylamide-treated cells that were co-treated with an inhibitor of glutathione synthesis and the response was decreased in cells co-treated with a sulfhydryl donor, implicating oxidative stress. Moreover, acrylamide treatment decreased glutathione levels in these cells, providing further evidence that acrylamide was generating cell transformation at least in part via altered glutathione homeostasis. In this study, ABT-treatment (an inhibitor of cytochrome P450) increased the basal level of DNA damage in the thyroid and acrylamide treatment did not further increase this degree of DNA damage. No other studies that evaluated the role of acrylamide metabolism by CYP2E1 on DNA damage in thyroid cells were identified. However, Puppel et al. (2005) showed that DNA damage induction in acrylamide-treated cell lines in vitro does not correlate with the level of CYP2E1 activity, since DNA damage was similar in rat hepatocytes that have CYP2E1 activity and other cells that lack significant CYP2E1 activity. These results would also argue against implicating glycidamide, the product of CYP2E1-mediated metabolism, in the observed DNA damage. Consistent with the hypothesis that DNA damage from acrylamide exposure is not primarily due to interaction with glycidamide, Mei et al. (2007) found that both glycidamide and acrylamide are clastogenic and mutagenic (causing large deletions) in the mouse lymphoma assay system (which lacks CYP2E1), but glycidamide acts via a DNA adduct and acrylamide acts via a mechanism not involving glycidamide adducts.

The potential importance of oxidative stress as a mechanism of acrylamide-induced DNA damage has been further explored directly in thyroid cells (Chico-Galdo et al. 2006). The authors hypothesized that acrylamide acts by increasing hydrogen peroxide levels in thyroid cells, but found no such increase at the concentrations tested (concentrations that are comparable to blood

concentrations observed in rats treated with 10 mg/kg-day acrylamide); there was no effect on either hydrogen peroxide production or destruction. Acrylamide treatment generated DNA damage as measured in a comet assay in rat thyroid cells lines as well as human primary thyrocytes. This DNA damage response was similar to that observed for various treatments designed to increase thyroid cell hydrogen peroxide levels or decrease glutathione levels, including treating cells with an inhibitor of glutathione synthesis. Although no experiments were done in this study to test whether acrylamide would enhance the effect of the glutathione inhibitor, the results are consistent with those of Park et al. (2002) in implicating oxidative stress (and possibly dysregulation of glutathione homeostasis) as a key event in acrylamide-induced DNA damage.

The results of Chico-Galdo et al. (2006) show that the effects of treatments that increase oxidative stress, including treatments that deplete glutathione, cause DNA damage as does acrylamide treatment. Puppel et al. (2005) also showed that glutathione depletion of rat hepatocytes increased the DNA damaging effect of acrylamide treatment in vitro. Together, these in vitro studies provide evidence that glutathione binding of acrylamide protects cells from acrylamide-related DNA damage. Since glutathione binding is saturable, the implication is that such a process would lead to non-linear dose-response kinetics for some DNA damaging events.

The modified Hill criteria presented by U.S. EPA (2005) result in specific predictions for a mutagenic MOA. First, agents with a mutagenic MOA will induce mutations following a relatively short exposure time, prior to the appearance of preneoplastic lesions and/or tumors. This follows directly from the Hill criterion of temporality, which states that a cause of an effect must occur before that effect. In contrast, an agent with a non-mutagenic MOA might only increase mutations in the target tissue (e.g., increased mutations secondary to cell proliferation) after a relatively long exposure time, if at all, and such increases would not necessarily occur prior to the appearance of preneoplastic lesions and or/tumors. Therefore, the current analysis evaluated the timing of point mutation induction to help characterize the MOA for acrylamide-induced thyroid tumors. The second prediction is that when point mutation is the key event for carcinogenesis, these mutations will occur at doses lower than or equal to the dose required to produce tumors. If positive responses for tumors are observed at doses lower than required to cause point mutations, the MOA is more likely to be non-mutagenic. Therefore, a dose-response concordance analysis of mutation with tumor incidence was conducted to evaluate the MOA.

The *in vivo* genotoxicity studies of acrylamide by Maniere et al. (2005) and Manjanatha et al. (2006) are selected for further analysis because of adequate data for dose response modeling, and a relevant route of exposure and clear association with genotoxicity (see Table 2). Maniere et al. (2005) measured DNA damage (using the comet assay) and DNA adducts in leukocytes, brain, bone marrow, liver, testes, and adrenals of Sprague Dawley rats exposed to a single oral dose of acrylamide. Statistically significant increases were reported in results from the comet assay (Olive tail moment and/or % DNA in the tail) for leukocytes, brain, and testes after 24 hours, and so these endpoints are modeled here. Manjanatha et al. (2006) exposed Big Blue mice (which have been genetically altered to allow determination of mutagenicity frequency in all tissues *in vivo*) to acrylamide in drinking water for 3-4 weeks. Mutation frequency was statistically significantly increased in lymphocytes and liver (neither of which had increased tumor incidence in the rat studies). As for the tumor bioassays (described below), adjustments for control doses

are made with these genotoxicity bioassays prior to modeling, based on the expected or measured contamination of acrylamide in feed (see Table 2 footnote).

Dose-response modeling was done for these genotoxicity data, and compared with the tumor dose-response data, as described in the Methods section.

The probabilities of an "adverse" genotoxic response are shown in Figures 1 and 2. Satisfactory fit was obtained with the power model for each of the data sets (p-values all greater than 0.1). As further described in Methods, we compared the genotoxicity and tumor response from acrylamide administrations, controlling for the background levels of both. Figures 3 and 4 show the comparative probabilities of response for the selected genotoxicity endpoints and thyroid tumors. Each plot shows the same four tumor dose-response curves, representing the thyroid tumor observations in males and females from the two cancer bioassays (Johnson et al., 1986 and Friedman et al., 1995). The fits of the BMDS multistage models to the thyroid tumor responses are satisfactory (all p-values greater than 0.10). The general shapes of the predicted tumor dose-response behaviors are very similar for the four data sets. Figures 3 and 4 differ with respect to the genotoxicity endpoint for which probabilities of adversely high response are shown and to which the tumor probabilities are compared. Figure 5 shows these same data, but plotted linearly as percent of control change with the addition of a data from Klaunig and Kamendulis (2005) for the comet bioassay and thyroid effects, and from Abramsson-Zetterberg (2003) for a murine micronucleus assay. With respect to the comet assays shown in Figures 4 and 5, we note that the DNA damage endpoints may not be maximized after one or seven days of exposure, and that mutations may continue to increase even when steady state has been reached between DNA damage induction and DNA repair. Other issues related to interpretation of the implications of the modeling results are addressed in the Discussion.

Several lines of evidence support a mutagenic mode of action (consistent with linear low-dose extrapolation) for the development of thyroid tumors. These lines of evidence include:

- Glycidamide is mutagenic and adducts with glycidamide are formed in many tissues (as cited above).
- Statistically significant *in vivo* genotoxicity as described by Abramsson-Zetterberg (2003), Butterworth et al., (1992), Klaunig and Kamendulis (2005), Maniere et al. (2005), and Manjanatha et al. (2006), occurs after dosing durations ranging from a single dose through 4 weeks of dosing (although the data are from a variety of tissues in both rats and mice), consistent with the Hill criterion of consistency. These dosing durations are much shorter than those needed for thyroid tumors to occur (Johnson et al., 1986; Friedman et al., 1995). This is consistent with the Hill criterion of temporality in the U.S. EPA (2005) guidelines.

However, several lines of evidence do not support a linear mutagenic mode of action for the development of thyroid tumors. These lines of evidence include:

- Acrylamide is clastogenic and DNA adduct formation is seen in a number of tissues that are not targets for tumors (as cited above).
- Thyroid tumor responses are consistent with previous work that shows follicular cell tumors have a dose response curve that is curvilinear upward (U.S. EPA, 1998).

- Statistically significant *in vivo* genotoxicity (studies as cited above) has only been observed at doses higher than those that caused increases in tumors in the rat bioassays (Johnson et al., 1986; and Friedman et al., 1995). Some of these genotoxic doses are significantly higher than those that cause tumor formation and several of these high doses are without any genotoxicity. To the degree that the endpoints evaluated reflect the maximal gene mutation frequency in the thyroid, this result is not consistent with the criterion of dose response concordance in the U.S. EPA (2005) guidelines.
- When selected sets of these genotoxicity data are modeled based on incidence of an "adverse" response, 8 out of 9 dose response curves show tumor increases occurring at doses below doses that cause genotoxicity (see Figures 3 and 4). To the degree that the endpoints evaluated reflect the maximal gene mutation frequency in the thyroid, this result is also not consistent with the criterion of dose response concordance in the U.S. EPA (2005) guidelines.
- An alternative analysis in which these, and other, genotoxicity data sets are plotted along with tumors as multiples of control response also shows this same behavior: tumors lead genotoxicity in dose response (Figure 5). To the degree that the endpoints evaluated reflect the maximal gene mutation frequency in the thyroid, the result shown in Figure 5 is also not consistent with the criterion of dose response concordance in the U.S. EPA (2005) guidelines. The gene mutation data from the Big Blue assays are much more likely to have maximized the mutational response than the DNA damage (comet) assays.

The strongest evidence for a mutagenic MOA comes from the *in vitro* and *in vivo* data that clearly show that glycidamide is mutagenic. Furthermore, this genotoxicity occurs with shorter dosing duration than the tumor response, which is consistent with the Hill criterion for temporality in the evaluation of MOAs (U.S. EPA, 2005). However, these same *in vivo* studies offer the strongest evidence that the tumor dose response is not being caused by genotoxicity, a necessary step for demonstrating a mutagenic MOA, because of the lack of dose response concordance. This lack of dose response concordance, indicated in figures 3, 4 and 5, suggests that most high dose tumors, and perhaps even those at low doses, are evoked by alternative MOAs.

We conclude that this MOA, that acrylamide is evoking thyroid tumors as a result of glycidamide-induced mutations, only partially applies, and that data exist to exclude this MOA operating as a sole basis of the observed thyroid tumors.

Assessment for thyroid follicular cell growth stimulation. As described in the Methods, we used U.S. EPA (1998), Capen (2001) and others for evaluation of thyroid follicular cell growth stimulation as a MOA for the tumorigenesis of acrylamide. Chronic reduction in circulating thyroid hormone levels will increase TSH levels, which will result in increased size and numbers of thyroid cells, increased thyroid gland weight, and, finally, evoke tumors of the thyroid. This pattern of changes can result from a number of different mechanisms.

The most direct evidence for testing this MOA would be the evaluation of thyroid histopathology and thyroid hormone levels at tumorigenic doses. However, the available studies are somewhat limited.

Khan et al. (1999) administered 0, 2, or 15 mg/kg-day acrylamide by gavage in water to groups of six, 42-day-old female Fischer-344 rats, the same strain as used in the tumor bioassays, for 2 or 7 days. The animals were acclimatized by handling for 14 days prior to dosing to minimize stress. The animals were killed 24 hours after the last dose. Statistically significant increases in thyroid follicular cell height and decreases in thyroid follicular colloid area (the storage area for thyroid hormone in follicles of the gland) were observed at both exposure times, but responses at both doses were similar. No statistically significant effects were observed on plasma levels of T_4 or TSH. However, the authors noted several subtle changes that did not reach statistical significance. A dose-related increase in adjusted T_4 was observed in the groups dosed for 7 days. Adjusted TSH was also decreased at the high dose, but was increased at the low dose. No statistically significant changes in pituitary TSH levels were observed at either dose or exposure duration, but adjusted pituitary TSH was lower when measured as ug/gland (but not ug/mg of pituitary tissue) at 7 days. Khan et al. (1999) suggested that the changes in follicular cell structure are consistent with the thyroid being in an active state, and that the absence of a significant effect on TSH may have been due to the short exposure duration, or that a short window of decreased T_4 and compensating increases in TSH was missed. They also suggested that the decreased colloid area and hypertrophy of follicular cells reflected persistent TSH stimulation.

Freidman et al. (1999) gave acrylamide to Fischer 344 rats (10/group/sex, age of ~8 weeks) in drinking water for 28 days, resulting in measured doses of 0, 1.4, 4.1, 12, 19 and 25 mg/kg-day for the males and 0, 1.3, 4.3, 9.0, 19 and 24 mg/kg-day for the females. Blood was collected after 14 days, and at study termination, and serum levels of T3, T4, TSH, and several other hormones were determined. Three high-dose males died during the fourth week of dosing. The authors stated that five males and four females in the high-dose group were also killed prior to study termination, because they were in poor condition. Body weights, food consumption, and water consumption in the highest dose group were generally lower than in the controls. Decreased body weight was also observed in males at 12 mg/kg-day, and in both sexes at 19 mg/kg-day. Acrylamide ingestion at 19 mg/kg-day or above in both sexes was associated with neurotoxicity. After 14 days, T3 and T4 levels exhibited a positive linear trend with dose in males, with the change in T4 being statistically significant at the high dose. TSH was unaffected. In females, changes in the T3 and T4 levels were similar to those seen in males, but the differences were smaller and the trends were less significant. In contrast to the results in males, TSH was significantly decreased at the high dose in females. After 28 days of dosing, these hormone changes had reversed; statistically significant, dose-related *decreasing* trends were found in T3 and T4, as were statistically significant *decreases* in comparisons to controls observed in males at 19 mg/kg-day and above for T3 and at the high dose for T4. A higher TSH level was also seen at the high dose in males when compared with controls, but this increase was not statistically significant. There were no dose-related changes in T4 or TSH in females at 28 days; a non-statistically significant lower T3 level was seen at the high dose. Thyroid weights were similar among all groups, except for a small, not statistically significant, increase in absolute and a statistically significant increase in relative mean thyroid weight in the high dose females. There were no histological findings in the thyroid attributed to treatment. The study authors noted large variability in testosterone, TSH, and prolactin levels, and suggested that this may have been due to the changes in these hormones with circadian rhythms, and the time-span over which sacrifice and collecting was conducted.

Lafferty et al. (2004) exposed male F344 and Sprague-Dawley rats to acrylamide via drinking water at either 0, 2, or 15 mg/kg-day for 7, 14, or 28 days. In both rodent strains, statistically significant increases in DNA synthesis were observed in the thyroid at the high dose and at all times. A statistically significant, and dose related, increase was also seen at the low dose at all times in F344 rats. This increase in synthesis could be due to either DNA repair or growth stimulation. Non statistically significant increases in the mitotic index were also seen in the thyroids of both strains at nearly all doses and times, and a decrease in the apoptotic index that was not statistically significant but dose related, was observed in F344 rats. These effects are more likely to be related to growth stimulation. No significant differences in body or tissue weights were found when compared to controls at either dose of acrylamide and at any time points tested.

Johnson et al. (1984) reported cystic dilatation of the thyroid follicle after two years of acrylamide exposure as 5.0, 3.4, 6.8, 1.7, and 12% in males; and 0.0, 0.0, 1.7, 1.7, and 5.0% in females at doses of 0.002, 0.012, 0.10, 0.50, and 2.0 mg/kg-day, respectively. Trend tests for either sex and pooled results are statistically significant.[3] This lesion is considered by some to be follicular cell hyperplasia (Boorman, 1983; as cited in Johnson et al, 1984). Dulak (1989) also showed thyroid hyperplasia as 2.0, 2.0, 3.4, 3.0, and 2.7% at doses of 0.002, 0.002, 0.10, 0.50, and 2.0 mg/kg-day for males; and 0.0, 2.0, 5.0, and 1.0% at doses of 0.002, 0.002, 1.0, and 3.0 mg/kg-day for females. These modest increases, however, were not dose related, nor statistically significant. Imail et al. (2005) did not find proliferative changes in the thyroid gland after 30-week exposures in rats given acrylamide at doses of 3.3 or 5.6 mg/kg-day via drinking water and also given other chemical treatment. This latter observation is consistent with the fact that the proliferative lesions found by Johnson et al. (1986) after two years were not evident up to 18 months in interim kills (Gorzinski et al., 1984).

Consideration of the cellular targets of acrylamide suggests a possible molecular mechanism by which acrylamide might cause the observed changes in thyroid hormones. As mentioned in numerous reviews (e.g., reviewed by Shipp et al., 2006), acrylamide is known to interfere with microtubule function. Thus, the possibility exists that acrylamide might lead to the loss of integrity of microfilaments and microtubules in thyroid follicular development. Chemicals that disorganize these organelles block thyroid follicle formation (Capen, 1996). This loss of integrity of both microfilaments and microtubules from acrylamide exposure in young rodents might lead to problems in the maintenance of follicle structure and adequate hormone release. Furthermore, at least in the rat, volumetric fractions of different thyroid cells, including follicular cells, change dramatically over the first 120 days of life (Capen, 1996), which is the same age as when the Fisher 344 rats are started on the long-term tumor bioassays.

Alternatively, the *in vitro* studies of Chico-Galdo et al. (2006) provide evidence that effects of acrylamide on growth stimulation may not originate in the thyroid gland. For example, these investigators showed that acrylamide treatment had no effect on several measures of thyroid cell proliferation or activity *in vitro*. Acrylamide treatment did not activate the cyclic AMP growth pathway, nor did it enhance the activation of this pathway by TSH in rat thyroid cells. Acrylamide treatment had no affect on the normal formation and removal of hydrogen peroxide

[3] $P < 0.05$ Cochran-Armitage trend test conducted by us.

(a physiological substrate for iodide oxidation in thyroid hormone synthesis) in response to growth stimulation in several thyroid cell lines. Furthermore, acrylamide did not alter thyroid cell proliferation (as measured by BrdU incorporation into newly synthesized DNA) in thyroid cells. Together these data suggest that acrylamide's effect on thyroid tumor formation is not due to altered growth signaling at the level of the thyroid cell itself, and, thus, might result from changes outside the thyroid gland – possibly involving changes in endocrine regulation, such as stimulation by TSH.

Due to the complexities of endocrine physiology, several plausible ex-thyroid mechanisms can be explored. For example, it is well established in the rat, that the loss of circulating thyroid hormone resulting from increased liver metabolism will lead to an increased release of thyroid stimulating hormone (TSH) from the pituitary gland (e.g., Capen, 1992). This loss of circulating thyroid hormones may come about by an increased breakdown of these hormones by the rat liver because of enzyme induction resulting from an administered chemical.

For acrylamide exposure, some minimal evidence exists that an increased catabolism of thyroid hormones might be occurring in the liver. For example, Johnson et al. (1984) specifically measured liver weights, a common but nonspecific indication of increased liver metabolic capability. These weights were statistically significantly increased[4] in both male and female rats at the dose of 2.0 mg/kg-day, a dose at which thyroid tumors are also found; no analysis was conducted of levels of specific liver metabolic enzymes or enzyme classes. Burek et al. (1980) also found statistically significant increases in relative and absolute liver weight in male rats given acrylamide in water at a dose of 5 mg/kg-day as part of the 90 day range-finding study for the Johnson et al. (1986) study, and significantly increased liver weights at 20 mg/kg-day with histopathology of the liver after recovery. In contrast to these results, Dulak (1989) also measured liver weights and found either no change or a statistically significant decrease. Imai et al. (2005) also measured liver weights and found that such weights did not change, at least after 30 weeks of acrylamide exposure.

A second mechanism by which acrylamide might affect thyroid responses via a site outside of the thyroid is via perturbation of endocrine signaling as a secondary consequence of neurotoxicity or altered neurotransmitter levels in the hypothalamus. This mechanism is a logical avenue for examination since neurotoxicity is a sensitive non-cancer effect of acrylamide and regulation of thyroid hormones occurs via neurotransmitters such as dopamine in the hypothalamic-pituitary-thyroid axis. Although the literature on acrylamide-induced toxicity is robust, specific data on the ability of acrylamide to induce toxicity in the hypothalamus or alter dopamine responsiveness in the brain are more limited. Nevertheless, numerous studies (reviewed in LoPachin et al., 2006) have demonstrated that acrylamide inhibits CNS neurotransmission at the level of the nerve terminal by adduction with sulfhydryl groups on critical proteins involved in fusion of nerve terminal vesicles with target membranes and neurotransmitter uploading into presynaptic vesicles. In rats exposed orally to 21 mg/kg-day acrylamide for 21 days or injected with 50 mg/kg-day acrylamide for five days, neurotoxicity (abnormal gait) and nerve terminal degeneration occurred in a dose and duration-dependent manner. Decreased dopamine uptake into striatal synaptic vesicles and nerve terminal release of dopamine from striatal synaptosomes were also observed. Significant changes in striatal nerve

[4] $P < 0.05$ ANOVA pairwise comparison conducted by us.

glutathione levels were not caused by *in vivo* treatment with acrylamide, showing that general oxidative stress mechanisms were not responsible for the observed neurotoxicity. However, *in vitro* treatment of striatal vesicles with acrylamide or sulfhydryl reactive agents decreased dopamine uptake. Based on these studies the authors suggest that acrylamide toxicity is initiated in a dose-response sequence as: disruption of presynaptic nerve terminal uptake and release of dopamine via sulfhydryl adduction to critical nerve terminal proteins, morphological changes observed by silver staining (argyrophilic changes), nerve terminal degeneration, and clinical signs of neurotoxicity.

While the mechanistic linkage between acrylamide exposure, perturbation of dopamine-dependent neurotransmission, and endocrine regulation of the thyroid is intriguing, direct measurement of the effects of acrylamide on the hypothalamus are limited. Moreover, no studies were identified that directly measured both hypothalamus function and thyroid hormone levels. Lehning et al. (2003) found that 50 mg/kg-day acrylamide administered by intraperitoneal injection caused widespread nerve terminal degeneration within 5 days, including in the hypothalamus. An alternative dosing regimen of 21 mg/kg-day administered orally for 21 days affected some brain regions, but not the hypothalamus. Ali et al. (1983) reported that injection of 20 mg/kg-day acrylamide for 10 days had no effect on levels of serotonin or dopamine in the hypothalamus, in contrast to an earlier study by Dixit et al. (1981) who reported a decrease in dopamine levels in the brain following a similar treatment protocol. Other studies have reported increased dopamine receptor binding in some brain regions of acrylamide-treated rats (Bondy et al., 1981). Each of these mechanistic studies was of short duration and used relatively high doses. However, low doses of acrylamide might perturb neuroendocrine function with longer-durations of exposure. This possibility is consistent with the histopathology observations in the chronic tumor bioassays (Johnson et al., 1986; Friedman et al., 1995), which show non-neoplastic nervous system pathology (tibial or sciatic nerve degeneration) at doses that also cause thyroid tumors. Overall, the specific pattern of effects caused by acrylamide on neuroendocrine regulation in the hypothalamus is difficult to decipher due to the paucity of data and the complexity of mapping neuroregulation in various brain regions. However, the data show that acrylamide can perturb normal hypothalamus structure and possibly function (at least at high doses). Furthermore, LoPachin et al. (2006) concluded that presynaptic nerve function is a likely target of acrylamide throughout the central and peripheral nervous system.

Thus, several lines of evidence for acrylamide are available to support or refute portions of the areas of emphasis by U.S. EPA (1998) for a thyroid-pituitary hormone disruption MOA. These lines of evidence are shown below:

Increases in cellular growth:

- Khan et al. (1998) observed statistically significant thyroid follicular hypertrophy (increased follicular cell height) in female F344 rats at 2 and 15 mg/kg-day for 2 or 7 days, and corresponding statistically significant loss of colloid.
- Friedman et al. (1999) observed a small, statistically significant, increase in relative thyroid weight in female rats dosed with 24 mg/kg-day for 28 days. However, this effect did not occur in males.
- Lafferty et al. (2004) showed a statistically significant, and dose related increase in thyroid follicular DNA labeling in F344 rats at 2 and 15 mg/kg-day and at 7, 14 and 28

days (Table 2). A non-statistically significant increase in the mitotic index was also observed in 5 of 6 groups, which was not dose related. A non-statistically significant, but dose related decrease in apoptotic index was also observed in all but one of 6 groups.

- No increase in cell proliferation was evident in acrylamide-treated thyroid cell lines *in vitro* (Chico-Galdo et al. 2006), but this study design does not provide information on acrylamide effects on TSH levels via mechanisms outside of the thyroid cells.
- A statistically significant increase in cystic dilatation of the follicles in the thyroid occurred in both male and female rats (Johnson et al., 1984) after 2 years. A modest increase in thyroid hyperplasia was also seen by Friedman et al. (1995) in both male and female rats after 2 years, but this increase was neither statistically significant nor dose related.

Thus, evidence is available for cellular growth in vivo in short-term studies, but this evidence is equivocal in long-term studies.

Hormone changes:

- Khan et al. (1998) did not observe any statistically significant changes in TSH or T_4 in rats exposed for either 2 or 7 days; T3 was not measured. However, at 7 days slight dose-related increases in the adjusted plasma T4 were seen, with a corresponding slight decrease in the adjusted TSH.
- After 14 days, Friedman et al. (1999) observed a statistically significant increase in T4 (males) and T3 (females). The latter observation was accompanied by a statistically significant decreased TSH (as might be expected).
- After 28 days, however, Friedman et al. (1999) showed that these hormone changes had disappeared or reversed. A statistically significant decrease was seen in T_3 and T_4 (males) with the expected increase in TSH, but this latter increase was not statistically significant. Females showed only a decrease in T3 at the high dose, but this change was not statistically significant.

Thus, evidence is available in short-term studies of hormone levels changing in a manner consistent with the proposed MOA based on other measures of growth stimulation. The data at the longest time point are consistent with thyroid growth stimulation. Longer-term studies have not monitored thyroid hormone levels.

Site of action:

- Increases in liver weight evident in several studies might indicate increased metabolic capability, leading to loss of thyroid hormone. Evidence on this increased liver weight is mixed, and studies on the enhancement of liver metabolism by acrylamide were not located.
- Loss of integrity of microfilaments and microtubules will lead to problems in the development and perhaps maintenance of thyroid follicle structure and hormone release. The *in vivo* development of follicles is still occurring in rats up to 120 days of age (Capen, 1996), after the time of the start of the cancer bioassays. Acrylamide is known to damage these organelles in other organs (Lapadula et al, 1989), but no direct evidence is available for thyroid microfilament and microtubule damage.

- Neurotoxicity is a sensitive non-cancer effect of acrylamide and regulation of thyroid hormones occurs via the hypothalamic-pituitary-thyroid axis. Although the literature on acrylamide-induced neurotoxicity is robust, specific data on the ability of acrylamide to induce toxicity in the hypothalamus or alter dopamine responsiveness in the brain are more limited.

Thus, several sites of action are plausible and may be interacting, but definitive information on any of them is not available.

Dose Correlations:

- Thyroid follicular cell tumors were statistically significantly increased at 2 mg/kg-day in males and females in the Johnson et al. (1986) study, and at 2 mg/kg-day in males and at 3 mg/kg-day in females in the Friedman et al. (1995) study. These tumorigenic doses are in the range of doses that show:
  - Statistically significant thyroid histological changes in Khan et al. (1999) and Johnson et al. (1984). Histological changes were also seen by Friedman et al. (1995), but these were neither statistically significant nor dose related. Such histological changes were not evident in Imai et al. (2005), although the timing in this latter study may not have been long enough to evoke the change.
  - Dose related increases in thyroid DNA labeling at 3 time points (Lafferty et al., 2004),
  - Increases in mitotic index and decreases in apoptotic index (Lafferty et al., 2004).
- These tumorigenic doses are below the general range of doses that cause hormone changes after short-term exposure (Khan et al., 1999; Friedman et al., 1999). Hormone changes after long-term exposure have not been monitored.

Thus, evidence is available on dose correlations, but the data are not all consistent.

Reversibility of effects when chemical dosing stops:

- No data on reversibility studies related to thyroid effects or other growth stimulation were located.

Thus, as summarized in Table 3, short-term acrylamide exposure causes changes to thyroid histology, hormones, DNA labeling, mitotic indices and apoptotic index. Not all of these changes are statistically significant, nor consistent, but together these effects show thyroid growth stimulation. Minimal data on several sites for acrylamide action exist. Based on this modest amount of information, we conclude that acrylamide is evoking thyroid tumors to some extent as a result of follicular cell growth stimulation. Our conclusion is consistent with evidence from two of the examples in U.S. EPA (1998), also shown in Table 3. The evidence we summarize for acrylamide more closely matches the evidence for a bis-benzenamine, where U.S. EPA (1998) concluded that both a mutagenic and growth stimulation mode of action was occurring, rather than a nitrosamine, where U.S. EPA (1998) concluded that only a mutagenic mode of action was occurring.

Summary of MOAs. It appears that a mutagenic MOA caused by exposure to glycidamide may be operating to a limited extent. A thyroid follicular cell growth stimulation MOA based on a

liver, thyroid or hypothalamus specific event caused by acrylamide also appears to be operating to some extent. Table 4 summarizes information on these 2 MOAs. Based on the tumor observations, which show little, if any, increase from a low dose of approximately 0.002 mg/kg-day until doses of 1.0 or above, we conservatively assume that mutagenicity determines the low-dose response and that growth stimulation dominates the response at higher doses. Thus, as per U.S. EPA guidelines we "consider the respective contribution of each mode of action in different dose ranges."

Specific evidence for a threshold-like part of this modeling for thyroid tumors in the high dose range includes:

- Tumors occur primarily in endocrine tissues, including the thyroid (Johnson et al., 1986; Freidman et al., 1995), occur late in life, and are mostly benign. Thyroid tumor responses in 3 of these studies are consistent with U.S. EPA (1998) that these types of thyroid tumors have curvilinear upward slopes.
- Acrylamide is indirectly genotoxic, such toxicity is generally expected to have a threshold.
- The tissue pattern of DNA adduct formation resulting from acrylamide exposure (presumably due to the glycidamide metabolite) does not correlate well with the observed tumor response.
- Statistically significant *in vivo* genotoxicity in a variety of model systems occurs at doses that are generally much higher than doses causing thyroid tumors in the rat (Abramsson-Zetterberg, 2003; Butterworth et al., 1992; Klaunig and Kamendulis, 2005; Maniere et al., 2005; Manjanatha et al., 2006). Plots of genotoxicity data with adequate dose response and relevant routes of exposure, along with male and female thyroid tumors from both long-term tumor bioassays, show that tumors precede mutations in dose response in all but one comparison (Figures 3, 4 and 5). In light of the general consistency of the results from this variety of assays, the overall weight of evidence is not consistent with the Hill criterion of dose response concordance for a mutagenic MOA (U.S. EPA, 2005).
- Growth stimulation appears to be occurring as evidenced by thyroid histological changes at tumorigenic doses by Khan et al. (1998) and Johnson et al. (1984), hormone changes at 28 days (Friedman et al., 1999) and by DNA labeling and mitotic index increases and apoptotic index decreases by Lafferty et al. (2004) (Table 3).

Evidence for a linear-like part of this modeling for thyroid tumors in the low dose range includes:

- Some tumors occur in tissues that are not hormonally dependent (nervous system, oral cavity) (Johnson et al., 1986), and some tumors evoked are malignant (Johnson et al., 1986; Freidman et al., 1995).
- Glycidamide is mutagenic and DNA adducts with glycidamide are formed in many tissues.
- *In vivo* genotoxicity occurs in time before tumors occur (Abramsson-Zetterberg, 2003; Butterworth et al., 1992; Klaunig and Kamendulis, 2005; Maniere et al., 2005; Manjanatha et al., 2006). This information is consistent with the Hill criterion of temporality for a mutagenic MOA (U.S. EPA, 2005).

Because the data are most consistent with both MOAs being relevant, but at different parts of the dose response region, several approaches are considered for the dose-response modeling, as described in the next section.

Thyroid Tumor Dose Response Assessment. We first show multistage modeling of dose-response relationships for tumors for the Friedman and Johnson studies described in Table 1, and include evaluations for the four study-by-sex combinations, the two sex-specific pooled data, and for the set of all pooled doses (see Table 5). The pooled doses are merely a larger data set; we did not combine groups by dose. We show BMDs and BMDLs and their associated slope factors (SFs) at either 10 or 2%, as per U.S. EPA (2001) guidance and software; the rationale for the choice of benchmark response is given in the following paragraphs.

Based on our MOA understanding and EPA guidance (2005), we "decoupled" the different parts of the dose response assessment curve by comparing all responses with the low dose responses that were neither statistically or biologically significant (i.e., those doses less than the apparent experimental threshold of 1.0 mg/kg-day). This comparison is shown in Table 5 as "All Doses" or "Decoupled Low Dose." All results shown in Table 5 have acceptable model fits (data not shown, but available upon request). Pooled responses at BMD_{10} for "All Doses" are comfortably among the individual data set responses; a comparison of ratios between BMD_{10}s and their corresponding $BMDL_{10}$s shows that the ratio of BMD_{10} to $BMDL_{10}$ for "pooled all" was the smallest among all ratios, suggesting greater confidence in the pooled data; this led us to further investigate pooled responses.

Based on our MOA conclusion, we anticipated a difference between multistage model slope factors (SFs) of pooled "All Doses" and "Decoupled Low Dose." Table 5 shows such a difference. SFs at "All Doses" BMD_{10}s are higher than those at "Decoupled Low Dose" BMD_{02}s, which is consistent with our understanding of the underlying dual MOA biology. However, SFs at the "Decoupled Low Dose" BMD lower limit, the $BMDL_{02}$, are slightly higher than the SF for $BMDL_{10}$ when all doses are modeled. These latter observations reflect the much wider confidence limits of the "Decoupled Low Dose" analysis, which is likely due to the use of less data in the modeling. While the use of SFs at the "Decoupled Low Dose" BMD_{02}s might appear reasonable for low dose extrapolation based on our MOA understanding, the SFs at the "Decoupled Low Dose" $BMDL_{02}$s, based on much wider confidence limits, are not. The expectation would be that these latter slopes are smaller than corresponding values at $BMDL_{10}$ with "All Doses."

This inability to fully use the "Decoupled Low Dose" analysis led us to estimate BMD/L_{02}s using the multistage model and "All Doses." These values are also shown in Table 5. Slope Factors (SFs) at BMD_{02} for two of three pooled "All Doses" more closely approximate those found at BMD_{10}, "All Doses" rather than those found at "Decoupled Low Dose" BMD_{02}. This observation indicates that the multistage model has difficultly modeling data in the low dose range when "All Doses" are modeled. The use of these latter results of the multistage model is not biologically reasonable given our understanding of the dual MOA.

Because the multistage model with decoupled or all data was not able to give fully or biologically reasonable answers, and because EPA (2001 and 2005) also considers other models,

another U.S. EPA BMDS software model, the probit, was investigated. The use of the probit model has the advantage of being more consistent with discontinuous dose response behavior, or possible thresholds, exhibited by the thyroid tumor data, because the model can accommodate a shallower slope at low doses with a steeper rise for the higher doses. As for the multistage model, the BMD and its Slope Factor (SF) and its corresponding BMDL and its SF were determined for each pooled data set with the probit model. These values are shown in Table 6. The adequacy of the probit model was also evaluated and compared with the multistage model using goodness of fit statistics, AIC values, plots of scaled residuals, comparisons of ratios of BMD to BMDL and visual fit.

The goodness-of-fit statistics for either the multistage or probit models are all acceptable and fairly close across the three pooled data sets; AICs are essentially identical; the plots of scaled residuals also indicate that the two models are very similar in terms of fitting the data---except for the pooled female data where the probit model has somewhat better residuals (all of these data are not shown, but available upon request). Comparisons of BMD to BMDL ratios show differences, however, with the probit model yielding smaller ratios and thus more confident estimates. Plots of the models overlaid on the data also show differences. The multistage model (Figure 6a) gives a poorer visual fitting of the lower dose data than the probit model (Figure 6b), mainly due to the inability of the multistage model to visually fit the 3 data points at the inflection dose of 0.5 mg/kg-day. Perhaps more importantly, the probit model consistently yields lower slopes at BMD_{02} than at BMD_{10} (Table 6) with all data, unlike the multistage model (Table 5). These probit-based slopes are similar to those from the multistage modeling with only low dose data (Table 5). These findings with the probit model fit our analysis of the underlying biology in suggesting that different MOAs, one possibly linear in the low dose region and one likely nonlinear in the high dose region, predominate in different parts of the dose response curve.

As a check of the results of a probit model, we also fit a Weibull model as suggested by U.S. EPA (1998) for thyroid tumors, with a fixed power of 2.0 as suggested by U.S. EPA software. The resulting BMD_{02} of 0.82 mg/kg-day for pooled all data from the Weibull model was nearly identical to a BMD_{02} of 0.81 mg/kg-day determined by the probit model found in Table 6. Comparable $BMDL_{02}$s were 0.72 and 0.69 mg/kg-day, respectively.[5] As a further check of the use of a probit model, a weighted linear regression was applied to the low-dose data only as shown in Table 7. A weighted linear regression is a standard tool in medical research and we consider its use here to be more policy neutral. The SF values from the weighted linear regression shown in Table 7 are roughly equivalent for the BMDs of the probit model and about 2 to 3-fold higher for the BMDLs of the probit model than comparable values found in Table 6. The similarity for BMDs indicates agreement between the probit and weighted linear regression in general; the disparity in the BMDLs indicates that the probit is a better fitting model with all data than the weighted linear regression with only low dose data. Interestingly, the high p-values for the weighted linear regression on the low-dose data indicate that no statistically significant

[5] Allowing the Weibull model to determine the power based on the given data was not possible in EPA's software without zeroing out one or more "control" doses. When such "control" doses were zeroed out, the powers of individual and pooled data sets varied from 0.95 to 6.2, encompassing the default value of 2.0. Full results of the Weibull model runs are not shown here, but are available upon request.

slope or dose effect exists. This conclusion is consistent with a threshold dose for tumor development that is higher than the dose range of the low dose data set (i.e., 1.0 mg/kg-day), as well as with a potential very low slope due to a mutational MOA. Figure 6c shows the pooled-all low dose data plotted with upper and lower 95% confidence bounds of this weighted linear regression.

The evaluation of various BMD, BMDLs, SFs from Tables 5, 6 and 7, cannot be used alone to distinguish the most reasonable choice of SF for purposes of dose response assessment. This is because any one mathematical model is unlikely to pattern the complexities of the biological response. However, one of these models is more scientifically appropriate when considered with our understanding that different MOAs for acrylamide-induced thyroid tumors are operating at different parts of the dose response curve. Overall, we judge that the most scientific appropriate choice of regression is that of the probit model of pooled-all data. This choice is most appropriate because:

- The use of pooled-all data increases the number of data points and the number of doses, resulting in statistically significant regressions even when restricted to the low-dose data. Pooling the responses of the same sex from both studies is supported by the similarities in the two data sets, whether for males or females.
- The probit model extrapolates the full range of tumor data in a manner that is consistent with two MOAs predominating in different parts of the dose response curve, that is mutagenicity in the low dose region and growth stimulation in the high dose region. Specifically, the probit model has an appropriately smaller SF for BMD_{02} than for BMD_{10}, when compared with the multistage model; the probit model also has better BMD to BMDL ratios and is also able to better visually fit the inflection point of 0.5 mg/kg-day.
- The probit model is confirmed by both a Weibull model [another of U.S. EPA's models, and one specifically mention by U.S. EPA (1998) for thyroid tumors], and by a simple weighted linear regression model, which is a standard tool in medical research. This latter model has much practical experience.

While upper bounds are useful for determining acceptability of model estimates, such bounds are not good predictors of population response and their use for risk prediction can be highly misleading. Thus, the slope value chosen here for the linear part of the dose response assessment is 0.025 $(mg/kg\text{-}day)^{-1}$, derived as the best (median) statistical estimate, that is the BMD_{02}, based on all the pooled-all data as shown in Table 6. An upper bound estimate of 0.029 $(mg/kg\text{-}day)^{-1}$, based on this best value, is also shown in Table 6.

The risk value chosen for the non-linear part of this dose response assessment could be found by taking the $BMDL_{10}$ of 1.5 mg/kg-day (i.e., pooled-all data of Table 6), and dividing this by an uncertainty factor of roughly between 30 and 100. This overall factor represents uncertainties for human variability, including children, and extrapolation of rat thyroid data to humans. The resulting RfD for tumors by this MOA would be in the range of 0.05 to 0.02 mg/kg-day.

Extrapolation from Experimental Animals to Humans. The chosen slope factor of 0.025 $(mg/kg\text{-}day)^{-1}$ from the pooled-all rat data needs to be adjusted to reflect a human equivalent slope factor. This might best accomplished by a PBPK model and we are further investigating the recent publication by Young et al. (2007) and an earlier PBPK model by Kirman et al.,

(2003) in this regards. However, comparative toxicokinetics information from humans and Fisher 344 rats are available on which prior extrapolations from rats to humans have been made (OEHHA, 2005; Shipp et al., 2006). We use these same data here as well.

Appropriate data for calculating a rat to human interspecies toxicokinetic factor, referred to as a Chemical Specific Adjustment Factor (CSAF)[6], are presented by Fennell et al. (2005), who gave human male volunteers a single oral dose of labeled acrylamide in water in doses of 0.5, 1.0, or 3.0 mg/kg and dosed male Fischer 344 rats with 3.0 or 50 mg/kg labeled acrylamide by gavage in water. The study found that the acrylamide and glycidamide hemoglobin adducts in blood increased in a dose-dependent manner. In this case, hemoglobin adducts are a useful dosemetric since the extent of adduct formation is proportionally related to the concentration of the respective chemicals in blood. The dose administered to humans in this study is not quite ideal, because it is likely well above both the dietary intake of acrylamide and likely regulatory levels. However, Fennell et al. (2005) showed that both acrylamide and glycidamide hemoglobin adducts in humans have a linear response between 0 and 3 mg/kg when plotted by orally administered acrylamide dose, indicating that nonlinear kinetics would not be an issue in using this study to calculate a CSAF. Thus, using the area under the curve (AUC) for hemoglobin adducts in blood to determine an interspecies toxicokinetic adjustment factor is appropriate. When normalized for dose, the resulting human AUC values are 2.75-3.7 fold higher than the AUC values for rats; the normalized values are used in calculating the CSAF. Using the human AUC value for the low dose group (0.5 mg/kg) (i.e., the human dose closest to the RfD), and the rat AUC value for 3 mg/kg (the dose closest to the BMD), results in an interspecies toxicokinetic adjustment factor of 3.5 for acrylamide; the corresponding value for glycidamide is 1.2. These factors would be applied to effects attributed to acrylamide and glycidamide, respectively. Shipp et al. (2006) also calculated a CSAF for glycidamide of 1.2, but calculated a CSAF for acrylamide of 2.6, by comparing the adducts at the same dose in humans and rats (3 mg/kg), rather than using the lowest dose in humans.

Adequate studies could not be located to develop a chemical-specific rat to human interspecies toxicodynamic factor. However, a toxicodynamic interspecies extrapolation factor of 1 is appropriate based on the following information:

- Goodman and Wilson (1991) consider the best estimate of the interspecies factor to be lognormally distributed around a value of 1.

[6] The International Programme on Chemical Safety (IPCS) released harmonized guidelines for the application of chemical-specific adjustment factors (CSAF) for interspecies differences and human variability in dose response assessment (IPCS, 2005) The IPCS guidelines list a number of considerations that should be evaluated to determine whether the data support the development of a CSAF, which we have followed here: (1) identify the active moiety (typically considering parent vs. metabolite); (2) an appropriate dose metric should be identified (typical dose metrics are measures of overall exposures (AUC or 1/Clearance) or the peak concentration in blood, C_{max}); (3) experimental data should be adequate – (a) humans in study should be representative of the "at risk" population; (b) in vivo study route should be the same as exposed human route; if not, the implications of route to route extrapolation should be considered; (c) ideally, toxicokinetic data in animals should be based on a dose similar to the BMD or NOAEL and under the same dosing conditions, and toxicokinetic data in humans should be obtained at doses similar to the estimated or potential human exposure level; (d) the study should have enough subjects to reliably estimate central tendency (typically at least 5), and the standard error should be <20% of the mean

- Our unpublished observations on a number of comparisons of experimental animals and human slope factors for a variety of chemical carcinogens also indicate that the most likely value of the toxicodynamic factor between experimental animals and humans is a value of 1, although variation around this value exists.
- Williams (1995) states that tumors in humans do not form in the presence of mutagens if TSH-stimulated growth is prevented.
- U.S. EPA (1998) guidelines on use of an overall default factor of 1 for extrapolating the results of thyroid tumors in rats to humans in the absence of specific data to the contrary.

Thus, the combined toxicokinetic and toxicodynamic factor is 1.2 (i.e., 1.2 x 1 = 1.2) for the linear portion of the dose-response range (which is conservatively attributed for the purposes of this analysis to direct DNA mutation for which only glycidamide is implicated). This leads to the best statistical estimate of the adjusted human slope value of 0.030 $(mg/kg\text{-}day)^{-1}$ [i.e., 0.025 $(mg/kg\text{-}day)^{-1}$ (SF) x 1.2 = 0.030 $(mg/kg\text{-}day)^{-1}$]. For comparison with policy-based values, the upper bound estimate of 0.035 $(mg/kg\text{-}day)^{-1}$ can be used [i.e., 0.029 $(mg/kg\text{-}day)^{-1}$ (SF) x 1.2 = 0.035 $(mg/kg\text{-}day)^{-1}$].[7]

Discussion

We briefly reviewed relevant studies on acrylamide's DNA toxicity, tumor formation and the manner of its tumor formation. We then presented evidence on two modes of action (MOAs) for acrylamide-induced thyroid tumors: mutagenicity and thyroid growth-stimulation. Key components of this analysis are data on the genotoxicity of acrylamide and glycidamide, with particular emphasis on applying the modified Hill criteria of temporality and dose-response concordance from U.S. EPA (2005), and data related to thyroid growth stimulation following the framework of U.S. EPA (1998) and Capen (2001).

We believe that data exist to exclude heritable mutations as the sole MOA with confidence. This is because although surrogate mutations occur prior to the development in tumors in time, the available surrogate mutation do not proceed tumors in dose. In addition, tumors found from acrylamide exposure are not of early onset and are more often associated with hormonally-active organs, which are not characteristics of mutagenic thyroid tumorigens (for example, see EPA 1998, case study 4). Moreover, sufficient evidence exists to support the conclusion that at least some of the tumors in the high dose area are being evoked by thyroid growth stimulation.

In contrast, we feel that the growth stimulation cannot be excluded as the sole MOA, because the observed tumor response is also consistent with a biological threshold. For example, Figure 6c shows a weighted linear regression of low dose data. A horizontal line cannot be excluded as a possible fit for these data, suggesting that a threshold is possible. Indeed, a recent publication by Swenberg et al. (2008) shows that whereas biomarkers of exposure can be extrapolated down to zero, biomarkers for effect, such as mutations, can only be interpolated back to the background incidence. This same threshold might also apply for tumors based on mutagenicity, or based on indirect genotoxicity due to mechanisms such as oxidative stress. However, the data shown in

[7] Note that EPA (2005) guidelines request that an lower bound to the slope associated with this risk also be given However, EPA (2001) BMD software does not enable the estimation of this lower bound with a probit model, and thus we do not show a value here.

Figure 6c are also consistent with, and indeed indicate, a low-dose linearity, suggesting that at least some of the tumors in the low dose area may be evoked by mutagenicity.

Since the information is available to support both MOAs, we turn to EPA (2005) for guidance and find that "If there are multiple modes of action at a single tumor site, one linear and one nonlinear, then both approaches are used to decouple and consider the respective contributions of each mode of action in different dose ranges." Our analysis builds on the typical practice of tumor weight of evidence and dose-response modeling in several ways that are consistent with the complexities of acrylamide's tumorigenicity and U.S. EPA (2005) guidelines. First, a comparison of evidence is shown for the thyroid growth stimulation as per EPA (1998). Second, a semi-quantitative approach of evaluating genotoxicity modes of action is shown for comparison to the Hill criteria described in EPA (2005). Third, we use biology-based judgments to select empirically-derived dose response models, in the absence of data for a biologically-base dose-response model as per U.S. EPA (2005). Fourth, we apply chemical specific analysis for potential adjustments to the dose-response assessment for childhood sensitivity (U.S. EPA, 2005). The salient issues related to the application of each of these steps are described below

Thyroid Growth Stimulation Evidence. Acrylamide administered for 2 or 7 days, at doses in the range that evoke tumors, decreases thyroid colloid and increases follicular cell height (Khan et al., 1999). Both of these changes indicate release of thyroid hormones, which appears to occur at 7 days; all three effects indicate changes to thyroid homeostasis. Acrylamide for 14 days shows statistically significant increases in plasma thyroid hormones (Friedman et al., 1999), which is consistent with thyroid hormone releases at 2 and 7 days, although these changes in thyroid hormones are reported only at doses above those that evoke tumors in the longer-term bioassays. Accompanying TSH levels are statistically significant decreased, as expected. However, such hormone changes reverse after acrylamide exposure for 28 days, with a statistically significant trend in both T3 and T4 decrease, and an increase (not statistically significant) of plasma TSH at the highest dose. These latter changes at 28 days are evidence of thyroid growth stimulation (Friedman et al., 1999). Thyroid DNA labeling is also statistically significantly increased at doses that evoke tumors, mitotic indices are increased (not statistically significant), and the apoptotic index is decreased (not statistically significant) (Lafferty et al., 2004). These latter three effects also suggest thyroid growth stimulation and may be more useful than thyroid weights for assessing growth in rats caused by small to moderate increases in TSH (Capen, 2001). Thus, the results of longer-term acrylamide administration might be a prolonged disruption in thyroid-pituitary homeostasis, leading to tumor development in rats. In addition, tumor development has been confirmed from long-term acrylamide exposure and one of the two available long-term tumor bioassays shows thyroid hyperplasia, which would be expected with this MOA. The long-term effects of acrylamide on thyroid-pituitary hormone homeostasis have not been monitored, however. Finally, the site of action for acrylamide may be in the liver with an enhanced catabolism of thyroid hormone (although the evidence for this is equivocal), or in the thyroid with microfilament and microtubule damage (acrylamide damages these organelles in other organs, but no thyroid-specific data are available), although other data suggest that it is not directly acting on the thyroid, or via neurotoxicity in the hypothalamus. Although acrylamide is a known neurotoxicant, sufficient data on its specific toxicity to the hypothalamus are not available.

Table 3 compares EPA's five areas of evidence for thyroid growth stimulation for acrylamide matched with two examples from U.S. EPA (1998). EPA concluded that example 3, a bis-benzenamine, evokes thyroid tumors by both an antithyroid and mutagenic MOA. As a result, U.S. EPA used both a linear and nonlinear method in the dose response assessment. U.S. EPA concluded that example 4, nitrosamine, evokes tumors by a mutagenic MOA only. As a result, U.S. EPA used a linear dose response assessment. We believe that the evidence for acrylamide more closely matches the findings of example 3 from U.S. EPA (1998), rather than example 4. This supports our conclusion that both MOAs are relevant for acrylamide and that it is appropriate to consider both a linear and nonlinear dose response assessment.

Concordance of Genotoxicity and Thyroid Tumor Data. If the genotoxicity results from the assays analyzed here are surrogate measures of a mutational event responsible for the formation of thyroid tumors, one would expect the data to be consistent with the modified Hill criteria for evaluating whether a precursor event plays a causative role in tumorigenesis (U.S. EPA, 2005). Specifically, one would expect that the mutations occur before the observed tumors (temporal concordance) and that increases in mutations occur at doses below those that cause tumors (dose-response concordance). The Hill criterion of biological plausibility is readily met, since mutation is a common MOA; however, the Hill criterion for specificity cannot be met for acrylamide for this same reason. The strength of the relationship and consistency of various measures of genotoxicity are addressed in the context of the dose-response relationship.

Ideally, one would compare a direct precursor, such as mutations in the same tissue, species, sex, and strain as that in which tumors are observed, and preferably in a gene relevant to tumorigenesis (e.g., an oncogene). In this ideal case, lack of concordance is an indication that that measured mutagenicity is not consistent with the observed tumorigenicity and suggests that mutagenicity, in and of itself, does not account for or explain the presence of the thyroid tumors. When other explanations for the occurrence of the thyroid tumors can be offered (and the data are more supportive of those other explanations), then support for a mutagenic mode of action is diminished, perhaps to the point that it would be rejected. However, when there are differences between the biological context of the mutagenicity and tumor data, the implications of these differences need to be considered.

The expected relationships between the dose-response behavior of a mutagenic response and a corresponding tumor response include the following. First, at any given dose, the probability of the triggering mutagenic response should be at least as great as that of the tumor response. In the analyses reported here, the probability of the genotoxic responses have been "scaled" so that the probability of response, in the absence of exposure, is approximately the same as the probability of thyroid tumors in the absence of exposure. The effect of this scaling is to transform the genotoxic surrogate to a metric that is essentially measuring the probability of thyroid-tumor-producing mutations. If thyroid tumors are produced *solely* by a mutagenic mechanism, then this metric should "track" well with the thyroid tumor probabilities; it should neither be much greater nor much less than the tumor probabilities at any given dose.

Second, because of the "tracking" mentioned above, if thyroid tumors are produced solely by a mutagenic MOA, the predicted genotoxic response probabilities should be increasing rapidly in the dose range where the tumor responses are increasing rapidly, and vice versa. Specifically,

the probability dose-response of a precursor genotoxic response should not be predicted to be flat when tumor rates are increasing, if a genotoxic insult is solely responsible for the tumor formation. Conversely, and perhaps more importantly, one should not expect that the probability of genotoxic insult would increase dramatically when tumor formation rates are constant; such a pattern is indicative that the measured genotoxicity is not a contributor.

Figures 3 and 4 are relevant to the evaluation of how well the above expectations are satisfied for acrylamide and therefore of concordance between the genotoxicity and tumorigenicity observations. Note that, with respect to "tracking," none of the genotoxicity endpoints do a very satisfactory job. The MF measurements from male mouse lymphocytes (Big Blue assay from Manjanatha et al., 2006; Figure 3a) are the only ones for which the predicted probabilities of genotoxic insult are at least as great as those for thyroid tumor production, for any given dose (attention restricted to the range of the doses applied in the cancer bioassays). For all of the other genotoxicity endpoints, significant portions of the dose range exist for which the predicted probabilities of genotoxic insult are less than the probabilities of tumor. Indeed, for the dose ranges in question, the predicted dose-response for those other genotoxicity endpoints is essentially flat in most cases, even though the tumor rates are observed to be significantly increasing with dose. Thus, those results are discordant with expectations that one would have when genotoxicity is the sole mechanism driving tumorigenicity. Figure 3a shows a pattern that might suggest that genotoxicity is not a factor at all (substantially increasing probability of genotoxic insult at low doses where tumor rates are predicted not to increase very much at all). However, over-interpretation of that plot should be avoided because a genotoxicity dose-response that is more consistent with the tumorigenicity dose-response (i.e., with MF being linearly related to dose) cannot be rejected with a high level of confidence. Moreover, a simpler way to model this response, by using multiples over background incidence and linear regression, shows that this effect in males, when pooled with the female response, occurs after the tumor response (Figure 5).

Nevertheless, for all but one of the genotoxicity endpoints examined in Figures 3, 4 and 5, one does not observe the behavior expected when a mutagenic MOA is producing the thyroid cancer response. Given that the genotoxicity endpoints are measured at much higher dose levels than those employed in the cancer bioassays, and that the dose-response curves as shown in Figures 1 and 2 are essentially flat in the range of tumor bioassay doses, the preponderance of the evidence suggests that the lack of concordance is not an artifact of the dose-response modeling. Rather, differences between mutagenicity and tumorigenicity suggest that genotoxicity is not a precursor to tumor formation. Moreover, these large differences (easily more than 10 fold) shown in Figures 3, 4 and 5 will likely be robust to suggested alternative choice of models and uncertainty in the exact dose response shape. These issues, of course, should be subjects of additional investigation as the approach for discerning concordance or its absence is further developed and refined, but we note that similar observations are described by Allen et al. (2005) in a categorical regression investigation of acrylamide genotoxicity data, and by Shipp et al (2006) in an extensive review of genotoxicity data.

As noted, the ideal data sets to compare mutagenicity data with tumor data are not available, so surrogate data were used in this analysis. The following considerations apply in evaluating the implications of using the surrogate data. The gene mutation data of Manjanatha et al. (2006)

have the advantage that heritable gene mutations (i.e., mutations that could be transmitted to daughter cells) are measured in an *in vivo* study. In addition, exposure is in drinking water for 3-4 weeks and up to 3 weeks were allowed to fix any mutational event. Based on limited data, this duration is sufficient for hazard identification studies using transgenic mutation assays (Thybaud et al., 2003); however, it is not known whether this duration is sufficient for direct comparisons of dose-response curves for gene mutation and tumors. Indeed, for the weakly mutagenic chemical dichloroacetic acid (DCA), the mutation frequency at a given dose has been observed to increase from 4 weeks to 10 weeks to 60 weeks of exposure (Leavitt et al. (1997). More importantly, the gene mutation data used in our analysis are from mice, while the tumor data are from rats. No chronic acrylamide tumor bioassays are available in the mouse, and the tissue targets, if any, in the mouse are unknown. In particular, it is not known whether the targets in the mouse would be expected to be the same as those in the rat. Furthermore, the only available gene mutation data from this study are in tissues other than those in which tumors are seen in the rat. Since one would not expect the dose-response for tumors in different tissue targets to be identical, this consideration limits the degree to which one would expect concordance between the available gene mutation from the Manjanatha et al. (2006) study and the tumor data. Overall, both of these considerations (study duration and differences in species/tumor target) limit the quantitative conclusions that can be drawn from direct comparisons of the dose-response curves for gene mutations from this study and tumors, although valid qualitative conclusions can be drawn, as further discussed below.

The Maniere et al. (2005) study has the advantage that it is conducted in rats, although it is conducted in Sprague-Dawley rats, while the tumor data are from Fisher 344 rats; metabolic and other differences among strains can lead to significant quantitative and qualitative differences in tumor response. The Maniere et al. (2005) study also had the advantage of evaluating several tissues in which one or both of the bioassays found tumors. In addition, for Figure 5 the Klaunig and Kamendulis (2005) data point was conducted in the Fisher 344 rat and in the thyroid. Other differences between these two studies and the ideal study design noted above, however, also affect the interpretation of these data. Both studies measured DNA damage, based on the comet assay rather than heritable gene mutations. This damage is less useful than gene mutations as surrogates for the mutations of interest, since DNA damage can arise from mechanisms that do not generate mutations, and the measured DNA damage may not result in a proportionate amount of gene mutation. Indeed, there is at least some evidence that acrylamide induces DNA damage via mechanisms other than direct DNA reactivity. Such mechanisms include disruption of DNA-associated proteins or generation of oxidative stress. Either of these two mechanisms could adequately explain the body of genotoxicity data and would be likely to yield non-linear dose-response functions. Furthermore, while the exposure duration of 1 day (for Maniere et al., 2005) or 7 days (for Klaunig and Kamendulis, 2005) is appropriate for the comet assay, it may not be sufficient to ensure that the gene mutation response is sufficiently maximized to compare with the tumor response after a 2-year exposure.

Based on these considerations, definitive quantitative concordance of the tumor and genotoxicity dose-response curves over the entire range of evaluation should not be expected. Nonetheless, comparisons of the timing and more general dose-response trends are appropriate. Increases in genotoxicity endpoints are observed after short exposure durations, well before tumors appear. However, evaluation of all but one of the many different measures of *in vivo* mutagenicity and

DNA damage endpoints found that the probability of triggering a mutagenic or DNA damage response is much lower than the tumor probability for all or for almost all endpoints over the entire dose-response curve. Despite the caveats noted for more quantitative comparisons, the weight of evidence from these multiple comparisons supports the conclusion that gene mutation cannot fully explain the dose response for thyroid tumors. This conclusion is further supported by consideration of the tissue distribution of tumors. In particular, it is not clear why the liver, with its high metabolic capability to form glycidamide, does not develop tumors in the rat, whereas other tissues, collectively with less metabolic capability, and some of which are hormone-responsive, do generate tumors. MOAs separate from or coupled with DNA reactivity would be required to explain the observation of thyroid, and perhaps other tumors in animals following acrylamide exposure. Ultimately, however, such caveats guided us to make the conservative assumption of linear low dose response assessment and gave support to our low dose decoupling approach. This dual approach has been suggested by several other investigators (e.g., CIR, 2005; Shipp et al., 2006; Allen et al., 2005; Klaunig and Kamendulis, 2005). In particular, the review by Shipp et al (2006) exhaustively lists alternative MOAs for thyroid tumorigenesis and supporting data.

Choice of Model for Tumor Response. Calleman et al. (1993) showed that the formation of glycidamide from acrylamide appears to be convex downward as measured by the formation of glycidamide-hemoglobin adducts, perhaps due to a more efficient conversion to glycidamide from acrylamide in the low dose range. Young et al. (2007) also note this observation, describing it as due to a more efficient conversion of acrylamide to glycidamide in the low dose range. However, this convex downward behavior is in apparent contrast to the formation of thyroid tumors after acrylamide exposure, which is better fit by a convex upward plot as described by U.S. EPA (1998) (see also Figure 6b and discussion below), supporting a mechanism of tumor formation in addition to, or perhaps other than mutagenesis caused by glycidamide formation.

When two MOAs operate in the same tissue or organ, U.S. EPA (2005) guidelines recommend that biological data should be used to decouple the two MOAs in order to determine the contribution of each MOA to the tumor risk, but do not specify how to do this. Such biphasic modeling of tumor data based on several MOAs is not a new idea. In addition to U.S. EPA (2005), others have described situations that would be appropriate for such modeling (e.g., Butterworth and Bogdanffy, 1999; Butterworth, 2006; Liehr, 2000), and in the case of formaldehyde a biologically-based dose response model resulted in such biphasic modeling and has been used in regulatory settings (e.g., Conolly et al., 2004). As stated above, multiple MOAs have also been suggested for acrylamide specifically (Klaunig and Kamendulis, 2005; Shipp et al., 2006); two of these MOAs lend themselves to biphasic modeling as we show here.

Modeling of the individual thyroid tumor data sets and emphasizing the low-dose region is complicated by less consistency and fewer data points. In contrast, pooling the data increases the number of data points and the number of doses, resulting in statistically significant regressions even when restricted to the low-dose data. Pooling the responses of the same sex from both studies is supported by the similarities in the two data sets, whether for males or females as per EPA (2005) guidelines. Although the Friedman et al. (1995) data have a slightly higher response than the Johnson et al. (1986) data for the same doses, the consistency in dose-response shape

suggests that the differences can be viewed as representations of sample variation (see Table 2 and Figure 6). Pooling the responses of different sexes from both studies also seems reasonable because these same figures show that the pooled responses in males and females intertwine.

Although the multistage model generated statistically meaningful estimates of slope factors (SFs) for pooled data applicable to the BMD_{02} when "All Doses" were modeled, these slope factors are generally larger than those determined by this model with "Decoupled Low Dose" (Table 5). These higher slopes indicate that the multistage model does not fit the dual nature of the underlying MOAs when all data are modeled. And while the "Decoupled Low Dose" BMD_{02}s might be useful for determining relevant slope factors for extrapolation to the low dose region, SFs applicable to the corresponding $BMDL_{02}$ are precluded from such extrapolation because they are generally higher than those generated from the $BMDL_{02}$s from "All Doses." In brief, the use of these higher slope factors from the "Decoupled Low Dose" $BMDL_{02}$ from the multistage model, does not make biological sense because the low dose data are where tumors are not yet statistically significant and slopes in this area should be correspondingly lower than in high dose areas. In contrast, the use of the probit model is successful in generating SFs that are lower in the low dose region than in the high dose region with all data modeled as shown in Table 6. These differences are consistent with the underlying tumor observations and the expected dual biology based on the MOA analysis.

As mentioned previously, the evaluation of BMD, BMDLs, SFs from various models cannot be used alone to distinguish the most reasonable choice of SF because any one mathematical model is unlikely to pattern the more complex biological response. However, one of these models, the probit, is able to interpolate the full range of tumor data in a manner that is consistent with two MOAs operating in different parts of the dose response curve. In addition, the probit model, which is widely used in toxicology, is confirmed by a simple weighted linear regression, which is likewise widely used. The use of this probit model is also confirmed with the Weibull model, the use of which is mentioned by U.S. EPA (1998) for thyroid tumors. Moreover, the probit model has also been used for modeling the induction of thyroid tumors by acrylamide by the FAO (2006).

While useful for determining acceptability of model estimates, upper bounds of SFs are not good predictors of expected response. One of the difficulties in interpreting upper bounds is that they can be highly uncertain as population descriptors and thus their use for risk prediction can be highly misleading. This is because the upper bound is influenced by the variance of the slope parameter as well as the variance of the background parameter. Thus, for assessing human health risk, a SF associated with an expected value is preferred over a SF associated with an upper bound. While many agencies commonly employ conservative assumptions and steps in order to produce protective risk estimates (Jones-Otazo et al., 2005), such approaches represent science policy, not scientific prediction. Risk predictions, often called "most likely" or "median" estimates, usually are the most data intensive, imposing stronger data requirements than those of protective approaches (Sexton et al., 1995), including mechanistic understanding of toxicity at high and low doses.

Possible Childhood Susceptibility. U.S. EPA (2005) encourages the review of age-dependent adjustment factors when the dose response assessment is based on a mutagenic mode of action.

Since we propose a mutagenic MOA in the low dose region, we investigated the metabolism of acrylamide to glycidamide by cytochrome P450 2E1 (CYP2E1) by age. Several studies in humans have shown CYP2E1 levels to be absent or minimal during gestation, followed by a rapid increase immediately following birth, and a gradual increase into adulthood (e.g., Vieira et al., 1996, Hakkola et al., 1998, Johnsrud et al., 2003). The metabolism of acrylamide to glycidamide is likely necessary for a mutagenic MOA. Because CYP2E1 levels are lower in the fetus or child than in adults, we do not anticipated any heightened tumorigenic response by the young to acrylamide in this low dose region due to toxicokinetics. In fact, we expect that the young would have less glycidamide-induced mutations, potentially leading to a lower slope factor, than adults given the same dose of acrylamide. However, information on toxicodynamics between the adult and child is not available for acrylamide. If such information were to indicate that children are more susceptible than adults, this would justify increasing the slope factor. Because these points operate in the opposite direction, one based on data in hand which serves to lower the SF, and one based on data to be acquired which might raise the SF, we judge that the best overall factor is a value of 1. Our judgment is similar to that described by U.S. EPA (2005) for chemicals undergoing CYP2E1 metabolism in general.

In contrast, if a threshold estimate for thyroid tumor response is envisioned based on the high dose data, it might be reasonable to explore the added sensitivity of children. This is because the tumorigenicity at high dose is expected to be due to growth stimulation by acrylamide, for which the lack of CYP2E1 enzymes in children would not be protective. The appropriate place to explore this potential increased susceptibility would be further in-depth consideration of the uncertainty factor for within human variability, for which we used the default of 10-fold in the determination of the RfD.

Summary. A recent dose response screening assessment for acrylamide has been developed by OEHHA (2005); Shipp et al (2006) has developed a more comprehensive dose response assessment. For thyroid tumors specifically, OEHHA (2005) used a linear dose response assessment, whereas Shipp et al. (2006) presented both an RfD and a linear extrapolation. Both groups acknowledged the dual nature of the likely MOAs for acrylamide. Shipp et al. (2006) gave an excellent review of thyroid physiology and potential thyroid MOAs.

Building on the work of OEHHA (2005), Shipp et al. (2006) and U.S. EPA (2005), we find that two MOAs are likely to be operating in different parts of the dose response curve of thyroid tumors from acrylamide exposure. Specifically, in the low end of the dose range, a mutagenic MOA, presumably from the glycidamide metabolite, has some supporting and detracting data; in the high end of the dose range, a thyroid growth stimulation MOA, presumably from the acrylamide parent compound, has more supporting and less detracting data. While neither set of supporting or detracting data for either MOA is definitive, both sets of data are consistent with a biphasic or "decoupled" approach. Therefore, based on the best evidence available and U.S. EPA (2005) guidelines, we determine a SF of 0.030 $(mg/kg\text{-}day)^{-1}$ for the linear, low dose, part of the "decoupled" approach and a RfD of 0.05 to 0.02 mg/kg-day for the non-linear, high dose, part of the approach.

Additional work on extrapolation of thyroid tumor results from experimental animals to humans might be useful to further enhance the degree of certainty in this analysis. Specifically this work might include:

- Investigation of mutagenic damage to the thyroid from *in vivo* studies in the rat at doses similar to those that have evoked tumors.
- Histopathology of thyroid for determination of acrylamide-specific damage, perhaps focusing on microfilaments and microtubules, known to be necessary for adequate follicular formation.
- Pituitary and thyroid hormone analysis after long-term acrylamide administration.

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Figure 1a. Data and Model Fit for Big Blue Mutant Frequency Assay in Male Mouse Lymphocytes.

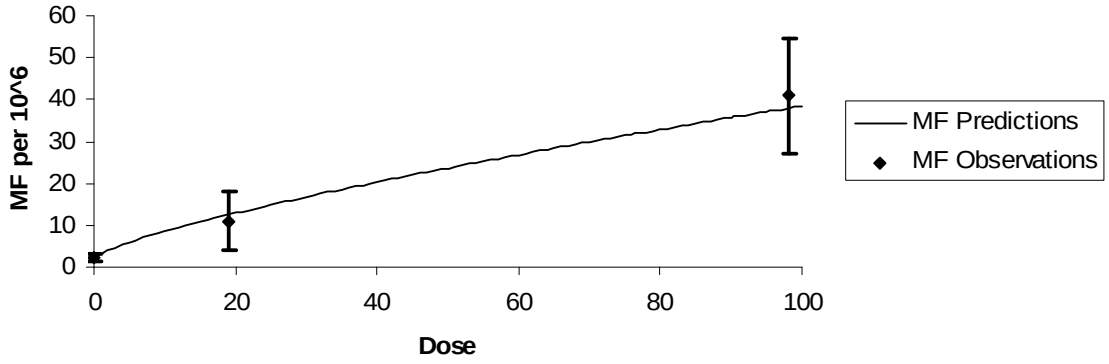
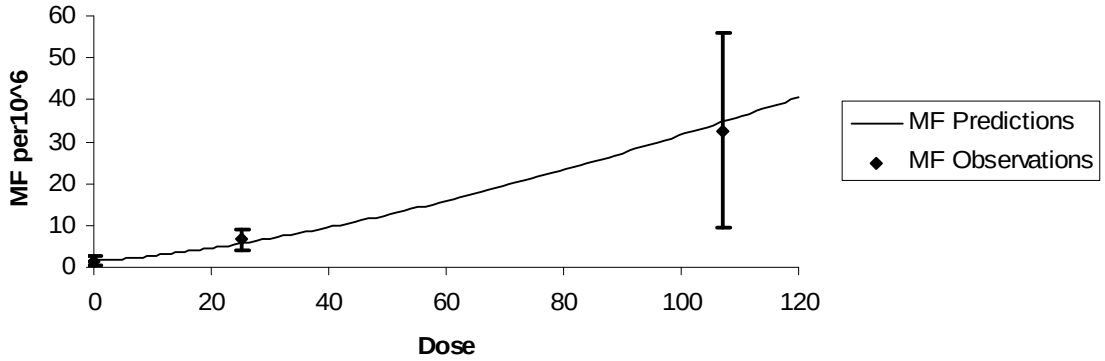


Figure 1b. Data and Model Fit for Big Blue Mutant Frequency Assay in Female Mouse Lymphocytes.



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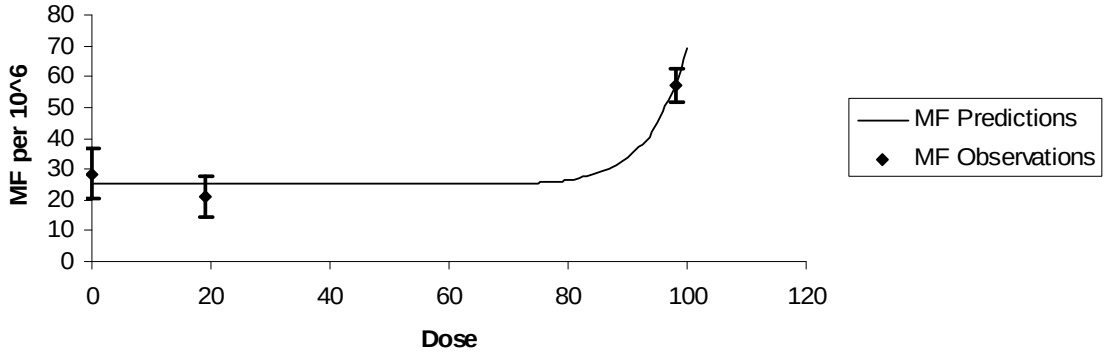

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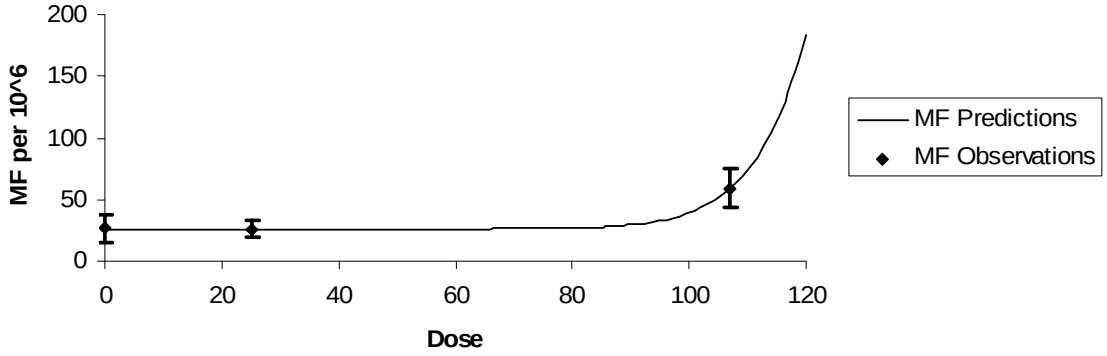
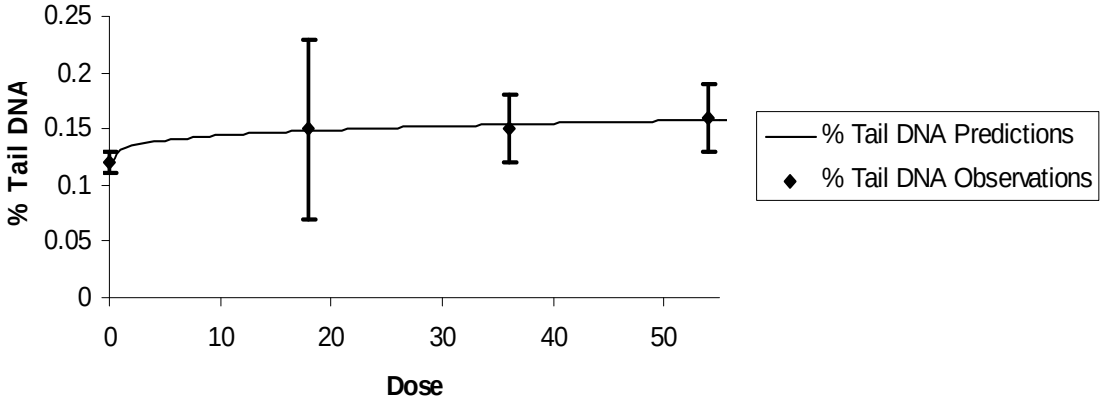


Figure 2a. Data and Model Fit for Comet Assay of Percent Tail DNA in Leukocytes of Male Sprague Dawley Rats.



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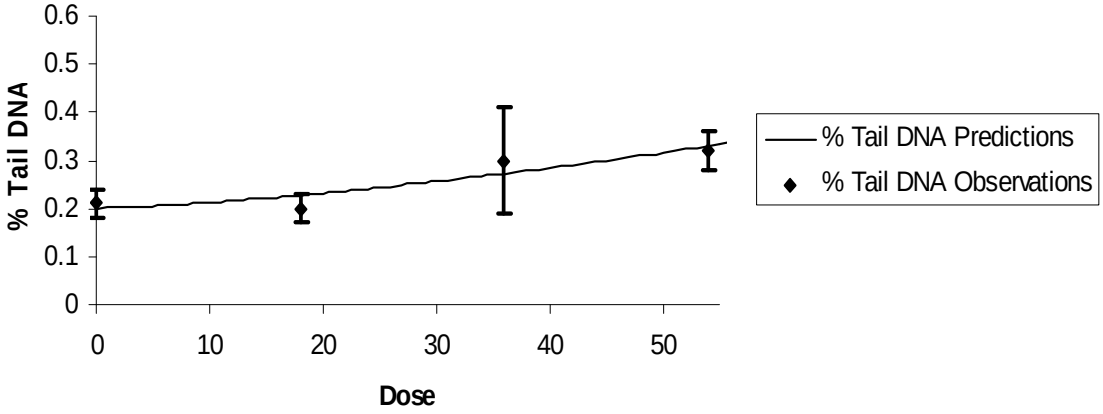

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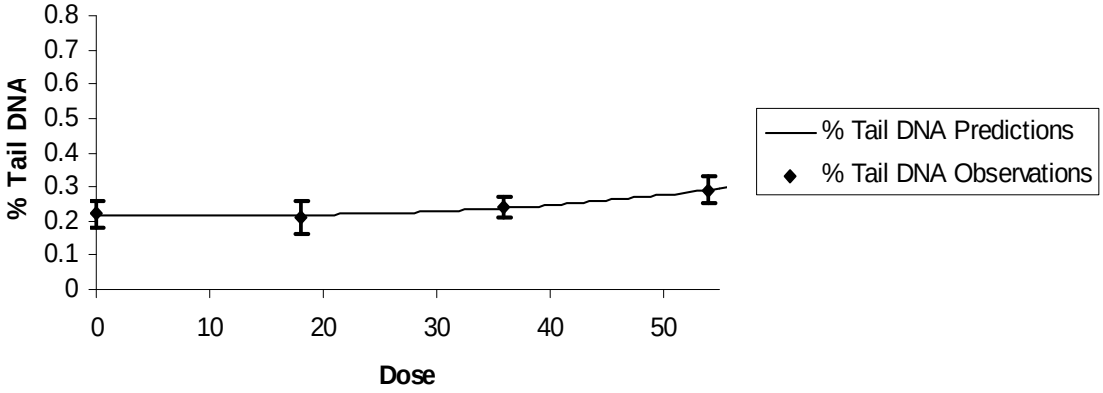
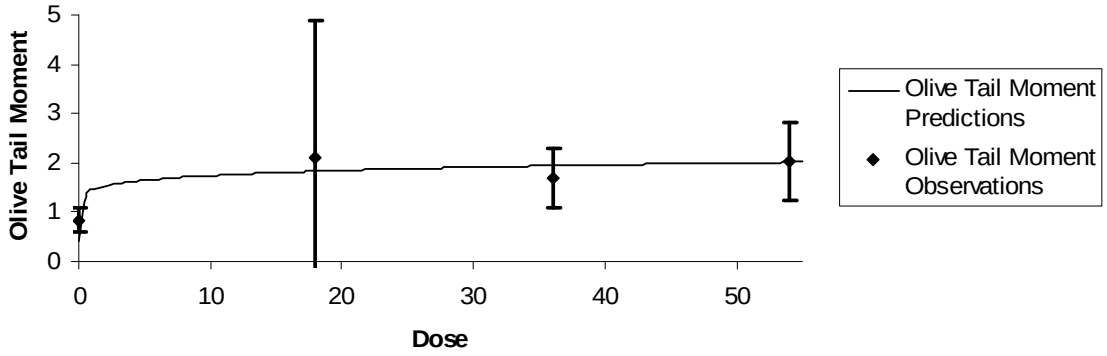


Figure 2d. Data and Model Fit for Comet Assay of Olive Tail Moment in Leukocytes of Male Sprague Dawley Rats.



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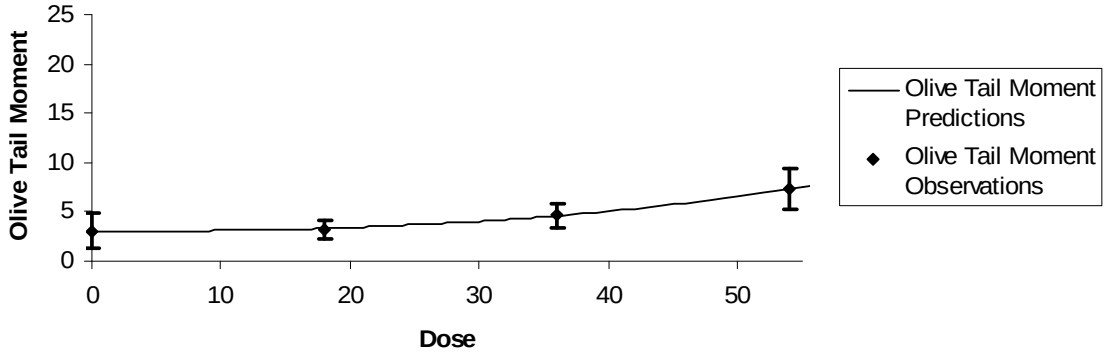


Figure 3a. Dose-Related probability of response for thyroid tumors in the rat compared to predicted probability of male mouse lymphocyte mutant frequency

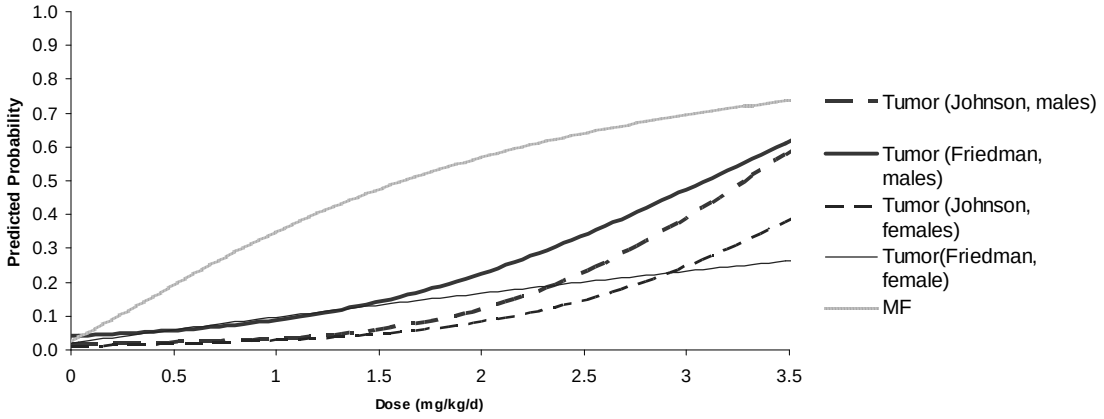


Figure 3b. Dose-Related probability of response for thyroid tumors in the rat compared to predicted probability of female mouse lymphocyte mutant frequency

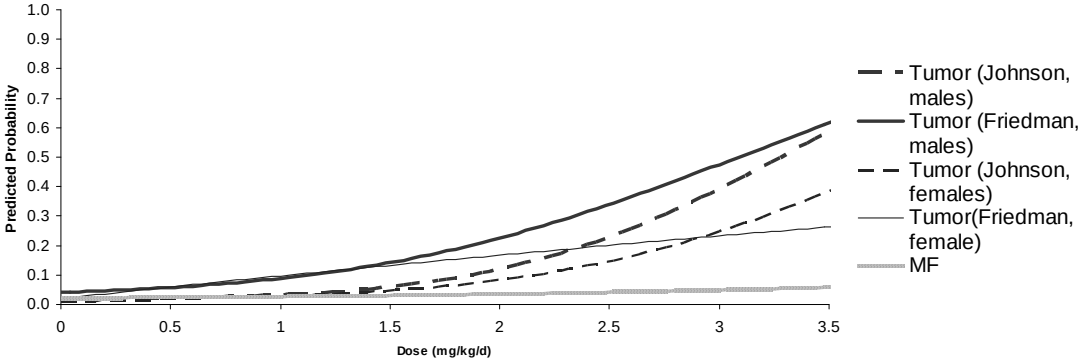
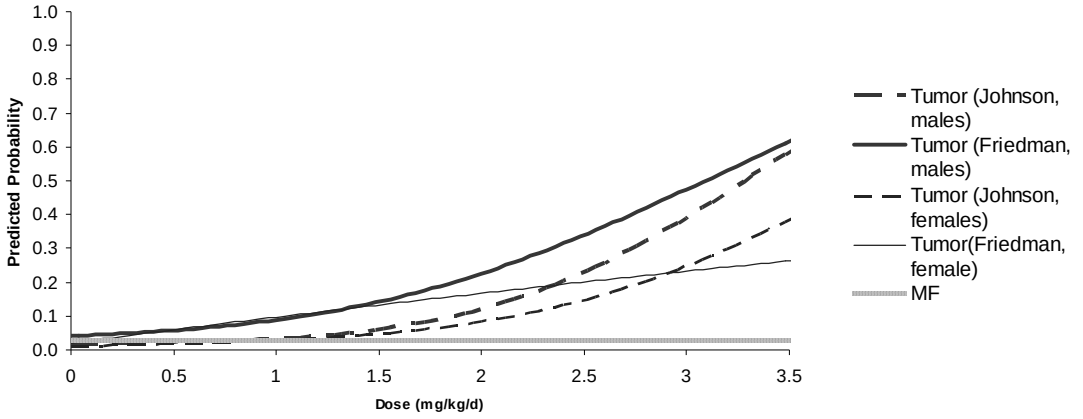


Figure 3c. Dose-Related probability of response for thyroid tumors in the rat compared to predicted probability of male mouse liver cell mutant frequency



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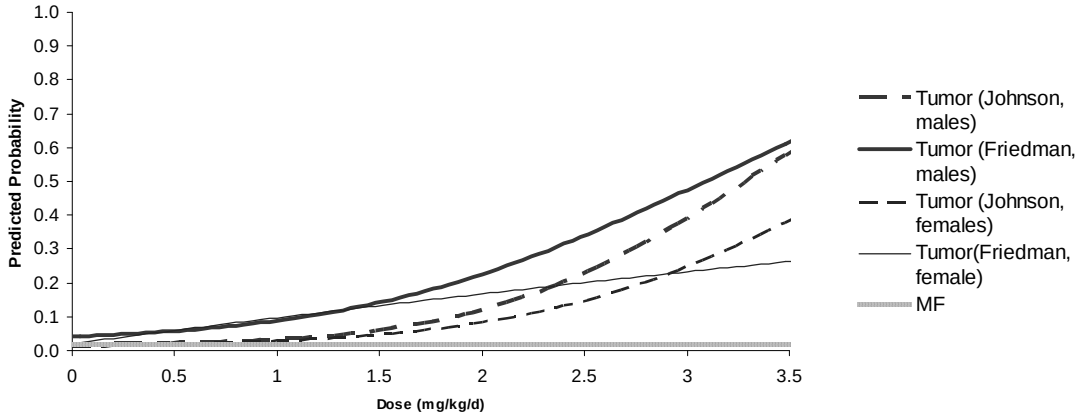


Figure 4a. Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of % Tail DNA in Comet assay in male rat leukocytes

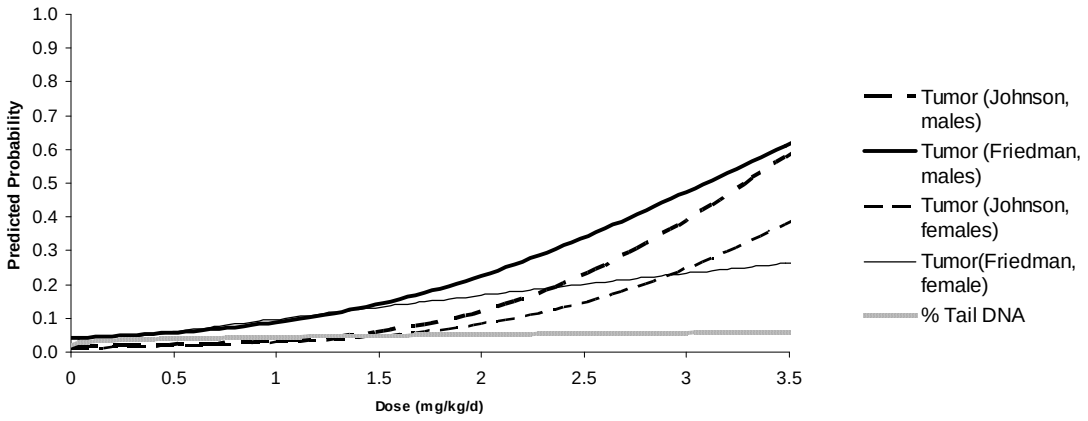


Figure 4b. Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of % Tail DNA in Comet assay in male rat brain cells

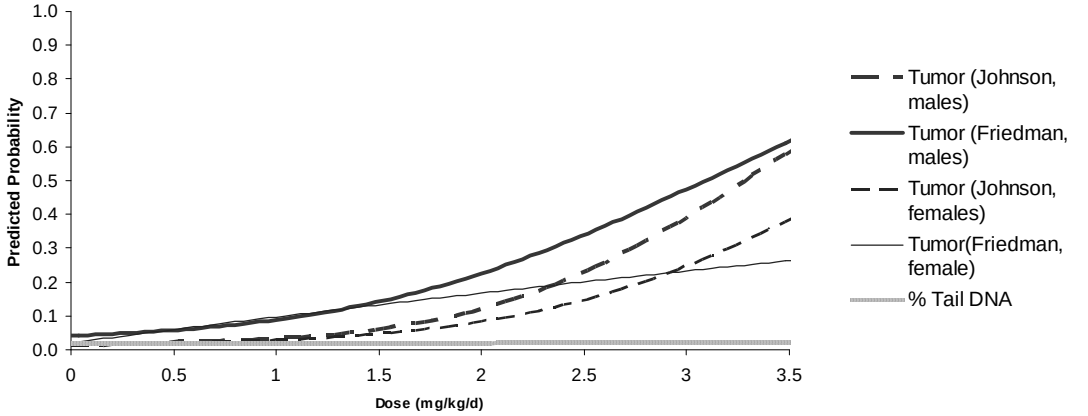


Figure 4c. Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of % Tail DNA in Comet assay in male rat testes cells

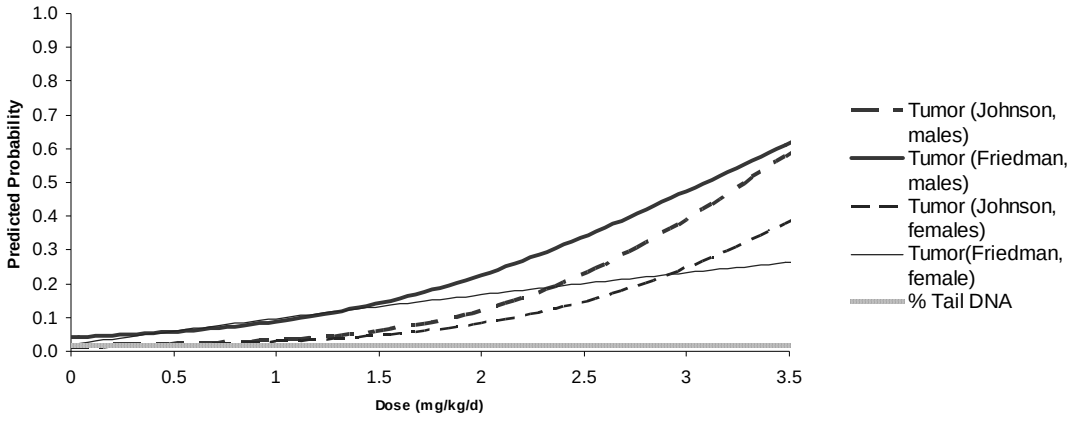


Figure 4d. Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of Olive Tail Moment in Comet assay in male rat leukocytes

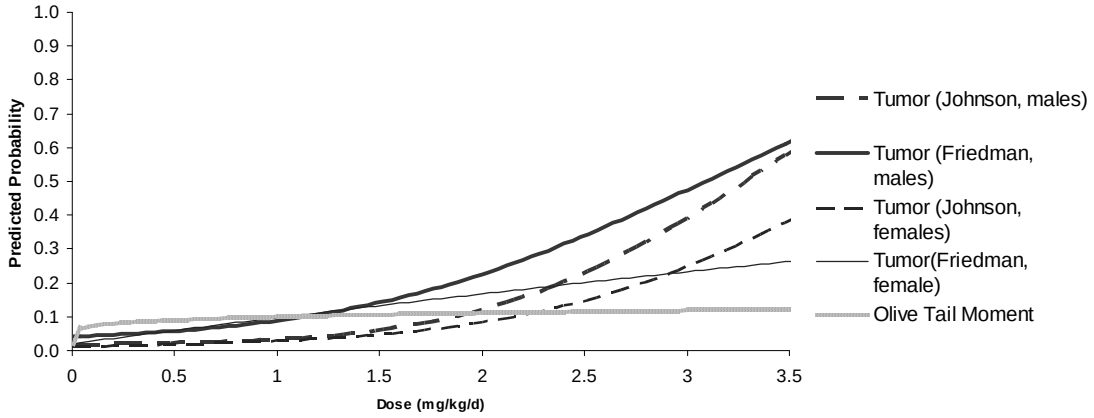
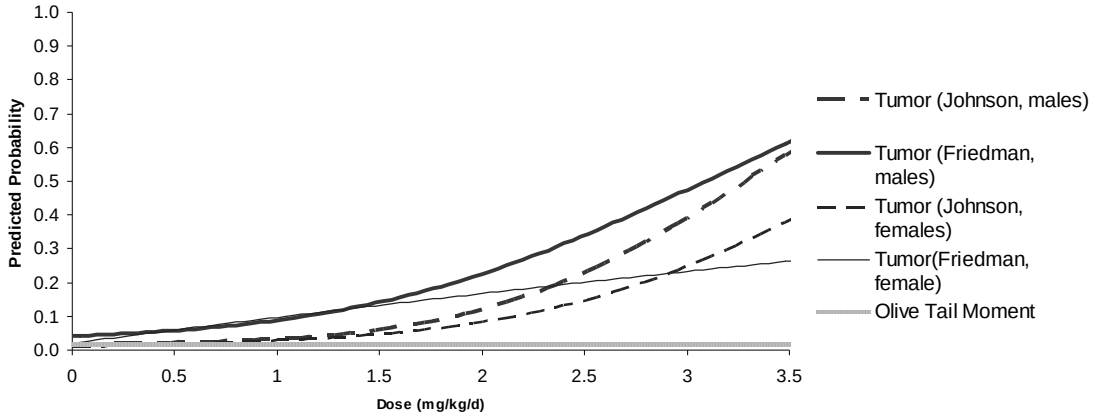


Figure 4e. Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of Olive Tail Moment in Comet assay in male rat brain cells



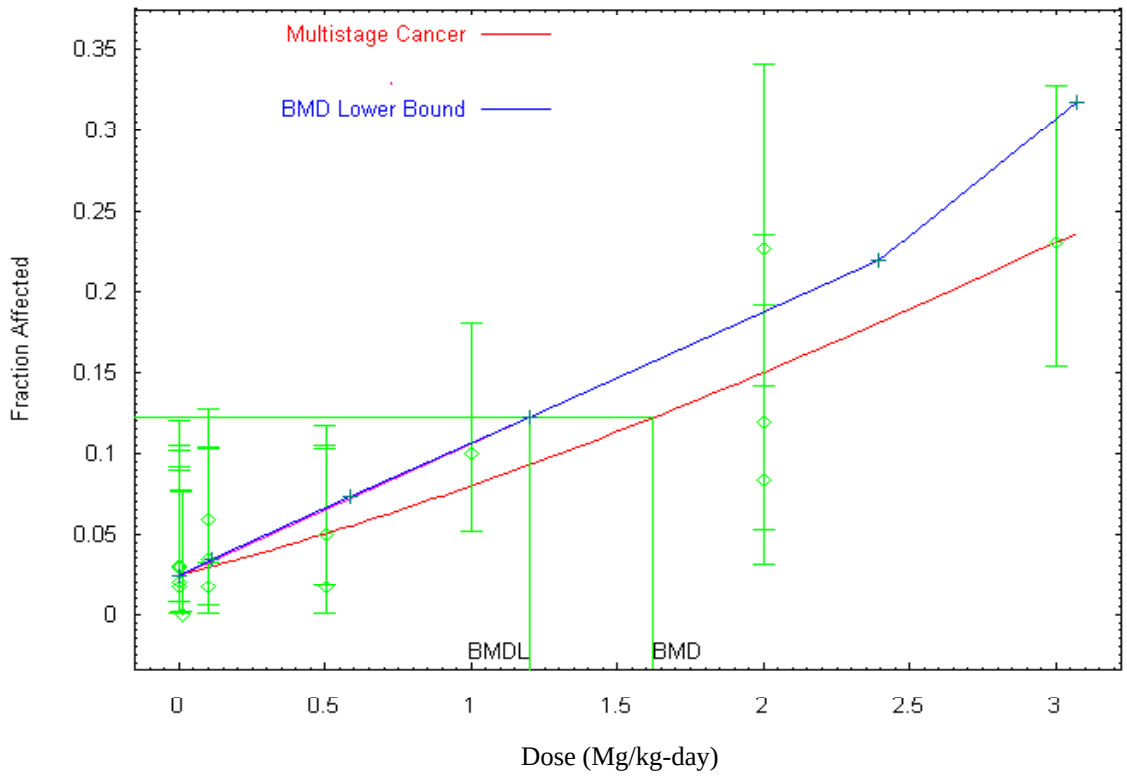


Figure 6a. Multistage model fitted to pooled-all thyroid tumor data, showing little change in slope between the low and high dose regions.

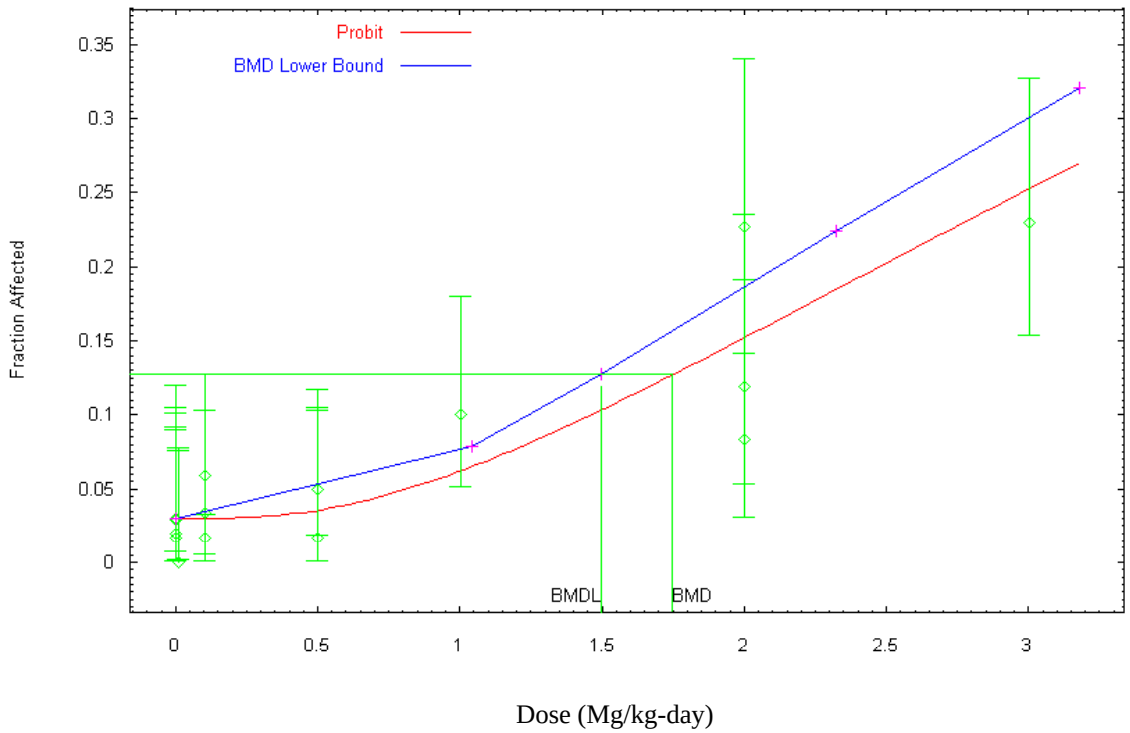


Figure 6b. Probit model fitted to pooled-all thyroid tumor data, showing differing slopes between the low and high dose regions.

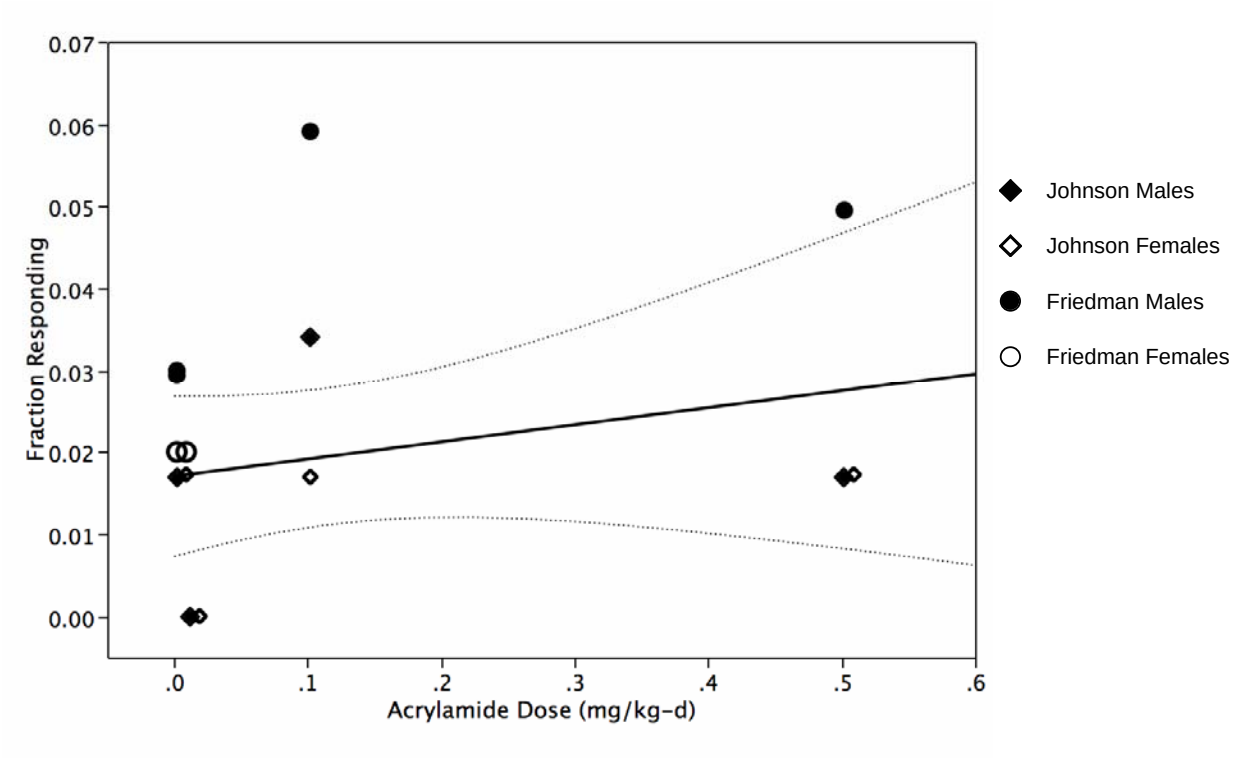


Figure 6c. Weighted linear regression on low-dose points, for pooled data (male, female, both studies) with 95% upper and lower confidence curves for the model. Displayed dose is jittered to show all 14 data points.

Figure 5. Multiples above background for tumors, DNA labeling, and several measures of in vivo genotoxicity

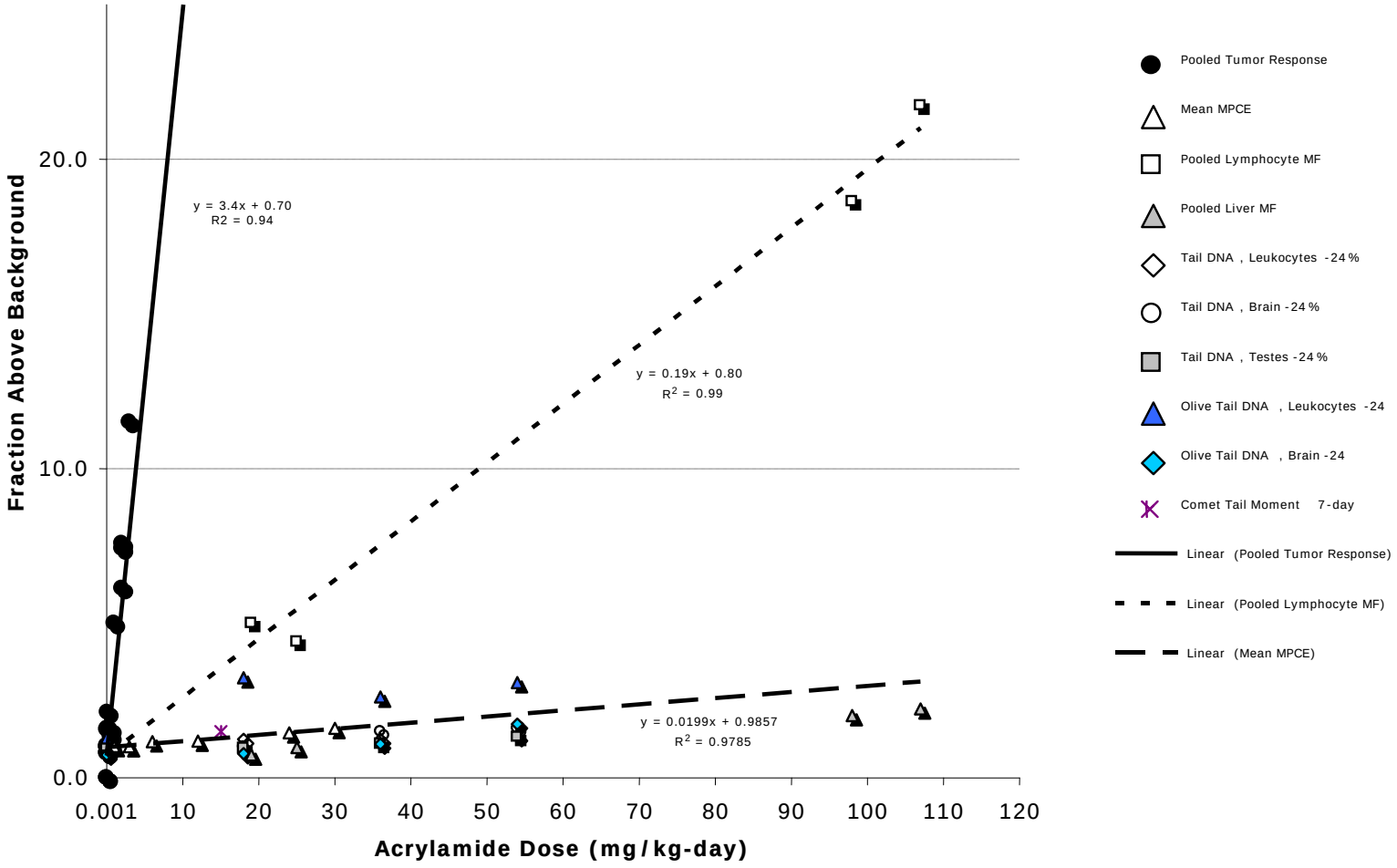


Table 1. Thyroid tumor incidence and fraction as a function of acrylamide dose in the Johnson et al. (1986) and Friedman et al. (1995) 2 year drinking water studies. [1]

Acrylamide dose	Johnson Males		Johnson Females		Friedman Males		Friedman Females	
mg/kg-day[2]	Incidence	Fraction	Incidence	Fraction	Incidence	Fraction	Incidence	Fraction
0.002	1/60	0.017	1/58	0.017	3/100	0.030	1/50	0.020
0.002					3/102	0.029	1/50	0.020
0.012	0/58	0.0	0/59	0.0				
0.10	2/59	0.034	1/59	0.017	12/203	0.059		
0.50	1/59	0.017	1/58	0.017	5/101	0.050		
1.0							10/100	0.10
2.0	7/59*	0.12*	5/60*	0.083*	17/75*	0.23*		
3.0							23/100*	0.23*

[1] Asterisks (*) indicate authors' designation of statistical significance.

[2] Control doses with dietary acrylamide have been estimated. See text for discussion.

Table 2. Lymphocyte and liver mutagenicity in male and female big blue mouse, follicular cell labeling in Fisher 344 male rats, and DNA damage from the comet bioassay in Sprague Dawley male rats. [3]

Acrylamide dose mg/kg-day[4]	Big Blue Mouse				Fisher 344 Male Rat			Sprague Dawley Rat									
	Lymphocyte Hprt MF x 10^-6		Liver cII MF x 10^-6		Follicular Cell Labeling (%)			% Tail DNA @ 24 hours			Olive Tail Moment @ 24 hours		% Tail DNA @ 2 hours		% Tail DNA @ 5 hours		
	Male	Female	Male	Female	7 Day	14 Day	28 Day	Leukocytes	Brain	Testes	Leukocytes	Brain	Testes	Adrenals	Bone marrow	Liver	Adrenals
0.001								0.12	0.21	0.22	0.84	3.1	0.18	0.20	0.22	0.17	0.20
0.002	2.2	1.5	28.4	26.5	3.0	2.7	2.6										
2					7.6*	5.6*	4.7*										
15					7.9*	6.1*	5.6*										
18								0.15	0.20	0.21	2.1	3.3					
19	11.0*		21														
25		6.6*		26													
36								0.15	0.30*	0.24	1.7*	4.6					
50																	
54								0.16*	0.32*	0.29*	2.0*	7.3*	0.32*	0.25*	0.33*	0.26*	0.29*
98	41.0*		57.2*														
107		32.6*		59*													

[3] Asterisks (*) indicate authors' designation of statistical significance.

[4] Control doses with dietary acrylamide have been estimated. See text for discussion Fisher 344 rats. For Big Blue Mice these values are estimated as 0.002 mg/kg-day based on Manjanatha et al (2006) measurement of acrylamide in diet and food feeding factors for mice (EPA, 1988)

Table 3. Comparison of U.S. EPA's Required Data Demonstrating Antithyroid Activity for Acrylamide and Two Examples (quoted text is from U.S. EPA, 1998).

Data/Chemical	Bis-benzenamine	Acrylamide	Nitrosamine
Cancer Findings	The incidences of benign and malignant thyroid follicular cell tumors were significantly increased in the mid- and high-dose male and female F344 rats in roughly equal proportions in this 2-year drinking water study. No interim kills were conducted.	The incidences of more benign and fewer malignant thyroid follicular cell tumors were significantly increased in high-dose male and female F344 rats in roughly equal proportions in two, 2-year drinking water studies. Interim kills in one study showed no increase in tumors by 18 months.	The incidences of thyroid follicular cell tumors were significantly increased in high-dose Wistar male rats in a 26-week drinking water study. A second study in male and female Wistar rats by i.p. injection for 30 weeks showed tumors at mid and high dose males and high dose females.
Increases in Cellular Growth	"The incidence of diffuse hyperplasia of the thyroid gland in the 2-year bioassay was significantly increased in the mid- and high-dose rats of both sexes and the high-dose mice of both sexes." "Administration of 400 ppm in tap water to male Wistar rats for 20 weeks. At cessation of treatment, the mean values for thyroid weight were statistically significantly different in treated (30+6 mg) as compared with control (18+8 mg) groups (21 rats per group), respectively."	Statistically significant increase in relative thyroid weight in female rats for 28 days; effect did not occur in males. Statistically significant thyroid follicular hypertrophy in female F344 rats for 2 or 7 days, and corresponding loss of colloid. Statistically significant increase in thyroid follicular DNA labeling in F344 rats at 7, 14 and 28 days. An increase in the mitotic index and decrease in apoptotic index were also observed. A statistically significant increase in focal cystic dilatation of the follicles occurred in both male and female rats after 2 years. An increase in thyroid hyperplasia was seen in a second study, but this increase was not statistically significant.	"Thyroid weights were recorded at 30 weeks in the i.p. injection cancer study of compound 4. Although thyroid follicular cell tumors were present in the mid- and high-dose males and in the high-dose females, thyroid weights overall showed no statistically significant difference among groups and therefore no correlation with the incidences of thyroid tumors. This lack of statistical significance remained when the thyroid weights were calculated relative to body weights. At the histological level, the thyroids from treated groups showed a dose-response relationship for the incidence of follicular cell hyperplasia. The hyperplasia, however, was not of the diffuse form typical of antithyroid compounds, but manifested as small solitary foci, presumably representing the first stage in the continuum of hyperplasia to adenoma to carcinoma."

Hormone changes	"Following 20 weeks of treatment, hormone levels are altered in rats, including decreases in both T4 and T3 and increases in TSH."	No statistically significant changes observed in TSH or T_4 in rats exposed for either 2 or 7 days; T3 was not measured; at 7 days, dose-related increases in the adjusted plasma T4 were seen, with a slight decrease in the adjusted TSH. After 14 days, a statistically significant increase was observed in T4 (males) and T3 (females), accompanied by a statistically significant decreased TSH in females. After 28 days, statistically significant decrease was seen in T_3 and T_4 (males) with an increase in TSH, (not statistically significant); females showed only a decrease in T3 at the high dose (not statistically significant).	"No difference in serum T4 or TSH levels in treated groups when compared with levels in control animals." (T3 levels not measured.)
Site of Action	No data, but: "Some close analogues are known to reduce radioactive iodine accumulation in the thyroid; presumably this is due to an inhibition of thyroid peroxidase activity."	Increases in liver weight is evident in several, but not all, studies; such increase might result in thyroid hormone loss. Loss of integrity of microfilaments and microtubules will block follicle formation; although acrylamide is known to damage these organelles in other organs, no information is available for the thyroid. Neurotoxicity is a sensitive non-cancer effect of acrylamide and regulation of thyroid hormones occurs via the hypothalamic-pituitary-thyroid axis, but specific data on the ability of acrylamide to induce toxicity in the hypothalamus are limited.	"No studies were available that have investigated the effects of compound 4 on thyroid peroxidase, the deiodination pathway, or effects on thyroid metabolism and excretion in the liver. However, the chemical is without goitrogenic or thyroid-pituitary hormone effects, indicating that such studies are not needed."

Dose Correlations	"Significant dose-related goitrogenic effects (e.g., increase in thyroid weight, diffuse hyperplasia) are noted after subchronic and chronic dosing in rats and mice."	Thyroid follicular cell tumors were statistically significantly increased at 2 mg/kg-day in males and females in the first study, and at 2 mg/kg-day in males and at 3 mg/kg-day in females in a second study. These tumorigenic doses are in the range of doses that show: o statistically significant thyroid histological changes in two studies, although such histological changes were not evident in two other studies o Dose related increases in thyroid DNA labeling at 3 time points o Increases in mitotic index and decreases in apoptotic index. These tumorigenic doses are generally below doses that cause hormone changes after short-term exposure.	"The chemical is without goitrogenic or thyroid-pituitary hormone effects, indicating that such studies are not needed. Obviously, there are no significant dose correlations to consider."
Reversibility	No data.	No data.	No data.

Conclusions	“In sum, these observations constitute an adequate database to evaluate antithyroid effects, but there is a lack of specific information on the site of action.” “Characterization of cancer dose-response relationships should primarily rely on mutagenic considerations for the thyroid and liver tumors using a low-dose linear procedure. However, the thyroid tumor responses may be due to both its mutagenic and antithyroid properties. Other chemicals with both mutagenic and antithyroid effects also have led to high thyroid tumor incidences, as have combinations of mutagenic and antithyroid stimuli. Because it is not possible to totally discern the relative impacts of these influences for compound 3, threshold considerations should be used in addition to a linear extrapolation so as to estimate the lower bound on the thyroid cancer risk.”	We conclude that this MOA, that acrylamide is evoking thyroid tumors as a result of follicular cell growth stimulation, is likely to be correct to some extent. It appears that a mutagenic MOA caused by exposure to glycidamide may also be operating to a limited extent. Based on the available evidence we conclude that both MOAs are operating at different doses. Thus, these MOAs were “decoupled” and several approaches are considered for the dose-response modeling. See text for additional discussion.	"Compound 4, a nitrosamine, produces thyroid follicular cell tumors in rats after a very short latency period. It also produces lung, liver, and kidney tumors in rats after a short latency period and pancreatic, liver, and lung tumors in Syrian hamsters. Compound 4 is mutagenic in various short-term tests. Because compound 4 is mutagenic, causes both thyroid and other tumors with a short latency, and does not cause antithyroid effects, the thyroid follicular cell tumors appear to be caused by a mutagenic mode of action. Dose-response relationships for the thyroid tumors should be evaluated using a low-dose linear default procedure."

Table 4. Key events, related evidence and plausibility of tumor modes of action.

Event	**Summarized evidence in animals (see text for additional details)**	**Is this key event in the MOA plausible in humans?**	**Taking into account kinetic and dynamic factors, is this key event in the MOA plausible in humans**
An heritable mutation to thyroid follicular cell DNA	• Glycidamide is directly mutagenic. • DNA-glycidamide adducts are found in multiple tissues, but overall tumor response does not match distribution. • Mutations lead tumors in timing of response, but not in dose response.	The MOA is plausible in humans, but not without subsequent growth stimulation.	This MOA is plausible and comparative kinetic information between experimental animals and humans allows specific choices of adjustment factors (see text for discussion).
Follicular cell growth stimulation	• Increased DNA synthesis in thyroid at multiple time points; increased mitotic index; decreased apoptotic index • Decreased thyroid colloid and increase follicular cell height after 2 or 7 days; equivocal changes in thyroid histology after long-term exposure. • Increased thyroid hormone release up to 14 days, but hormone decrease & thyroid weight increase after 28 days; no long-term studies monitoring hormone data are available. • Equivocal changes in liver weights occur after long-term exposure, suggesting increase catabolism of thyroid hormones; other possible sites of action are microfilament and microtubule development in the thyroid and neurotoxicity in the hypothalamus.	The MOA is known to occur in humans.	This MOA is plausible, but less likely to occur in humans given the same dose of acrylamide as in rats, based on well-recognized differences in thyroid hormone homeostasis. A conservative assumption is to use a factor of 1-fold when extrapolating rat data to humans.

Table 5. Multistage model estimates of slope factors (SF) for the rat thyroid tumor data.

BMD/L values are in mg/kg-day; SF values are in $(mg/kg\text{-}day)^{-1}$.

Data set[a] (All doses)	BMD 10	SF at BMD 10	BMDL 10	SF at BMDL 10
J female	2.2	0.045	1.5	0.067
J male	2.0	0.051	1.2	0.085
F female	1.3	0.077	0.94	0.11
F male	1.6	0.065	0.76	0.13
Pooled female	1.8	0.057	1.2	0.085
Pooled male	1.7	0.058	1.1	0.095
Pooled all	1.6	0.062	1.2	0.083

(Decoupled Low Dose[b])	BMD 02	SF at BMD 02	BMDL 02	SF at BMDL 02
Johnson female[c]	1.2	0.016	0.18	0.11
Pooled male	0.84	0.024	0.19	0.11
Pooled all	0.80	0.025	0.23	0.088

(All doses)	BMD 02	SF at BMD 02	BMDL 02	SF at BMDL 02
Pooled female	0.37	0.054	0.22	0.089
Pooled male	0.76	0.026	0.20	0.099
Pooled all	0.39	0.052	0.23	0.087

a. F = Friedman, et al., 1995; J = Johnson, et al., 1986.
b. Only the data with dose<1.0 mg/kg-day were modeled. Only BMD02 values are presented with these low dose data. BMD10 values are not calculated by BMDS because of unacceptable extrapolation (BMD larger than three times maximum input dose).
c. Only one dose was lower than 1.0 mg/kg-day in Friedman female study; thus these values are from the Johnson study only.

Table 6. Probit model estimates of slope factors (SF) for pooled male and female rat data on thyroid tumors. BMD/L values are in mg/kg-day; SF values are in $(mg/kg\text{-}day)^{-1}$.

Data set	BMD 02	SF at BMD 02	BMDL 02	SF at BMDL 02
Pooled female	0.82	0.024	0.67	0.03
Pooled male	0.97	0.021	0.58	0.034
Pooled all	0.81	0.025	0.69	0.029
	BMD 10	SF at BMD 10	BMDL 10	SF at BMDL 10
Pooled female	1.8	0.057	1.5	0.069
Pooled male	1.7	0.059	1.3	0.079
Pooled all	1.7	0.057	1.5	0.067

Table 7. Slope Factors (SF) from weighted linear regression on just the low dose data (dose<1.0) on thyroid tumors. BMD/L values are in mg/kg-day; SF values are in (mg/kg-day)$^{-1}$.

Data set	BMD 02	SF at BMD 02[a]	BMDL 02[b]	SF at BMDL 02[b]	p-value[c]
Pooled female	1.53	0.013	0.35	0.057	0.61
Pooled male	0.84	0.024	0.18	0.111	0.58
Pooled all	0.92	0.022	0.33	0.061	0.41

a. Values calculated as 0.02/BMD02.
b. BMDL values read from the graph of the upper bound response. BMDL values have precision of roughly +/- 0.005. SF values have precision of roughly +/- 0.004. Inverse prediction of confidence values is not presented in JMP 6.0 software because of lack of statistical significance of the model.
c. Observed p-values (significance probabilities) of 0.05 or less are often considered evidence of a regression effect. High p-values suggest the specified model fits no better than the overall response mean and is consistent with evidence of a threshold in tumor response.