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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 point mutations, but acrylamide does not. We also find that thyroid tumors are most consistently sensitive in rats, being evoked in each of four 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 likely dominates the response at higher doses. Following US EPA guidelines, we determined that the probit model best reflects the overall data set; this model is also preferred because it better reflects the underlying “decoupled” biology of contributions from potentially two modes of action. We use the probit model to identify a health-protective, linear cancer slope factor (SF) of $0.030 \text{ (mg/kg-day)}^{-1}$ for the low area of the dose–response curve associated with possible mutagenicity. We also identify a Reference Dose (RfD) in the range of 0.05–0.02 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. This dose–response assessment is briefly summarized in light of other related work.

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1. Introduction

A number of investigators have conducted dose–response assessments for acrylamide due to its widespread occurrence in foods (e.g., Dybing and Sanner, 2003; OEHHA, 2005; FAO, 2006; Shipp et al., 2006). Some of these investigations have modeled tumors in a more screening level assessment, which is useful as an initial means of determining whether risks are of sufficient concern to warrant further study. Other assessments were more comprehensive, which often provides the necessary analysis and data for enhancing the confidence in risk management decisions.¹

Due to the growing concern over dietary exposure to acrylamide, a number of epidemiological studies have evaluated various cancer endpoints associated with acrylamide exposure. 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 (SMR = 1.38, CI = 0.28–4.02), 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.

Several 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; Hogervorst et al., 2007). Mucci et al. (2005), Pelucchi et al. (2006), and Hogervorst et al. (2007) evaluated women's dietary acrylamide intake through a food frequency questionnaire and found no evidence for an elevated risk of breast cancer. Hogervorst et al. (2007) did, however, observe an increased risk in the highest quintile of acrylamide exposure for both endometrial and ovarian cancer in never smokers, and borderline

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E-mail address: dourson@tera.org (M. Dourson).¹ U.S. Environmental Protection Agency (EPA) is also developing an assessment (see: <http://www.regulations.gov/fdmspublic/component/main?main=DocketDetail&d=EPA-HQ-ORD-2007-1141>).

increase was observed for ovarian cancer in the total group. In addition, Olesen et al. (2008) evaluated women's dietary intake of acrylamide through acrylamide-hemoglobin (AA-Hb) and glycidamide-hemoglobin adducts, unlike the previous studies, and found, after fully adjusting their model for smoking and other confounding factors, that women with the highest AA-Hb concentrations had a 2.7 times elevated risk of estrogen-receptor positive (ER+) breast cancer compared to women with lower concentrations. Maier et al. (submitted for publication) discusses these studies on mammary tumors for acrylamide in more depth. Overall, the epidemiological literature does not provide convincing evidence that dietary exposure to acrylamide is a significant risk for cancer.

In contrast to these mostly negative human studies, acrylamide carcinogenicity studies in rats have reported increases in combined benign and malignant tumors of the thyroid, mammary tissues, and tunica vaginalis of the testis, as well as sporadic findings of other tumors.² 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 showed that the onset of tumors was generally late in the experiment (at nearly 2 years). Interim kills were not conducted 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 well-established 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, 2008).

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 (T₄), is shorter in rodents than in humans (12 h versus 5–9 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 (US 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.

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). In addition, 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, which is not consistent with the observed tumor distribution. These results might be expected if tumors were evoked by modes of action not related to DNA adduct formation. Alternatively, these results might be expected since adducts represent a biomarker of exposure, and are not as related to tumor formation as are mutations, a biomarker of effect (Swenberg et al., in press).

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 purpose of this article is to briefly review relevant experimental animal studies on acrylamide for thyroid tumorigenicity, genotoxicity, and thyroid growth stimulation. We then follow the US 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.

2. Methods

US 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 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 proceeds by a default procedure informed by specific data, either a linear or a non-linear model based on one or more combinations of relevant tumors. The guidance also notes that “If there are multiple modes of action at a single tumor site, one linear and another non-linear, 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.

We follow these guidelines by first describing our methods for analyzing mutagenicity and growth stimulation modes of action. Afterwards, we are guided by EPA (2005) to model tumors by

² Mammary tumors and testicular vaginalis mesotheliomas are discussed in companion papers (Maier et al., 2008 and Haber et al., 2008). They are not the focus of our work here.

either a mutagenic or thyroid growth stimulation mode of action, or to “consider the respective contribution of each mode of action in different dose ranges.”

2.1. Mode of action

The mode of action (MOA) framework within the cancer risk assessment guidelines of the U.S. Environmental Protection Agency (US EPA, 2005) is built in part 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 life stages that could be more susceptible for dose–response analysis. Furthermore, as per US 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.

US 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. We did not use DNA adducts for this quantitative evaluation because adducts are generally seen as biomarkers of exposure, whereas mutagenicity is a biomarker of effect that is more directly related to tumor endpoints (e.g., Swenberg et al., in press).

In order to represent the tumor dose–response for this comparison, we fit a multistage model to the thyroid tumor data. 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 US EPA software, BMDS (version 1.4.1; US 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:

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

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 $d = 0$,

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

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 Section 4. 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 Section 4.

We then evaluate data to determine the extent to which growth stimulation mode of action is responsible for the thyroid tumors observed in the acrylamide cancer bioassays. For this MOA, we referred Capen (2001) and US EPA (1998) among several resources to evaluate thyroid tumorigenesis.³

For example, US EPA (1998) asks for evidence in the following five areas in order to show a thyroid–pituitary MOA:⁴

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.

³ The role thyroid growth stimulation in the development of thyroid follicular cell tumors has been reviewed many times (e.g., Capen, 1992, 2001; US 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.

⁴ Although stated otherwise, not all of these criteria are required. For example, in three of four examples that US 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.

2.2. Method for modeling tumors via mutagenic and thyroid growth stimulation MOAs

The US 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 (US 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 US EPA cancer guidelines (US 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, US 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.

The US 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 four 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, US EPA describes a non-linear 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 US EPA for non-cancer toxicity, with the BMDL commonly divided by one or more uncertainty factors (US EPA, 2002).

However, if supporting data exist, US 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 US EPA's array that best fits the most rele-

vant 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).

3. Results

We first describe tumor observations in experimental animals, focusing on combined benign and malignant thyroid tumors because each of four experiments (two studies each testing males and females) 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.

3.1. 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 (six sections), spinal cord (three sections), peripheral nerves (three 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 3–130 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 conserva-

tively 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) × 0.086 food feeding factor for a chronic study in F344 rats (US 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 (three levels), spinal cord (three 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.

As per the Section 2, 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

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^a

Acrylamide dose mg/kg-day ^b	Johnson males		Johnson females		Friedman males		Friedman females	
	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
0.012	0/58	0.0	0/59	0.0	3/102	0.029	1/50	0.020
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 [*]

^a Asterisks (*) indicate authors' designation of statistical significance.

^b Control doses with dietary acrylamide have been estimated. See text for Section 3.

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.

3.2. 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; European Union, 2002; OEHA, 2005; Shipp et al., 2006; Carere, 2006; Besa-
ratina and Pfeifer, 2007; Swenberg et al., in press), 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, for example, in mouse lymphoma cells (Knapp et al., 1988), or negative, for example, with Chinese hamster V79H3 cells (Tsuda et al., 1993), or are negative but tested to insufficiently high doses. Mei et al. (2008) showed that acrylamide and glycidamide generate mutations through a clastogenic mode of action in mouse lymphoma cells, and also reported that glycidamide induces mutations via DNA adducts whereas acrylamide does not. The slopes of the mutation frequencies dose-response curves for these two chemicals are different, with acrylamide showing an apparent threshold, or very shallow slope. Koyama et al. (2006) also showed clear differences in genotoxicity between acrylamide and glycidamide, stating that the former was clastogenic and the latter was mutagenic. Results are also available from *in vivo* somatic cell gene mutation assays. For example, Neuhaeuser-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. Ghanayem et al. (2005) showed that CYP2E1 metabolism is needed to evoke genetic damage in somatic and germ cells of mice treated with acrylamide at doses of 25 mg/kg-day or greater.

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). Manjanatha et al. (2006) also showed that repeated oral doses of 19 mg/kg-day or higher 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 (Knapp et al., 1988; Tsuda et al., 1993). Studies on spindle disturbances suggest a protein-interactive mode of action (Adler and Parry, 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; Knapp et al., 1988; Russo et al., 1994). European Union (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 (Segeberback 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 was reduced 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. (2008) 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 US 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

Table 2
Lymphocyte and liver mutagenicity in male and female Big Blue[®] mouse, DNA damage from the comet bioassay in Sprague–Dawley male rats, and of micronucleated polychromatic erythrocytes (PCE) in peripheral blood of CBA-CA male mice^a

Acrylamide dose mg/kg-day ^b	Big Blue [®] mouse				Sprague–Dawley rat								CBA-CA mouse ^c		
	Lymphocyte Hprt MF × 10 ⁻⁶		Liver cII MF × 10 ⁻⁶		% Tail DNA @ 24 h			Olive tail moment @ 24 h	% Tail DNA @ 2 h		% Tail DNA @ 5 h			Mean fMPCE (per milliliter)	
	Male	Female	Male	Female	Leukocytes	Brain	Testes	Leukocytes	Brain	Testes	Adrenals	Bone marrow	Liver	Adrenals	Male
0.001					0.12	0.21	0.22	0.84	3.1	0.18	0.20	0.22	0.17	0.20	1.18
0.002	2.2	1.5	28.4	26.5											1.21
1															1.20
3															1.39
6															1.41*
12															
18					0.15	0.20	0.21	2.1	3.3						
19	11.0*		21												1.73*
24															
25		6.6*		26											1.90*
30															
36					0.15	0.30*	0.24	1.7*	4.6						
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*											

^a Asterisks(*) indicate authors' designation of statistical significance.

^b For the Sprague–Dawley rats of Maniere et al. (2005) these control values are estimated as 0.001 mg/kg-day based on estimations of dietary acrylamide level as for Fisher 344 rats (see text for Section 3). For Big Blue[®] Mice of Manjanatha et al. (2006) these values are estimated as 0.002 mg/kg-day based on measurement of acrylamide in diet, and food feeding factors for mice (US EPA, 1988). We have also applied this latter estimate to CBA-CA mice of Abramsson-Zetterberg (2003).

^c fMPCE is the frequency of micronucleated polychromatic erythrocytes (PCE) in peripheral blood (Abramsson-Zetterberg 2003).

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 acrylamide dose of 18 mg/kg or higher. 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 h, 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 at estimated doses of 19 mg/kg-day or higher. 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 Section 2

The probabilities of an “adverse” genotoxic response are shown in Figs. 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 Section 2, we compared the genotoxicity and tumor response from acrylamide administrations, controlling for the background levels of both. Figs. 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. Figs. 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. Fig. 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 Figs. 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 Section 4.

In summary, several lines of evidence support or refute a mutagenic mode of action (consistent with linear low-dose extrapolation) for the development of thyroid tumors. These lines of evidence include:

- o Acrylamide is clastogenic for which the underlying mechanisms, such as cross-linking chromosomes or chromosomal-associated proteins are threshold-based, non-linear events (e.g., Carere, 2006).
- o Glycidamide is mutagenic and DNA adducts with glycidamide are formed in many tissues with a linear dose–response (as cited above). However, DNA adduct formation is seen in a number of tissues that are not targets for tumors (as cited above).
- o 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). These dosing durations are much shorter than those needed for thyroid tumors to occur (Johnson et al., 1986; Friedman et al., 1995), which is consistent with the Hill criterion of temporality in the US EPA (2005) guidelines.
- o Statistically significant *in vivo* genotoxicity (studies as cited in the previous bullet) has only been observed at doses higher than those that caused increases in tumors in the rat bioassays (e.g., compare doses causing tumors in Table 1 with doses causing genotoxicity in Table 2). Some of these genotoxic doses are

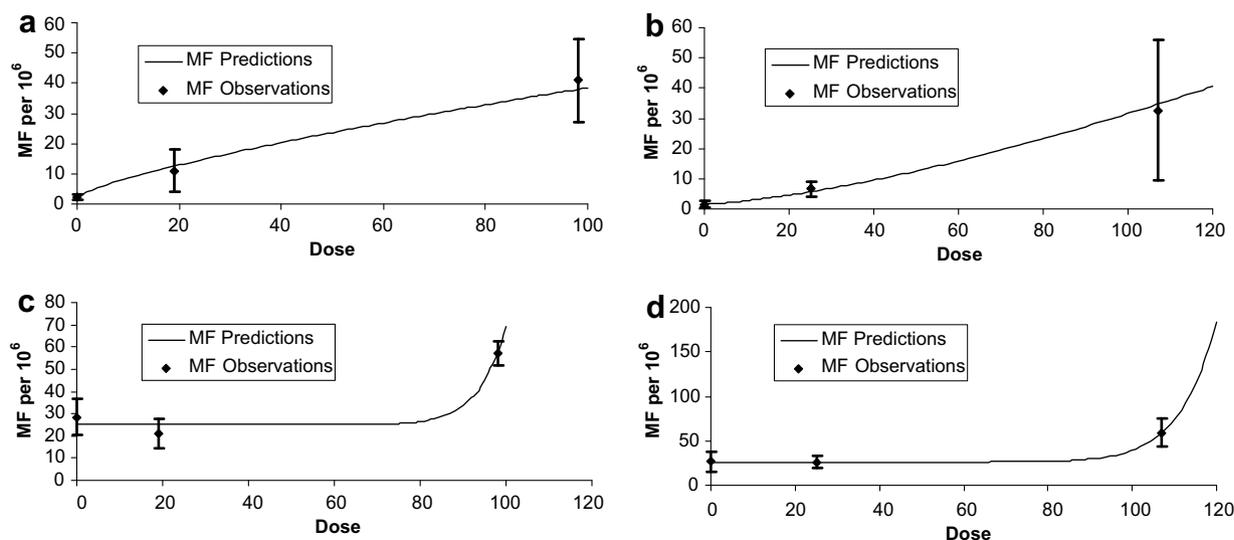


Fig. 1. (a) Data and model fit for big blue mutant frequency assay in male mouse lymphocytes. (b) Data and model fit for big blue mutant frequency assay in female mouse lymphocytes. (c) Data and model fit for big blue mutant frequency assay in male mouse liver cells. (d) Data and model fit for big blue mutant frequency assay in female mouse liver cells.

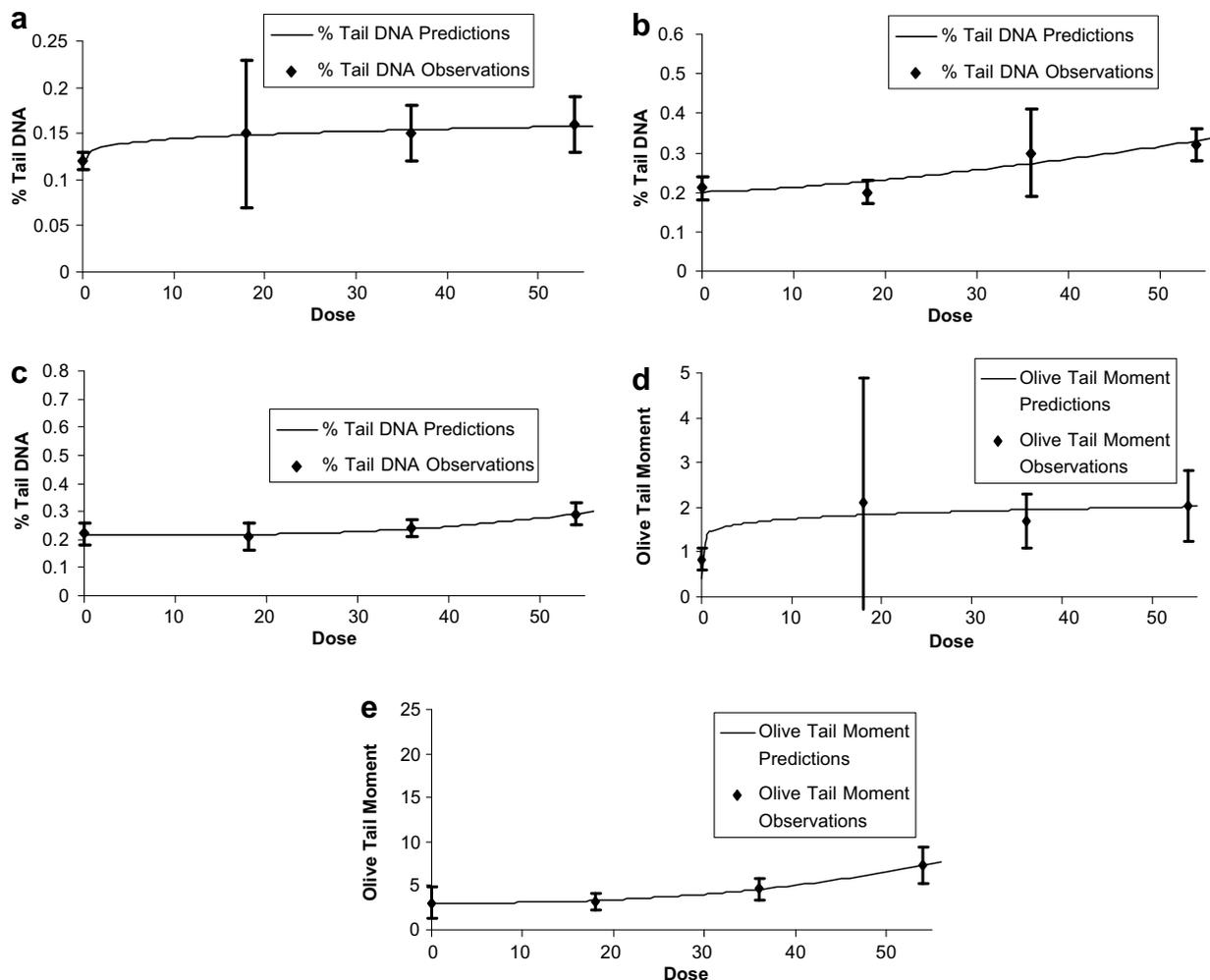


Fig. 2. (a) Data and model fit for comet assay of percent tail DNA in leukocytes of male Sprague–Dawley rats. (b) Data and model fit for comet assay of percent tail DNA in brain cells of male Sprague–Dawley rats. (c) Data and model fit for comet assay of percent tail DNA in testes cells of male Sprague–Dawley rats. (d) Data and model fit for comet assay of olive tail moment in leukocytes of male Sprague–Dawley rats. (e) Data and model fit for comet assay of olive tail moment in brain cells of male Sprague–Dawley rats.

significantly higher than those that cause tumor formation and several of these high doses are without any genotoxicity. In addition, when these genotoxicity data are modeled based on incidence of an “adverse” response, eight out of nine dose–response curves show tumor increases occurring at doses below doses that cause genotoxicity (see Figs. 3 and 4). Furthermore, an alternative analysis in which these, and other, genotoxicity data sets (including one thyroid data set) are plotted along with tumors as multiples of control response also shows that tumors lead genotoxicity in dose–response (Fig. 5). While not all of these genotoxicity endpoints might be expected to be good surrogates for the potential thyroid mutagenic event, at least some of them are expected to be good surrogates, especially when adducts appear to be formed in all tissues in nearly equal proportions. These results are not consistent with the criterion of dose–response concordance in the US EPA (2005) guidelines.

Thus, 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 (US EPA, 2005). However, these same *in vivo* studies offer strong 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 Figs. 3–5, suggests that most high-dose tumors, and perhaps even those at low doses, are evoked by alternative MOAs.

We conclude that the available data can be used to exclude this MOA as a sole basis of the observed thyroid tumors.

3.3. Assessment for thyroid follicular cell growth stimulation

As described in the Section 2, we used US 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

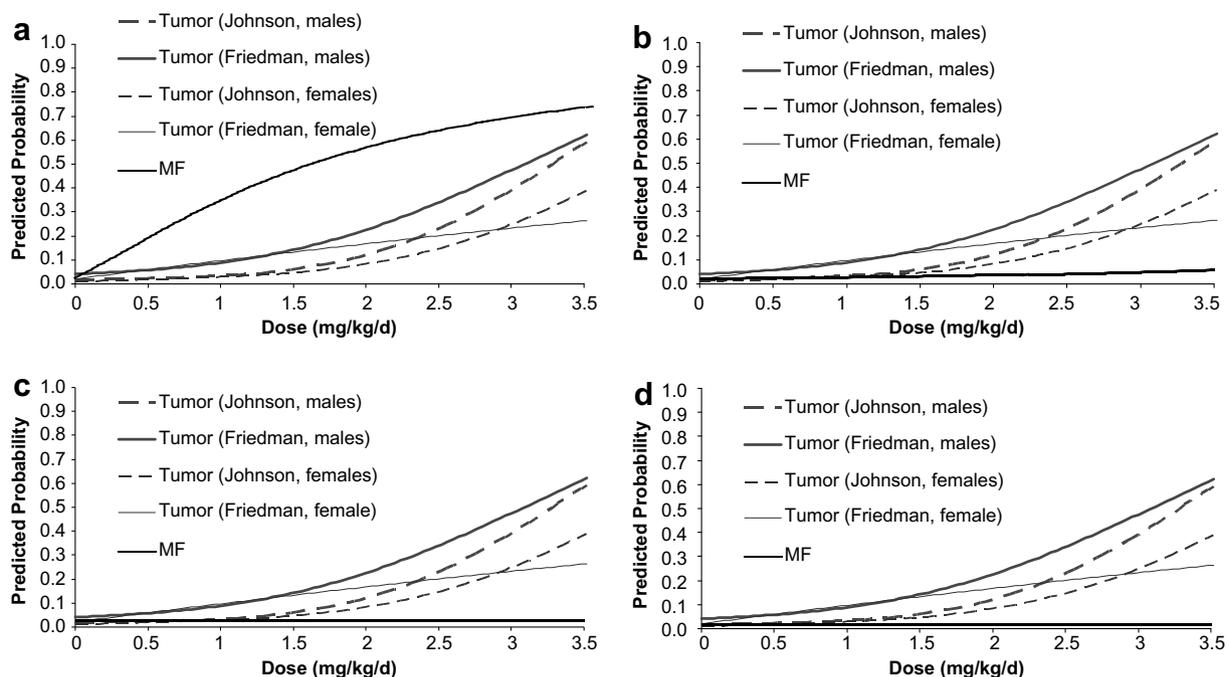


Fig. 3. (a) Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of male mouse lymphocyte mutant frequency. (b) Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of female mouse lymphocyte mutant frequency. (c) Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of male mouse liver cell mutant frequency. (d) Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of female mouse liver cell mutant frequency.

days. The animals were acclimatized by handling for 14 days prior to dosing to minimize stress. The animals were killed 24 h 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 T4 or TSH. However, the authors noted subtle changes that did not reach statistical significance. A dose-related increase in adjusted T4 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 $\mu\text{g}/\text{gland}$ (but not $\mu\text{g}/\text{mg}$ 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 T4 and compensating increases in TSH was missed. They also suggested that the decreased colloid area and hypertrophy of follicular cells reflected persistent TSH stimulation.

Bowyer et al. (2008) gave acrylamide to a large number of male Fisher 344 rats (at approximately 70 days of age) in drinking water at control, 25, 100, or 500 $\mu\text{g}/\text{L}$ for 14 days. We estimate that the corresponding doses are 0, 2.4, 10, and 43 $\text{mg}/\text{kg}\text{-day}$. Endpoints monitored at the end of 14 days included gene expression in hypothalamus, pituitary, and thyroid; levels of neurotransmitters, including dopamine and its metabolites in the hypothalamus and pituitary; serum levels of selected pituitary and thyroid hormones; and histology of pituitary and thyroid glands with an emphasis on evaluating the presence of cell proliferation. Body weights and locomotor activity in rats dosed with either the first two doses were unaffected, but rats at the high-dose had reduced body weights (92–93% of control values) and clear impairment of motor

activity. A statistically significant increase in serotonin was seen in the hypothalamus at the low- and intermediate-doses, but not the high-dose. A principal metabolite of serotonin, and levels of dopamine and its metabolites in the hypothalamus were unaffected. None of these chemistries were affected in the pituitary. A statistically significant decrease in T4 was seen at the high-dose. Dose related, but not statistically significant increases were seen in triiodothyronine (T3), and dose related, but not statistically significant decreases were seen in reverse T3. TSH was unaffected at any dose. A thyroid-releasing-hormone challenge indicated that acrylamide did not affect thyroid hormone release. No evidence was observed for cell morphology, cell proliferation or apoptosis at high-dose when compared with control with standard stained sections, but immunohistochemical staining for Ki-67 antigen, a marker of cell proliferation, in thyroid follicular cells showed a statistically significant and dose related decrease. Ten of 10 markers for thyroid hormone production and cell proliferation in the thyroid and pituitary were above control values; one of these increases, for Type II 5' deiodinase, was statistically significant. The authors report that gene expression cDNA microarrays in the pituitary and hypothalamus appeared to be unaffected.

In an unpublished study, Friedman 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 $\text{mg}/\text{kg}\text{-day}$ for the males and 0, 1.3, 4.3, 9.0, 19, and 24 $\text{mg}/\text{kg}\text{-day}$ 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 $\text{mg}/\text{kg}\text{-day}$, and in both sexes at 19 $\text{mg}/\text{kg}\text{-day}$. Acrylamide ingestion at 19 $\text{mg}/$

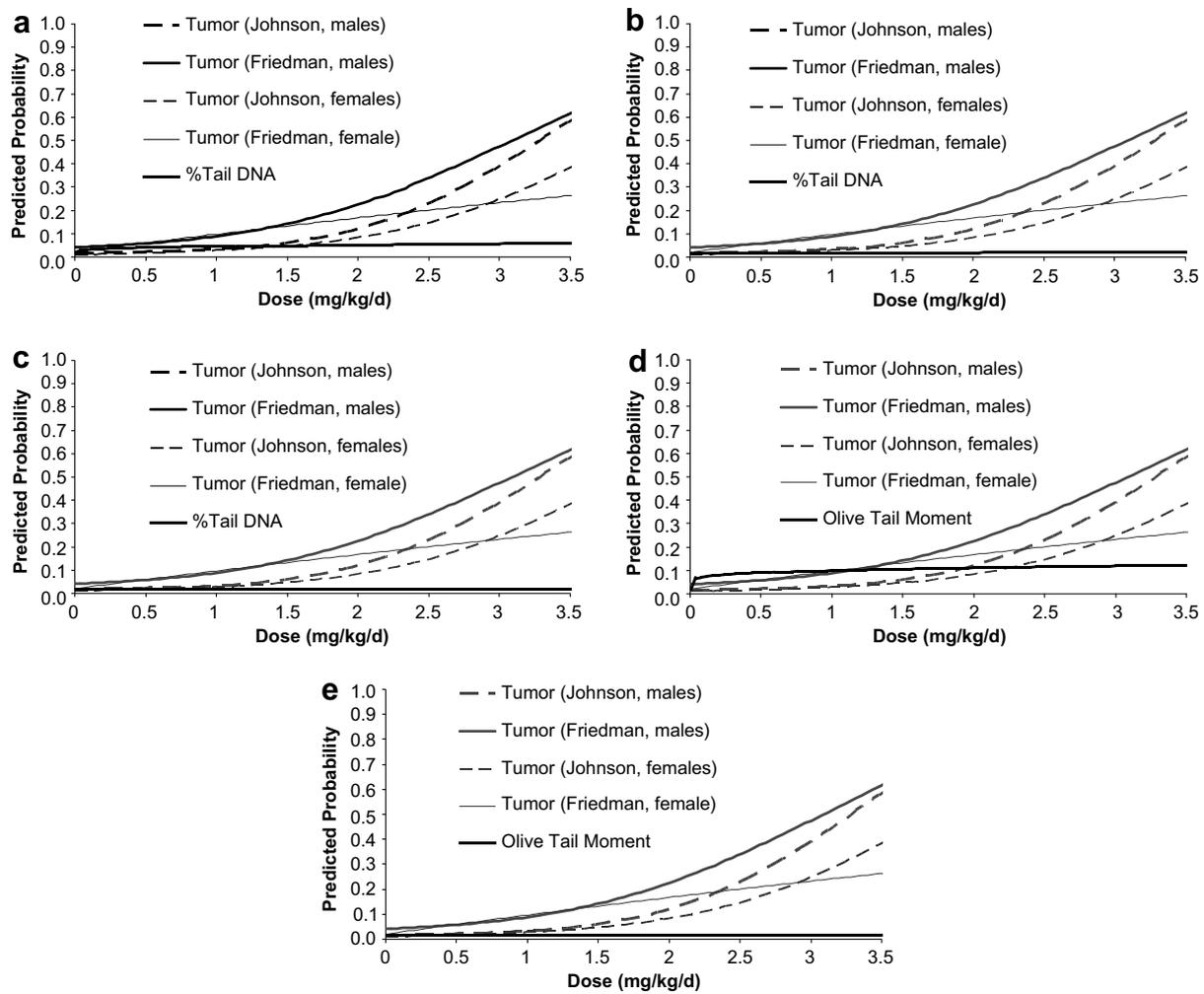


Fig. 4. (a) Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of % tail DNA in comet assay in male rat leukocytes. (b) Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of % tail DNA in comet assay in male rat brain cells. (c) 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 (d) Dose-related probability of response for thyroid tumors in the rat compared to predicted probability of live tail moment in comet assay in male rat leukocytes. (e) 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.

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 for the purpose of measuring DNA synthesis in selected rat tissues. Osmotic pumps were used to infuse 5-bromo-2'-deoxyuridine (BrdU) 7 days prior to kill. For the thyroid, statistically significant increases in DNA synthesis measured by labeling index of BrdU, a well known measure for cell proliferation, were observed in both rodent strains 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. Changes in the mitotic and apoptotic indices were also in the direction consistent with cell proliferation in the F344 rat, but not statistically significant. 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.

In an unpublished study on juvenile F344 rats, Imai et al. (2008) did not show any thyroid histopathology in males or females after 12 weeks of acrylamide exposure in drinking water at either 1.0, 2.1, or 4.4 mg/kg-day in males, or 1.2, 2.5, or 4.9 mg/kg-day in females, but did show a statistically significant increase in relative thyroid weight in high-dose females.

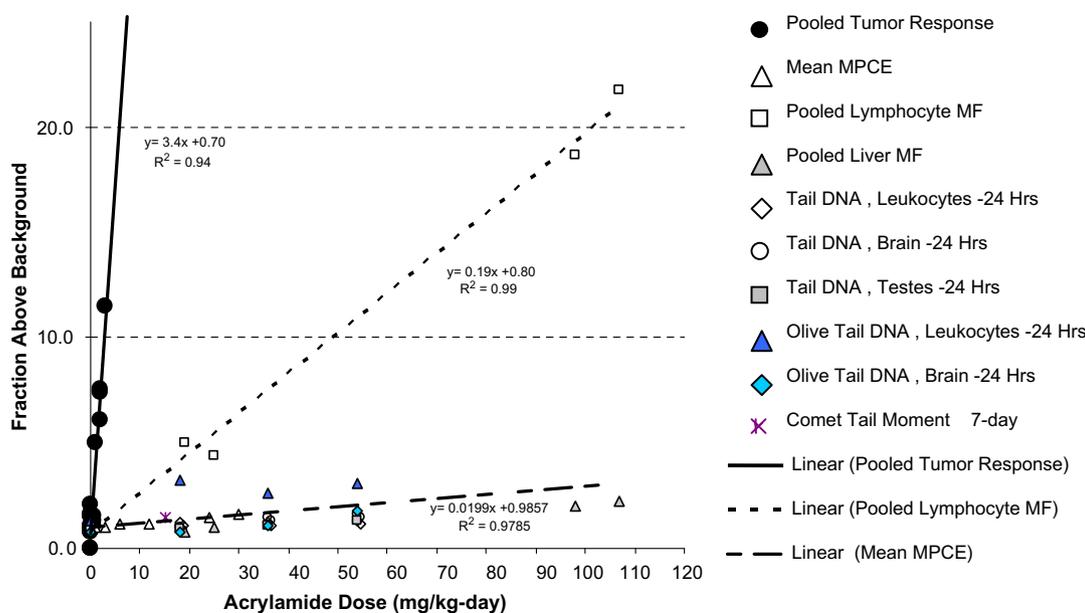


Fig. 5. Multiples above background for tumors, DNA labelling, and several measures of *in vivo* genotoxicity.

In an experiment testing the promotion capabilities of acrylamide in mammary tumorigenesis, Imai et al. (2005) did not find proliferative changes in the thyroid gland after 30-week exposures in Sprague–Dawley female rats (6–7 weeks of age at start) given acrylamide at doses of 3.3 or 5.6 mg/kg-day via drinking water. Thyroid weights were similarly unaffected.

Johnson et al. (1984) reported cystic dilatation of the thyroid follicle in F344 rats 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 or pooled results are statistically significant.⁵ This lesion is considered by some to be follicular cell hyperplasia (Boorman, 1983). These proliferative lesions were not evident up to 18 months in interim kills (Gorzinski et al., 1984).

Friedman et al. (1995) reported thyroid hyperplasia in F344 rats 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, or statistically significant. Interim kills were not conducted in this study.

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, a possibility exists that acrylamide in the thyroid may 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 effect on the normal formation and removal of hydrogen peroxide (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. In light of the positive BrdU findings of Lafferty et al. (2004), the data of Chico Galdo et al. (2006) 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. The fact that Khan et al. (1999) demonstrated statistically significant histological changes in the thyroid that are consistent with persistent TSH stimulation is supportive of this suggestion.

Due to the complexities of endocrine physiology, several additional plausible outside of 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 non-specific indication of increased liver metabolic capability. These weights were statistically significantly increased⁶ 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

⁵ $p < 0.05$ Cochran-Armitage trend test conducted by us.

⁶ $p < 0.05$ ANOVA pairwise comparison conducted by us.

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, 2008) also measured liver weights and found that such weights did not change, at least after 12–30 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. This hypothesis was directly tested by Bowyer et al. (2008) as described more fully above. Twenty-one measures of gene expression in the hypothalamus were shown, several of which were directly related to thyroid function; the authors concluded that there were no significant changes from acrylamide exposure. Serotonin was statistically significantly increased at the low- and intermediate-dose, but not at the high-dose. Dopamine was not affected at any dose.

Additional literature on acrylamide-induced toxicity in the hypothalamus or alter dopamine responsiveness in the brain are 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 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 LoPachin et al. (2006) 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.

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, Dixit et al. (1981) reported a decrease in dopamine levels in the brain following a similar treatment protocol and Bowyer et al. (2008) showed increases in serotonin but not dopamine after oral exposures. Other studies have reported increased dopamine receptor binding in some brain regions of acrylamide-treated rats (Bondy et al., 1981).

Many of these mechanistic studies were 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. Furthermore, LoPachin et al. (2006) concluded that presynaptic nerve function is a likely target of acrylamide throughout the central and peripheral nervous systems. Overall, the specific pattern of effects caused by acrylamide on neuroendocrine regulation in the hypothalamus is difficult to decipher due to the complexity of mapping neuroregulation in various brain regions, leading us to conclude that the data are mixed regarding the effect of acrylamide on normal hypothalamus–pituitary structure and function.

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

Increases in cellular growth:

- o At 2 and 7 days, 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, and corresponding statistically significant loss of colloid.
- o At 7, 14 and 28 days, Lafferty et al. (2004) showed a statistically significant, and dose related increase in thyroid follicular DNA labeling in male F344 rats at 2 and 15 mg/kg-day and at 7, 14 and 28 days (Table 2); changes in the mitotic and apoptotic indices were consistent with growth stimulation, but not statistically significant.
- o At 14 days, Bowyer et al. (2008) showed a statistically significant, and dose related, decrease in Ki-67, a marker of cell proliferation, in male F344 rats; thyroid histopathology was not evident.
- o At 14 days, Bowyer et al. (2008) observed that 10 of 10 markers for thyroid hormone production and cell proliferation in the thyroid and pituitary were above control values; one of these, Type II 5' deiodinase, was statistically significant.
- o At 28 days, Friedman et al. (1999) observed a statistically significant increase in relative thyroid weight in female rats; this effect did not occur in males.
- o At 12 weeks, Imai et al. (2008) observed a statistically significant increase in relative thyroid weight in female F344 rats. This effect did not occur in males and thyroid histopathology was not evident in either sex. Imai et al. (2005) did not show any of these effects in a different rat strain.
- o At 2 years, Johnson et al. (1984) observed a statistically significant increase in cystic dilatation of the follicles in the thyroid occurred in both male and female rats; this increase was not evident at 6, 12, and 18-month interim kills.
- o At 2 years, Friedman et al. (1995) observed a small increase in thyroid hyperplasia in male and female rats, but this was neither statistically significant nor, in the case of females, dose related.

Thus, evidence is available for cellular growth in many short- and long-term studies. Some evidence is negative, equivocal or absent in several studies.

Hormone changes:

- o At 2 and 7 days, Khan et al. (1998) did not observe any statistically significant changes in TSH or T4 in F344 female rats; T3 was not measured; at 7 days, the authors report a slight dose-related increase in the adjusted plasma T4 with a corresponding slight decrease in the adjusted TSH.

- o At 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.
- o At 14 days, Bowyer et al. (2008) showed a statistically significant decrease in T4 at high-dose; such an effect was not seen at two lower doses.
- o At 28 days, Friedman et al. (1999) showed a statistically significant decrease in T3 and T4 (males) with the expected increase in TSH, but this latter increase was not statistically significant; females showed only a non-significant decrease in T3 at the high-dose.

Thus, evidence in short-term studies appears to show an initial increase in thyroid hormone levels and then a decrease. This decrease is consistent with thyroid growth stimulation. Longer-term studies have not monitored thyroid hormone levels.

Site of action:

- o Loss of integrity of microfilaments and microtubules will lead to problems in the development and perhaps maintenance of thyroid follicle structure and hormone release (Capen, 1996). 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.
- o Increases in liver weight evident in several studies (as cited above) 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.
- o 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 (as cited above), specific data monitoring the ability of acrylamide to induce gene expression or neurotransmitter changes in the hypothalamus as reported by Bowyer et al. (2008) generally showed no effects; gene expression changes in the pituitary and thyroid appeared to offer some support for growth stimulation, although neurotransmitter changes in the pituitary were unaffected.

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

Dose correlations:

- o 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, 2008), nor in Bowyer et al. (2008).
 - Increased thyroid weights in females, but not males (Imai et al., 2008); such increased weights were not seen in Imai et al. (2005) or in Friedman et al. (1999) except in females at high-dose.
 - Dose related and statistically significant increases in thyroid DNA labeling at 7, 14, and 28 days using a 7 day sampling period for DNA synthesis (Lafferty et al., 2004); Bowyer et al. (2008) showed a statistically significant and dose related decrease in Ki-67 labeling at 14 days.

- o These tumorigenic doses are below the general range of doses that cause hormone changes after short-term exposure (Khan et al., 1999; Bowyer et al., 2008; Friedman et al., 1999). Hormone changes after long-term exposure have not been monitored.

Thus, evidence is available on dose correlations, but not all data are available at relevant doses.

Reversibility of effects when chemical dosing stops: No data on reversibility studies related to thyroid effects or other growth stimulation were located.

As summarized in Table 3, short-term acrylamide exposure causes some changes to thyroid histology, thyroid hormones, thyroid DNA labeling/mitotic indices/apoptotic index, and gene expression in the thyroid and pituitary glands. Not all of these changes are statistically significant, nor consistent, but together these effects show modest thyroid growth stimulation. Taken together with minimal data on several sites for acrylamide action, we conclude that acrylamide is evoking thyroid tumors in part due to follicular cell growth stimulation. Our conclusion is consistent with evidence from two of the examples in US EPA (1998), also shown in Table 3. The evidence we summarize for acrylamide more closely matches the evidence for a bis-benzenamine, where US EPA (1998) concluded that both a mutagenic and growth stimulation mode of action was occurring, rather than a nitrosamine, where US EPA (1998) concluded that only a mutagenic mode of action was occurring.

3.4. Summary of MOAs

Glycidamide is mutagenic; acrylamide is genotoxic. Both toxicities appear at exposures shorter than those needed to develop tumors, but only at doses that are in excess of tumorigenic doses. It thus appears that a mutagenic MOA may be operating, but only to a limited extent. Information on increases in cell growth, hormone changes, sites of action, and dose correlations are available, suggesting that thyroid follicular cell growth stimulation is occurring. A liver, thyroid and/or pituitary specific event(s) might be involved.

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 linear response. This is consistent with the fact that glycidamide is mutagenic and DNA adducts with glycidamide are formed in many tissues. In addition, *in vivo* genotoxicity occurs in time before tumors, which is consistent with the Hill criterion of temporality for a mutagenic MOA. This linear response is conservative, however, since genotoxicity does not lead tumors in dose, and therefore is not consistent with the Hill criterion of dose–response concordance. Nor are tumors found in many tissues with DNA adducts. Furthermore, evidence exists that while low-dose linearity may exist in DNA adduct formation, this may not be true with genotoxicity.

For the high-dose region, growth stimulation dominates the response, although we continue to assume that a modest amount of tumors may be formed by a mutagenic MOA. This is consistent with the fact that tumors occur primarily in endocrine tissues at the high-dose, including the thyroid, occur late in life, are mostly benign, and, specifically for the thyroid, have curvilinear upward slopes consistent with US EPA (1998). Furthermore, growth stimulation is occurring as evidenced by statistically significant changes in thyroid histology at several time points, hormone changes at 14 and 28 days, and by increased DNA labeling at 7, 14 and 28 days. Note, however, that not all data from these studies show growth stimulation, and one datum, Ki-67, shows a decrease in growth at one time point.

Table 3
Comparison of US EPA's required data demonstrating antithyroid activity for acrylamide and two examples (quoted text is from US 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	Statistically significant increase in relative thyroid weight in female rats for 28 days and 12 weeks; effect did not occur in males; effect was not measured at other times	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
	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 thyroid follicular BrdU-DNA labeling in male F344 rats at 7, 14 and 28 days. Another measure of cell proliferation, Ki-67, was statistically significantly decreased in male F344 rats after 14 days, however.	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	Statistically significant thyroid follicular hypertrophy in female F344 rats for 2 or 7 days, and corresponding loss of colloid. Male F344 rats after 14 days, or male and female F344 rats after 12 weeks, did not show changes in thyroid histopathology. A statistically significant increase in focal cystic dilatation (authors note: thyroid hyperplasia) of the follicles occurred in both male and female rats after 2 years. An inconsistent increase in thyroid hyperplasia was seen in a second study, but this increase was not statistically significant No statistically significant changes observed in TSH or T4 in rats exposed for either 2 or 7 days; T3 was not measured; according to the authors at 7 days, the adjusted values showed slight dose-related increases in plasma T4 and decreases in plasma TSH levels After 14 days, a statistically significant increase was observed in T4 (males) and T3 (females), accompanied by a statistically significant decreased TSH in females. A statistically significant decrease was seen in T4 in males at the highest dose After 28 days, statistically significant decrease was seen in T3 and T4 (males) with a non-significant increase in TSH; females showed only a non-significant decrease in T3 at the high-dose	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	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 Increase in liver weight is evident in several, but not all, studies; such increase might result in thyroid hormone loss 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 and gene expression or neurotransmitter changes in the hypothalamus do not appear to be affected; gene expression changes in the pituitary and thyroid lend some support to growth stimulation	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

Table 3 (continued)

Data/ Chemical	Bis-benzenamine	Acrylamide	Nitrosamine
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: <ul style="list-style-type: none"> o statistically significant thyroid histological changes in two studies, although such histological changes were not evident in three other studies o Dose related increases in thyroid DNA BrdU labeling at 3 time points, but decreases in Ki-67 labeling at 1 time point o Increases in mitotic index and decreases in apoptotic index that are not inconsistent with growth stimulation in one study, but not another 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 Conclusions	No data 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	No data We conclude that acrylamide is evoking thyroid tumors as a result of follicular cell growth stimulation to some extent. It appears that a mutagenic MOA caused by exposure to glycidamide may also be operating to a limited extent. Thus, these MOAs were “decoupled” and several approaches are considered for the dose–response modeling. See text for additional Section 4	No data 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

Because the data are most consistent with both MOAs being relevant, but at different parts of the dose–response curve, we are guided by EPA (2005) to “consider the respective contribution of each mode of action in different dose ranges” as described in the next section.

3.5. 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 study and 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 US 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 BMD₁₀ for “All Doses” are comfortably among the individual data set responses; a comparison of ratios between BMD_{10s} and their corresponding BMDL_{10s} shows that the ratio of BMD₁₀ to BMDL₁₀ for “pooled all” was smaller than any individual ratio and all but one pooled ratios, suggesting greater confidence in these 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_{10s} are higher than those at “Decoupled Low Dose” BMD_{02s}, which is consistent with our understanding of the underlying dual MOA biology. However, SFs at the “Decoupled Low Dose” BMD lower limit, the BMDL₀₂, are slightly higher than

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	<ul style="list-style-type: none"> 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 known to occur in humans, but growth stimulation is also needed	This MOA is plausible and comparative kinetic information between experimental animals and humans allows specific choices of adjustment factors (see text for Section 4)
Follicular cell growth stimulation	<ul style="list-style-type: none"> Increased DNA synthesis measured by BrdU labeling in thyroid at multiple time points; a second study shows decrease cell proliferation measured by Ki-67at one time point Decreased thyroid colloid and increase follicular cell height after 2 or 7 days; changes in thyroid histology after longer-term exposure in some studies but not others Increased thyroid hormone release up to 14 days, but hormone decrease at 14 and 28 days; thyroid weight increase at 28 days and 12 weeks in females but not males; no long-term studies monitoring hormone data or thyroid weight are available Equivocal changes in liver weights occur after long-term exposure, suggesting increased catabolism of thyroid hormones; other possible sites of action are microfilament and microtubule development in the thyroid and neurotoxicity in the hypothalamus or pituitary; gene expression and neurochemistries are apparently unaffected in the hypothalamus, but gene expression may be affected in the thyroid and pituitary 	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-day)⁻¹

Data set ^a	BMD	SF at BMD	BMDL	SF at BMDL
<i>(All doses) (10)</i>				
Johnson female	2.2	0.045	1.5	0.067
Johnson male	2.0	0.051	1.2	0.085
Johnson pooled	2.1	0.048	1.6	0.063
Friedman female	1.3	0.077	0.94	0.11
Friedman male	1.6	0.065	0.76	0.13
Friedman pooled	1.3	0.079	0.96	0.10
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
<i>(Decoupled low dose^b) (02)</i>				
Johnson pooled	1.4	0.014	0.24	0.083
Friedman pooled	0.38	0.053	0.12	0.17
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
<i>(All doses) (02)</i>				
Johnson pooled	1.1	0.019	0.31	0.066
Friedman pooled	0.24	0.083	0.18	0.11
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 Friedman et al. (1995); 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.

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_{02s} might appear reasonable for low-dose extrapolation based on our MOA understanding, the SFs at the “Decoupled Low Dose” BMDL_{02s}, based on much wider confidence limits, are not. The expectation would be that these latter slopes are smaller than corresponding values at BMDL₁₀ with “All Doses.”

This inability to fully use the “Decoupled Low Dose” analysis led us to estimate BMD/L_{02s} using the multistage model and “All Doses.” These values are also shown in Table 5. Slope Factors (SFs) at BMD₀₂ for three of five pooled “All Doses” more closely approximate those found at BMD₁₀, “All Doses” rather than those found at “Decoupled Low Dose” BMD₀₂. This observation indicates that the multistage model has difficulty 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.

Table 6
Probit model estimates of slope factors (SF) for pooled study and sex data on rat 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	BMDL 02	SF at BMDL 02
Johnson pooled	0.99	0.020	0.76	0.026
Friedman pooled	0.75	0.027	0.62	0.032
Pooled female	0.82	0.024	0.67	0.030
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
Johnson pooled	2.2	0.046	1.6	0.061
Friedman pooled	1.6	0.061	1.4	0.074
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

the SF for BMDL₁₀ when all doses are modeled. These latter observations reflect the much wider confidence limits of the “Decoupled

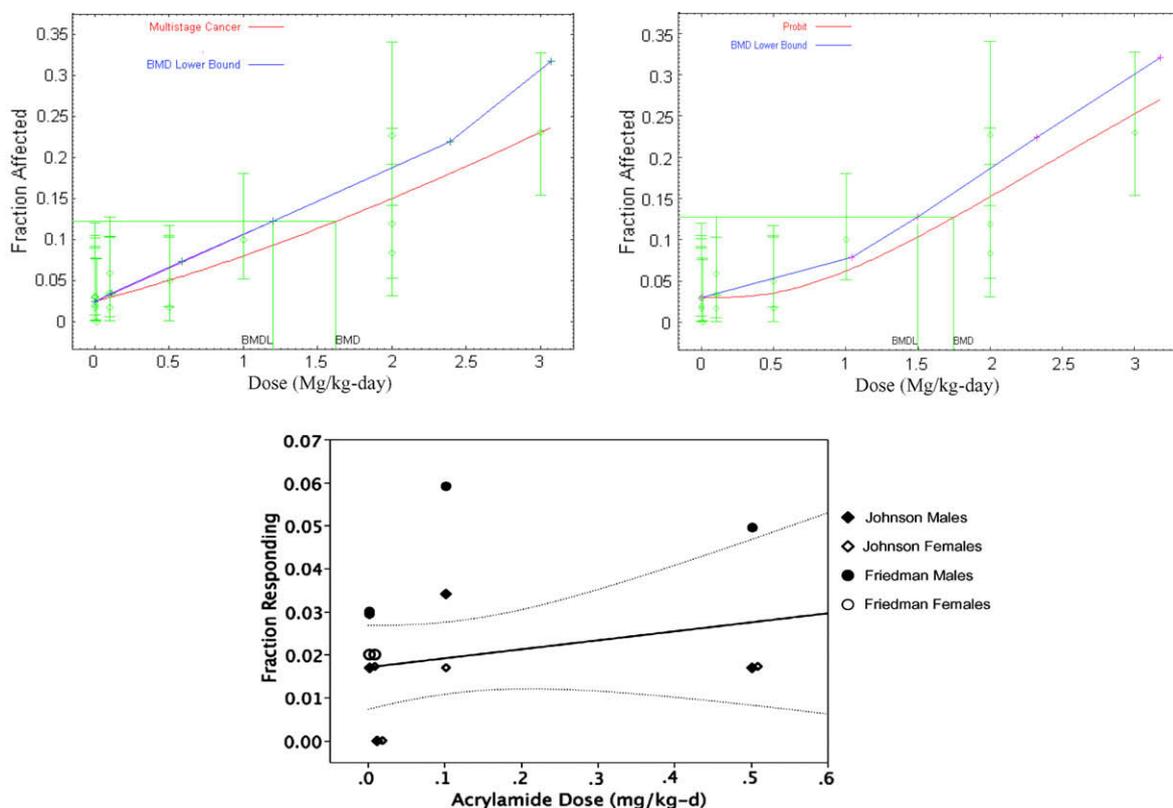


Fig. 6. (a) Multistage model fitted to pooled-all thyroid tumor data, showing little change in slope between the low- and high-dose regions. (b) Probit model fitted to pooled-all thyroid tumor data, showing differing slopes between the low- and high-dose regions. (c) Weighted linear regression on low-dose 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.

Because the multistage model with decoupled or all data was not able to give fully or biologically reasonable answers, and because EPA (2001, 2005) also considers other models, another US 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 (Fig. 6a) gives a poorer visual fitting of the lower dose data than the probit model (Fig. 6b), mainly due to the inability of the multistage model to visually fit the three 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 non-linear 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 US EPA (1998) for thyroid tumors, with a fixed power of 2.0 to allow comparison with previous EPA thyroid model results. 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.⁷ 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. The SF values from the weighted linear regression shown in Table 7 are roughly equivalent for the BMDs of the probit model and about 2–3-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 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

⁷ 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.

Table 7
Slope factors (SF) from weighted linear regression on just the low-dose data (dose < 1.0) on thyroid tumors

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

BMD/L values are in mg/kg-day; SF values are in (mg/kg-day)⁻¹.

^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.

data set (i.e., 1.0 mg/kg-day), as well as with a potential very low slope due to a mutational MOA. Fig. 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 5–7, 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:

- o 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.
- o 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 BMD₀₂ than for BMD₁₀, 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.
- o The probit model is confirmed by both a Weibull model [another of US EPA's models, and one specifically mentioned by US EPA (1998) for thyroid tumors], and by a simple weighted linear regression model.

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-day)⁻¹, derived as the best (median) statistical estimate, that is the BMD₀₂, based on all the pooled-all data as shown in Table 6. An upper bound estimate of 0.029 (mg/kg-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 BMDL₁₀ 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 0.05–0.02 mg/kg-day.

3.6. Extrapolation from experimental animals to humans

The chosen slope factor of 0.025 (mg/kg-day)⁻¹ from the pooled-all rat data needs to be adjusted to reflect a human equivalent slope factor. This might best be accomplished by either a PBPK model such as the ones published by Young et al. (2007) or Kirman et al. (2003). Until the results of these models are integrated, however, comparative toxicokinetics information from humans and Fisher 344 rats are available, and on which prior extrapolations from rats to humans have been made (OEHHA, 2005; Shipp et al., 2006). We use these comparative data here as well.

Appropriate data for calculating a rat to human interspecies toxicokinetic factor, referred to as a Chemical Specific Adjustment Factor (CSAF)⁸, are presented by Fennel 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 dose metric 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, Fennel 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 non-linear 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. Fuhr et al. (2006) suggested a rat to human toxicokinetic adjustment as low as ~0.5 based a comparison of

⁸ 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 five), and the standard error should be <20% of the mean.

urinary excretion of glycidamide metabolites. A similar value could be calculated from an unpublished comparison of human and rat urinary excretions of glycidamide metabolites (Kopp and Dekant, 2008). Additional considerations would be needed prior to using these suggestions.

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

- Allen et al. (1988) compared experimental animal and human TD25s (tumor dose at 25% tumor incidence) for 23 chemicals and found that sensitivity was on average equal with one method or that humans were on average less sensitive than experimental animals by a second method. Variation around the best estimates for these two methods was most often within 10-fold. These results indicate that the most likely value of the toxicodynamic factor between experimental animals and humans is a value of 1, or less, because the results of this study include variations in both toxicokinetics and toxicodynamics.
- Goodman and Wilson (1991) compared published human and experimental animal cancer potencies (including those of Allen et al., 1988) and considered the best estimate of the overall interspecies factor to be lognormally distributed around a value of 1 with most deviations falling within an order of magnitude. These results also indicate that the most likely value of the toxicodynamic factor between experimental animals and humans is a value of 1, or less, because the results of this study include variations in both toxicokinetics and toxicodynamics.
- Williams (1995, 2008) states that tumors in humans do not form in the presence of mutagens if TSH-stimulated growth is prevented. This statement supports a toxicodynamic factor of 1, or less, because rats are known to be more susceptible to thyroid growth stimulation than humans (*v. supra*). Furthermore, US EPA (1998) guidelines recommend the 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., $1.2 \times 1 = 1.2$) 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 $0.030 \text{ (mg/kg-day)}^{-1}$ (i.e., $0.025 \text{ (mg/kg-day)}^{-1} \text{ (SF)} \times 1.2 = 0.030 \text{ (mg/kg-day)}^{-1}$). For comparison with policy-based values, the upper bound estimate of $0.035 \text{ (mg/kg-day)}^{-1}$ can be used (i.e., $0.029 \text{ (mg/kg-day)}^{-1} \text{ (SF)} \times 1.2 = 0.035 \text{ (mg/kg-day)}^{-1}$).⁹

4. 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 US EPA (2005), and data related to thyroid growth stimulation following the framework of US 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 data do not precede 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, which is also summarized in Table 3). Moreover, sufficient evidence exists to conclude 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, Fig. 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.

In addition, a recent publication by Swenberg et al. (*in press*) shows that whereas a biomarker of acrylamide exposure, DNA adducts, can be extrapolated linearly down to zero dose, a biomarker for effect, clastogenicity as measured by micronucleus induction, is not different than existing background incidence at tumorigenic doses. Differences in slopes have also been shown previously between adducts, and micronuclei, with glycidamide exposure (Paulsson et al., 2003). The implication of these findings is that a threshold, or a slope different than linear, might also apply for tumors based on mutagenicity, or based on indirect genotoxicity due to mechanisms such as oxidative stress.

Since information is available to support both MOAs, we turn to US EPA (2005) for guidance and find that “If there are multiple modes of action at a single tumor site, one linear and one non-linear, 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 US EPA (2005) guidelines. First, a comparison of evidence is shown for the thyroid growth stimulation. Second, a semi-quantitative approach of evaluating genotoxicity modes of action is shown for comparison to the Hill criteria described in US 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 US EPA (2005). Thus, although we were not able to mathematically decouple the contribution from the two potential modes of action, the biological mode of action analysis was used to inform the choice of model used to fit the data, and to develop quantitative values applying to different portions of the dose–response curve. Fourth, we apply chemical specific analysis for potential adjustments to the dose–response assessment for childhood sensitivity (US EPA, 2005). The salient issues related to the application of each of these steps are described below.

4.1. 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. 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 significantly decreased. At similar doses, Bowyer et al. (2008) did not show these hormone changes

⁹ Note that US EPA (2005) guidelines request that a lower bound to the slope associated with this risk also be given. However, US 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.

at 14 days but did show a statistically significant decrease in T4 at the highest dose, which is consistent with the observation by Friedman et al. (1999) at 28 days of statistically significant decreases in both T3 and T4, and an increase (not statistically significant) of plasma TSH at the highest dose. This pattern of hormone change at 28 days suggests that TSH levels would further increase in response and, if sustained, would stimulate thyroid growth.

Female thyroid weights are increased at 28 days in Friedman et al. (1999) and at 12 weeks in females of Imai et al. (2008), indicating growth stimulation, but an increase in thyroid weight is not seen by Imai et al. (2005) in a different rat strain. Thyroid weights were not measured at other times. Thyroid DNA labeling is statistically significantly increased at doses that evoke tumors when measured by BrdU uptake in a process that maximizes sensitivity of the test. This labeling increase occurs at 7, 14, and 28 days and is consistent with growth stimulation and not DNA repair because of the intensity of cell labeling seen (J. Klaunig, personal communication with M. Dourson). Mitotic and apoptotic indices are also changed in a direction consistent with growth stimulation, but the changes are not statistically significant (Lafferty et al., 2004). These latter three endpoints may be more useful than thyroid weights for assessing growth in rats caused by small to moderate increases in TSH (Capen, 2001). In contrast, the results of Bowyer et al. (2008) show a decrease in Ki-67 labeling, another marker of cell proliferation, after 14 days of exposure. The differences between the results of Bowyer et al. (2008) and Lafferty et al. (2004), using different markers of cell proliferation, may be due to the sensitivity of the tests employed. The measure used by Bowyer et al. (2008), Ki-67, does not stain an actively replicating cell (Krohn et al., 2003), unlike the marker used by Lafferty et al. (2004), BrdU incorporation into DNA.

Thus, the results of longer-term acrylamide administration appear to be a prolonged and modest disruption in thyroid–pituitary homeostasis. Such disruption is consistent with tumor development from two long-term acrylamide exposure studies, and consistent with one of the two available long-term tumor bioassays showing thyroid hyperplasia. The long-term effects of acrylamide on thyroid–pituitary hormone homeostasis have not been monitored, however. 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 the data of Chico Galdo et al. (2006) suggest that it is not directly acting on several other pathways in the thyroid; or via neurotoxicity in the hypothalamus. Bowyer et al. (2008) specifically investigated this latter hypothesis and found that gene expression or neurotransmitter changes in the hypothalamus did not suggest any related activity, but that gene expression changes in the pituitary and thyroid might be related. 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 hormone growth stimulation for acrylamide matched with two examples from US EPA (1998). EPA concluded that example 3 of its case studies, a *bis*-benzenamine, evokes thyroid tumors by both an anti-thyroid and mutagenic MOA. As a result, US EPA used both a linear and non-linear method in the dose–response assessment. US EPA concluded that example 4, nitrosamine, evokes tumors by a mutagenic MOA only. As a result, US EPA used a linear dose–response assessment. We believe that the evidence for acrylamide more closely matches the findings of example 3 from US 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 non-linear dose–response assessment.

4.2. 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 (US 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 the 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 unless a rate-limiting step occurs after the genotoxic event.

Figs. 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; Fig. 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. Fig. 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 (Fig. 5).

Nevertheless, for all but one of the genotoxicity endpoints examined in Figs. 3–5, 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 Figs. 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.

Alternatively, it might be that such differences are due to the timing of the genotoxicity studies (from one day to several weeks) when compared with the 2-year cancer bioassays. This is because toxicity is often seen at lower doses with longer durations of exposure. However, the large differences shown in Figs. 3–5 are easily more than the 10-fold factor commonly used for such adjustments between exposure durations, and some of these differences would not be resolved with the use of any adjustment factor because the overall extent of genotoxicity is not as great as that for tumors (e.g., as shown in Fig. 5). Thus, these differences 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 was 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 (as also discussed above). 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 10–60 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 Fig. 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 (as also discussed above).

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 hor-

none-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; Exon, 2006). In particular, the review by Shipp et al. (2006) exhaustively lists alternative MOAs for thyroid tumorigenesis and supporting data.

4.3. 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 US EPA (1998) (see also Fig. 6b and Section 4 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, US 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, however. In addition to US 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); the two MOAs we describe lend themselves to biphasic modeling.

Modeling of the individual thyroid tumor data sets and emphasizing the low-dose region is complicated by less consistency in the data 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 US 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 1 and Fig. 6). Pooling the responses of different sexes from either study also seems reasonable and is statistically supported.

Although the multistage model generated statistically meaningful estimates of slope factors (SFs) for pooled data applicable to the BMD₀₂ 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” BMD₀₂s might be useful for determining relevant slope factors for extrapolation to the low-dose region, SFs applicable to the corresponding BMDL₀₂ are precluded from such extrapolation because they are generally higher than those generated from the

BMDL₀₂s from “All Doses.” In brief, the use of these higher slope factors from the “Decoupled Low Dose” BMDL₀₂ 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, and 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 US 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), where it was found to be a conservative choice.

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.

4.4. Possible childhood susceptibility and other potentially susceptible populations

Under the US EPA (2005) guidelines, variability in cancer response, including variability related to life-stage, should be considered in quantitative assessments, based on chemical-specific data when possible. For chemicals acting via a mutagenic mode of action, the guidelines also include default age-dependent adjustment factors, for use in the absence of chemical-specific data. Since we propose a mutagenic MOA in the low-dose region, and since the metabolism of acrylamide to glycidamide is likely necessary for a mutagenic MOA, we investigated the implications of age-related differences in metabolism of acrylamide to glycidamide by cytochrome P450 2E1 (CYP2E1). 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). This is also apparently true between neonatal and adult mice (Gamboa et al., 2003). However, the parameter of interest is amount of glycidamide formed, not the amount of CYP2E1 available. For many chemicals, there is a significant excess of CYP2E1 in the liver, and metabolism is limited by blood flow into the liver, not CYP2E1 levels (e.g., Lipscomb et al., 2004). Therefore, it is not known whether CYP2E1 levels would be limiting in the

fetus or child, and what the impact of lower CYP2E1 levels would be on glycidamide dose to the child. Additional research and modeling, such as that conducted recently by Walker et al. (2007), would be helpful in the determination of specific adjustment factors, if needed, for acrylamide. Walker et al. (2007) used Monte Carlo modeling and a PBPK model to estimate that the median child dose of glycidamide is about half that of the median adult, with the maximum difference in dose between the median and 99th percentile child ranging from about 2 to about 4. Although further evaluation and refinement of the model would be necessary for its use in risk assessment, these results support the consideration that the dose of glycidamide is lower in children than in genetically comparable adults, and that a decrease in the slope factor might be supported. However, information on toxicodynamic differences between the adult and child is not available for acrylamide carcinogenicity. The US EPA (2005) and its supplemental guidelines provide several conceptual reasons that children may have greater dynamic sensitivity. If such dynamic information were to indicate that children are more susceptible than adults, or if it was assumed that children were more dynamically more sensitive, this would justify increasing the slope factor. Because these concepts operate in the opposite direction, toxicokinetic information, which serves to lower the SF, and toxicodynamic information, 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 US EPA (2005) for chemicals undergoing CYP2E1 metabolism in general.

It is also reasonable to explore the added sensitivity of children, if a threshold estimate for thyroid tumor response is envisioned based on the high-dose data. This is because the tumorigenicity at high-dose is expected to be due to growth stimulation, perhaps by acrylamide, for which the lower CYP2E1 activity in children may 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.

Walker et al. (2007) also considered other sources of kinetic variability, including the implications of variability in glutathione levels, which was included in the age-related analysis described above, and in glutathione transferase (GST). The latter is of particular interest, since 20–50% of the population, depending on ethnicity, has a GSTM1 null mutation, which is devoid of activity. The authors found that the population with the highest glycidamide dose would be those with the GSTM1 null mutation, but also noted that it is not known which, if any, GST is responsible for conjugating acrylamide and glycidamide; if other GSTs are involved, the impact of the GST polymorphism would be decreased or removed.

4.5. Summary

Dose–response assessments for acrylamide has been developed by Dybing and Sanner (2003), OEHHA (2005); FAO (2006) and Shipp et al. (2006). For thyroid tumors specifically, OEHHA (2005) used a linear dose–response assessment resulting in an average upper bound SF of $0.33 \text{ (mg/kg-day)}^{-1}$. FAO used a margin of exposure approach; their BMDL₁₀ value for thyroid tumors from Friedman et al. (1995) was 0.62 mg/kg-day , which would result in either an RfD comparable to the one derived by Shipp et al. (2006), or the one we derived, depending on choice of uncertainty factor. Shipp et al. (2006) presented both an RfD and a linear extrapolation for thyroid tumors; their RfD was 0.0015 mg/kg-day , and their average upper bound SF for thyroid tumors was $0.098 \text{ (mg/kg-day)}^{-1}$. Although several of these groups acknowledged the potential dual nature of the MOAs for acrylamide, none of these groups conducted a dose–response assessment in a decoupled fashion, along the lines suggested by US EPA (2005). In contrast, our MOA

analysis decoupled the contribution from these two MOAs and informed our choice of the final dose–response model.

Building on the work of OEHHA (2005); FAO (2006) and Shipp et al. (2006), 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 US EPA (2005) guidelines, we determine a SF of $0.030 \text{ (mg/kg-day)}^{-1}$ for the linear, low-dose, part of the dose–response curve [upper bound estimate of $0.035 \text{ (mg/kg-day)}^{-1}$], with an RfD of $0.05\text{--}0.02 \text{ mg/kg-day}$ for the portion of the dose–response driven by growth stimulation, a non-linear, high-dose effect. Together, these values might define lower and upper bounds on exposures of concern for subsequent risk management decisions, consistent with the guidance of US EPA (1998) when both linear and non-linear techniques are used in the dose–response assessment.

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:

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

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