



Evaluation of Human Relevance and Mode of Action for Tunica Vaginalis Mesotheliomas Resulting from Oral Exposure to Acrylamide

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Abstract

The human relevance and mode of action of acrylamide-related tunica vaginalis mesotheliomas (TVMs), a tumor of the scrotum, was evaluated based on the available data on acrylamide and general biology considerations. TVMs are found almost exclusively in F344 rats, suggesting an association with the hormonal milieu unique to F344s, and suggesting an association with Leydig cell tumors (LCTs), which occur in F344 rats at a very high incidence. These hypotheses are biologically plausible, but direct data on acrylamide were lacking for several key events; some of the gaps could be addressed based on other biology information. The data were not sufficient to identify a single definitive MOA. Multiple MOAs may apply, and some contribution from mutagenicity is plausible, along with a likely influence from LCTs or from the same hormonal changes that result in higher LCT incidence in F344 rats. Other MOAs, such as oxidative stress, may also apply. The data reviewed are not sufficient to distinguish between a causal relationship between LCTs and TVMs, and the hypothesis that these tumor types reflect a response to some shared influence (e.g., hormonal milieu of the F344 rat). Some of the plausible MOAs are not relevant to humans, while others are. In light of the very low incidence of TVMs in humans and the MOA data reviewed, the most appropriate upper bound estimate of the risk of acrylamide-related TVMs in humans is below de minimis levels.

Introduction

Mesotheliomas of the tunica vaginalis (TVMs) were reported in both of the available cancer bioassays conducted with acrylamide (Friedman et al., 1995; Johnson et al., 1986). These tumors, which occur on the cell layer lining the epididymis, testis, and scrotum, are most common in F344 rats, but are also found occasionally in other strains (particularly after i.p. injection, which results in direct exposure of the tunica vaginalis), and in other species. These tumors have been reported in humans, but they are very rare.

This manuscript evaluates the data related to the mode of action (MOA) of one of the key tumors associated with acrylamide in rats, with the aim of developing an improved scientific basis for the qualitative and quantitative cancer assessments of acrylamide. This manuscript is one of a series of three evaluating in depth the data related to the MOA of each of the key tumors associated with acrylamide in rats (see also, Dourson et al., 2008 for an evaluation of thyroid tumors and Maier et al., 2008 for an evaluation of mammary tumors). This series considers both the possibility that a common MOA (e.g., direct DNA reactivity) plays a causal role in the observed tumor responses in rats (Johnson et al., 1986; Friedman et al., 1995), as well as the possibility that tissue-specific processes play key roles. After consideration of human relevance of the determined MOA(s), the MOA-based analysis can then be used to guide dose-response analyses for either individual tumor types or combined tumor incidences. A similar type of analyses would need to be completed for tumors observed in the ongoing NTP bioassays in rats and mice (NTP, 2007).

This manuscript briefly reviews the data on acrylamide-related tumors in rats, and provides some background information on the morphology and physiology of the tunica vaginalis. It then explores the MOA data relevant to TVMs, with particular attention to the hypothesis that the TVMs are secondary to Leydig Cell Tumors (LCTs, also known as interstitial cell tumors). The MOA data, together with information on TVMs in humans, are used to evaluate the potential MOA for acrylamide-related TVMs in rats, and the human relevance of these tumors. The human relevance is evaluated based on biological/MOA considerations, as well as taking into account the available information on the incidence of TVMs in humans. Finally, the data are used to develop a quantitative assessment for TVMs. Other investigations have evaluated a variety of tumor endpoints, including evaluation of TVMs (e.g., OEHHA, 2005; Shipp et al., 2006). U.S. Environmental Protection Agency (EPA) is also developing a comprehensive assessment for this chemical.

Tumor Data in Animal Studies

TVMs were reported in both of the available cancer bioassays conducted with acrylamide. In the first study, Johnson et al. (1986; unpublished version is Johnson et al., 1984) conducted a 2-year chronic/carcinogenicity study with F344 rats, in which groups of 90 rats/sex/dose group were administered acrylamide in drinking water at doses of 0, 0.01, 0.1, 0.5, or 2.0 mg/kg-day. Statistically significant increases in TVMs were reported at the two highest doses, although there was an inconsistent dose-response (Table 1; Figure 1). Increases were also noted in tumors of the mammary gland of females (positive trend in adenocarcinomas, significant increases in fibromas and combined benign tumors) and thyroid gland of both sexes. The authors of the second study (Friedman et al., 1995; unpublished version is Dulak, 1989) stated that it was

conducted to address the atypical dose-response relationship for the TVMs and to enhance the statistical power, in addition to other reasons not relevant to this analysis. The doses tested in males were 0, 0.1, 0.5, and 2.0 mg/kg-day, and included all of the doses tested by Johnson et al. (1986), except for the lowest dose. An unbalanced study design was used, with additional animals in the male control and low-dose groups, in order to have sufficient power to detect a 5% increase in tumor incidence over a 1.3% “background” incidence of TVMs. In addition, two separate control groups were used in order to better determine the variability of low-incidence background tumors. This study reported a statistically-significant increase in TVMs only at the high dose of 2.0 mg/kg-day. Thus, the two bioassays consistently reported increased TVMs, although there were differences in the dose-response. Friedman et al. (1995) also reported increases in thyroid tumors in males and females (as discussed by Dourson et al., 2008), and in mammary tumors (fibroadenomas) in females (as discussed by Maier et al., 2008). No full cancer bioassay in a second species has been completed, although studies are in progress in rats and mice through the National Toxicology Program (NTP, 2007). Dourson et al. (2008) evaluated the data on acrylamide found in rat chow reported by Twaddle et al. (2004). The acrylamide concentration in rat chow is typically approximately 20 ppb or less, but some diets had high concentrations, resulting in an average of 27 ppb based on analysis of several unaltered diets (Dourson et al., 2008). Based on a food factor of 0.086 for a chronic study in F344 rats (US EPA, 1988), the control diet can be estimated to contribute approximately 0.002 mg/kg-day to acrylamide intake. This background level of acrylamide intake is primarily relevant for dose-response assessments, but in considering mechanistic studies, it is noteworthy that all animals received some minimal dose of acrylamide.

Tunica Vaginalis Mesotheliomas and Other Mesotheliomas

The tunica vaginalis is derived from the peritoneum, and consists of a single layer of mesothelial cells that line the epididymides, testes, and scrotum. The mesothelium both provides a limiting layer to adjoining serosal tissues, and provides a frictionless surface to facilitate movement within the peritoneal cavity (Whitaker et al., 1982). In the rat, spontaneous mesotheliomas are found most commonly in the tunica vaginalis in males, and ovary of females (Ilgren, 1993). Of the reports of spontaneous or chemical-related TVMs compiled by Ilgren (1993), the preponderance of the studies was in F344 rats. However, TVMs have also been reported in several other rat strains, including Sprague-Dawley, Buffalo, CD, Wistar, and White (Porton strain) rats. Hall (1990) stated that almost all mesotheliomas in the F344 rat are thought to arise from the tunica vaginalis, and then may spread from there to the peritoneum. Independent evaluation of this statement is complicated by the consideration that authors often use general terms such as mesotheliomas or peritoneal mesotheliomas, and then either do not provide more specific location information, or later note that the tumors were localized to the TVM. Therefore, although this report focuses on TVMs, peritoneal mesotheliomas in general are also addressed, consistent with the recommendation by McConnell et al. (1986) that most neoplasms of the same histomorphogenic type are combined even if they occur in different anatomic sites.

Increased TVMs associated with chemical exposure (Ilgren, 1993; NTP, 1999) have been reported for chemicals that act via several MOAs, including TVMs in rats following exposure to chemicals that are mutagenic (e.g., 2-acetylaminofluorene, methyl(acetoxy methyl) nitrosamine, methylnitrosourea, and ethylene oxide); or act primarily via oxidative damage either directly

(e.g., potassium bromate) or by the generation of oxygen radicals (pentachlorophenol). TVMs have also been seen in dogs following exposure to a hormonally-active chemical, stilbesterol).¹ The NTP historical control database for drinking water and feeding studies reports that mesotheliomas (tissue unspecified) occurred in rats at an incidence of 2-3%, depending on the time period (the feed used varied with time period) and whether the study administered the chemical in drinking water or feed. Damjanov and Friedman (1998) reported that mesotheliomas occur at a rate of 1.3% in the F344 rat animal colony used by Friedman et al. (1995), and that overall the background rate is 1-4%. In a review of more than 300 NTP bioassays (51,230 treated and control rats), Mitsumori and Elwell (1988) reported an incidence of TVM of 1.5% in male F344 rats; studies with treatment-related increases in neoplasms of the testis or accessory sex organs were excluded from their review. The authors noted that actual incidences may have been underestimated, due to mortality occurring prior to 2 years. TVMs have occasionally been reported in other strains of rats besides the F344 rat, but such reports are rare, and most commonly associated with i.p. exposure (see below).

The individual animal data from the unpublished versions of the two acrylamide bioassays (Johnson et al., 1984, unpublished; Dulak, 1989, unpublished) were evaluated to identify the total incidence of mesotheliomas in these two studies. No mesotheliomas were reported in females in either study. As shown in Table 1, the incidence of animals with TVMs was virtually identical to the incidence of animals with any mesothelioma. The sole exception was that one control rat in Johnson et al. (1984) had a mediastinal mesothelioma but no TVM. Several TVM-bearing animals in both studies had mesotheliomas in multiple organs. In the Dulak et al. (1989)

¹ Note that multiple MOAs may be possible for several of these chemicals; only the predominant MOA is listed here.

study, consideration of other mesotheliomas also did not affect the total incidence of animals with mesotheliomas.

In a supplemental histopathology evaluation of the TVMs identified in the Friedman et al. (1995) study, Damjanov and Friedman (1998) found no differences in size, histology, or ultrastructure between the TVMs in the control and exposed groups, suggesting that acrylamide exposure may act to enhance spontaneous tumors, rather than initiating tumor formation. Damjanov and Friedman (1998) also described the tumors as histologically benign and noted that metastases did not occur in the study. In characterizing the tumors as histologically benign, the authors noted that there are no established criteria for distinguishing between benign and malignant mesotheliomas, a fact confirmed by other investigators (Swenberg, personal communication). The authors also noted several potential experimental studies that could be used to distinguish whether the tumors were benign or malignant, but that it is not possible, based on the current data, to state whether the acrylamide tumors were malignant.

It is not known why mesotheliomas in F344 rats particularly occur on the tunica vaginalis. As part of a detailed review of the pathology of TVMs induced by potassium bromate, a chemical that induced mesotheliomas in a variety of tissues, Crosby et al. (2000) suggested that the mesorchium (a fold of the tunica vaginalis between the testis and epididymis) is the primary mesothelial target for bromate-induced carcinogenesis. They further suggested a number of potential factors that may contribute to the development of tumors at the convergence of the mesorchium and mesothelium. These factors were: (1) blood flow; (2) direction of flow of peritoneal fluid; (3) heating and cooling processes; (4) lymphatic drainage; (5) enervation; and

(6) other physiological properties of the target tissue combined with one or more of the other factors. Among these physiological factors, Crosby et al. (2000) noted that mesothelial cells have high plasticity and easily immortalize spontaneously, and speculated that mesothelial cells in general may be missing a tumor suppressor function. Several of the studies they cited were conducted with human mesothelial cells, although it is reasonable to expect that there are differences in growth control among mesothelial cells located in different tissues, and between human and rat tunica vaginalis mesothelial cells, particularly in light of the differences in TVM frequency. Crosby et al. (2000) also reported unpublished data that mesothelial cells in vitro contain lower levels of reduced and total glutathione compared to nontarget cells (compared to HepG2 cells, nontarget cells for bromate carcinogenesis). This finding supports susceptibility to oxidative stress as a potential MOA for the TVMs. The particular susceptibility of the tunica vaginalis may also be explained by the report that the cell division of this tissue is up to 10x the rate in the mesothelium of other areas of the serosa (Whitaker et al., 1982). This increased cell division rate was reported as occasional, with small clusters of replicating cells, and was hypothesized to possibly be related to local, intermittent stimuli.

Another possible reason for the susceptibility of the tunica vaginalis has been proposed in the context of acrylamide (Shipp et al., 2006). As described more fully later in this paper, this hypothesis suggests that the increased size of the testis in animals with LCTs results in increased pressure and irritation on the tunica vaginalis, resulting in promotion of tumors of the tunica vaginalis. The pressure and irritation would be expected to be highest at the mesorchium, consistent with the observations of Crosby et al. (2000) that the mesorchium is the most common site on the tunica vaginalis for TVMs.

TVMs have been reported in humans, but are very rare. The data related to TVMs in humans are presented after the synthesis of MOA information, as part of an evaluation of the implications of quantitatively extrapolating from the rat data to humans.

Morphologically, the spontaneous TVMs observed in rats are consistent with epithelial mesotheliomas observed in humans (Tanigawa et al., 1987). Kim et al. (2006) concluded that rat mesotheliomas were similar to mesotheliomas in humans, at least at the cellular and molecular level, based on an evaluation of gene expression data from the broader category of peritoneal mesotheliomas induced by *o*-nitrotoluene and bromochloroacetic acid. In light of the difference in TVM and mesothelioma incidence between F344 rats and humans, this similarity at the cellular and molecular level suggests that neighboring tissues play a role in the development of mesotheliomas.

Despite these similarities, there is an anatomical difference between the rat and human scrotal cavity. In the rat, the scrotal cavity is continuous with the peritoneal cavity, while in the human the scrotal and peritoneal cavities are separated (Crosby et al., 2000; Wall et al., 2006). This means that rat TVMs are much more likely to extend into the peritoneal cavity than human TVMs. The lower propensity of human TVMs to spread to the peritoneal cavity would mean that the severity in humans is lower.

Thus, TVMs in rats and humans appear to be similar at the morphological, cellular and molecular level, and mesothelial tissue in general may have a particular susceptibility to tumor

induction. However, the susceptibility of male F344 rats to TVMs, in contrast to the low incidence of TVMs in humans and other strains of rats, suggests species- and strain-specific differences in growth control. Tissue-specific toxicodynamics would also be expected to play a role, in light of the wide tissue distribution of acrylamide and lack of correlation in general between tissue distribution and tumor targets.

Evaluation of MOA for TVMs

Three broad MOA possibilities might be considered for acrylamide-related TVMs. Note that these potential MOAs are not mutually exclusive, and more than one MOA may apply. It is also recognized that each of these hypotheses consider multiple MOAs, due to the potential for different permutations or combinations of some steps. To enhance readability of this manuscript, the discussion of each hypothesis focuses on the central MOA, and discusses possible alternatives in the context of data gaps, but does not rigorously evaluate each MOA within each hypothesis.

Hypothesis A: Acrylamide-related TVMs are secondary to the enhancement of the size or incidence of LCTs in F344 rats. This relationship between TVMs and LCTs could be endocrine, paracrine, or the result of a physical interaction (e.g., the presence of the LCT resulting in an enlarged testis and physically irritating the mesothelium).

Hypothesis B: Acrylamide-related TVMs result from direct mutagenicity by glycidamide, or by other DNA reactivity of acrylamide due to indirect gene damage, such as by oxidative stress or by interaction with chromatin proteins. The impact of such damage would be enhanced by

endocrine, paracrine, or physical influences, based on the specificity of the observed tumor sites vs. the location of mutations.

Hypothesis C: Acrylamide-induced TVMs result from some other (as-yet-unidentified) hormonal signal that may also play a causal role in LCT development, but the TVMs occur in parallel with the LCTs, rather than being secondary to them.

Shipp et al. (2006) proposed that the TVMs in the acrylamide studies were secondary to the high incidence of LCTs in F344 rats. The overall hypothesis (K.S. Crump Group, 1999; Shipp et al., 2006) is based on the recognition of the high incidence of LCTs in F344 rats, and the preponderance of TVMs being in this same strain. The authors hypothesized that

“acrylamide produces a centrally-mediated cascade of hormonal alterations that exacerbate the already stressed testicular hormonal capacity [of the F344 rat]. Further decompensation of hormone responsiveness and production of localized hormones results. Background rates of TVMs already are formed in response to this localized reduction in androgenic hormones through a growth factor receptor-mediated autocrine response. Further decreases in the regional androgen levels would accelerate and extend the spontaneous rate of tumor formation, even in the absence of exogenous genetic damage in these cells.”

Alternatively, the authors (K.S. Crump Group, 1999) also suggested that acrylamide could act as a clastogen or cause aneuploidy, altering chromosomes of the mesothelial cells themselves.

Age-related hormonal changes occurring in F344 rats could trigger growth factor signals, leading to expression of the chromosomal effects and cell transformation. Further autocrine stimulation

could then lead to tumors. The authors also noted that the genotoxic and hormonal components could both be occurring, but stated that in all of these possibilities, formation of LCTs and the resulting hormonal changes are a necessary precursor to TVMs.

Two MOAs were proposed for this connection, and are noted as subsets of Hypothesis A. The first hypothesis suggests that the TVMs are due to a hormonal imbalance, that there is an association between the production of LCTs and TVMs related to the hormonal milieu of F344 rats, and that acrylamide stimulates LCTs in F344 rats, thus indirectly increasing the incidence of TVMs. Spontaneous mesotheliomas have been attributed to hormone imbalance (Crosby et al., 2000). The second hypothesis is based on the work of Tanigawa et al. (1987), and suggests that the relationship between LCTs and TVMs is physical, with enlargement of the testis from the LCT resulting in physical stimulus (pressure) on the mesothelium similar to a solid state/foreign body response. These MOAs are not mutually exclusive, and both could apply. These MOAs are explored further in the following text.

Hypothesis B is that carcinogenicity is due to direct or indirect mutagenicity. According to the U.S. EPA (US EPA, 2005), data evaluating the potential for mutagenicity should always be considered. As discussed in more detail below, the acrylamide metabolite glycidamide is mutagenic, but in vivo data do not support the conclusion that mutagenicity is the primary MOA for tumor formation. Mutations could also result from indirect effects on DNA, such as oxidative stress; this latter mechanism is only briefly discussed in this paper, due to minimal data investigating this possibility. Indirect mutagenicity would generally be addressed differently by the U.S. EPA than direct mutagenicity, although it is not entirely clear how oxidative stress

would be handled under the EPA guidelines, in light of background levels of oxidative stress and the rapidity of the response.

Hypothesis C takes into account that there may be other possibilities not addressed by either of the first two hypotheses. While limited data preclude the consideration of other potential MOAs in detail, these other potential MOAs are noted for completeness, as part of the consideration of all the data and consideration of all potential MOAs.

To evaluate the hypotheses, we used the approach described in the U.S. EPA Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005) to evaluate MOA, along with the approach described by U.S. EPA (2005) and Meek et al. (2003) to evaluate human relevance of the observed tumors. The approach for evaluation of MOA considers (1) the strength, consistency, and specificity of the association; (2) dose-response concordance; (3) temporal relationship; and (4) biological plausibility and coherence. Consideration of human relevance involves (1) identification of the MOA in animals; (2) consideration of the plausibility of the key events in the animal mode of action in humans; and (3) consideration of the plausibility of the animal MOA in humans, taking into account kinetic and dynamic considerations. In addition, the U.S. EPA (2005) framework addresses whether there are populations or life stages that can be particularly susceptible to the hypothesized mode of action. As part of the evaluation of each potential MOA, the relevant biology is discussed. In particular, because the data directly relevant to addressing the MOA for acrylamide-related TVMs are rather limited, other relevant information on the biology of tumors and pathways of interest is also reviewed.

As described below, the data are insufficient to demonstrate conclusively a non-mutagenic MOA, due to insufficient precursor information. The data are also insufficient to support a conclusion that any one MOA is responsible for the rat TVMs. Therefore, the data as a whole were evaluated for a weight of evidence determination of the potential human cancer risk related to the rat TVMs. Unlike the MOA and human relevance framework described in the previous paragraph, which are framed in terms of either/or propositions, this weight of evidence evaluation takes into account the potential for multiple MOAs, and considers (semi-quantitatively) the *degree* to which MOAs may contribute, not only *whether* they may contribute.

Hypothesis A

Development of LCTs in F344 Rats

To address the hypothesis that the development of LCTs is a necessary precursor to the development of TVMs, we first review the MOAs of LCT formation and then evaluate the data on acrylamide relative to these MOAs. Substantial information on the formation of LCTs in rats and humans is available. Excellent reviews on the mechanisms of LCT formation and relevance to humans were published by Cook et al. (1999) and Clegg et al. (1997). The latter review reported on the results of a workshop convened to review the available data and to reach consensus about the relevance of the tumors for human risk assessment. Clegg et al. (1997) focused on seven hormonal modes of induction of LCTs: androgen receptor antagonism, 5 alpha-reductase inhibition, testosterone biosynthesis inhibition, aromatase inhibition, estrogen

agonism, gonadotropin releasing hormone (GnRH) agonism and dopamine agonism. With the exception of GnRH agonists, which act directly on the Leydig cells, all of the MOAs involve disruption of the hypothalamus-pituitary-testis (HPT) axis and compensatory increases in luteinizing hormone (LH) levels. Of these seven MOAs, the first five were considered to be relevant or potentially relevant to humans, although quantitative differences may exist across species, with rodents being more sensitive. In contrast, the latter two MOAs, GnRH agonism and dopamine agonism, were considered not relevant to humans, because human Leydig cells do not respond to decreased prolactin with downregulation of LH receptors and do not have luteinizing hormone releasing hormone (LHRH) receptors.

Since all but one of these MOAs act via disruption of the HPT axis, we first review the HPT axis, and its key control points and feedback loops, prior to addressing the impact of acrylamide. This review is based primarily on the reviews of Cook et al. (1999) and Shipp et al. (2006) (Figure 2). In brief, testosterone production in humans and rats is under the control of the hypothalamus and pituitary. The hypothalamus secretes GnRH, which stimulates the synthesis and release of LH from the pituitary. LH binds to Leydig cells in the testis, initiating a cascade of events that stimulates testosterone production. Testosterone receptors on neurons in the hypothalamus provide feedback control of GnRH production.

In the normal F344 rat, GnRH acts on the pituitary to stimulate the release of LH, and LH stimulates Leydig cells to synthesize testosterone. If testosterone levels are low, a feedback signal stimulates GnRH and compensatory increase in serum LH levels in order to maintain testosterone at physiological levels. Dopamine agonists decrease prolactin release from the

pituitary. Decreased prolactin causes a decrease in the number of LH receptors on Leydig cells in rats, decreasing testosterone levels. This stimulates GnRH production, resulting in a compensatory increase in serum LH levels in order to maintain testosterone at physiological levels. Decreased prolactin may also directly result in increased GnRH levels, because high levels of prolactin inhibit release of GnRH release. It has been proposed that this sustained increase in LH results in Leydig cell hyperplasia and LCTs (Prentice and Meikle, 1995).

There are several differences between rats and humans in the molecular control of this pathway. Human (and mouse) Leydig cells do not have GnRH or prolactin receptors, and have fewer LH receptors than do rat Leydig cells. This means that decreased prolactin does not affect LH receptor numbers on human (or mouse) Leydig cells, and so the dopamine mechanism is not considered relevant to humans. Similarly, since GnRH agonists act directly on Leydig cells, the absence of GnRH receptors in humans and mice means that the MOA of GnRH agonism is not believed to apply to these species. The smaller number of LH receptors on Leydig cells also leads to quantitative differences between rats and humans for the other five MOAs discussed above.

F344 rats have a very high background of LCTs, of more than 80% in a 2-year study (Boorman and Chapin, 1990). This high incidence is believed to be related to high basal levels of LH in this strain. The molecular reason for such high basal levels is not known, nor is it known whether the altered control occurs at the level of the pituitary, the hypothalamus, or elsewhere.

Therefore, consistent with the framework for evaluating human relevance (US EPA, 2005; Meek et al., 2003), the first step in evaluating the human relevance of any LCTs associated with acrylamide exposure is to evaluate the connection between acrylamide exposure and LCT formation, as well as to evaluate the relevant MOA data.

No experimental data were located on the potential for the first five MOAs for LCT development being relevant to the effect of acrylamide on Leydig cells. In the absence of experimental data, we considered the chemical structure, and noted that there is no structural similarity between acrylamide and the chemicals reported by Clegg et al. (1997) to act via these MOAs. Although mechanistic similarity can exist in the absence of structural similarity, the differences between the relative size/structure of acrylamide and those of the chemicals for which these MOAs apply suggest that acrylamide may not act via these MOAs. However, such structural comparisons are insufficient for a definitive conclusion. Similarly, no data were available specifically regarding the potential that acrylamide acts as a GnRH agonist. Therefore, these potential MOAs could not be further evaluated.

Table 2 lays out the key events in the development of LCTs by dopamine agonists. As shown, acrylamide-specific data are not available for each key event, but such data are available for most of the key events, and non-chemical specific information on basic physiology can be used to supplement the analysis.

The first step in Table 2 is increased dopaminergic activity. Several studies are available on the effect of acrylamide on the dopamine system, but direct acrylamide binding to dopamine

receptors has not been shown. Overall the data are complex and on the surface appear to be contradictory, with some studies supporting an agonist effect, and others appearing not to.

Ali (1983) reported dose-related statistically significant increases in dopamine in the caudate nucleus, but not the hypothalamus, of male F344 rats receiving 10 or 20 mg/kg-day acrylamide i.p. for 20 days. In contrast, several studies involving exposure for up to 20 days or lactational exposure to acrylamide reported decreases in dopamine in whole brain or particular regions of the brain. Specifically, male Wistar rats gavaged with 50 mg/kg-day for 5 days had decreases in the cerebellum, pons medulla, midbrain, and hypothalamus (Dixit et al., 1981). In another study, male Wistar rats exposed on postnatal days 0-21 to mothers gavaged with 25 mg/kg-day had decreased whole brain dopamine levels; young Wistar rats (age 12-60 days) exposed for 5 days to 25 mg/kg-day had decreases in the pons medulla, midbrain, and hypothalamus (Husain et al., 1987). Male F344 rats given a single i.p. dose of up to 100 mg/kg or up to 20 daily i.p., doses with up to 20 mg/kg-day had a decrease or no effect in dopamine levels in the frontal cortex (Ali et al., 1983; Ali, 1983), but no effect on the striatum or hypothalamus (Ali et al., 1983; Ali, 1983). Shipp et al. (2006) proposed an explanation for this apparent contradiction, noting that the caudate nucleus contains the D₂ dopamine receptor, and that this receptor is also thought to be the primary dopamine receptor in the pituitary. Shipp et al. (2006) noted that, in contrast, the frontal cortex contains relatively high levels of the D₃ dopamine receptor, which acts as an autoreceptor in some areas of the brain and decreases production of dopamine when activated. This difference in dopamine receptor in different regions of the brain may explain the apparent inconsistency between decreases in dopamine levels in several brain regions, but increases in the caudate and a hypothesized dopaminergic effect of acrylamide on the pituitary.

The hypothesis that acrylamide enhances LCT formation via a dopaminergic MOA suggests that acrylamide exposure would increase dopamine activity in the pituitary, inhibiting release of prolactin. This leads to decreased testosterone levels and a compensatory increase in LH receptors and LH levels and, ultimately, enhanced LCT formation. The hypothesis is biologically plausible, but no data are available to directly test it. No data were located on dopamine levels in the pituitary after acrylamide exposure, nor on the potential of acrylamide to bind directly to the dopamine receptor and to act as an agonist or antagonist. However, the binding of spiroperidol, a dopamine antagonist, to brain tissue rapidly increased after a single gavage dose of 25 - 100 mg/kg or repeated (10-30 mg/kg-day for 10 days) doses of acrylamide, although there was not a clear dose-response (Agrawal et al., 1981b; Agrawal and Squibb, 1981; Agrawal et al., 1981a; Bondy et al., 1981; Uphouse and Russell, 1981). Although the chemical used to measure binding is a dopamine antagonist, the observed changes serve as a marker of overall binding capacity, and suggest that binding of dopamine and dopamine agonists may also increase. This would result in an overall increase in dopaminergic activity. This increase in binding has been attributed to alteration of the receptor binding characteristics (Agrawal et al., 1981b), as well as to upregulation of dopamine receptors from a previously inaccessible pool (Agrawal et al., 1981b; Uphouse and Russell, 1981), and possibly to damage to dopamine neurons and denervation supersensitivity of the postsynaptic cell (Agrawal et al., 1981a). The dopamine receptor system was much more sensitive than muscarinic or serotonergic receptors. Another potential mechanism for an acrylamide effect on dopaminergic pathways was suggested by LoPachin et al. (2006), who presented evidence for an effect of acrylamide on dopamine signaling. They found that acrylamide inhibits dopamine release to synapses, and that this

inhibition occurs as a result of acrylamide interacting with sulfhydryl groups on specific proteins involved in pre-synaptic vesicle loading or membrane fusion.

Overall, although the exact mechanism of the effect of acrylamide on the dopamine system has not been elucidated and definitive support is not available, the data are consistent with the hypothesis that acrylamide exposure increases dopamine activity in the pituitary. Therefore, acrylamide may increase dopaminergic activity by increasing the affinity of dopamine for the receptor, increasing the number of receptors, or increasing the sensitivity of postsynaptic cells (which may occur through either of the first two mechanisms or via a third mechanism).

Data are strong that acrylamide causes the next key event, decreased serum prolactin levels in male rats in short-term studies (Ali et al., 1983; Friedman et al., 1999; Uphouse et al., 1982). Friedman et al. (1999) administered acrylamide in the drinking water to male and female F344 rats for 28 days, resulting in calculated doses of 0, 1.4, 4.1, 12, 19, or 25 mg/kg-day (males) or 0, 1.5, 4.3, 9, 19 or 24 mg/kg-day (females). There was a dose-related decrease in prolactin in males at 14 days, with a much smaller decrease at 29 days. At 14 days, serum prolactin levels at the three highest doses were 64%, 19%, and 13% of control, respectively; only the top two doses were statistically significant. At 28 days, decreases were observed at the top two doses, but neither change was statistically significant. There was no clear effect at the two doses in the range of the bioassays (1.4 and 4.1 mg/kg-day), although a nonsignificant decrease of about 17% was observed at 4.1 mg/kg-day. Ali et al. (1983) also reported clear dose-related decreases in serum prolactin in male F344 rats receiving 20 daily i.p. injections of 10 or 20 mg/kg-day, but only the decrease at 20 mg/kg-day was statistically significant, due to high variability in the

control group. A single gavage dose of 100 mg/kg also decreased prolactin levels (Uphouse et al., 1982). These studies show that short-term exposure to acrylamide does decrease prolactin levels. However, in light of the short exposure durations, complexity of the duration response, observation of effects only at doses well above those that cause tumors, and overall complexity of hormonal feedback mechanisms, it is difficult to determine the relevance of these observations to the increased LCT response.

The next key event in Table 2 for which data are available is decreased testosterone production. Although no data are available directly addressing the effect of acrylamide on testosterone production, the data support the conclusion that short-term exposure to acrylamide decreases serum testosterone levels. Friedman et al. (1999) measured testosterone levels as well as prolactin levels. At 14 days, they found that testosterone levels at the high dose were 56% of control, but this decrease was not statistically significant. At 28 days, testosterone levels were 45%, 27%, 9% of control, respectively, at the three highest doses. Only the decreases at the two highest doses were statistically significant, and there was no effect at doses in the range of the bioassays. Unlike the results for prolactin levels, the effect was larger at 28 days than 14 days, suggesting that an even-longer exposure could result in an effect at lower doses. However, the interplay between these results and those for prolactin is difficult to interpret, since the time points at which effects were seen were not consistent. A dose-related decrease in testosterone, which was statistically significant at 20 mg/kg-day but not at 10 mg/kg-day, was also reported by Ali et al. (1983) following 20 daily i.p. injections of F344 rats.

The next key event is a compensatory increase in LH levels. No information was located directly evaluating LH levels in rats (or other species) exposed to acrylamide. However, in light of the physiology of the HPT axis, it is reasonable to expect that sustained decreases in testosterone levels would result in compensatory increases in LH levels.

The final event, an increase in LCT incidence or size, is difficult to evaluate in F344 rats, due to the high background level of LCTs in this strain. No data are available regarding the effect of acrylamide on LCTs (or on the earlier key events) in other strains of rat. The incidence of LCTs in the control rats was 95% and 82% in the two acrylamide bioassays (Johnson et al., 1986; Friedman et al., 1995), respectively, and was not increased in the exposed animals. Although the *incidence* of LCTs did not increase in either of the bioassays, there is some evidence that acrylamide increased the *size* and volume of the LCTs, and therefore of the testicle. In particular, in an unpublished study, Iatropoulos et al. (1998) conducted a blind histopathology review of 38 TVMs that occurred in the Friedman et al. (1995) study; key data from this study are presented in Table 3. The LCTs for these animals were graded as occupying 25%, 50%, 75%, or 100% (by volume) of the testes. Table 3 presents their compilation of LCT size, along with the mesothelioma diagnosis and degree of progression (benign vs. malignant)². As shown by the average LCT size for each dose group, there was no dose-response for LCT size in the animals evaluated (i.e., only animals initially diagnosed with TVMs). However, the degree of

²The terminology of “benign” vs. “malignant” used here is adopted directly from that used by the study authors, recognizing that there is disagreement in the approach used by various acrylamide investigators. In contrast to the terminology used by Iatropoulos et al. (1998), Damjanov and Friedman (1998) considered all of the tumors in this study to be benign, while cautioning that there are no established criteria for distinguishing between benign and malignant mesotheliomas. Iatropoulos et al. (1998) did not report the criteria they used, but stated that they used “preestablished morphologic criteria pertaining to the location, extent, severity, pattern, and shape of the proliferative lesions of the mesothelium.” Even if the latter study were not actually distinguishing between benign and malignant tumors, the study does show that mesotheliomas of greater severity or further progression were associated with the larger LCTs.

progression of the TVMs correlated closely with the size of the LCTs. All cases of malignant mesotheliomas were accompanied by LCTs occupying $\geq 75\%$ of the testis (i.e., grades of 3 or 4) and all LCTs of grades 3 or 4 that were analyzed were accompanied by malignant mesotheliomas (when the tissue was available). Conversely, benign mesotheliomas were accompanied by LCTs occupying $\leq 50\%$ of the testis (i.e., grade of 2 or less). A limitation of the study is that only the animals with mesotheliomas were evaluated. Because the authors did not evaluate all of the animals, a full evaluation of the relationship between acrylamide exposure and size of the LCT is not possible. However, since there was a dose-related increase in TVMs, and TVM malignancy correlated with LCT size, this provides some support for a relationship between acrylamide and LCT size. Interpretation of this study is also complicated because it is an unpublished, non-GLP report, and the diagnosis of a number of the lesions differed from that in the original (Friedman et al., 1995) report, with a total of nine diagnoses across all dose groups changed from mesotheliomas to focal mesothelial hyperplasia or mesothelial data with no lesions.

Based on the above evaluation, the overall evidence supporting the conclusion that acrylamide increases LCT severity is weak to moderate; evidence for earlier key events is stronger. If acrylamide does affect LCT severity, the strongest evidence is that it does so by acting on levels of prolactin and/or testosterone. The data are insufficient to determine how these effects on prolactin and testosterone occur. Increased dopaminergic activity is consistent with the observed effects on prolactin and testosterone, and interactions of acrylamide with the dopaminergic system have been documented, but the data regarding the role of acrylamide are inconsistent and there is no clear evidence showing acrylamide to be a dopamine agonist. However, acrylamide does alter the binding capacity of dopamine receptors, inhibit dopamine release to synapses, and

increase dopamine levels in the caudate, a brain region that, like the pituitary, contains D2 dopamine receptors. Conversely, an analysis of the chemical structure of acrylamide suggests that acrylamide would not increase LCT severity via the other known MOAs, since direct endocrine activity is not suspected, although there are no experimental data directly testing other MOAs.

Considering the data in the light of the modified Hill criteria, overall, the proposed MOA is biologically plausible, based on the observed effects of acrylamide and well-established pathways for the development of LCTs. For some key events, acrylamide-specific data are not available, but the event is plausible based on what is known about the biology of the dopaminergic pathway for LCT development. Much of the available data are coherent and supported by multiple studies, as illustrated in Table 2. The data are generally consistent, if the differences between effects of dopamine in different regions of the brain can be explained by differences in dopamine receptor types. Only short-term data are available, and the effects occur prior to the observed tumors, satisfying the criterion of temporality. No data on tumor precursors are available to compare with the timeline for key events to further investigate temporality. Only one study tested a dose in the range of the bioassay doses (Friedman et al., 1999), and no effect was seen at those doses in the short term assay, although effects could occur at lower doses following longer exposures. This means that the data are insufficient to evaluate dose-response concordance for the proposed key events. Similarly, the magnitude of the response for the key events at the tested doses was nontrivial, at least partially meeting the criterion of strength, but data in the range of the tumor doses would be needed to fully evaluate this criterion. Finally, the observed changes are not specific to LCTs, but specificity is often not met.

If acrylamide does increase TVMs via increasing dopaminergic activity, this MOA is not relevant to humans, as described above. The other MOAs described by Clegg et al. (1997) are all nonmutagenic, and if acrylamide were to increase LCT severity via any of these MOAs, a nonlinear approach would be used for low-dose extrapolation. Thus, analysis of any LCTs resulting from acrylamide exposure would conclude that these tumors are either not relevant to humans, or occur via a nonmutagenic MOA.

Relationship Between LCTs and TVMs

The previous section addressed the issue of LCT induction by acrylamide, and the possible MOA of this induction. However, we are interested not in acrylamide-related increases in LCTs, but in acrylamide-related increases in TVMs. If LCT induction is a necessary precursor step to the induction of TVMs, then the conclusions regarding LCT induction would also apply to TVM induction. Therefore, the next step in the analysis was to investigate the relationship between LCTs and TVMs; this approach hypothesizes that LCT development is a key event in the development of TVMs.

The first step in the investigation was to evaluate biological plausibility of LCTs as a precursor to TVM development. As noted above, F344 rats differ from other strains of rats in having a very high incidence of LCTs (>80% in controls) and a much higher incidence of TVMs (approximately 1%). These high incidences compared with other rat strains, together with the physical proximity of the Leydig cells in the testis and the tunica vaginalis in the scrotum,

suggest an association between these two tumor types. The association could be causal, with LCTs being a necessary precursor to TVMs, or both tumor types could be reflecting a third alteration. Another possibility is that an effect external to the testis predisposes F344 rats to the development of TVMs, and this predisposition is enhanced by the LCT. TVMs cannot be a necessary precursor to LCTs, since TVMs occur at a lower incidence. In addition, not every LCT leads to development of TVM, given the much lower incidence of the latter tumor type.

The hypothesis that the TVMs result from LCTs suggests two sets of associations. If TVMs *only* result as secondary to LCTs, then all reported cases of TVMs should be in animals with LCTs; this association would be weakened to the degree that there is a multifactorial cause of TVMs. Similarly, chemicals that cause increases in LCT incidence or size should consistently cause increases in TVMs. Thus, it is valuable to investigate whether the chemical-related TVMs in the acrylamide studies resulted only from LCTs.

To address this issue, the literature on LCTs and TVMs were reviewed to determine the degree of concordance of reports of LCTs and TVMs. Our review found that there was a substantial degree of concordance, although TVMs were occasionally reported in the absence of LCTs, and conversely, there were some reports of an increased incidence of LCTs in the absence of TVMs. Data were not available to correlate size of LCTs and incidence or progression of TVMs for chemicals other than acrylamide.

The following lines of evidence support the LCT/TVM connection. As noted above, a compilation of reports of spontaneous or chemical-related TVM found that the F344 rat was the

most frequently affected strain (Ilgren, 1993). Shipp et al. (2006) surveyed more than 400 NTP bioassays, and found that the chemicals that caused increased TVMs in male F344 rats did not cause increases in TVMs in male B6C3F1 mice that were exposed via the same route and following a similar protocol, indicating a species- or strain-specificity (or both). Similarly, no increase in mesothelial tumors was reported in the female F344 rats in the acrylamide bioassays (Friedman et al., 1995; Johnson et al., 1986); this absence was confirmed by a review of the individual animal data from the unpublished studies (Dulak, 1989, unpublished; Johnson et al., 1984, unpublished). The finding that only males developed mesotheliomas following acrylamide exposure of F344 rats supports the conclusion that the TVMs do not reflect a general tumorigenic influence on mesothelial tissue, but instead reflect some sex-related difference. These considerations suggest an association between TVMs and F344 rats, and, in light of the high incidence of LCTs in F344 rats and the proximity of the tissues, suggest an association between these two tumor types.

To evaluate the relationship between TVMs and LCTs in rats exposed to acrylamide, we reviewed the individual animal data in the unpublished versions of the acrylamide bioassays (Dulak, 1989, unpublished; Johnson et al., 1984, unpublished). In the Johnson et al. (1984, unpublished) study, one rat at 0.1 mg/kg-day had TVM but no LCT. In the Dulak et al. (1989, unpublished) study, three rats with TVMs but not LCTs were also identified, two at 0.5 mg/kg-day and one at 2.0 mg/kg-day (see Table 3)³. As shown in Table 3, two of these three animals were reclassified as focal mesothelial hyperplasia, rather than mesothelioma, in the Iatropoulos et al. (1998) unpublished reanalysis of the Dulak/Friedman pathology data. One animal was

³ Table 3 also shows one animal for which the reanalysis determined no mesothelial tissue change and no LCT. This animal is shown as having both TVM and LCT in the unpublished individual animal data from the original study.

classified as a benign mesothelioma. All of the analysis by Iatropoulos and colleagues was conducted blind using pre-established criteria. The TVM reported in the Johnson et al. (1984, unpublished, 1986) study in the absence of LCT was confirmed by a recent pathology working group re-evaluation of that study by Wall et al. (2006) and supporting individual animal data provided by Wall (personal communication). Results from the 2006 pathology working group did not change the mesothelioma diagnosis for this rat. Thus, our analysis of the unpublished data suggests that TVM in the absence of LCT is possible. Similarly, if the hyperplasia is a precursor to mesothelioma, and if LCT is a necessary precursor to TVMs, one would expect the hyperplasia to occur only in the presence of LCT, contrary to the observations. The lack of complete concordance suggests that some TVMs can arise independently of LCTs.

Alternatively, both tumor types may be responding to the same external signal. This latter hypothesis is consistent with the finding by Iatropoulos and colleagues of focal mesothelial hyperplasia in the absence of LCTs. Overall, the small number of male rats without an LCT made this evaluation difficult. Even if there were a few animals with TVM but not LCT, it is not known whether these specific tumors were related to acrylamide exposure, in light of the background incidence of TVMs.

As noted above, Iatropoulos et al. (1998) evaluated the animals initially diagnosed with proliferative mesothelial lesions in the Friedman et al. (1995) study, and reported that the degree of progression of the lesion correlated with the size of the LCT, with benign TVMs associated with LCTs occupying $\leq 50\%$ of the testis, and malignant TVMs associated with LCTs occupying

75% or more of the testis⁴. While these data suggest an association between LCT and TVM in acrylamide-exposed rats, a definitive determination on this association in the acrylamide-exposed animals is limited by the absence of information on LCT size in the animals without TVMs. In addition, it should be noted that correlation is insufficient to show causation, and both the LCTs and TVMs could be responding to the same stimulus, without there being a direct causative relationship between LCTs and TVMs. Additional limitations to using these data were noted in the previous section. Similar studies on the relationship between LCT size and TVM progression were not located for other chemicals.

To further investigate the relationship between LCTs and TVMs, we surveyed the literature on TVMs to determine whether TVMs can occur in the absence of LCTs, and if so, under what conditions. As part of this evaluation, the occurrence of TVMs in strains other than the F344 rat was also investigated. A number of situations were located in which chemicals increased TVMs without increasing LCTs, in several different strains. Berman and Rice (1979) administered a single i.p. injection of methyl(acetoxymethyl)nitrosamine (DMN-OAc) to F344, Sprague-Dawley, and Buffalo rats, and observed TVMs in all strains. Although “numerous” LCTs were reported, particularly in F344 rats, the authors stated that there was no strong correlation between TVMs and LCTs in all of the rat strains, and only a slight correlation (chi square $p = 0.1$) in F344 rats. The authors noted that the tunica vaginalis was uniquely susceptible over other mesothelial tissue in this study, and that fluid given by i.p. injection readily reaches the testes, since the peritoneal extension that covers the testes is patent (exposed) in the rat. In another i.p. study, nitroso-5,6-dihydrouracil (NO-DHU) caused mesothelioma of the testes (implied to be TVMs) in

⁴As noted above, regardless of whether Iatropoulos et al. (1998) were truly distinguishing between benign and malignant mesotheliomas, the tumors described as falling into the latter category were of greater severity or further progression, and were associated with the larger LCTs.

Wistar rats (Pelfrene and Garcia, 1975). Thus, although the use of i.p. injection may have increased the exposure (and thus susceptibility) of the tunica vaginalis, this study shows that TVMs can arise in multiple strains, and in the absence of LCTs. It is also noted that these two chemicals are potent mutagens, and so might be causing TVMs via a different MOA from other chemicals. TVMs were also increased in MRC rats fed nitrosopyrrolidine for 67 weeks; half of the animals with TVMs also had LCTs (Greenblatt and Lijinsky, 1972).

Several other chemicals were reported to increase TVMs in F344 rats, with no associated reported increase in the incidence of LCTs, although the high background of LCTs in this strain may have precluded the detection of effects on LCTs. Data were not available to evaluate a correlation with LCT size. Inhalation exposure to ethylene oxide (a classic point mutagen structurally related to glycidamide) caused increases in peritoneal mesotheliomas (described as being generally present on the tunica vaginalis), along with leukemia and brain tumors in F344 rats (Lynch et al., 1984; Snellings et al., 1984). In another study in F344 rats, inhalation exposure to ethylene dibromide (EDB, dibromoethane) increased TVMs in males (associated with testicular degeneration) and mammary fibroadenoma and adenocarcinoma in females, as well as hemangiosarcomas and nasal cavity tumors in both sexes (NTP, 1982); neither the TVMs nor the mammary tumors were reported in the parallel inhalation mouse study (NTP, 1982), nor in a gavage study with EDB in Osborne-Mendel rats (NCI, 1978), although the gavage study did also report increased thyroid follicular cell adenomas. Bromate also increased TVMs in F344 rats (Crosby et al., 2000; Kurokawa et al., 1983), as well as in a variety of other tissues, including the thyroid and kidney. Bromate appears to act primarily via oxidative stress, although it may also have some direct DNA reactivity. Other chemicals reported to cause increases in

TVMs in F344 rats in NTP studies include glycidol (NTP, 1990), o-nitrotoluene (NTP, 2002), and cytembena (NTP, 1981). Thus, there have been several reports of TVMs without associated increases in the incidence of LCTs. However, in light of the high background of LCTs, detecting an effect of LCTs is difficult, and an effect may have been missed. No other studies were located that evaluated the association between LCT size and TVMs, as done by Iatropoulos et al. (1998).

Finally, review of a compendium of mesothelioma data revealed that virtually all reported TVMs in rats, with the few exceptions noted here, were in F344 rats (Ilgren, 1993). (There may have been some additional cases, since some mesotheliomas are noted as peritoneal mesotheliomas, but are really TVMs.) TVMs were also noted in dogs, but no mesotheliomas were reported in mice (Ilgren, 1993), and the incidence of mesotheliomas (benign or malignant, not otherwise specified) in the NTP historical control database for *mice* was 0.17% in males and 0% in females. Thus, the vast majority of chemical-related TVMs occur in male F344 rats. The exceptions were TVMs associated with intraperitoneal exposure (a non-environmentally-relevant route that results in high exposure of the tunica vaginalis), and ones caused by nitroso compounds.

We also attempted to evaluate the converse, whether increases in the incidence of LCTs have been reported without accompanying increases in TVMs. The review on LCTs by Cook and colleagues (Cook et al., 1999) formed the starting point for this analysis. This review presents a compilation of chemicals that caused Leydig cell hyperplasia or adenoma, broken down by MOA and chemical class, along with a listing of other tumor sites reported for each respective study.

These authors also noted the difficulty of identifying effects on LCTs in F344 rats, and that their judgments of effects on LCT incidence in this strain were equivocal. None of the chemicals listed in the review were reported as also inducing TVMs. Spot-checking of a small number of studies confirmed that the selected published studies did not report any increase in TVMs or in mesotheliomas in general. However, TVMs may have been missed in standard histopathology analyses, since the inside surface of the scrotum is not typically evaluated. Thus, even in F344 rats, it is not clear whether an increase in LCTs necessarily leads to an increase in TVMs. If LCTs occurred without an increase in TVMs, this would suggest that some additional influence(s) is needed for increased LCTs to lead to increases in TVMs; this additional factor is not currently known. Furthermore, an increase in LCT incidence may occur without an increase in LCT size, and it is not known whether increased LCT size occurred in any of these studies.

Thus, the overall data on LCTs and TVMs suggest that there is substantial consistency, but not full concordance; TVMs can occur without LCTs, and LCTs (e.g., in strains other than F344 rats) can occur without a corresponding increase in TVMs. It appears that there is stronger concordance for the acrylamide data, but this cannot be fully evaluated in the absence of information on LCT size in animals that did not have TVMs. Conversely, the data are not available to evaluate the possibility of an association between LCT size and TVMs for other chemicals. Overall, the available information on concordance supports the idea that LCTs may be a precursor to TVMs both in F344 rats and in acrylamide-exposed rats. However, the incomplete nature of the concordance suggests the possibility of additional causative factors. LCTs may be one of multiple pathways for development of TVMs (i.e., one of multiple potential precursors), with contributions from mutagenicity and/or other hormonal influences.

Alternatively (or in addition), some other causative factor may be responsible for both the increase in LCTs and in TVMs. One way for this to occur would be if both tumor types are regulated by the same hormones. Another possibility is that an effect of acrylamide external to the testis predisposes F344 rats to the development of TVMs, and this predisposition is enhanced by increased testis size associated with LCTs. This two-part effect might explain the much lower incidence of TVMs compared to LCTs. All of these hypotheses are consistent with the finding of less severe or precursor lesions (benign mesothelioma and focal mesothelial hyperplasia) in animals with no LCTs.

Communication Between the Tunica Vaginalis and Leydig Cells and Other Tissues

Shipp et al. (2006) noted two possible mechanisms for TVM formation, both related to Hypothesis A, above. One hypothesis is that TVMs result from hormonal imbalance. This hypothesis builds on the observation of O'Shea and Jabara (1971) that subcutaneous exposure of dogs with stilbesterol resulted in proliferative lesions and papillary growths of the genital serosa. The authors attributed nongenital serosal proliferative lesions to metastases.

This idea of a hormonal mechanism for TVM formation is consistent with both the idea that TVMs are secondary to LCTs, and with the suggestion that both LCTs and TVMs may reflect the changes in the same hormones. Since both Leydig cells and the tunica vaginalis occur in hormonally active tissue, we investigated the possibility of direct hormonal communication. The tunica vaginalis fluid contains elevated levels of a number of hormones, suggesting the possibility of both endocrine and paracrine regulation of the tunica vaginalis tissue. Based on an

analysis of the tunica vaginalis fluid in infertile men, Gerris and Shoysman (1984) found that levels of testosterone and other androgens were higher in tunica vaginalis fluid than in serum, while levels of LH, follicle-stimulating hormone (FSH), and prolactin were lower in the tunica vaginalis fluid. They suggested that intratesticular steroid concentrations are directly related to the concentrations in the tunica vaginalis fluid, due to a direct continuity between the peritubular interstitial space in the testis, the rete testis fluid and the interstitium around the vasa efferentia and epididymal duct. Rat and human mesothelial cells respond to and/or produce growth factors such as PDGF, EGF, and TGF- β 1, although the direction and magnitude of the response differed between species (Gabrielson et al., 1988; Gerwin et al., 1987; Walker et al., 1991). These growth factor responses suggest the possibility of paracrine and autocrine regulation of TVMs, although no data specifically on growth factor response of tunica vaginalis cells were located. Overall, these data support the idea that tunica vaginalis cells receive hormonal input from a number of sources, but no evidence was located for any direct hormonal communication between Leydig cells and the tunica vaginalis.

The overall pattern of tumors following acrylamide exposure also suggests hormonal involvement, since the three target tissues for which tumors were consistently reported by both Johnson et al. (1986) and Friedman et al. (1995) are in hormonally-responsive tissues (thyroid, mammary gland, and tunica vaginalis). However, even in the presence of such a hormonal MOA, there is still the possibility that acrylamide acts as a weak mutagen in a sensitive tissue. Tissues could be sensitive due to lower DNA repair capacity, high cell proliferation under endocrine or other control, or local metabolism that leads to proportionally greater activation at the tumor site. The data for acrylamide are not sufficient to eliminate any of these possibilities.

The second alternative under Hypothesis A for a relationship between LCTs and TVMs is based on the work of Tanigawa et al. (1987). This hypothesis suggests that the relationship between LCTs and TVMs is physical, with enlargement of the Leydig cells resulting in physical stimulus on the mesothelium similar to a solid state/foreign body response.

The suggestion that LCTs influence TVM development based on a physical interaction is plausible, in light of the close physical relationship of the tissues. As rats age, their increase in body weight puts increased weight on the scrotum, resulting in increased pressure and irritation on the tunica vaginalis. Development of LCTs would be expected to increase this pressure. The pressure and irritation would be expected to be highest at the mesorchium, which has been reported as the most common site on the tunica vaginalis for TVMs and associated preneoplastic lesions (Crosby et al., 2000). Physical pressure can also induce mesothelial cells to release growth factors (Waters et al., 1997), which could lead to tumor production. The upright position in which humans locomote would lead to much less pressure at the mesorchium in humans. Although there are no data available to directly test this hypothesis, such data could be obtained using tissue from the Johnson et al. (1986) and Friedman et al. (1995) bioassays, or from the currently-ongoing NTP study of acrylamide, to measure the testes and determine if increased testis size is associated with TVM in the same testis. This information could not distinguish between a paracrine interaction and direct pressure, but it could provide data countering an endocrine MOA, and, if a correlation were found, would strengthen the preliminary findings from the unpublished Iatropoulos (1998) study. Conversely, increased testis size associated with TVM in the testis on the other side would indicate a decoupling of the LCT and TVM.

Based on these considerations, the data are strongest for the hypothesis that both LCTs and TVMs reflect a broader hormone imbalance. The exact nature of this imbalance has not been described, and so key events are not sufficiently known to fully evaluate this as a potential MOA. While there are exceptions to the hypothesis that LCTs are a necessary precursor to TVM formation, the data are consistent with the hypothesis that an influence external to the testis predisposes the tunica vaginalis to tumors, and that this predisposition is promoted by increased LCT size.

Hypothesis B

Genotoxicity as a Potential MOA

A number of reviews have summarized the available genotoxicity data on acrylamide (Dearfield et al., 1988; Dearfield et al., 1995; EU, 2002; OEHHA, 2005; Shipp et al., 2006). Rather than describing the genotoxicity data in detail here, the reader is referred to the companion paper by Dourson et al. (2008), who conducted a detailed evaluation of the genotoxicity data on acrylamide with particular attention to the in vivo data and consideration of the modified Hill criteria with respect to genotoxicity. Although Dourson et al. (2008) evaluated the data with respect to MOA for thyroid tumors, the same general considerations apply to TVMs. Overall, the genotoxicity data indicate that acrylamide is clastogenic, and that its metabolite glycidamide is a mutagen in vivo and in vitro.

In light of the clear evidence that the acrylamide metabolite is mutagenic, the potential for a mutagenic MOA cannot be ruled out based on a classical analysis of genotoxicity data.

However, the finding that a chemical (or its metabolite) is mutagenic is not sufficient to show that the chemical causes specific tumors via a mutagenic MOA (U.S. EPA, 2007). For that determination, it is necessary to evaluate the mutagenic MOA in light of the modified Hill criteria (U.S. EPA, 2005)

The data showing glycidamide mutagenicity are consistent, with clearly positive results, as summarized in the above reviews. In vivo studies show that oral dosing with [¹⁴C]-acrylamide results in the formation of DNA adducts of glycidamide in a wide range of tissues, but no clear relationship between adduct formation and the sites at which acrylamide causes tumors has been observed. Specifically, studies in mice and rats have found similar levels of DNA adducts following in vivo exposure to acrylamide in the target organs for tumor development in rats (thyroid, mammary gland and testes) and non-target tissues (liver, lung, kidney, spleen, and brain); the target tissues in the mouse are not known, but no tissue specificity of DNA adducts was reported in mice (Doerge et al., 2005; Maniere et al., 2005; Segerback et al., 1995).⁵ The lack of association of DNA adduct formation with tumor formation suggests that events other than DNA adduct formation (at least the specific adducts identified) are needed to explain the pattern of tumors. These toxicodynamic processes could be further evaluated by evaluating site concordance between mutations (not merely adducts) and tumors; such data are not currently available.

⁵ Note that, while the testis is listed here as a target tissue, the tunica vaginalis comprises a very small proportion of the total testicular cell content, and no acrylamide-related tumors were reported at other testicular locations. Therefore, even if adduct formation were increased in the tunica vaginalis, it is unlikely that it would have been detected in an assay of the whole testis.

To evaluate dose-response and temporal considerations, results from transgenic animals were considered. These test systems have easily retrievable markers for detecting mutations, allowing the in vivo detection of somatic cell gene mutations. Small, but consistent and statistically significant increases over controls in mutant frequencies have been reported in several in vivo gene mutation assays with acrylamide (Hoorn et al., 1993; Manjanatha et al., 2006; Myhr, 1991). These studies provide qualitative confirmation that in vivo exposure to acrylamide results in gene mutations. However, it is more problematic to evaluate whether these mutations were a key event in the development of tumors following acrylamide exposure (or a marker for such an event), because the available studies were in mice. In addition, the tissues evaluated in the mouse (bone marrow, lymphocytes, liver) are not tumor targets for acrylamide in the rat. Preliminary gross pathology data from the ongoing NTP study in the mouse point to the liver, Harderian gland, and possibly the lung as tumor targets (Doerge, 2008). Tumors in the Harderian gland are associated with a mutagenic MOA in general. Conversely, it is of interest that increased mutation frequency in the liver of Big Blue® mice was observed only at 107 mg/kg-day, but not at 25 mg/kg-day (Manjanatha et al., 2006), a dose well above the high dose reported by Doerge (2008) as being associated with increased liver tumors. (See Table 2 of Dourson et al., 2008). This appears to violate the dose-response criterion for mutations causing liver tumors.

Klaunig and Kamendulis (2005) treated male F344 rats with acrylamide in drinking water at 15 mg/kg-day for up to 28 days (for measurement of unscheduled DNA synthesis) or 7 days (for the Comet assay, which evaluates single strand breaks and alkali-labile sites). Increased DNA

synthesis was seen in target tissues (thyroid, adrenal medulla, and testicular mesothelium), but not in nontarget tissue (liver). DNA damage was also seen in the thyroid and adrenal, but not in the liver; DNA damage was not measured in the testicular mesothelium. The mechanism for these tissue-specific DNA reactivities is not clear. In a similar experiment by the same group, (Lafferty et al., 2004), inhibition of oxidative metabolism of acrylamide reduced acrylamide-induced DNA synthesis only in the adrenal medulla. In the testicular mesothelium, this effect was not apparent (Lafferty et al., 2004). Effects in the thyroid were equivocal; the metabolic inhibitor itself increased DNA synthesis, and acrylamide in the presence of inhibitor did not further increase DNA synthesis. Overall, these data suggest the involvement of glycidamide in the induction of DNA synthesis and presumably adrenal medullary pheochromocytomas, but that the observed DNA synthesis in the testicular mesothelium was not related to glycidamide, the presumed mutagenic metabolite; results regarding the role of glycidamide in the thyroid were inconclusive. Lafferty et al. (2004) noted that the induced DNA synthesis could reflect either DNA repair or cell proliferation, although Klaunig and Kamendulis (2005) stated that the data suggest that DNA reactivity and cell proliferation may both contribute to the observed tumors.

Acrylamide has also been shown to be genotoxic to male germ cells (Dearfield et al., 1988; Dearfield et al., 1995; EU, 2002; OEHHA, 2005; Shipp et al., 2006), an effect that may be due both to acrylamide reacting with protein or protamines and direct DNA reactivity of glycidamide. Although these data show that acrylamide reaches cells in the vicinity of the tunica vaginalis, the germ cells are physically separate from the tunica vaginalis and result from different tissue, and so these results are not directly relevant to the issue of a potential mutagenic MOA for TVMs.

Overall, the data are clear that DNA damage and DNA adducts are formed by the acrylamide metabolite, glycidamide, but the degree to which these lesions are formed in vivo, and the relationship between these lesions and tumors, is unclear. The broad distribution of DNA adducts does not provide a direct explanation of the observed tumor targets, but a role of DNA mutagenicity cannot be ruled out. Interestingly, ethylene oxide, a mutagenic carcinogen that is structurally related to glycidamide, also causes TVMs (along with other tumor types not seen with acrylamide).

Overall, a mutagenic MOA is biologically plausible, based on the mutagenicity of glycidamide and the known relationship between mutations and tumors. Inconsistencies in the database (related to the criterion of coherence) were noted above. Insufficient data are available to fully evaluate dose-response, particularly for the TVM. However, the dose-response data are not consistent for liver tumor induction in mice by gene mutations. Although glycidamide is a clear mutagen (strength), the absence of a clear relationship between the tissues in which DNA adducts are formed and the sites at which acrylamide causes tumors shows that specificity is not met. The data are insufficient to evaluate temporality for a mutagenic MOA.

In summary, TVM induction by a mutagenic MOA is plausible in general. However, the body of evidence for acrylamide and its metabolite glycidamide reviewed above suggests that although mutagenicity cannot be ruled out, to the extent that mutagenicity contributes to tumor formation, it likely acts in concert with other MOAs. To the extent that a mutagenic MOA may contribute to the tumor response in rats, a mutagenic MOA is plausible in humans.

Oxidative Stress

Only very limited data are available to evaluate the potential contribution of oxidative stress, but these data are consistent in suggesting that oxidative stress may play a role in acrylamide carcinogenesis. These data are discussed more fully by Dourson et al. (2008) and Maier et al. (2008). In vitro studies have found that acrylamide reduces glutathione levels (Klaunig and Kamendulis, 2005; Park et al., 2002) and induces DNA damage similar to the damage induced by oxidative stress (Chico-Galdo et al. 2006). In addition, oral exposure of Sprague-Dawley rats to acrylamide resulted in up-regulation of genes related to cellular redox in the testis; separate data were not available for the tunica vaginalis (Yang et al., 2005). Finally, as noted in the next section, potassium bromate, a chemical for which oxidative stress appears to play a significant role in carcinogenicity, also causes increased TVMs. While these data suggest that further exploration of an oxidative stress hypothesis would be of interest, the data are insufficient for evaluation of the modified Hill criteria.

Hypothesis C

Other MOAs

In analyzing the data on TVMs and LCTs, several interesting similarities between acrylamide and several chemicals that caused TVMs were noted. Although we were not able to identify any unifying hypotheses, these associations are noted here. For example, potassium bromate causes increases in both TVMs and thyroid tumors, as well as kidney tumors in F344 rats (Kurokawa et

al., 1986; Kurokawa et al., 1983). As noted earlier, oxidative stress and glutathione depletion appear to play important roles in bromate carcinogenicity. TVMs were also increased in F344 rats exposed to pentachlorophenol (NTP, 1999). This chemical is negative or weakly positive in genotoxicity assays, and also causes oxidative stress (ATSDR, 2001).

Maier et al. (2008) raised the possibility of a consistent unifying hormonal control mechanism that is related to development of tumors in the three primary targets for acrylamide in the F344 rat (thyroid, mammary gland, and tunica vaginalis). They noted that acrylamide could act 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. Specific data on the ability of acrylamide to induce toxicity in the hypothalamus are limited, and the specific pattern of effects caused by acrylamide on neuroendocrine regulation in the hypothalamus is difficult to decipher due to the paucity of data and the complexity of mapping neuroregulation in various brain regions. However, the data show that acrylamide can perturb normal hypothalamus structure and possibly function (at least at high doses). As for oxidative stress, the data are insufficient for evaluation of these other potential MOAs relative to the Hill criteria.

Synthesis

The above discussion supports the following conclusions regarding TVMs:

1. TVMs occur in humans at a very low frequency.

2. In rodents, TVMs occur almost exclusively in F344 rats and following direct exposure of the tunica vaginalis in other strains following i.p. injection. This suggests that TVMs are related to the unique characteristics of the hormonal milieu in F344 rats. TVMs have also been reported occasionally in other species.
 - a. The observation that TVMs occur preponderantly in F344 rats means that some aspect of the biology of this strain makes it particularly susceptible to this tumor type. The specific factor making the F344 rat susceptible is not known.
 - b. No increase in mesothelial tumors were reported in the female F344 rats in the acrylamide bioassays, indicating that the acrylamide-related TVMs do not reflect a general tumorigenic influence on mesothelial tissue. Based on an analysis for this assessment, there was a statistically significant increase in total mesothelial tumors in the Friedman et al. (1995) study, and a statistically significant increase in the Johnson et al. (1986) study that was not dose-related. However, in both studies, all of the acrylamide-exposed rats with mesothelial tumors also had TVMs.
3. Hypothesis A is that TVMs seen after acrylamide exposure are secondary to the enhancement of LCTs in F344 rats. This relationship between TVMs and LCTs could be endocrine, paracrine, or the result of a physical interaction, such as pressure from the increased size of testes bearing LCTs.
 - a. The background incidence of LCTs is too high to observe an effect of acrylamide on LCT incidence. It may be possible to evaluate the effect of acrylamide on LCT size, but this study has not been conducted.

- b. Data are available suggesting that acrylamide increases LCT size, but the data are weak to moderate.
- c. The evidence is stronger for an effect of acrylamide on earlier key events in the development of LCTs. The strongest data support the hypothesis that acrylamide affects LCT development by acting on levels of prolactin and/or testosterone, but the data are insufficient to definitively determine how these effects occur. Increased dopaminergic activity is consistent with the observed effects on prolactin and testosterone, and interactions of acrylamide with the dopaminergic system have been documented, but there is no clear evidence showing acrylamide to be a dopamine agonist.
- d. If acrylamide does affect LCTs via increasing dopaminergic activity, that MOA for LCT development is not relevant to humans.
- e. The other MOAs for the formation of LCTs described by Clegg et al. (Clegg et al., 1997) are all nonmutagenic, and if acrylamide were to increase LCT incidence or size via any of these MOAs, a nonlinear approach would be used for low-dose extrapolation for an effect on LCTs.
- f. The data regarding a causal connection between LCTs and TVMs are weaker than the data supporting an effect of acrylamide on LCTs, but an association is observed. The physical proximity of the tumors and substantial concordance between the size of LCTs and progression of tunica vaginalis tumors following acrylamide exposure suggests a relationship between the two tumor types, but there are several limitations to that study. TVMs are found almost exclusively in the presence of LCTs, although there is not complete concordance between these

two tumor types in the overall literature. Concordance appears to be stronger for acrylamide than in the overall literature.

- g. The data reviewed are not sufficient to distinguish between there being a causal relationship between LCTs and TVMs, and the hypothesis that these tumor types respond to some other influence (e.g., hormonal milieu of the F344 rat). Both mechanisms may apply. For example, an effect external to the testis may predispose F344 rats to the development of TVMs, with this predisposition being enhanced by increased testis size due to an effect of acrylamide on LCTs. This is consistent with the observation that some TVMs or precursors are found in the absence of LCTs.
 - h. Based on the MOA(s) for LCT formation, the proportion of TVMs that are secondary to LCT formation would either (1) not be considered relevant to humans (if they result from increased dopaminergic activity) or (2) a nonlinear approach would be appropriate for extrapolation to low doses.
 - i. No evidence of direct hormonal communication between Leydig cells and the tunica vaginalis was located.
4. Hypothesis B is that TVMs seen after acrylamide exposure result from mutagenicity or other DNA reactivity of acrylamide or its metabolite glycidamide on the tunica vaginalis. This effect may be enhanced by endocrine or paracrine influences.
- a. The overall data on mutagenicity do not support mutagenicity being the primary cause of the TVMs, but a small contribution of mutagenicity to the development of these tumors is plausible.

- b. Acrylamide could also cause mutations via indirect mechanisms of reaction with DNA, such as resulting from oxidative stress. Linear low-dose extrapolation would not be expected to be appropriate for an oxidative stress MOA.
5. Hypothesis C is that TVMs seen after acrylamide exposure result from some other (as-yet-unidentified) hormonal signal that may also play a causal role in LCT development, with the TVMs occurring in parallel with the LCTs, rather than being secondary to them. Insufficient data are available to evaluate this hypothesis.
6. Finally, LCTs may be one of multiple pathways for development of TVMs (i.e., one of multiple potential precursors), with contributions from mutagenicity and/or endocrine influences (Hypotheses A, B, and C).
7. The data are insufficient to definitively show any one MOA occurs.
8. The relevance to humans of the TVMs remains a possibility, but if the tumors occur in humans, the potency would be expected to be much lower than in F344 rats.
9. Overall, these data suggest that a mutagenic MOA cannot be ruled out, and may be responsible for a small percentage of the total tumor response, but a nonmutagenic MOA is more likely driving the tumor response.

Quantitative Considerations

As a test of approaches for extrapolation from the F344 rat TVM data to humans, we compared the incidence of TVMs predicted based on linear extrapolation from the rat data and average dietary intake of acrylamide with data on the reported incidence of TVMs in humans. Because registry data on TVMs per se are not available, the incidence of TVMs was estimated based on case reports of TVMs. In addition, comparisons were done for mesotheliomas in general, in case

the target tissue is mesothelial tissue in general, rather than mesothelial tissue of the tunica vaginalis. This latter comparison was done first using the SEER cancer registry of NCI, either directly, based on peritoneal and retroperitoneal mesotheliomas (to exclude asbestos-related pleural mesotheliomas) (Young et al., 2007). SEER data were also used indirectly, based on the analysis by Greenberg et al. (2002) of the background (non-asbestos related) mesothelioma incidence, based on the SEER data.

The incidence of TVMs was not located through any standard database, but the SEER database of the NCI collects data on total mesotheliomas, broken down into mesotheliomas of the pleura and lung, and mesotheliomas of the peritoneum and retroperitoneum. All mesotheliomas are considered rare tumors in humans. Based on reporting of all tumors in people aged 20 and older in a geographic area representing about 14% of the U.S. population, Young et al. (2007) reported the number of mesotheliomas in the period 1988-2001. The authors reported 3148 mesotheliomas of the lung and pleura. These tumors result primarily from asbestos exposure, although there may also be contributions from other causes. The authors reported 354 peritoneal and retroperitoneal mesotheliomas (212 in males and 142 in females); separate data for peritoneal mesotheliomas alone were not available. The total mortality from human mesothelioma that is not related to occupational exposure to asbestos and other chemicals is estimated at about one in a million (Greenberg et al., 2002).

In the absence of a registry collecting data specifically on TVMs, information on this tumor type was identified from the literature and a published review. Approximately 80 cases have been reported in the literature in the period from 1966 through 1996, with about a third of these cases

associated with asbestos exposure (Plas et al., 1998; Spiess et al., 2005). Although not every case necessarily results in a published case report, the fact that individual case reports merit publication indicates the rarity of these tumors. The actual incidence of TVMs in humans is not known, but TVMs are estimated to account for less than 5% of the malignant mesotheliomas in humans (Serio et al., 1992).

One could develop a very conservative estimate of the risk of TVMs in humans based on the rat data using either a linear or nonlinear extrapolation from an LED10. If one considers that the rat TVMs are relevant to humans and that a linear extrapolation to low doses is appropriate, then the TVM risk in humans can be estimated using the potency estimate and estimated acrylamide intake. CalEPA (2005) calculated upper bound human potency estimates for TVM of 0.58 and 0.4 per mg/kg-day from the Johnson et al. (1986) and Friedman et al. (1995) studies. JECFA (2005) estimated that average acrylamide intake at the national level ranged from 0.3 to 2.0 $\mu\text{g}/\text{kg}\text{-day}$. For high percentiles consumers (90th to 97.5th percentiles), intake estimates ranged from 0.6 to 3.5 $\mu\text{g}/\text{kg}$ bw per day, and up to 5.1 $\mu\text{g}/\text{kg}$ bw per day for the 99th percentile consumer. JECFA stated that children appeared to ingest approximately two to three times the adult intake when expressed on a body weight basis.

Using the average of the two slope factors calculated by CalEPA (2005) of 0.49 per mg/kg-day and an average intake of 2 $\mu\text{g}/\text{kg}\text{-day}$ would result in a risk of TVMs in the human population of 0.00098, or a risk of almost 1 in a thousand. Of the available epidemiology studies of acrylamide and cancer, studies of the Marsh cohort (Marsh et al., 1999; Marsh et al., 2007) investigated the incidence of cancer of the testis and male genital tract. No effect was seen,

although the absolute numbers of observed and expected cancers was very low (1-2 in a cohort of up to 8508), and the statistical power to detect an increase was low, as reflected by the broad confidence limits (Erdreich and Friedman, 2004). However, the very low background incidence of TVMs in humans would make a small increase on the order of 10^{-4} or 10^{-5} easily detectable. Furthermore, an increase of 1 in a thousand would be quite evident, and likely would be reported in the literature, independent of any association with acrylamide exposure. Thus, this screening-level evaluation based on cancer risks extrapolated from the TVM data in rats is inconsistent with the human data on TVM incidence. This inconsistency could reflect a biological difference between F344 rats and humans. Alternatively, the discrepancy could be because the screening-level quantitation used a linear extrapolation, while a biphasic approach may be more appropriate.

The data on TVM incidence in humans can be used to provide some bounds on the acrylamide-associated risk of TVMs, using the report of Plas et al. (1998) that a total of approximately 80 TVMs have been reported in humans in the world literature. Although not every TVM will have been reported in the literature, the value of 80 is reasonable as a bounding estimate, since at least a third of the TVMs are associated with asbestos exposure (Plas et al., 1998; Spiess et al., 2005). It is also noted that the human cases are identified predominantly by palpation or as the result of infertility problems, while the animal cases were identified by histopathology. This would lead to an underestimation of TVMs in humans relative to the incidence in rats. However, for risk assessment comparison purposes, a factor of three would be a reasonable approximation of the magnitude of this underestimation. As possible support of the idea that this underestimation is not large, more than 50% of the TVMs in the acrylamide bioassays were macroscopically

detectable (Damjanov and Friedman, 1998; our review of the unpublished individual animal data). There is considerable uncertainty in the estimate of the actual number of TVMs not associated with asbestos exposure, but this estimate is sufficient to provide a reasonable bounding estimate on the prevalence of TVMs related to acrylamide exposure.

The denominator for the population associated with the 80 reported TVMs is not known, but it is reasonable to estimate that the physicians in the U.S. and Western Europe are most likely to publish identified cases of TVMs in humans. Therefore, the denominator was estimated as the U.S. population of 300 million (United States Census, 2007) plus the EU population of 490 million (CIA, 2007). Males are slightly less than half the population (Intute, 2007), and so the relevant male population in the U.S. and the EU can be estimated at about 395 million. Thus, a reasonable bounding estimate of the frequency of TVMs related to acrylamide exposure is 80/395 million, or a risk of 2 per 10,000,000. This is more than three orders of magnitude smaller than the risk estimated directly from the rat data using a linear extrapolation, and below de minimis levels. Based on these considerations, quantitative extrapolation from the rat TVMs to risk in humans is not appropriate. In light of the generally similar toxicokinetics of acrylamide in rats and humans, the quantitative differences can be attributed to toxicodynamic differences.

Similar calculations were also conducted for the broader class of mesotheliomas, in case the target tissue is mesothelial tissue in general, rather than mesothelial tissue of the tunica vaginalis. Considering mesotheliomas as a whole has the advantage that SEER cancer registry data are available for mesotheliomas, but the disadvantage that mesotheliomas are broken only into (1)

pleural and lung mesotheliomas and (2) peritoneal and retroperitoneal mesotheliomas. Because much of the total mesotheliomas incidence in humans is due to asbestos exposure, assuming that all observed mesotheliomas result from acrylamide exposure would not make sense.

Based on extrapolation from the SEER data on mesotheliomas, and correcting for the percentage of the population covered by the SEER reporting (14%), one can estimate 2529 peritoneal and retroperitoneal mesotheliomas in the U.S. in the period 1988-2001, an incidence much higher than that reported for only tunica vaginalis mesotheliomas. Based on an average U.S. population of 263 million during that period, and 28% of the population being below 20 years of age, the at-risk population can be estimated at 189 million. Thus, the incidence of peritoneal and retroperitoneal mesotheliomas over the 13-year period is estimated at 1.3×10^{-5} , corresponding to a lifetime risk of 7×10^{-5} . As shown in Table 1, the incidence of TVMs in the acrylamide bioassays was virtually identical to the total incidence of mesotheliomas. Based on total mesotheliomas in the Friedman et al. (1995) study, the BMDL₁₀ can be estimated as 0.848 mg/kg-day; this corresponds to a dose of 0.23 mg/kg-day after adjusting by body weight^{3/4}. The corresponding slope factor for mesotheliomas is 0.43 per mg/kg-day, a value similar to that calculated by CalEPA (2005) for TVMs only,⁶ corresponding to a risk of 9×10^{-4} for mesotheliomas at an acrylamide daily dose of 0.002 mg/kg-day. Although the difference between the bounding estimate based on the human data for total mesotheliomas and the risk estimate from the rat data is smaller than the difference based on TVM cases alone, the difference is still large, even making the very conservative assumption that all non-asbestos-related mesotheliomas are due to acrylamide. No acceptable fit could be obtained to the Johnson et al. (1986) mesothelioma data, even after dropping the high dose.

⁶ It appears that CalEPA did not adjust for the background intake of acrylamide by the control group.

There are a number of uncertainties in these estimates. The biggest uncertainty relates to the contribution of asbestos exposure to the incidence of human mesotheliomas. Because a large percentage (but not all) of lung and pleural mesotheliomas are associated with asbestos exposure, these tumors were excluded from the above analysis. Conversely, some percentage of peritoneal mesotheliomas can be attributed to asbestos exposure. For example, Plas et al. (1998) reported a history of asbestos exposure in 34% of the TVM cases, and noted that the real prevalence of asbestos exposure may have been higher. In light of these uncertainties, perhaps a more relevant estimate of the human mesotheliomas not associated with occupational exposure to asbestos or other chemicals is that of Greenberg et al. (2002), who estimated a background (i.e., non-asbestos-related) mesotheliomas mortality rate of 1-2 in a million based on back-extrapolation from SEER data in men and women. (This “background” rate is comparable to the rate of 1 in a million that is often considered a de minimis risk in environmental risk assessment.) Comparing the projections from the rat data with the mortality from non-occupational mesotheliomas, it is clear that the risk of almost 1 in a thousand estimated from the rat data considerably overestimates the potential for acrylamide-related mesotheliomas, particularly since it would not be reasonable to expect that all non-occupational mesotheliomas are due to acrylamide.

In an analysis based on MOA, it is appropriate to focus on tumors related to the MOA under consideration, in this case TVMs or possibly total mesotheliomas. However, if a generic MOA such as genotoxicity is the primary determinant of the tumor response, the potential for the absence of tissue concordance should be considered. In such cases, one could evaluate tumor risk based on the combined incidence of all tumor types. However, such an analysis does not

appear to be appropriate (Vater et al., 1993), based on the conclusion presented above that the TVM response is predominantly not due to genotoxicity, as well as the determination that the thyroid and mammary tumor incidence at high doses is largely due to a non-genotoxic MOA (see Dourson et al., 2008; Maier et al., 2008).

The overall weight of the evidence concerning the MOA leads to the conclusion that the most appropriate estimate of human cancer risk based on the rat TVMs associated with acrylamide exposure is either de minimis or nil. Multiple MOAs are likely in the rat and some small contribution from a mutagenic MOA is plausible. However, the MOAs that most likely are driving the tumor response are either not relevant to humans or, if the risk to humans were estimated quantitatively, would be properly modeled with a nonlinear dose-response, with additional important differences in toxicodynamics leading to quantitative differences in response. Although the mutagenic MOA may explain some tumors, estimates of the incidence of human TVMs and total non-asbestos mesotheliomas, along with evidence supporting a nongenotoxic MOA, indicate that the risk to humans from the small fraction of tumors possibly attributable to mutagenicity would be de minimis.

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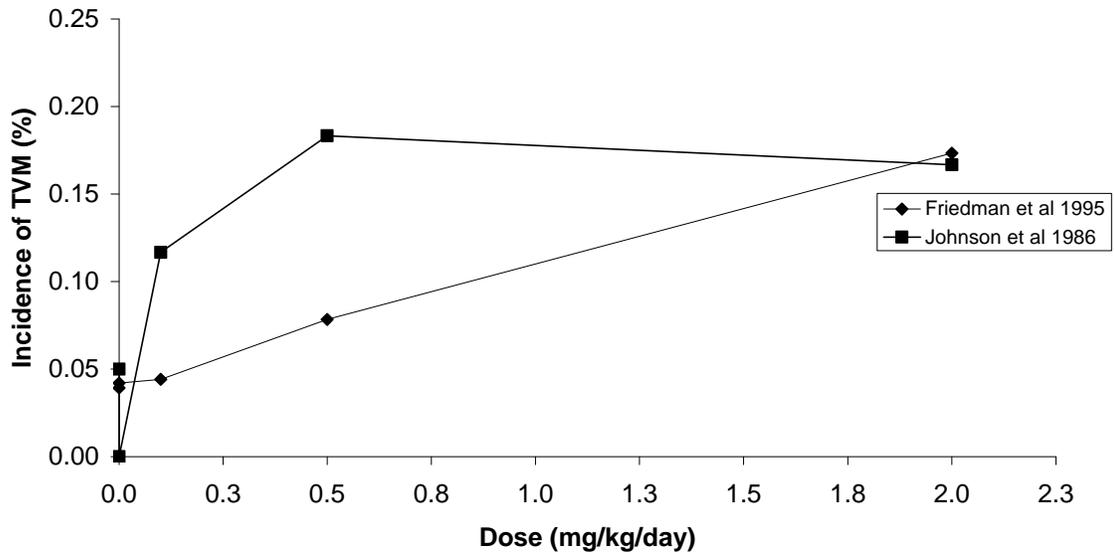
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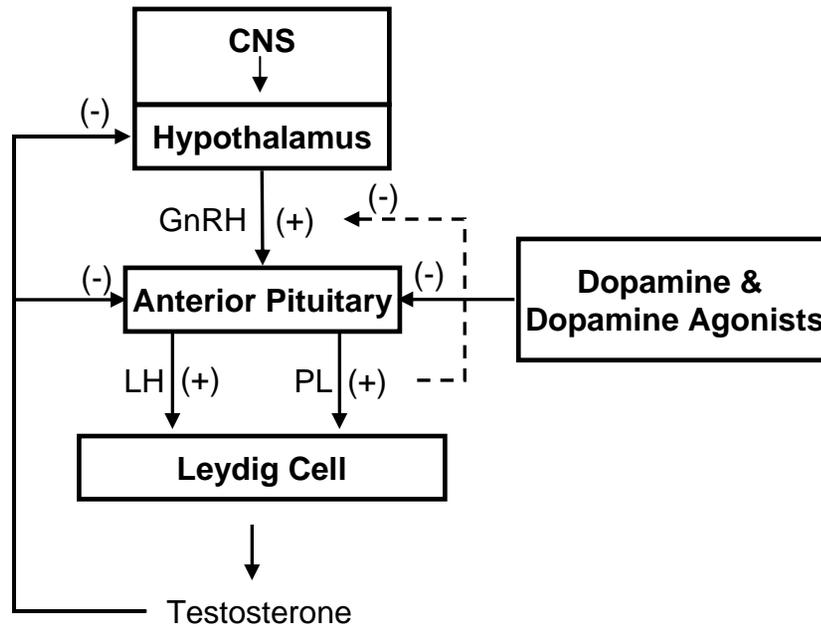
Figure Legends

Figure 1. Doses reported by the study authors were increased by 0.002 mg/kg-day to account for acrylamide levels in the basal diet (Dourson et al., 2008). In addition, the response % in the second control dose in the Friedman et al. (1995) study was adjusted from 0.039 to 0.042% so that it does not overlap with the response in the controls from the Johnson et al. (1986) study.

Figure 2. Hypothalamus-pituitary-testis pathway in normal rats and potential site of action of dopamine agonists.

Figure 1. Tunica Vaginalis Mesothelioma (TVM) in Male F344 Rats





- - - ➔ Denotes hypothesized feedback loop

Table 1. Incidence (% response) of TVMs and all Mesotheliomas in Male F344 Rats Exposed to Acrylamide

Study	Dose (mg/kg/day) ¹					
	0.0	0.0	0.01	0.1	0.5	2.0
Friedman et al. 1995						
n	102	102	--	204	102	75
Incidence (TVM only)	4 (3.9%)	4 (3.9%)	--	9 (4.4%)	8 (7.8%)	13* (17.3%)
Incidence (All mesotheliomas)	4 (3.9%)	4 (3.9%)	--	9 (4.4%)	8 (7.8%)	13¶ (17.3%)
Johnson et al. 1986						
n	--	60	60	60	60	60
Incidence (TVM only)	--	3 (5.0%)	0 (0.0%)	7 (11.7%)	11† (18.3%)	10† (16.7%)
Incidence (All mesotheliomas)	--	4 (6.7%)	0 (0.0%)	7 (11.7%)	11¶ (18.3%)	10¶ (16.7%)

¹Doses as reported by authors. Taking into account the background level of acrylamide, the actual doses were 0.002 mg/kg-day higher than shown (Dourson et al., 2008)

†Statistically significant, $\alpha=0.05$, as reported by study authors

**Statistically significant, $p<0.001$, as reported by study authors

¶Statistically significant, $p<0.05$, based on chi-square test conducted for this assessment

Table 2. Evaluation of Human Relevance for Leydig Cell Tumors Following Acrylamide Exposure

Event (Step #)	Evidence in animals	Reference for animal data	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?
Increased dopaminergic activity (#1)	Indirect. No data in pituitary, but 10 mg/kg-day for 20 days i.p. increased dopamine in caudate, which also has D2 receptors. Also data showing effect of acrylamide on dopamine receptor capacity at doses as low as 10 mg/kg-day for 10 days	Ali (1983) Agrawal et al. (1981a; 1981b); Agrawal and Squibb (1981); Bondy et al. (1981); Uphouse and Russell (1981)	No data but plausible.	No data but plausible.
Decreased prolactin (#2)	Yes. Decrease at ≥ 12 mg/kg-day for 28 days	Uphouse (1982); Ali et al. (1983); Friedman et al. (1999)	Yes. Prolactin release is controlled by dopamine in humans (Kreek 2002)	Yes.
Downregulation of LH receptors on Leydig cells (#3)	No data, but plausible based on observed decreases in prolactin	--	No. Human Leydig cells appear not to have prolactin receptors (Cook et al. 1999). Decreased prolactin does not decrease LH receptors in humans (Clegg et al. 1997)	No.
Increased GnRH (#4)	No data, but plausible based on observed decreases in prolactin and observation of downstream events	--	No data but plausible.	No data but plausible as an independent step. Not plausible in light of nonplausibility of Step 3 in humans

Decreased testosterone production (#5)	Yes – decreased testosterone levels at 14 and 28 days	Uphouse (1982); Ali et al. (1983); Friedman et al. (1999)	No data but plausible, based on GnRH action on pituitary.	No data but plausible as an independent step. Not plausible in light of nonplausibility of Step 3 in humans
Increased LH (#6)	Indirect. Increases in dopamine observed in caudate, and dopamine at high doses increases serum LH.	--	No data but plausible.	No data but plausible as an independent step. Not plausible in light of nonplausibility of Step 3 in humans.
Leydig cell tumors (LCT) (#7)	Suggestive. High background in controls, but larger size in presence of acrylamide	Iatropoulos et al. (1998)	No data but plausible.	Unlikely, based on human disease states (Clegg et al. 1997; Klaunig et al. 2003)

Table 3. Comparison of Mesothelial and Leydig Cell Diagnoses from Iatropoulos et al. (1998) Re-Read of Friedman et al. (1995)

Dose Group	Dose (mg/kg-day) ¹	Animal Number	Mesothelioma	Leydig Cell Tumor Size ²
Control 1	0	126	No mesothelial tissue present	3
		134	Benign mesothelioma, focal	1
		170	Malignant mesothelioma	3
		179	Benign mesothelioma, focal	2
Control 2	0	257	Malignant mesothelioma	4
		301	<i>Focal mesothelial hyperplasia</i>	1
		335	<i>Focal mesothelial hyperplasia</i>	1
		353	Malignant mesothelioma	3
			average	2.6
			average malignant	3.3
Low	0.1	432	No mesothelial change	0
		457	Malignant mesothelioma	4
		473	Malignant mesothelioma	3
		484	Malignant mesothelioma	4
		514	<i>Focal mesothelial hyperplasia</i>	1
		564	Malignant mesothelioma	3
		594	<i>Focal mesothelial hyperplasia</i>	1
		603	Malignant mesothelioma	3
		606	<i>Focal mesothelial hyperplasia</i>	1
				average
		average malignant	3.4	
Mid	0.5	729	Malignant mesothelioma	3
		732	Malignant mesothelioma	3
		756	Benign mesothelioma, focal	1
		758	Benign mesothelioma, focal	0
		762	Malignant mesothelioma	4
		767	<i>Focal mesothelial hyperplasia</i>	0
		780	Benign mesothelioma, focal	2
		783	Benign mesothelioma, focal	2
				average
		average malignant	3.3	
High	2	810	Benign mesothelioma, focal	1
		813	Malignant mesothelioma	3
		814	Benign mesothelioma, focal	1
		816	Malignant mesothelioma	3
		821	<i>Focal mesothelial hyperplasia</i>	0
		824	<i>Focal mesothelial hyperplasia</i>	1
		832	Malignant mesothelioma	3
		841	Benign mesothelioma, focal	2
		844	Malignant mesothelioma	3
		847	Benign mesothelioma, focal	2

850	Benign mesothelioma, focal	2
868	Malignant mesothelioma	3
878	Benign mesotheliomas	2
	average	2.3
	average malignant	3

Legend: Only animals initially diagnosed as having TVM were evaluated. Some of these diagnoses of mesotheliomas were changed to focal mesothelial hyperplasia, shown in italics.

¹Doses as reported by authors. Taking into account the background level of acrylamide, the actual doses were 0.002 mg/kg-day higher than shown (Dourson et al., 2008)

²Leydig cell size was reported as -- (no LCT); +(25%), ++(50%), +++(75%), or ++++ (100%) of testes. The number of pluses was converted to numbers for presentation here.