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

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

Dear Colleagues:

We have completed our analysis on mammary tumors and tunica vaginalis mesotheliomas (TVMs) and have developed a detailed mode of action analysis for each of these tumors and a dose-response assessment for mammary tumors (Haber et al., 2008; Maier et al., 2008). These texts have been submitted to Regulatory Toxicology and Pharmacology. We have received permission to share the attached documents with you.

We are in agreement with EPA's draft acrylamide text in a number of places. Specifically:

- We agree with EPA on page 150 that acrylamide shows an increase in both DNA synthesis and DNA damage in mammalian tissues and cells, suggesting that a combination of DNA reactivity and cell proliferation may contribute to the observed acrylamide-induced carcinogenicity in the rat target tissues. In fact, based on EPA (2005), we have determined it is likely that multiple modes of action (MOA) are occurring with the mammary tumor and TVM endpoints.
- We agree with EPA on pages 190 & 191 that tumors with statistically significantly elevated incidences in both of the available rat bioassays (mammary gland tumors in females and tunica vaginalis mesotheliomas in males, specifically) should be considered for dose response assessment and that other tumors should be excluded.

Despite our agreements, it is our opinion that the document would be greatly enhanced by a more thorough consideration of alternative cancer MOAs, including the possibility that multiple MOAs (including a mutagenic MOA) apply. This evaluation would identify each of the key events for each of the alternative MOAs and evaluate them against the modified Hill criteria. Additional consideration of the nonmutagenic MOAs is possible and desirable, based both on the acrylamide data and the underlying biology of the target tissues. Haber et al. (2008) and Maier et al. (2008) have done these analyses for



## ***TERA* Statement of Purpose**

Toxicology Excellence for Risk Assessment (***TERA***) is a non-profit, 501(c)(3) corporation organized for scientific and educational purposes. Our mission is to protect public health by developing and communicating risk assessment information, improving risk methods through research, and educating the public on risk assessment issues. Some specific activities of ***TERA*** are listed below.

- Establish high-quality risk assessment values based on the latest scientific data and methods through the **Verifiable Estimates for Risk Assessment (*VERA*)** program
- Provide a unique side-by-side comparison of hazard values, information and dose response from organizations and independent parties worldwide through the **International Toxicity Estimates for Risk (*ITER*)** Database
- Conduct **research** to improve the underlying methods for human and ecological risk assessment
- **Peer Review and Consultation** of risk information, methods and study designs through an independent and public process
- **Educate** diverse groups on risk assessment issues, through training courses, scientific support and the State Hazard Evaluation Lending Program (**State HELP**)
- Improve the practice of **risk assessment** through independent and objective guidance and advice

***TERA*** is a non-profit corporation organized under section 1702.01 of the Ohio Revised Code, and is classified as a **501(c)(3) organization** under the Internal Revenue Service Code. Corporations, companies, associations, individuals and foundations may support the work of ***TERA*** through tax-deductible contributions.

TVMs and mammary tumors, respectively. Their conclusions are consistent with the discussion in EPA's risk characterization recommending additional studies of hormone disruption, as well as with EPA's MOA conclusion that a mixed MOA is possible. These analyses can significantly enhance the overall understanding of MOA in the context of the underlying biology, as well as the understanding of the potential interplay between different MOAs. At a minimum, these additional considerations warrant a more thorough discussion to enhance the document.

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

- Presentation of the MOA for tumors endpoints, which we find overly simplistic. Many mechanisms exist within and beyond the two broad categories that EPA labels "genotoxicity" and "hormone-related."
- Combination of mammary tumors for hazard identification in Table 4-33 (page 141), which we find as inherently inconsistent. Tumors are being combined by EPA authors that other scientists (including EPA scientists, e.g., for the hazard identification for atrazine) do not normally combine. In addition, certain statements on statistical significance are incorrect, or lacking, based on tables presented in the Johnson et al. (1986) and Friedman et al. (1995) papers.
- Use of TVM tumors for dose response assessment. Based on a consideration of MOA data and a screening level comparison of TVM incidence in humans, and acrylamide-related incidence of these tumors in rats, quantitative linear extrapolation of the TVM incidence from F344 rats exposed to acrylamide to humans is clearly inconsistent with the observed human tumor data, and thus not appropriate.

*TERA's* mission, as is the EPA's, is to protect public health. We encourage EPA to adhere to this mission and their recently published guidelines (2005) to ensure the available and extensive information on acrylamide is used in a scientifically appropriate fashion, which will enable credible risk management decisions.

Sincerely,

Lynne Haber, Ph.D., DAB I  
Manager, Research Program

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

**Evaluation of Human Relevance and Mode of Action for  
Tunica Vaginalis Mesotheliomas Resulting from Oral  
Exposure to Acrylamide<sup>1</sup>**

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<sup>1</sup>  
This manuscript has been submitted to Regulatory Toxicology and Pharmacology for publication

## **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. Overall, the data indicate that some relatively minor fraction of the TVMs in rats exposed to acrylamide may be relevant to humans. This conclusion is based on mode of action (MOA) considerations and the very low incidence of TVMs and other mesotheliomas in humans. 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). 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 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). These 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 was considered both on biological/MOA considerations, and 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, as well as for other reasons not relevant to the current 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).<sup>1</sup> The NTP historical control database for drinking water and feeding studies reports that mesotheliomas (tissue unspecified) occurred 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

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

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 the former study had a mediastinal mesothelioma but no TVM. Several TVM-bearing animals in this study had mesotheliomas in multiple organs. In the latter study, several of the animals with TVMs also had mesotheliomas in a variety of other tissues, but consideration of these other mesotheliomas 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, a finding 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. The authors stated that there are no established criteria for distinguishing between benign and malignant mesotheliomas, a fact confirmed by

other investigators (Swenberg, personal communication). They noted several experimental studies that could be used to distinguish whether the tumors were benign or malignant, but that such studies have not been performed, and 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 IVMs 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 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). Morphologically, the spontaneous TVMs observed in rats are consistent with epithelial mesotheliomas observed in humans (Tanigawa et al., 1987). Although there is a clear difference in incidence of TVMs in F344 rats and humans, Kim et al. (2006) evaluated gene expression data from the broader category of peritoneal mesotheliomas induced by *o*-nitrotoluene and bromochloroacetic acid, and concluded that the rat mesotheliomas were similar to mesotheliomas in humans, at least at the cellular and molecular level. 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.

**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.

Hypothesis C takes into account that there may be other possibilities not addressed by either of the first two hypotheses

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.

#### *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 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, thus 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. 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 number 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. However, we concluded 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 could 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 does not act via these MOAs. Similarly, no data were available specifically regarding the potential that acrylamide acts as a GnRH agonist.

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); male Wistar rats exposed on postnatal days 0-21 to mothers gavaged with 25 mg/kg-day had decreased whole brain dopamine levels (Husain et al., 1987); 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); and 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 D<sub>2</sub> 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 D<sub>3</sub> 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, thus leading 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, and no data were located 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, resulting in an overall increase of 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, inhibiting dopamine release to synapses, due to interaction 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. Thus, 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, although it is difficult to determine the relevance of these observations to the increased LCT response, 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.

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, but 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. 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 IVMs that occurred in the Friedman et al. (1995) study. 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)<sup>2</sup>. As shown by the average LCT size for each dose group, there was no

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<sup>2</sup>The terminology of “benign” vs. “malignant” used here is adopted directly from that used by the study authors, recognizing that 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

dose-response for LCT size in the animals evaluated (i.e., only animals initially diagnosed with TVMs). However, the degree of 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.

Thus, 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

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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.

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. Overall, the proposed MOA is biologically plausible. The data are generally consistent, assuming that 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. 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.

If acrylamide does increase TVMs via increasing dopaminergic activity, this MOA is not relevant to humans. 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 either conclude that these tumors are 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 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.

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 sex-specific differences in mesotheliomas following acrylamide exposure of F344 rats [in both Johnson et al. (1986) and Friedman et al. (1995), only the males were responsive] 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.

As noted above, Iatropoulos et al (1998) reported that in a blinded reanalysis of the tissues from the Friedman et al. (1995) study, among the animals initially diagnosed with proliferative mesothelial lesions, 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 testis<sup>3</sup>. 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. Similar studies on the relationship between LCT size and TVM progression were not located for other chemicals.

As an alternative approach to evaluating 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, two rats with TVMs but not LCTs were also identified,

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<sup>3</sup>As 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

one at 0.5 mg/kg-day and one at 2.0 mg/kg-day. However, this suggestion of TVMs in the absence of LCTs in the latter study is weakened by an unpublished reanalysis of the pathology data. In their re-analysis of the Friedman et al. (1995) tissues, Iatropoulos et al. (1998) reclassified the mesotheliomas as benign or malignant. In addition, several lesions originally classified as mesotheliomas were reclassified as focal mesothelial hyperplasia. Both of the animals originally identified as having TVMs but not LCTs were reclassified as being focal mesothelial hyperplasia, not TVMs. 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, but unpublished re-analyses of the pathology data suggest that the concordance may be tighter than apparent from the original unpublished studies. 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.

To further investigate this relationship, 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 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.

#### *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- $\beta$ 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.

#### *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), and Dourson et al. (2008) provides 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. 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 [<sup>14</sup>C]-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).<sup>4</sup> The lack of association of DNA adduct formation with tumor formation suggests that events other

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<sup>4</sup> 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.

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.

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. No bioassays of acrylamide in mice have been completed to date (although the one by NTP is nearing completion), and the tumor targets for acrylamide in the mouse, if any, are not known. In addition, the tissues evaluated in the mouse (bone marrow, lymphocytes, liver) are not tumor targets for acrylamide in the rat.

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 IVMs.

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.

Overall, the data are clear that DNA damage and DNA adducts are formed by the acrylamide metabolite, glycidamide, but 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). This observation, together with reports of TVM induction by other mutagenic carcinogens, indicates that 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.

#### *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). As discussed by Dourson et al. (2008) and Maier et al. (2008), oxidative stress may also contribute to acrylamide carcinogenicity, since 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.

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).

## 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. 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.

- 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.
  - 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.
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.

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.

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 µg/kg-day. For high percentiles consumers (90th to 97.5<sup>th</sup> percentiles), intake estimates ranged from 0.6 to 3.5 µg/kg bw per day, and up to 5.1 µg/kg bw per day for the 99th percentile consumer. JECFA stated that children appeared to ingest approximately two to three times the adult intake when expressed on a body weight basis.

Using the average of the two slope factors calculated by CalEPA (2005) of 0.49 per mg/kg-day and an average intake of 2 µg/kg-day would result in a risk of TVMs in the human population of 0.00098, or a risk of almost 1 in a thousand. Of the available epidemiology studies of acrylamide and cancer, studies of the Marsh cohort (Marsh et al., 1999; Marsh et al., 2007) investigated the incidence of cancer of the testis and male genital tract. No effect was seen, although the absolute numbers of observed and expected cancers was very low (1-2 in a cohort

of up to 8508), and the statistical power to detect an increase was low, as reflected by the broad confidence limits (Erdreich and Friedman, 2004). However, the very low background incidence of TVMs in humans would make a small increase on the order of  $10^{-4}$  or  $10^{-5}$  easily detectable. 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. As noted above, a total of approximately 80 TVMs have been reported in humans in the world literature, based on a search of the Medline database (Plas et al., 1998). Although not every TVM will have been reported in the literature, at least a third of the TVMs are associated with asbestos exposure (Plas et al., 1998; Spiess et al., 2005). Therefore, using the total of 80 TVMs as a bounding on the number of acrylamide-associated TVMs in humans appears reasonable. 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.

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. No data

specific to TVMs are reported in SEER. Because much of the total mesotheliomas incidence in humans is due to asbestos exposure, and assuming that all observed mesotheliomas result from acrylamide exposure would not make sense.

Based on extrapolation from the SEER data on mesotheliomas presented earlier in this paper, 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  $1.3 \times 10^{-5}$ , corresponding to a lifetime risk of  $7 \times 10^{-5}$ . 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 BMDL<sub>10</sub> 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<sup>3/4</sup>. 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,<sup>5</sup> corresponding to a risk of  $9 \times 10^{-4}$  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.

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<sup>5</sup> 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 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 over-estimates 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).

Based on these considerations, the MOA data indicate that some fraction of the TVM response related to acrylamide exposure in rats may be relevant to humans. Multiple MOAs are likely to apply, and some small contribution from a mutagenic MOA is plausible. However, in light of the very low incidence of TVMs in humans, the most appropriate estimate of the risk of TVMs from acrylamide exposure in humans at typical dietary levels is below de minimis levels.

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. 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. 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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## 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.

# **Reevaluation of Dose-Response Options for F344 Rat Mammary Tumors for Acrylamide – Additional Insights Based on Mode of Action<sup>2</sup>**

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action analysis

**Abstract**

A number of recent risk assessment documents or review articles have presented alternative analyses of the potential for dietary exposure to acrylamide to increase breast cancer risk. To help clarify the current scientific landscape, we conducted a critical review of data concerning the human relevance of mammary tumor findings from animal bioassays. We applied a systematic evaluation to alternative mode of action hypotheses, including 1) genotoxicity both direct and secondary to oxidative stress, 2) endocrine effects due to age-related hyperprolactanemia or secondary to neurotoxicity, and 3) paracrine regulation of mammary epithelial cell growth. Using a reasonable standard of scientific certainty and systematic weight of evidence approach, we evaluated the evidence for these alternative modes of action. Based on this evaluation we conclude that the dose-response assessment of mammary tumors observed in rats can be narrowed to two options. Option 1: Since a contribution of direct genotoxicity was not ruled out a conservative linear low-dose modeling approach for the adenomas and adenocarcinomas is used here, and the model selection procedure reflects the weight of evidence that other epigenetic modes of action likely play a significant role in the tumor response. Option 2: The combined fibroadenoma, adenoma and adenocarcinoma data from the bioassays is used as the basis for deriving a point of departure (POD) for tumor promotion potency. This POD should be carried forward using a non-linear approach based on the methods involved to estimate a Reference Dose (RfD).

## Introduction

This manuscript presents an evaluation of the options for completing a dose response assessment for a human cancer risk assessment of acrylamide focusing on mammary tumors. Two related manuscripts, Dourson et al. (2008) and Haber et al. (2008) discuss thyroid tumors and tunica vaginalis mesotheliomas (IVM), respectively. Other investigations have evaluated a variety of tumor endpoints, including evaluation of tumors of the mammary gland, (e.g.; OEHHA, 2005; Shipp et al., 2006). U.S. Environmental Protection Agency (EPA) is also developing a comprehensive assessment for this chemical (see: <http://www.regulations.gov/fdmspublic/component>; Docket Number EPA-HQ-ORD-2007-1141). The current analysis adds to these other assessments by presenting an updated and detailed evaluation of the weight of evidence for each of the leading mode of action hypotheses described in these other reviews applying the principles embedded in current guidance for assessing tumorigenic modes of action and human relevance of tumors (U.S. EPA, 2005; Sonich-Mullin et al., 2001; Meek et al., 2003).

While there has been interest in the potential carcinogenicity of acrylamide in occupational settings for some time, the finding that under some conditions the heating of carbohydrates can generate acrylamide, and thus contribute to dietary exposure, has generated additional public health interest. This interest has spurred epidemiology and mechanistic toxicology work that enhances the data upon which an evaluation of potential breast cancer risk can be conducted. Prior occupational epidemiology studies for acrylamide had limited use for evaluating breast cancer issues, since the populations studied were mostly males and the studies did not focus specifically on evaluating breast cancer risk (Marsh et al., 2007; Swaen et al., 2007). Several recent epidemiology studies have focused specifically on addressing the relationship between breast cancer and dietary acrylamide exposures in large cohorts, and these studies find little evidence of a higher risk of several cancers (Pelucchi et al., 2006; Mucci et al., 2003, 2004, 2005, 2006; Olesen et al., 2008; Hogervorst et al. 2007), including breast cancer. Two cohort studies found no association between acrylamide intake and breast cancer (Hogervorst et al., 2007; Mucci et al., 2006). Mucci et al. (2006) analyzed the dietary intake of acrylamide in a cohort of 43,404 women, 9 percent of which were postmenopausal, through a food frequency questionnaire. When compared to the lowest quintile of acrylamide intake, there was no significant increase in breast cancer in the higher quintiles (relative risk was 1.19 with a confidence interval of 0.91-1.55 when comparing quintiles 1 and 5) and no evidence of a linear dose response. Hogervorst et al. (2007) sampled 2589 postmenopausal women randomly drawn from the Netherlands Cohort Study (NLCS), 1796 of which were used in a breast cancer subcohort, where acrylamide intake was estimated through a food frequency questionnaire. The authors identified a significant increase in ovarian and endometrial cancer among never-smoking women, but no significant association was identified between acrylamide intake and postmenopausal breast cancer. Pelucchi et al. (2006) conducted a case-control study using information from hospital-based studies with the same design, food intake questionnaire and inclusion criteria. Of the patients included in this study, 2900 cases and 3122 controls were

identified with a median age of 55 and 56 years, respectively. An odds ratio of 1.06 (95% CI = 0.88-1.28) was identified for breast cancer, indicating that the authors found no evidence of increased risk of breast cancer in relation to dietary acrylamide.

Of the epidemiological studies identified, only one used biomarkers as an estimate of acrylamide exposure instead of food intake surveys. Olesen et al. (2008) conducted a nested case-control study on a cohort of 29,875 postmenopausal women (after exclusions, only 24,697 women were used for the study) using acrylamide-hemoglobin adducts as estimates for acrylamide intake. Neither acrylamide-hemoglobin (AA-Hb) nor glycidamide-hemoglobin (GA-Hb) levels were significantly associated with breast cancer without adjusting the model for smoking and other confounding factors. In a fully adjusted model, however, women with the highest AA-Hb concentrations had a 2.7 times increased risk of estrogen-receptor positive (ER+) breast cancer compared to women with lower concentrations. Overall, the current slate of epidemiology studies does not provide convincing evidence that typical dietary exposures to acrylamide are a significant risk factor for breast cancer. However, at least one study indicated a positive association, and there are limitations in using such data for dose-response assessment, since the statistical power of such studies to detect small increases in risk is limited.

When epidemiology data are too limited to serve as the basis for a quantitative risk assessment, data from well controlled chronic tumor bioassays in rodents informed by mechanistic toxicology information are often used as the centerpiece of a quantitative cancer risk assessment. Acrylamide carcinogenicity studies in rats have reported increases in both benign and malignant tumors of the thyroid, mammary tissues, and tunica vaginalis of the testis, as well as sporadic findings of other tumors. In particular, various types of mammary tumors were seen in both sexes of rats in the two available long-term bioassays of acrylamide (Johnson et al., 1986; Friedman et al., 1995), although the incidence of these tumors was not consistent between the studies. Johnson et al. (1986) conducted several interim kills that showed that the onset of tumors was generally late in the experiment (at nearly 2 years). Latency information was not reported in the Friedman et al. (1995) study. The implications of these findings are analyzed in detail for the current weight of evidence assessment.

As discussed more completely in Dousson et al. (2008), acrylamide, and its more DNA-reactive metabolite glycidamide, are widely distributed throughout all of the tissues of the body. Furthermore, while reaction with DNA has been proposed as a plausible mechanism of tumorigenesis, similar levels of DNA adducts have been observed in the various organs of rats exposed to acrylamide. In rats, no apparent kinetic explanation exists for the tissues that are targets for acrylamide-induced tumorigenesis, suggesting that the mechanism of acrylamide-induced carcinogenesis is driven by dynamic differences among the tissues, rather than the molecular dosimetry of DNA adducts. These findings are consistent with the perspectives of Swenberg et al. (2007) who conclude that DNA adducts are a biomarker of exposure and not a biomarker of effect. The pertinent biomarker of effect for carcinogenesis is mutation, which is subject to dynamic processes beyond adduction with DNA and is anticipated to have different dose-response characteristics than a biomarker of exposure like DNA or protein adducts. The fact that many of the tumor sites in experimental rats are also associated with hormone

production is consistent with a hypothesis that dynamic differences among tissues form a key determinant for acrylamide tumorigenesis. This analysis focuses on evidence for such key mode of action determinants.

A number of recent risk assessment documents or review articles have presented alternative interpretations of the current information related to the potential for dietary exposure to acrylamide to increase breast cancer risk. The purpose of this manuscript is to clarify the current scientific landscape on this issue. A detailed analysis was conducted to provide further critical review of arguments related to human relevance of mammary tumor findings from animal bioassays, weight of evidence for a variety of alternative potential modes of action for the mammary tumors observed in rodents, and a discussion of the appropriate dose-response models to employ for mammary tumor endpoints for use in human health risk assessments.

## Methods

Data analysis and dose-response modeling were conducted as described in Dourson et al. (2008) in a companion paper on evaluation of thyroid tumors. In brief, we use the mode of action (MOA) framework within the recent cancer risk assessment guidelines of the U.S. Environmental Protection Agency (U.S. EPA, 2005). In accordance with these guidelines, we consider whether each MOA is sufficiently supported by the existing human or experimental animal data, and whether the available evidence suggests these MOAs are relevant to humans. Furthermore, as per U.S. EPA (2005) guidelines, the model used for extrapolation to low doses is determined based on the most relevant MOA(s) given our current understanding of the science.

In order to represent the tumor dose-response for this analysis, we follow the standard U.S. EPA practice of fitting empirical models to the mammary tumor data (U.S. EPA, 2005), since we determined that data were not sufficient for acrylamide to develop a biologically-based dose response model. The U.S. EPA software, BMDS (version 1.4.1; U.S. EPA, 2003), is used to obtain and evaluate these empirical model fits.

The next step depends on the MOA that has been determined to apply to the tumor type of interest. For a mutagenic MOA, the typical conservative modeling assumption in the absence of more refined data is no threshold dose, and low-dose linearity. A line connects the point of departure (POD) to the origin, corrected for background. The slope of the line (the slope factor or SF) is used to estimate a risk per incremental increase in dose.

Using a bench-mark dose (BMD) based on extra risk, one calculates the slope factor (SF) directly from the desired benchmark response (BMR) level. The stability of the slope estimate is gauged by evaluating it for different BMR and BMD values. For example, if the BMD at 0.10 excess risk equals 7.1 mg/kg-day, then:

$$SF = BMR/BMD = 0.10 \text{ incidence}/7.1 \text{ mg/kg-day} = 0.014 \text{ (mg/kg-day)}^{-1}$$

Thus, for an exposure of 1 mg/kg-day of exposure the risk would be estimated as 0.014 or 14:1000.

As described by Swenberg et al. (2007) such a simplifying approach may not accurately reflect the anticipated biology for a chemical like acrylamide that forms DNA adducts that ultimately yield the same type of DNA damage (i.e., apurinic sites) as is produced endogenously (e.g., by oxidative stress). In such cases, low dose behavior is likely to be dominated by endogenous sources of mutation, while as dose increases exogenous DNA damage begins to drive the mutation rate and ultimately the tumor rate. Direct analysis of in vivo mutation rates at low doses would provide a more accurate reflection of the actual dose-response behavior. In the absence of such data for the mammary gland, the default modeling approach recommended in the EPA's cancer risk assessment guidelines was applied (U.S. EPA, 2005) to the tumor data, although the selection of data sets and models was informed by the underlying biology.

When the chemical acts via a non-mutagenic MOA for which the data suggest a biological threshold, U.S. EPA describes a nonlinear approach. In this case, the POD

(based on either tumors or a precursor endpoint) is used to develop a Reference Dose or Reference Concentration for oral or inhalation exposures, respectively, following the procedures prescribed by U.S. EPA for non-cancer toxicity, with the BMDL divided by uncertainty factors (U.S. EPA, 2005).

## Results and Discussion

### Summary of Animal Mammary Tumor Findings

A variety of toxicity study designs have provided evidence that acrylamide can either initiate or promote mammary tumors in rodents. Regulatory assessments to date have considered these tumor increases relevant for the purpose of human health risk assessment. However, this conclusion has not been fully vetted using U.S. EPA's (2005) most recent guidelines for carcinogen risk assessment and the question as to whether the tumors are specific to aged female F344 rats (and thus not relevant to humans) and whether the plausible modes of action would be most consistent with a linear or non-linear dose-response has continued to be a matter of scientific debate.

The tumorigenicity data relevant to the assessment of breast cancer potential includes two chronic bioassays in rats (Johnson et al., 1986; Friedman et al., 1995), and a tumor promotion study in rats that evaluated models for mammary tumors (Imai et al., 2005). Tumor screening studies in various mouse strains (Robinson et al., 1986; Bull et al., 1984a; Bull et al., 1984b), which did not include an evaluation of mammary tumors, assist the mode of action analysis.

In the earlier study by Johnson et al. (1986) the incidence of adenocarcinoma<sup>1</sup> showed a significant dose-related trend in females, although the incidence of this tumor type at the high dose (2 mg/kg-day) did not reach statistical significance compared to the control group. Fibromas, but not adenomas or fibroadenomas showed a significant increase, but only in the high dose group. Combined benign tumor incidence was also increased significantly in the high dose female group. The authors also noted that mammary tumors in the high dose group appeared to occur earlier than those in the control group, although the number of affected animals was small. The incidence of hyperplasia appeared to increase in some groups, but was not dose related. Study details and tumor incidence for females are described in Table 1a. Table 1b shows the tumor incidence for males. No treatment-related effects were noted in males. The incidence of fibromas at the high dose in males was similar to the incidence found at the high dose in females, but in contrast to females was not statistically significantly different than control male rats.

	Dose (mg/kg-day)				
	0.002 <sup>a</sup>	0.012	0.10	0.50	2.0
Number Examined	60	60	60	58	61
Adenocarcinoma	2	1	1	2	6 <sup>b</sup>
Adenoma	0	1	0	3	2
Adenoma or Adenocarcinoma	2	2	1	4 <sup>c</sup>	8
Fibroadenoma	10	11	9	17	16

<sup>1</sup> The tumor nomenclature reflects the nature of the cell types and malignancy of the observed neoplastic lesions. Adenocarcinomas are malignant tumors of epithelial cell origin. Adenomas are benign tumors of epithelial cell origin. Fibroadenomas are benign tumors containing epithelial and stromal cells (e.g., fibroblasts). Fibromas are benign tumors containing cells of stromal origin (Russo and Russo, 1996).

Fibroma	0	0	0	0	5 <sup>d</sup>
Fibroma or Fibroadenoma	10	11	9	17	21 <sup>e</sup>
Adenocarcinoma, Adenoma, Fibroadenoma or Fibroma	10 <sup>f</sup>	12 <sup>f</sup>	10	20 <sup>f</sup>	28
Hyperplasia	11	17	11	17	18
<sup>a</sup> Control “dose” determined as described by Dourson et al (2008)					
<sup>b</sup> Reported by authors as positive for trend (Mantel-Haenszel extension of the Cochran-Armitage test with alpha = 0.05)					
<sup>c</sup> One animal had both an adenoma and adenocarcinoma					
<sup>d</sup> Reported by authors as statistical difference from control group, mortality adjustment using the Mantel-Haenszel procedure with alpha = 0.05					
<sup>e</sup> Significantly different from the adjusted control, with p=0.02 by one-tail Fisher's Exact test					
<sup>f</sup> At dose=0.002, two animals had both an adenocarcinoma and fibroadenoma. At dose=0.012 and at 0.50, one animal had both an adenoma and fibroadenoma					

	Dose (mg/kg-day)				
	0.002 <sup>a</sup>	0.012	0.10	0.50	2.0
Number Examined	60	60	60	58	60
Adenocarcinoma	0	0	0	0	0
Adenoma	1	0	0	0	0
Adenoma or Adenocarcinoma	1	0	0	0	0
Fibroadenoma	3	2	4	2	3
Fibroma	3	4	6	4	7
Adenocarcinoma, Adenoma, Fibroadenoma or Fibroma	7	6	8 <sup>b</sup>	6	10
Hyperplasia	4	8	4	2	8
<sup>a</sup> Control “dose” determined as described by Dourson et al. (2008)					
<sup>b</sup> Two animals apparently had both a fibroadenoma and fibroma					

A second study was conducted by Friedman et al. (1995) to expand the dose-response range and confirm the findings reported by Johnson et al (1986). Double control groups and an unbalanced statistical design were employed to confirm the findings of the older study. Friedman et al. (1995) reported that the incidence of fibroadenomas was significantly increased above controls at both dose levels tested in females. The incidence of adenocarcinoma was low in the control and treated groups, although a slight increase that was not statistically significant was observed at the high dose group (relative to one of the control groups). No adenomas were found and fibromas were not reported. The incidence of hyperplasia was not increased in the acrylamide treated rats. No specific time-to-tumor analysis was presented. Table 2a shows the tumor incidences for females. Table 2b shows the tumor incidence for males; no effects were noted in males.

The incidences of the various mammary tumors between these two studies are not fully consistent. Friedman et al (1995) provided some information that might explain these differences. For example, Friedman et al. (1995) noted that the incidence of

fibroadenomas in their control animals was lower than the mid-range of historical background incidence, and was approximately 60% of the Johnson et al. (1986) control values. Even in the high dose, Friedman et al. (1995) observed that the incidence of fibroadenomas barely exceeded the average of the historical control incidence. Friedman et al. (1995) also noted that variability in tumor incidence was impacted by survival, since the background tumors are typically late onset. However, the percentage survival at the high doses do not appear to differ between Friedman et al. (1995) and Johnson et al. (1986) and, thus, survival differences do not appear to be the reason for the differences in tumor incidences.

In addition to the potential impact of background control incidence on interpreting the fibroadenoma dose-response, these tumor responses are further confused by possible misclassification of tumor types in Johnson et al. (1986). It is possible that the fibromas reported in Johnson et al. (1986) were misclassified fibroadenomas. Such a misclassification might occur in sectioning large tumors of mixed cell types. The significant increase in combined incidence of fibroadenomas plus fibromas in the Johnson et al. (1996) is consistent with the incidence of fibroadenomas reported in Friedman et al. (1995).

	Dose (mg/kg-day)			
	0.002 <sup>a</sup>	0.002 <sup>a</sup>	1.0	3.0
Number Examined	46	50	94	95
Adenocarcinoma	2	0	2	4
Fibroadenoma	5	4	20 <sup>b</sup>	26 <sup>b</sup>
Adenocarcinoma or Fibroadenoma	7	4	21 <sup>c</sup>	30
Hyperplasia	26	27	33	47

<sup>a</sup> Control “dose” determined as described by Dourson et al. (2008)  
<sup>b</sup> Reported by authors as statistical difference from control group, mortality adjustment using the Mantel-Haenszel procedure with alpha = 0.05  
<sup>c</sup> One animal had both an adenocarcinoma and fibroadenoma.

	Dose (mg/kg-day)				
	0.002 <sup>a</sup>	0.002 <sup>a</sup>	0.10	0.50	2.0
Number Examined	102	102	204	102	75
Adenocarcinoma	0	0	3	1	1
Adenoma	0	0	0	0	1
Adenocarcinoma or Adenoma	0	0	3	1	2
Fibroadenoma	1	4	1	2	4
Adenocarcinoma, Adenoma, or or Fibroadenoma	1	4	4	3	6
Hyperplasia	4	4	2	0	2

<sup>a</sup> Control “dose” determined as described by Dourson et al. (2008)

In a more recent mechanistic study, acrylamide administered in the drinking water of female Sprague-Dawley (SD) rats that were initiated with single doses of N-methyl-N-nitrosourea, a known, direct-acting mutagen, showed a rapid increase in incidence and multiplicity of mammary adenocarcinomas (Imai et al, 2005), but only a small and not statistically significant incidence of fibroadenomas in any of the groups. This rapid onset of mammary adenocarcinomas, is characteristic of a chemical that is known to cause tumors by mutation, and is in contrast to the observations in either of the longer-term bioassays of Johnson et al. (1986) or Friedman et al. (1995). The relatively short duration of dosing (30 weeks) or the difference in rat strain of Imai et al. (2005) as compared to these longer-term bioassays might explain the absence of fibroadenomas, which are hypothesized (Shipp et al, 2006) to result from age-related hyperprolactinemia in female F344 rats. Imai et al. (2005) also found that acrylamide did not increase mammary tumor incidence in animals initiated with 7,12-dimethylbenz(a)anthracene in combination with N-bis-(2-hydroxypropyl)nitrosamine as a mutagen. However, the incidence of mammary tumors was very high in the initiated group without acrylamide, and therefore, the opportunity for acrylamide to further significantly increase tumor incidence was limited.

No untreated controls or acrylamide-only groups without initiator treatment were included in the Imai et al. (2005) study. Thus, whether acrylamide acted as complete carcinogen or only as a promoter of adenocarcinomas in MNU-treated rats cannot be determined from this study, although acrylamide did not cause such mammary tumors at 6, 12 or 18 months in the Johnson et al. (1986) bioassay. This contrast between studies indicates that the mode of action for acrylamide may be different than a direct-acting mutagen. Study details and tumor incidence of Imai et al. (2005) are described in Table 3.

Treatment	Initiating Treatment: MNU <sup>a</sup>		
	Control	20 ppm acrylamide	40 ppm acrylamide
Number Examined	20	20	20
Adenocarcinoma	10	13	16 <sup>b</sup>
Fibroadenoma	0	0	1
	Initiating Treatment: DMBA/DHPN <sup>c</sup>		
	Control	20 ppm acrylamide	40 ppm acrylamide
Number Examined	19	20	20
Adenocarcinoma	16	20	18
Fibroadenoma	0	0	1
Fibroma	0	2	0

<sup>a</sup>Female SD rats were administered a single 50 mg/kg dose of N-methyl-N-nitrosourea by intraperitoneal injection followed by varying concentrations of acrylamide in drinking water for 30 weeks. Based in default body weight of 0.338 kg and water intake of 0.045 L/day for female rats the acrylamide doses are estimated as 2.5 mg/kg-day and 5 mg/kg-day for the low and high concentration groups respectively

<sup>b</sup>Statistically different from controls (p<0.05) using Fisher's Exact Test.

<sup>c</sup>Female SD rats were administered a single subcutaneous injection of 2800 mg/kg N-bis-(2-hydroxypropyl)nitrosamine and 1 day later a 50 mg/kg dose of 7,12-dimethylbenz(a)anthracene followed by varying concentrations of acrylamide in drinking water

for 22 weeks.

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, 2006). However, to supplement the studies in rats described above, data from screening bioassays conducted in mice (Bull et al., 1984a; Bull et al., 1984b; Robinson et al., 1986) can be used. These screening assays did not evaluate mammary tissues. Bull et al. (1984a) reported that oral or intraperitoneal dosing with high doses of acrylamide initiated skin tumors that were dependent on treatment with the promoter TPA and lung tumors that were not dependent on treatment with the TPA promoter in ICR-Swiss mice. The authors noted that the results were consistent with similar findings of increased lung tumors in the A/J mouse strain and skin tumors in SENCAR mice (Bull et al., 1984b). These studies provide at least some evidence of tumor initiation by acrylamide in susceptible mouse strains. However, since these strains are genetically susceptible to tumor formation, the observed increases in tumors even in the absence of a known promoter could reflect the ability of acrylamide to promote tumor formation from endogenously initiated cells.

Taken as a group, Johnson et al. (1986), Freidman et al. (1995), and Imai et al. (2005) provide evidence that acrylamide induces mammary gland tumors in female rats. However, increased tumor incidences in acrylamide-treated groups in the two chronic bioassays, Johnson et al. (1986) and Friedman et al. (1995), did not surpass those of historical controls for F344 rats and the spectrum of tumor types was not fully consistent across these two bioassays. Furthermore, the background incidence of fibromas in Johnson et al. (1984) males was higher than the incidence in females, calling into question the relevance of the high dose female response. Moreover, Freidman et al. (1995) noted that the background rate of fibroadenomas is highly variable among testing laboratories and was unusually low in the control group of their study – which might have contributed to the statistically-significant increase in this tumor type in their study. The authors suggest that this observation detracts from the toxicological relevance of the observed fibroadenomas – particularly since no increase in this tumor type was observed in the earlier study. However, although not statistically significant, Johnson et al. (1986) showed a comparable increase in fibroadenoma incidence at the high dose and the misclassification of fibroadenomas as fibromas is a possibility that if true would increase the consistency in the findings. Despite these differences among studies in incidence rates for specific tumor types, the available chronic studies do present a reasonably consistent picture of increases in total mammary tumors at high doses as shown in Tables 1 and 2. This conclusion is further supported by screening bioassays.

#### Implications of the Animal Tumor Findings for Mode of Action Assessment

Key findings from the observed patterns of tumor response that inform the mode of action analysis are described in this section – in particular attributes of the tumor response that give greater weight to either mutagenic or non-mutagenic modes of action are described. Many of these concepts are described in current EPA cancer risk assessment guidance (US EPA, 2005).

One aspect of the tumor response to consider is the degree of progression (i.e., the relative number of benign versus malignant tumors). A preponderance of benign tumors that occur with late onset as reported for acrylamide supports mechanisms other than mutagenicity. However, the contribution of direct DNA effects by a weak mutagen cannot be ruled out on this basis alone. Both chronic bioassays show some indication of a modest increase in adenocarcinomas at higher doses – although in neither study is the increase statistically-significant. The available studies show that mammary tumors typically occurred later in life. For example, the individual animal data from Johnson et al. (1986) show no significant increase in the incidence of mammary gland abnormalities for treated animals versus controls through the 18 month interim sacrifice. Based on these findings Johnson et al. (1986) commented that tumors occurred earlier in treated animals than in controls. A preponderance of benign tumors that occur late in life suggests that a non-mutagenic or at most a weak mutagenic response would be most consistent with the data.

A second aspect of the tumors that relates to assessing the likely mode of action is the nature of the tissue types affected. The mammary tumors observed in both studies are generally of the same type as the tumors in unexposed controls. The predominant cell type categorization (i.e., stromal vs. epithelial cells of origin) has unclear implications with regard to mode of action in the absence of dosimetry information at the cellular level. This matter is confused by the lack of concordance across the studies on the type of benign tumors that were observed as noted above. Many known directly mutagenic mammary tumorigens such as ionizing radiation and *n*-methyl-nitrosourea induce adenomas and adenocarcinomas to a higher degree than fibroadenomas (Russo and Russo, 1996). This conclusion is also consistent with the results described for Imai et al. (2005) described above. However, irradiation coupled with exogenous progesterone induced fibroadenomas in rats and irradiation coupled with estrogen induced adenomas (Yamanouchi et al., 1995) indicating the potential interplay of mutagenicity and endocrine growth signals. The reported increases in fibroadenomas and fibromas suggest that acrylamide is acting via modes of action distinct from (or in addition to) the mutagenic mechanism for classical direct acting mammary tumorigens – since adenocarcinomas did not predominate in the chronic bioassays.

A third aspect of the tumor response to consider is the array of organs affected. In the acrylamide cancer bioassays the observed tumors were predominately in endocrine-responsive tissues. In addition, mammary tumors are a common background tumor in female rats of the F344 strain used in these experiments. The spectrum of observed tumors sites supports mechanisms other than mutagenicity as contributing to the overall response, since a potent mutagen would not be expected to generate the observed pattern of target tissues given the observed wide tissue distribution of acrylamide and its genotoxic metabolite glycidamide (Doerge et al., 2005). However, this conclusion must be tempered, since the pattern of tumor targets does not eliminate the possibility that acrylamide is acting as a weak mutagen or causes other types of genotoxicity that are enhanced via other factors that make certain tissues sensitive (i.e., 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 or confirm any of these possibilities.

A fourth aspect of mode of action evaluation is the assessment of the shape of the dose-response curve for tumors. Because of limitations in statistical power for typical bioassays, only limited inferences about tumor mode of action can be gained from the evaluation of the tumor dose response. The empirical dose-response is not adequate to make conclusions about the shape of the dose-response in the low-dose range. Nevertheless, for completeness, this consideration is weighed along with others in gaining a full understanding of the mode of action. In Johnson et al. (1986) adenocarcinomas only show a small increase at the high dose (2 mg/kg-day), with no hint of increase at the second highest dose. This pattern is very similar to the observation in Friedman et al. (1995), which shows a slight (but not statistically-significant) increase at the high dose (3 mg/kg-day) compared to pooled controls, which is not observed at 1 mg/kg-day. This pattern of increased malignant tumors only at the high dose, does not provide support for a linear dose-response assumption. Fibroadenomas show an increase at the two highest doses in both studies, although this response is not statistically significant in Johnson et al. (1986). In the Johnson et al. (1986) the increase in fibroadenomas levels off, yielding a similar response at the two highest doses. The combined incidence of fibroadenomas and fibromas in the Johnson et al. (1986) study shows an increase in incidence with dose at the two highest doses. In the Friedman et al. (1995) study the high dose response is greater than the mid dose, but the increase is not linear. The fibroadenoma data are consistent with a threshold in the lower dose range and possibly a saturable mechanism at the high dose – neither observation of which is consistent with mutagenicity (i.e., the doses used in these chronic bioassay are unlikely to saturate the metabolic conversion of glycidamide to acrylamide). Fibromas were only increased in the females of the Johnson et al. (1986) study, and they showed a clear threshold, with no observed fibromas in controls or lower dose groups and no malignant tumors that would reflect direct progression from a fibroma. Hyperplasia was somewhat increased in Johnson et al. (1986), but not clearly increased in the Friedman et al. (1995) study. Overall, the tumor data show evidence of a threshold response, which is more consistent with an epigenetic than a mutagenic MOA.

A fifth aspect of the tumor analysis is the evaluation of tumor response observed in supplementary screening bioassays. In addition to the chronic bioassays, screening studies using initiation:promotion protocols can inform the mode of action evaluation, since tumor initiation is often associated with mutagens, while tumor promotion often occurs via non-mutagenic mechanisms. Several such studies have been conducted. Imai et al. (2005) found that acrylamide co-treatment in MNU-initiated rats increased the incidence of mammary adenocarcinomas, but did not increase fibroadenoma incidence. The increase was significant at the high dose (40 ppm acrylamide in drinking water or approximately 5 mg/kg-day estimated from EPA defaults for water consumption and body weight). Since no acrylamide only group was included in the study, it cannot be determined from this study whether acrylamide acted as both an initiator and promoter or only as a promoter of the adenocarcinoma response. However, the observation that only adenocarcinomas (but not fibroadenomas) were increased, and that MNU is a

demonstrated tumor initiator, provides evidence for tumor promotion by acrylamide. Moreover, Johnson et al (1986) study did not show any evidence of mammary tumors after 6, 12, and 18 months of acrylamide only exposure. Thus, the increase in mammary adenocarcinomas by acrylamide in the Imai et al. (2005) study appears to be from acrylamide's promotional activity, and not its ability to initiate tumors. In contrast, Bull et al. (1984a; 1984b) and Robinson et al. (1986) evaluated lung and skin tumor incidence in various strains of acrylamide-treated mice that were also exposed to a tumor promoter. These studies demonstrated the ability of high doses of acrylamide to initiate skin tumors that was dependent on addition of the promoter and the ability to initiate lung tumors without subsequent treatment with a promoter. The work of Bull and Robinson demonstrate that for some tissues in susceptible mouse strains short-term acrylamide dosing might act as a complete carcinogen. Overall, the screening bioassays are not adequate to resolve whether acrylamide is a tumor initiator, promoter, or both in mammary glands.

In summary, examination of the tumor patterns can be useful in evaluating potential mode of action hypotheses. As is typically the case, the data from the animal studies are not fully conclusive regarding any specific mode of action. Nevertheless, using a weight of evidence standard of “more likely than not” the tumor patterns suggest MOAs that are not mutagenic, or possibly non-mutagenic MOAs that include a smaller mutagenic component. This conclusion is based on the following observations:

- increases in mostly benign tumors,
- late age of tumor onset,
- cell-lines of origin that depart from classical mutagens,
- other tissues affected that are primarily hormone-responsive in origin,
- dose-response patterns that suggest thresholds,
- potential promotion of MNU-induced mammary tumors.

## MOA Hypotheses

### *Mutagenicity*

The genotoxicity of acrylamide and its oxidative metabolite, glycidamide, have been reviewed in detail in the companion paper of thyroid tumors by Dourson et al. (2008). There appears to be general agreement in the current assessments that acrylamide is not mutagenic in typical *in vitro* assays, while glycidamide is positive in such assays. Both chemicals induce DNA damage (e.g., cause DNA adducts and DNA strand breaks), although acrylamide does so only weakly. Acrylamide also affects chromosome integrity (e.g., increases micronuclei formation and chromosomal aberrations).

Very little data specific to evaluation of genotoxicity in the mammary gland are available. Doerge et al. (2005) found that 50 mg/kg acrylamide administered to mice and rats led to presence of glycidamide in serum and a range of tissues examined. Glycidamide-derived DNA adducts of adenine and guanine were formed in all tissues examined, with greater adduct formation in animals treated with glycidamide versus acrylamide. Adducts were observed in target and non-target tissues, including in the mammary gland of female rats

This lack of specificity of DNA adducts suggests that events other than DNA adduct formation (at least the specific adducts identified) are needed to explain the pattern of tumors. Klaunig and Kamendulis (2005) reported that in male F344 rats exposed to acrylamide, DNA damage as measured by the Comet assay (which measures single strand breaks and alkali-labile sites) and DNA labeling (which measures DNA synthesis that might reflect DNA damage or repair) occurred in the thyroid and adrenal glands, but did not occur in non-target tissues (i.e. the liver). Although these data enhance specificity arguments for DNA damage as a modulator of tumor response, these endpoints were not measured in the mammary gland. Butterworth et al. (1992) reported that acrylamide produced a slight response in the *in vitro* human mammary epithelial cell (HMEC) DNA repair assay in normal cells derived from discarded surgical samples from five different women, suggesting that acrylamide can induce DNA damage in human mammary tissue. Glycidamide produced a strong unscheduled DNA synthesis response in all cases in the same assay. No data were available to compare relative genotoxicity outcomes in different cell types in the mammary gland of rats or humans (i.e., fibroblasts versus myoepithelial cells versus epithelial cells across species).

Mechanistic studies have indicated lack of effects of CYP inhibition (Park et al., 2002) or relationship with CYP2E1 status in certain cell lines (Puppel et al., 2005) on genotoxicity outcomes in some tissues and cell types, which would suggest the acrylamide itself generates a genotoxic response in some cases. Studies also indicate that glutathione protects against acrylamide induced genotoxicity (Park et al., 2002; Klaunig and Kamendulis, 2005; Puppel et al., 2005), and that acrylamide induces DNA damage similar to the damage induced by oxidative stress (Chico-Galdo et al. 2006). None of these studies examined the mammary gland, but a role of oxidative stress as an indirect cause of the observed genotoxicity is plausible. Since glutathione binding is saturable and intracellular oxidative stress is tightly controlled, the involvement of these processes would increase the contribution of non-linear dose-response kinetics for DNA damaging events and would support the supposition that high acrylamide doses generate proportionately greater DNA damage than low acrylamide doses.

Specific detailed evaluation of genotoxicity outcomes in the rat mammary gland are largely absent, which hinders a full evaluation of mutagenicity as a viable mode of action hypothesis. This observation of DNA adducts in the mammary gland is mollified by the observation that doses that induce genotoxicity are generally greater than those that induce tumors as described in detail by Dourson et al. (2008) for thyroid tumors. A similar result is obtained for dose-response patterns of mammary tumors (data not shown). Moreover, the dose-response for biomarkers of effect (in vivo mutations or other measures of genotoxicity such as micronuclei) should be given greater weight, since the dose-response behavior for DNA adducts are not expected to correspond to those of mutations – the key precursor event for a mutagenic tumor mode of action (Swenberg et al., 2007). These observations are supported by the tumor patterns noted above, which indicate an important contribution of events other than mutagenicity in the generation of mammary tumors.

#### *Possible Role of Aged-related Hyperprolactinemia*

The observation that tumors that were significantly increased in both available rat bioassays were of endocrine origin has served as a starting point for mode of action hypotheses related to endocrine disruption. Moreover, the absence of significant increases in mammary tumors in male rats in either cancer bioassay implies that hormonal stimulation is required for mammary tumor growth in female rats. One hypothesis (K.S. Crump Group, 1999; Shipp et al., 2006) is that acrylamide acts as a dopamine agonist for the D1 receptors on F344 rat ovaries, and binding to such receptors increases prolactin levels. This acrylamide-stimulated prolactin release is leuteotrophic, stimulating the corpora lutea to secrete progesterone. Aging female F344 rats that have reached a pseudopregnant state have elevated basal prolactin and progesterone levels, which might be responsible for the high background of fibroadenomas in this strain of rats. Acrylamide may further enhance this age-related state of hyperprolactinemia through action at the D1 receptor increasing cell growth promotion in progesterone sensitive cells (such as the stromal cells in the rodent mammary glands). The result of this acrylamide-stimulated increase in progesterone is the production of fibroadenomas. Figure 1 highlights elements of the related neuroendocrine regulation of the mammary glands.

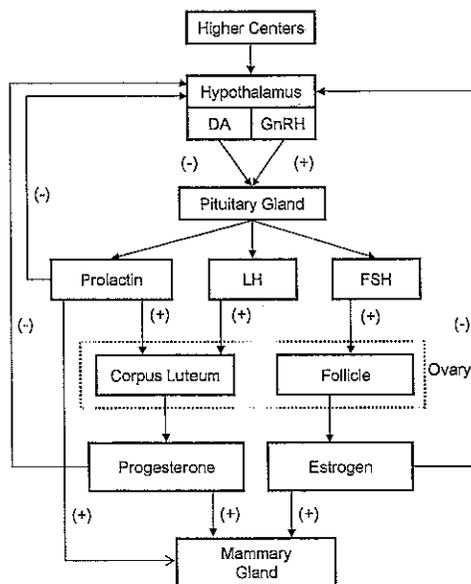


Figure 1 Neuroendocrine regulation of the Mammary Glands. DA = dopamine. GnRH = gonadotropin releasing hormone, LH = leutenizing hormone. FSH = follicle stimulating hormone (From K.S. Crump Group 1999)

A proposed key event in the cascade of events from acrylamide exposure to mammary tumors is the increase in mammary growth promoting hormones either prolactin or progesterone. Unfortunately, direct measurements of hormone levels are limited for acrylamide treated female rats. The two studies identified (Friedman et al., 1999; Khan et al., 1999) evaluated prolactin, but not progesterone levels in female rats. Neither study identified a significant change in circulating prolactin levels at doses significantly greater than those that induced tumors. It is not certain if this reflects the relatively short

duration of the studies (28 days or less) or the use of young adult animals that maintain hypothalamic control of prolactin secretion as opposed to aged or pseudopregnant rats, which have lost this hypothalamic control mechanism. Thus, current studies do not provide direct measurements of the proposed key event. Although direct confirmatory data are lacking for the proposed mode of action, it is noteworthy that many elements of the proposed mode of action remain biologically plausible and coherent based on the current understanding of the biology of these hormone responses.

Shipp et al. (2006) further argue that the observed increase in mammary tumors in F344 rats as developed due to the mode of action described above is not relevant to humans based on several considerations. First, there is a lack of interspecies concordance in hormone regulation. In humans, estrogen is the primary hormone thought to be increased in breast cancer, while in F344 rats progesterone and prolactin increases are most relevant to mammary tissue growth. Second, prolactin is not luteotrophic in primates – i.e., increased prolactin secretion in humans would not stimulate secretion of progesterone from the ovaries. Third, dopamine receptors in human ovaries (D1 receptors) are not involved in steroidogenesis, but in rats the D1 receptor activation increases progesterone levels (Shipp et al., 2006).

Recent data have provided evidence that some of the arguments against human relevance of rat mammary tumors may be incorrect. In particular, the suggestion that estrogen is important for human breast cancer, while prolactin is not important may not be correct. In a review on the relationship between prolactin and breast cancer noted that newer studies and reevaluation of the older data indicate that hyperprolactinemia is related to increased human breast cancer (Harvey, 2005). Meta-analyses show dopamine antagonists that increase prolactin release also increase breast cancer risk. Furthermore, dopamine agonists, which were hypothesized to decrease tumor growth by reducing prolactin release from the pituitary, may have had no significant effect on breast cancer treatment in prior studies because breast tumors produce prolactin independently of the pituitary-derived prolactin and are stimulated via paracrine regulation. This newer analysis by Harvey (2005) suggests that increased prolactin secretion is a risk factor for breast cancer in both rodents and humans.

The renewed insight into the important role of prolactin in human breast cancer does not negate the second and third points hypothesized by Shipp et al. (2006). These two points reflect on the important differences between humans and rodents with regard to age-related changes in hormone status and the likely impact of acrylamide in enhancing stimulatory hormone levels. Unfortunately, there are significant remaining data gaps with respect to these points. No data are available to indicate whether acrylamide would enhance progesterone or prolactin levels in humans, and no adequate data in animals were identified (as noted above). In addition, no data on acrylamide binding to dopamine receptors was identified from the published literature. Acrylamide is referred to as a “weak” dopamine receptor agonist by Shipp et al. (2006) and may act as other weak dopamine receptor agonists (e.g., apomorphine). No cancer bioassays in F344 rats were identified for other dopamine agonists to test the concordance in tumor responses between demonstrated dopamine agonists and acrylamide.

Some data on the ability of acrylamide to alter dopamine responsiveness at the level of the central nervous system has been described, but the data are limited and in some cases contradictory. Subacute exposures in male rats at 10 mg/kg-day for 10 days did not alter dopamine levels in the hypothalamus (Ali et al., 1983). More recently, Bowyer et al., (2008) reported that exposure of male F344 rats to acrylamide for 14 days had no effect on levels of dopamine or its metabolites in the hypothalamus even at doses as high as 50 mg/kg that caused clinical signs of neurotoxicity (lethargy and hind limb paralysis). The authors reported that no consistent treatment related effects consistent with altered hypothalamic regulation of hormone levels or hormone function were observed. However, as described in detail by Dourson et al. (2008), consistent with other investigators at least some indications of altered thyroid homeostasis were evident. The use of the Bowyer et al. (2008) study for the evaluation of mammary tumors is limited since female rats were not included in their study. It is also noteworthy that the absence of effect on neurotransmitter levels in various brain regions does not preclude an influence on neurotransmission. Recent studies showed that the effects of acrylamide in the nervous system may be as inhibitors of dopamine release to synapses caused by interaction with sulfhydryl groups on specific proteins involved in pre-synaptic vesicle loading or membrane fusion (LoPachin et al., 2006). Rats exposed orally to acrylamide 21 mg/kg-day for 21 days experienced nerve terminal degeneration in several brain regions, but not in the hypothalamus. However, in animals injected with 50 mg/kg-day acrylamide for five days, the effects were generally more severe and effects were also observed in the hypothalamus. The authors suggest that acrylamide toxicity is caused by nerve terminal effects that are initiated in a dose-response sequence as: disruption of dopamine uptake and release, morphological changes observed by silver staining (argyrophilic changes), nerve terminal degeneration, and finally clinical signs of neurotoxicity.

It is possible that with longer-term exposures not evaluated in the current studies, acrylamide-induced neurotoxicity might ultimately be seen to decrease dopamine responsiveness in the hypothalamus. Such a response would be consistent with the reversal of compensatory responses often observed in short-duration studies, and would also be expected to further augment the age-related loss of dopamine regulation observed in female F344 rats (independent of the effects on the ovaries). Thus, acrylamide-induced neurotoxicity alone might be sufficient to explain the enhancement by acrylamide of the natural background tumor response induced by age-related hyperprolactinemia and would serve as a unifying hypothesis to link the broad array of tumor responses observed in tissues regulated by the hypothalamus. Little data exist to examine this hypothesis, but it meets some aspects of the modified Hill criteria (U.S. EPA 2005), including biological plausibility and dose-response concordance (since measures of nerve damage were observed in the cancer bioassays at or below the tumorigenic doses). If the site of action in inducing mammary tumors is primarily at the level of the hypothalamus, then arguments related to the difference in D1 receptors in the ovaries would be given less weight, and human relevance of tumors would be considered more likely.

The proposed modes of action by Shipp et al. (2006) related to enhancement of prolactin-induced mammary tissue growth remain plausible, while the specific mechanism of toxicity is uncertain. At least one alternative hypothesis (neurotoxicity in the hypothalamus) would be consistent with the data and would suggest possible human relevance. Furthermore, as pointed out by Russo and Russo (1996) and Rudel et al. (2007), while the rodent mammary tissues endocrine response is different from that of humans in many ways, the model is considered sufficiently useful for application to human health risk assessment. This conclusion is borne out in current risk assessments for other mammary tumorigens. Thus, based on the various considerations noted above, the rat mammary tumors cannot be fully discounted and should be considered relevant to human health risk assessment. In either case; however, the effect of acrylamide on mammary tissue growth secondary to neurotoxicity at the level of the hypothalamus or due to dopamine agonist activity at the level of the ovaries would be most consistent with a non-linear dose response approach. Furthermore, based on age-related changes in neuroendocrine regulation of the mammary glands in female F344 rats, it is likely that this particular strain of rats would be more sensitive than humans to the mammary growth promoting effects of acrylamide regardless of whether acrylamide is augmenting the background process of providing tumor promotion through independent mechanisms.

#### *Direct Progression of Benign Tumors*

A second important consideration for assessing the relevance to human health of the observed array of tumors in the F344 rat mammary gland is whether they can progress to malignancy. U.S. EPA (2005) cancer risk assessment guidelines address evaluations of data based on only benign tumors. According to these guidelines, if only benign tumors are observed, there is no evidence for progression to malignancy, and there is no harmful effect of the benign disease itself, then such tumors are not considered in quantitative dose-response for the cancer risk assessment. Occasions where benign tumors may progress are to be evaluated on a case-by-case basis. Therefore, a key consideration for acrylamide is whether any of the benign tumors observed in the Johnson et al. (1986) or Friedman et al. (1995) studies may progress to malignant tumors and thus, should be included quantitatively in a cancer risk assessment.

Three distinct types of benign mammary tumors were identified in the chronic bioassays: fibromas, adenomas, and fibroadenomas. With regard to the fibromas, these tissues represent a distinct cell-type of origin (fibroblasts) and the lack of direct progression of these tumors is evidenced by the absence of fibrosarcomas. However, one caveat is that it is possible due to limited sectioning of large tumors to misclassify such tumors if only small or localized areas of epithelial tissue are involved. Due the possibility that this type of misclassification had occurred a conservative approach taken in our analysis is to consider fibromas as fibroadenomas. If it were known that the fibromas were distinct tumors (and not actually fibroadenomas) they would not be considered appropriate for direct combination with other tumors in the cancer assessment, since they would be considered to have derived from a histologically separate tissue and since no fibrosarcomas – the malignant tumor correlate of fibromas – were reported. This conclusion is consistent with current U.S. EPA (2005) guidelines.

The adenomas are a benign tumor of epithelial cell origin. Thus, one would reasonably make the argument that adenomas are a potential precursor tumor to the related malignant tumors (adenocarcinomas), and thus should be considered quantitatively in the cancer dose-response assessment.

The appropriate treatment of the fibroadenomas requires more complex consideration, since this tumor type is of mixed cell type origin and there is an increase in fibroadenomas incidence in both chronic bioassays (Johnson et al., 1986; Friedman et al., 1995), although not statistically significant in the earlier of these two studies. Some recent evaluations have concluded that the fibroadenomas observed in female rats should not be included in a quantitative cancer risk assessment for acrylamide (Shipp et al., 2006). This conclusion appears to have been reached on two grounds. First, the authors argued that tumors are induced by a mechanism that is not relevant to humans (i.e., acrylamide-induced hyperprolactinemia in aged rats). This first concern was discounted, at least in part, as described above. The second argument that has been levied against the inclusion of the fibroadenomas is that these tumors are histologically distinct from the only malignant tumors observed (adenocarcinomas) and that fibroadenomas do not progress to adenocarcinomas (or other malignant tumors). The potential evidence for progression of fibroadenomas and their relevance to human breast cancer is discussed in this section.

Several recent studies noted below comparing molecular markers from fibroadenomas and carcinomas support (at least in some cases) the conclusion that there is no (or limited) direct progression of fibroadenomas to adenocarcinomas. Marxfeld et al. (2006) found no overall similarities in gene expression profile between spontaneous fibroadenomas and adenocarcinomas in SD rats and concluded that on this basis there was no evidence of direct progression from fibroadenoma to adenocarcinomas – i.e. no evidence of clonal expansion. The authors note that this analysis did not test adenocarcinomas arising in fibroadenomas and the analysis does not account for Phyllode tumors. Franco et al. (2003) found no evidence that genetic changes (loss of heterozygosity) could discriminate fibroadenomas in 32 breast cancer cases from fibroadenomas in 26 patients without malignant disease. The same genetic changes were not found in the fibroadenomas as in malignant neoplasia within the fibroadenomas – indicating to the authors that genetic changes in fibroadenomas were not a risk factor for malignant disease. However, this conclusion does not consider the possibility that the difference in markers reflected those related to progression to malignancy, and thus other untested markers might have been shared between the benign and malignant tumors. Rizou et al. (2004) conducted cytogenetic analyses of short-term cultures of fifty-two samples of human fibroadenomas. Compared to carcinomas, fibroadenomas had less complex cytogenetic rearrangements and limited alterations in the three oncogenes studied (HER-2/neu, CCND1 and c-MYC). However, a subset of fibroadenomas had amplification of CCND1, supporting the hypothesis that some fibroadenomas display changes also found in carcinomas. The authors indicate that patients belonging to the group of individuals from which benign tumors show complex gene changes might have an increased risk for subsequent breast cancer. Kuijper et al. (2002) investigated the

clonality of both the stroma and the epithelium components of fibroadenomas. Nineteen fibroadenomas and nine phyllodes tumors (malignant tumors of mixed epithelial and stromal cells) were analyzed. Based on the patterns of clonal expansion, it was suggested that fibroadenomas can progress in an epithelial direction to carcinoma within the fibroadenoma and in a stromal direction to form phyllodes tumor. Thus, the available data from analysis of fibroadenomas for cell markers are mixed with regard to evidence for direct progression of fibroadenomas to adenocarcinomas based on analysis of the commonalities between various genetic markers and benign and malignant tumors. Direct evidence of progression of fibroadenomas does exist for production of phyllodes tumors or possibly carcinomas in fibroadenomas, but not for the formation of discrete adenocarcinomas (as seen in the rodent bioassays).

A second line of evidence for assessing the relevance of fibroadenoma to breast cancer is the evaluation of whether fibroadenoma is considered a risk factor for developing malignant disease in humans. This consideration has been explored by numerous investigators (Carter et al., 1988; Dupont et al., 1994; Carty et al., 1995; Ciatto et al., 1997; Markopoulos et al., 2004; Hartmann et al., 2005). In reviewing these and related studies the evidence indicates that only complex fibroadenomas or those with proliferative features are a risk factor for malignant disease. However, the positive correlations between benign and malignant disease do not necessarily provide sufficient evidence of direct cellular transformation, since the increase in both benign and malignant disease may arise independently from the same exposure. Moreover, translating these findings related to human breast tumor pathology and the evaluation of progression of fibroadenomas in the rat studies is complex.

Overall, the evidence does not indicate that benign non-proliferative lesions (such as simple fibroadenomas) increase the risk for malignant disease. Furthermore, direct molecular evidence of progression of fibroadenomas to malignant tumors is limited to unique tumor types: phyllodes tumors and carcinomas in the fibroadenoma itself. Thus, direct progression of fibroadenomas to independent (i.e., non-adjacent) adenocarcinomas has not been demonstrated on the basis of molecular studies or studies in human breast cancer patients. This conclusion is also supported by the tumor data reported in the chronic bioassays, since no animals were observed that had both fibroadenomas and adenocarcinomas.

#### *Potential Role of Stromal Epithelial Cell Interactions*

With evidence absent for the direct progression of fibroadenomas to adenocarcinomas, the use of combined tumor incidence data for fibroadenomas with adenoma and adenocarcinomas is not supported for a genotoxic mode of action hypothesis. To do so appropriately would require mutation and clonal expansion of fibroadenomas directly to adenocarcinomas. However, before discounting the evaluation of fibroadenomas for risk assessment, the potential for other relevant interactions between fibroadenomas and adenocarcinomas should be considered. Such an interaction might include the action of fibroadenomas to act via threshold mechanisms to promote the growth of adenomas and

adenocarcinomas. If such a mode of action were occurring, then combining benign and malignant tumors for a non-linear dose-response assessment could be appropriate. Recent understanding of stromal disruption (i.e., dysregulation of fibroblasts) as a promoter of epithelial tumors (such as adenocarcinomas) supports such an interaction as a plausible hypothesis in acrylamide tumor response.

Several authors have reviewed the molecular biology of stromal-epithelial cell communication (Wiseman and Werb, 2002; Kim et al., 2005; Barcellos-Hoff, 2005). The importance of these interactions has been tested directly for known mammary tumorigens. Tsai et al. (2005) exposed normal mammary stromal fibroblasts to ionizing radiation – an undisputed mutagen. The irradiated fibroblasts altered the growth of the normal epithelial cells and increased the invasive properties of transformed epithelial cells. Maffini et al. (2004) exposed mammary epithelial cells to MNU *in vitro* either to the mutagen or vehicle before transplanting the exposed cells into the fat pads of rats (cleared of epithelial cells) exposed to carcinogen or vehicle. Neoplastic transformation of these mammary epithelial cells occurred only when the stroma was exposed *in vivo* to MNU, regardless of whether or not the epithelial cells were exposed. This result indicates that the stroma is a crucial target of direct acting mutagens, and such effects on the stroma can induce epithelial tumors (such as adenocarcinomas).

This type of paracrine interaction would be consistent with the pattern of tumors in the cancer bioassays, where acrylamide affects stromal cells in rat mammary gland via an unknown mode of action, ultimately producing fibroadenomas. The fibroadenomas enhance the potential for pre-initiated epithelial cells to grow to form adenocarcinomas. This proposed mode of action would suggest that acrylamide is acting as a malignant tumor promoter via epigenetic mechanisms. This promotion mechanism is consistent with data for:

- 1) Both ionizing radiation and MNU treated mammary cells,
- 2) Acrylamide tumor promotion study by Imai et al. (2005), and for
- 3) Observation of tumors in tissues of high background incidence.

The specific mechanism for this promotion is unknown. Numerous possibilities exist that involve induction of cell signaling pathways in response to hormones, growth factors, or other stress responses in the stroma. Non-of these paracrine mediated mechanisms (even if originally caused by a genotoxic event in the stromal cell) would yield a linear dose-response.

### **Dose-response Options**

Using a reasonable standard of scientific certainty, the cancer risk assessment pertaining to mammary tumor dose-response can be narrowed to two options – the presentation of which would be consistent with current U.S. EPA (2005) practice.

Option 1: The mode of action for the adenomas and adenocarcinomas is uncertain and could be due to epigenetic mechanisms (i.e., endocrine control disruption), direct

genotoxicity, indirect genotoxicity, or due to a combination of these or other modes of action. Consistent with the default approaches of several organizations, one could conduct a linear low-dose response assessment. However, if this approach were selected, only the combined incidences of adenomas and adenocarcinomas should be used, since this reflects the development of tumors in one cell type that can progress to malignancy (McConnell et al, 1986)<sup>2</sup>. A contribution of direct DNA reactivity to the tumor dose-response cannot be ruled out, but the overall weight of evidence supports the use of a modeling approach that reflects the growing knowledge of cancer biology. The analysis by Swenberg et al. (2007) supports a role of processes beyond the initial DNA interactions that yield DNA adducts as modulators of the dose-response for genotoxicity. Genotoxicity does not lead mammary tumors in terms of the dose response (see for example the results of Dourson et al., 2008 for thyroid tumors, which also applies for mammary tumors since the same tumor doses were used). Such analyses show that a traditional linear approach probably overstates the risk, since at low doses endogenous DNA damage may be the driver for the cancer risk and other rate limiting mechanisms are likely to generate non-linear tumor responses as the dose increases. Such mechanisms include protection by glutathione, a requirement for ongoing growth promotion by hormones, or other epigenetic processes. Together, these considerations can be used to inform the dose-response assessment even if low-dose linear modeling is used.

Option 2: Combine adenoma, adenocarcinoma, fibroadenoma and fibroma data from the bioassays as the basis for deriving a point of departure for tumor promotion potency (tumor promotion event might be mutational or non-mutational in stromal cells). However, this POD should be carried forward using a non-linear approach, such as the judgment of a No Observed Adverse Effect Level (NOAEL) or benchmark dose (BMD), choice of uncertainty factors and estimation of a Reference Dose (RfD). This is because the combination of these 4 tumor types is not strictly in keeping with current guidelines (McConnell et al., 1986), and because linear modeling of these combined data violates the assumption of one molecule possibly leading to one tumor, since at least two cell types are involved. In contrast, the more general model of NOAEL divided by an uncertainty factor does not violate this assumption and can be used.

As a starting point for the dose-response analyses, the data for each tumor type was plotted as shown for Johnson et al. (1986) in Figure 2a and Friedman et al. (1995) in Figure 2b shown below.

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<sup>2</sup> Updates to these existing guidelines on combining neoplasms will maintain the recommendation to not combine adenomas and adenocarcinomas with fibroadenomas (McConnell, personal communication)

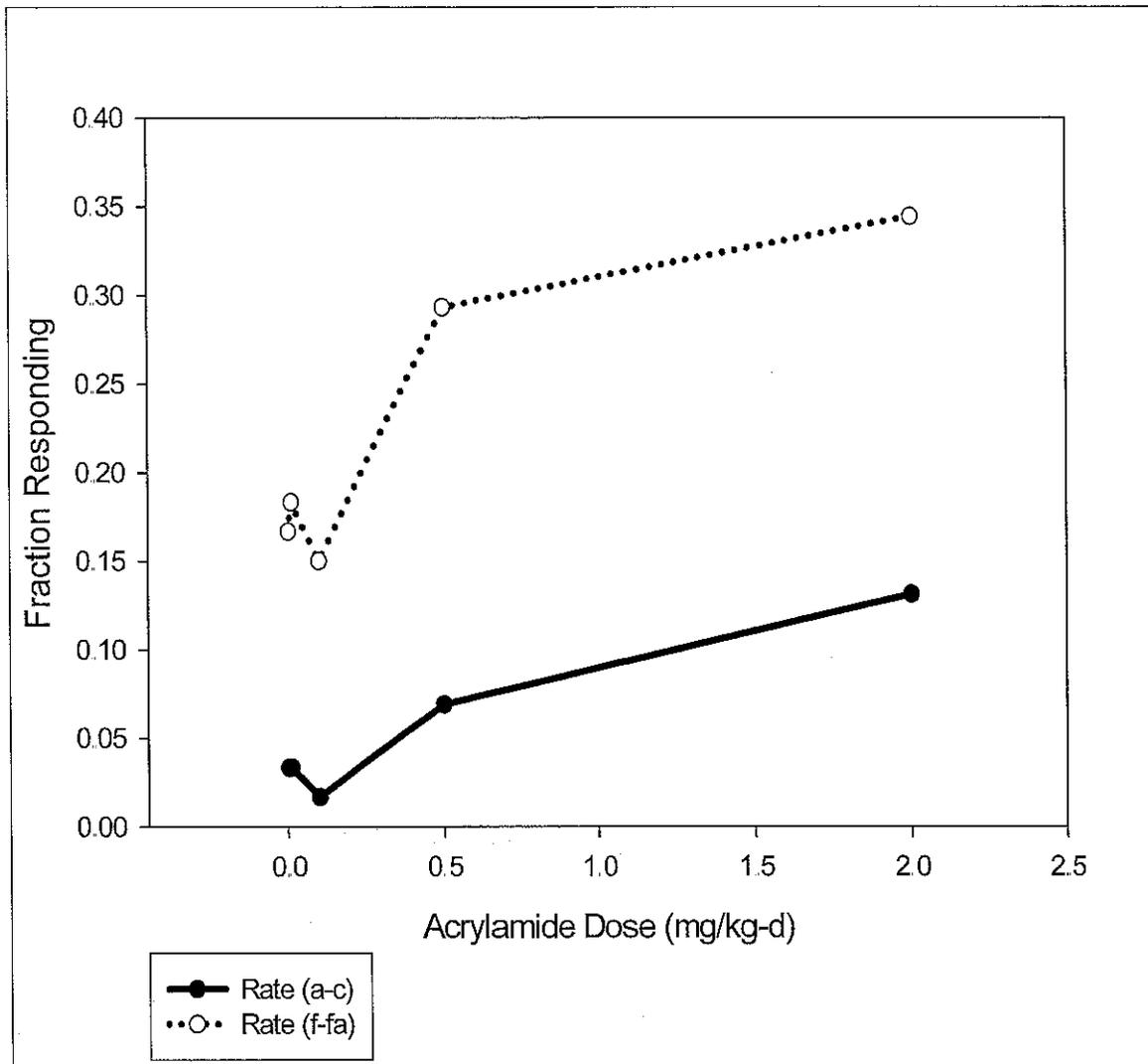


Figure 2a. Female mammary tumor data from Johnson et al., 1986. The lowest doses were jittered to make the different data points more visually distinguishable. Doses adjusted for dietary exposure of 0.002 mg/kg-d. a-c: adenomas & carcinomas; fa: fibroadenoma, f: fibroma.

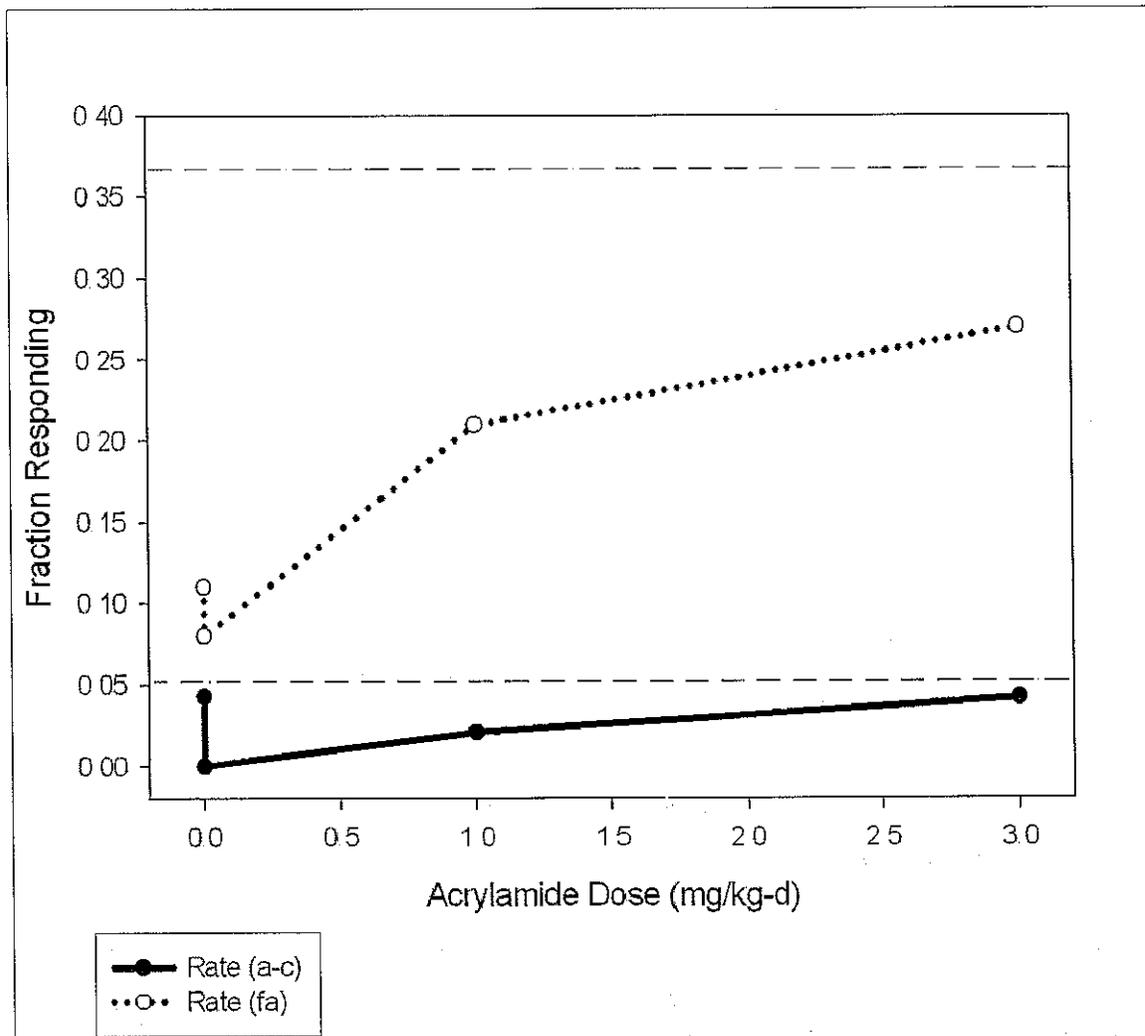


Figure 2b. Female mammary tumor data from Friedman et al., 1995. Doses adjusted for dietary exposure of 0.002 mg/kg-d. Fibromas were not reported. a-c: adenocarcinomas, fa: fibroadenoma. Historical controls from the National Toxicology Program (2008) are represented in the dashed lines at 5.2% for carcinomas or adenomas and 36.7% for fibroadenomas.

Data from the Johnson et al. (1986) study show an initial flat slope (or even a slight decrease) for adenomas and adenocarcinomas and then a gradual rise with dose (see Figure 2a), whereas those from the Friedman et al. (1995) study show a similar response at the two lower doses and a slight, but not statistically significant, rise at the high dose (see Figure 2b). Benchmark doses were estimated using U.S. EPA's Benchmark Dose software and involved the default multistage (MS) cancer model and the log(dose) probit model, both with non-negative parameters. The choice of these two models was based in part on the prior analysis for thyroid tumors from these same two data sets (Dourson et al., 2008). All fits were statistically acceptable ( $p$ -value > 0.10) except for the pooled data sets of the two studies (Table 4a). The Friedman et al. (1995) data set showed consistently lower response rates (higher BMD values) than the Johnson et al. (1986) data set, which might be expected since the tumor responses were not statistically significant. For both the Johnson et al. (1986) data and pooled data sets, the use of the MS model resulted in a one-stage process, i.e., only the background and linear parameter (coefficient of dose) were estimated. Consequently, the slope factor values from the lower bound on the benchmark dose estimates corresponding to a 10% increased tumor incidence ( $BMDL_{10}$ ) were very close to those based on the estimate of a 2% increase – the  $BMDL_{02}$  (data not shown). The MS model for the Friedman et al. (1995) data included only the background and cubic coefficients, which is reflected in the change in slope between the benchmark response (BMR) of 0.10 and 0.02 values (data not shown). The probit also exhibits pronounced curvature, which is consistent with the visual shape of the data and is similarly reflected in the change in slopes between the two BMR values.

The Johnson et al. (1986) data set gives the strongest results because of the number of dose groups and the larger tumor increase. The Friedman et al. (1995) data set is less useful for modeling because the two lower doses gave a similar response and the response of the only other dose was not statistically significant. The pooled data set did not produce an adequate fit from either model because the two data sets are not consistent with each other. The slopes are significantly different between these two models for the Johnson et al. (1986) data. For the  $BMR=0.10$ , the probit slope is about 80% that of the multistage, whereas for the  $BMR=0.02$ , the probit slope is about one-third the MS slope, both for the upper bound and the median slope values. The probit model is usually employed when there is a fairly flat slope that then increases with dose, as shown in the data. The multistage (MS) model can increase gradually if the linear coefficient is dominant, but can exhibit sharp increases if only higher order coefficients are present.

Tables 4a and 4b show individual tumor types, combinations of tumor types and pooled tumor data across the two studies modeled to estimate the dose associated with either a 10% or 2% increase in tumor response ( $BMD_{10}$  or  $BMD_{02}$ ), the lower bound estimates on the dose associated with these estimates ( $BMDL_{10}$  or  $BMDL_{02}$ ), and slopes of the lines from either the BMDs or the BMDLs to the origin ( $Slopes_{10}$  or  $02$ )<sup>3</sup>. Note that slope factors were not calculated for the datasets that combined adenomas, adenocarcinomas,

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<sup>3</sup> The choice of a  $BMD_{02}$  is consistent with EPA (2005) guidelines in that points of departure should be as close to the low dose part of the data as is feasible. See also Dourson et al. (2008) for additional discussion. Not all data are shown, but are available upon request.

fibroadenomas and fibromas since a non-linear dose-response approach (i.e., RfD) is most appropriate for the analysis of these combined data (as in option 2 discussed above).

For option 1, the slope factors derived from the adenoma plus adenocarcinoma data sets from a BMDL<sub>02</sub> probit model are considered more relevant for estimating the low-dose behavior of acrylamide mammary tumorigenesis if one elects a conservative linear dose-response procedure (i.e., a BMDL<sub>02</sub> of 0.6 mg/kg-day and a slope factor of 0.033). These slope factors are more relevant than those derived from the BMDL<sub>10</sub> multistage model, because:

- the multistage model is not as capable at visually fitting the observed low dose female mammary tumor data;
- the probit model is more consistent than the multistage model with the observation that genotoxicity does not lead tumorigenicity for mammary tumors, similar to this observation for thyroid tumors (Dourson et al., 2008); and the curvature of the probit model is more likely to better reflect the contribution of nonlinear processes, which are likely to be occurring even with these tumors.

**Table 4a. Benchmark Doses and Slope Factors from BMDS for Option 1, using combined adenoma + adenocarcinoma responses.**

BMR	Model	Data set <sup>a</sup>	BMD	SF at BMD	BMDL	SF at BMDL	p-value <sup>b</sup>
0.10	MS- Ca <sup>c</sup>	J ac	1.8	0.055	0.99	0.10	0.84
		F ac	5.1	0.019	3.5	0.029	0.35
		Pooled ac <sup>e</sup>	7.1	0.014	3.2	0.031	0.07
0.02	Probit <sup>d</sup>	J ac	0.90	0.022	0.60	0.033	0.58
		F ac	3.0	0.0070	1.7	0.011	0.15
		Pooled ac <sup>e</sup>	2.3	0.0090	1.4	0.014	0.03

<sup>a</sup>F: Friedman, et al., 1995; J: Johnson, et al., 1986. Pooled= both studies; ac = adenoma & carcinoma. Full BMD results are available upon request.

<sup>b</sup>Significance levels (p-values) greater than 0.1 indicate acceptable fit.

<sup>c</sup>Estimates derived using US EPA Benchmark Dose Software 1.4, Multistage-cancer model 1.5.

(SF at BMD)=BMR/BMD10. (SF at BMDL)=upper 95% bound on Slope.

<sup>d</sup>Estimates derived using US EPA Benchmark Dose Software 1.4, Probit log(dose) model.

(SF at BMD)=BMR/BMD02. (SF at BMDL)=upper 95% bound on Slope.

<sup>e</sup>Note that the Johnson and Friedman data sets are not consistent with each other and that the most plausible dose response assessment might involve the pooled data, despite the generally poorer model fit.

**Table 4b. Benchmark Doses and Slope Factors from BMDS for Option 2, using combined adenoma + adenocarcinoma + fibroma + fibroadenoma responses.**

BMR	Model	Data set <sup>a</sup>	BMD	SF at BMD	BMDL	SF at BMDL	p-value <sup>b</sup>
0.10	MS- Ca <sup>c</sup>	All tumors					
		Johnson	0.46	--	0.30	--	0.50
		Friedman	1.2	--	0.78	--	0.44
		Pooled <sup>e</sup>	1.0	--	0.71	--	0.02
0.02	Probit <sup>d</sup>	All tumors					
		Johnson	0.11	--	0.0048	--	0.75
		Friedman	0.093	--	0.00	--	0.27
		Pooled <sup>e</sup>	1.0	--	0.71	--	0.017

Option 2 as noted above would provide a non-linear assessment using the RfD approach (U.S. EPA 2005). This approach would use a BMDL<sub>10</sub> estimate for the combined data sets (ac-fa) of 0.30 mg/kg-day (the lowest combined BMDL<sub>10</sub>) followed by application of uncertainty factors as described in EPA cancer guidelines. This approach would yield

a higher safe dose than a risk specific dose of 1 in  $10^5$  individuals determined from option 1 and was not further derived here.

It might be possible to build a model that captures the potential contribution of mutagenicity in epithelial cells at the low dose and also considers the growth promotion influences caused by hormones and/or interactions from the stromal cells. Such a model is more likely to be biologically correct than either options 1 or 2. However, the ability to estimate the appropriate dose-response function for the Johnson et al. (1986) data is limited by the lack of data above the postulated threshold, which is any dose from 0.2 to 0.5. Moreover, the Friedman et al. (1995) study only has two doses level below threshold (the dietary background) and only four data points total. Taken together, insufficient data exist to derive a dose-response model that fully captures the biology. The probit modeling shown in Table 4 (and further described in option 1) is a reasonable approximation to this ideal model at this time.

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