Report Title

ACRYLAMIDE: A TWO-YEAR DRINKING WATER CHRONIC TOXICITY-ONCOGENICITY STUDY IN FISCHER 344 RATS

A HISTOPATHOLOGY PEER REVIEW AND PATHOLOGY WORKING GROUP REVIEW OF PROLIFERATIVE LESIONS INVOLVING THE MESOTHELIAL LINING CELLS OF THE TUNICA VAGINALIS OF THE TESTES IN A TWO-YEAR DRINKING WATER CHRONIC TOXICITY-ONCOGENICITY STUDY IN FISCHER 344 RATS WITH ACRYLAMIDE

PWG REPORT

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AND PATHOLOGY WORKING GROUP REVIEW
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IN A TWO-YEAR DRINKING WATER
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FISCHER 344 RATS WITH ACRYLAMIDE

CERTIFICATION OF GOOD LABORATORY PRACTICES

This histopathology peer review and pathology working group do not constitute a study. Therefore, Good Laboratory Practice Standards (40 CFR, Part 160) do not apply to this report. However, all appropriate documentation of the review process has been maintained.

Signature: ____________________________________________
Dr. Henry G. Wall
Author

Date
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# A HISTOPATHOLOGY PEER REVIEW
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EXECUTIVE SUMMARY

A Pathology Working Group (PWG) Review was convened to review pathology findings involving the mesothelial lining cells of the tunica vaginalis that were reported by the Study Pathologist and to examine the spectrum of proliferative or reactive changes involving mesothelial lining cells that were reported by the Study Pathologist or Reviewing Pathologist for the testes or epididymides; evaluate mechanistic data; and to provide advice to the study sponsor, SNF SAS, on the biological significance of mesothelial lining cell proliferative lesions in rats that occurred in the study reported by Johnson et al, (1984 and 1986) and the relative risk of mesothelial cell proliferative lesions in humans. The purpose of the PWG was to review diagnoses of proliferative or reactive lesions involving mesothelial lining cells of the tunica vaginalis, provide a consensus diagnosis for differences in diagnoses reported by the Study or Reviewing Pathologists, and to provide expert guidance concerning the biological significance of proliferative or reactive mesothelial lining cell lesions in Fischer 344 rats and the relative risk of these lesions in humans.

The Pathology Peer Review that was completed by Dr. Rodney A. Miller (Experimental Pathology Laboratories, Inc.) disclosed only three animals with different diagnoses for tunica vaginalis mesothelial lining cell effects than those rendered by the Study Pathologist. For two of these three animals, where the original diagnoses were “reactive mesothelium” and “mesothelioma”, the PWG panel consensus was “no mesothelial cell lesion observed.” For the other animal, whose original diagnosis was “no mesothelial cell lesion observed,” the consensus diagnosis was “reactive mesothelium.” The PWG panel also determined that “no mesothelial cell lesion observed” was the consensus diagnosis for six rats that were diagnosed by both pathologists as “reactive mesothelium.” These differences would not change the original interpretation of the chronic toxicity or carcinogenicity potential that was originally reported for the
study. The PWG Panel also evaluated and discussed published acrylamide toxicity assessments and toxicology research data; assessed the role of genotoxicity and hormonal imbalance in the induction of mesothelial cell tumors and the relevance of tunica vaginalis mesotheliomas (TVMs) in the F344 rat testes to human health risk assessment.

The Pathology Group concluded that:

- The Fischer 344 rat is not a good model for identifying chemically-induced testicular related effects because of the very high spontaneous rate of Leydig cell tumors

- The TVMs in this study appear to be rat specific and most likely not relevant to other species including man

- A genotoxic mechanism is not likely involved as:
  - the liver, which is the major site of metabolism, was not a target and no non-scrotal areas of the mesothelium were involved
  - the tumors have a late onset (noted after 92 weeks)
  - the mesothelial tumors are present in only one sex (males) and only affect one mesothelial site (tunica vaginalis)

- Hormonal profile, particularly as relates to prolactin in the Fischer 344 rat, is not relevant to man

- Hormonal imbalance (prolactin-related) is the most likely mechanism of tumor formation. Since the hormone profile is not relevant to man, this mechanism is not relevant to man

- Tunica vaginalis testes mesotheliomas are extremely rare in man but relatively common tumors in the Fischer 344 rat

- Acrylamide-induced TVM tumor morphology was not unique but the same as TVM tumor morphology in control animals
INTRODUCTION

Morphologic Characterization of Mesothelioma and Mesothelial Cell Reactive Lesions in Rodents

Mesotheliomas of the tunica vaginalis mesothelium of the testes and epididymis are not uncommon in Fischer 344 (F344) rats. Gould (1977) described nine occurrences of mesothelioma on the tunica vaginalis propria of the testis and epididymis of F344 rats from a group of 384 control (unexposed) male rats (2.3%) that had been maintained on various experimental diets in long-term feeding studies. Including females, about 700 rats were used in the three separate studies from which these animals were drawn. There were no occurrences of mesothelioma among the females. Among the nine rats, four had extension to the peritoneal cavity. The lesions were limited to the testis, epididymis, and spermatic cord in the least affected rats. The lesions ranged from 2 mm in diameter to large masses covering the visceral and parietal peritoneum. There was one instance of lymphatic invasion but no findings of distant metastasis. The lesions were described variably as confluent, rough superficial thickenings of the serosa, sessile broad-based nodules, and complex papillary structures. Papillary lesions had the most stroma. Cells were cuboidal to endothelioid in papillary tumors, and the more solid tumors had cuboidal cells on the surface and epithelioid cells with indistinct cytoplasmic borders more centrally. Nuclei with abundant chromatin and a prominent nucleolus were common to all tumor types. Pseudolobulated cells with peripheral spindle-shaped cells were noted in the centers of the more solid masses. Gould also noted the coincidence of Leydig cell tumors, but indicated there was no difference in the incidence of Leydig cell tumors between the mesothelioma and non-mesothelioma-bearing group. In his report, Gould reviewed ten studies wherein mesotheliomas had been reported and noted that the occurrence of mesotheliomas extending beyond the scrotum was unusual.

Mitsumori and Elwell (1988) reviewed the slides of mesothelial proliferative lesions in 30 rats that were randomly selected from 754 rats that had the diagnosis of mesothelioma in the NTP TDMS database of 51,230 F344 rats and proposed criteria to differentiate mesothelioma from mesothelial hyperplasia. They characterized hyperplasia as having a simple papillary growth pattern, cuboidal cells, no stratification, no pleomorphism, no stromal proliferation, no invasion, and indicated mitosis was uncommon. Mesothelioma was characterized by complex
papillary or glandular growth; cuboidal, columnar, or flattened cells that were sometimes stratified; pleomorphism, stromal proliferation, and as having mitosis and invasion present sometimes.

Hall (1990) described the normal peritoneum of F344 rats and pathologic conditions affecting the peritoneal cavity. He indicated that in the F344 rat, mesothelioma was the most common neoplasm of the peritoneal cavity; that almost all spontaneous mesotheliomas are thought to arise from the tunica vaginalis of male rats; and that mesotheliomas may occur in female rats around the ovary or elsewhere in the abdomen but were rare in comparison to occurrence in male rats. He provided descriptive criteria to distinguish mesothelial hyperplasia from mesothelioma. Mesothelial hyperplasia is characterized by focal thickening or single papillary (villous) projections of mesothelial cells without stromal proliferation. This lesion was considered a common lesion in the tunica vaginalis of males. Mesotheliomas are characterized as being predominantly papillary in form with several layers of mesothelial cells covering a fibrovascular stroma. Cells are polyhedral to cuboidal with abundant cytoplasm and round to oval nuclei with single or multiple nucleoli. Larger neoplasms can have prominent stroma and be covered by flattened or polyhedral mesothelial cells. Mesotheliomas may also have solid areas with a scirrhous response surrounding pleomorphic cells in clusters or forming tubular structures. Invasion of adjacent tissue is considered to be a characteristic of malignant tumors, but the invasion may be dependent on the type of adjacent tissue. Most mesotheliomas are thought to spread by direct extension, but some are thought to metastasize via the lymphatics. Hall also noted the microscopic classification of mesotheliomas in humans as epitheliomatous, sarcomatous, or mixed types; and provided the comparison that most observed in F344 rats are the epitheliomatous type, often with a stromal component.

The team of McConnell et al., (1992) developed and reported guidelines for diagnosing proliferative lesions of testes in rats. They briefly noted the much higher incidence of mesothelioma in the testes of F344 rats as compared to other rat strains and that most arise from the tunica vaginalis. They proposed morphologic criteria for distinguishing mesothelial hyperplasia from malignant mesothelioma, concentrating on pattern of growth and stroma with less characterization of cellular detail as compared to the description provided by Hall (1990).
Mesothelial Cell Proliferative Lesions in Acrylamide-Exposed Rats

Mesothelial cell proliferative lesions were reported and characterized for two chronic toxicity/oncogenicity studies of acrylamide administered to F344 rats in drinking water (Johnson et al., 1984 and 1986; Friedman et al., 1995) at doses from 0 to 2.0 mg/kg/day for 24 months. The first studies, by Johnson et al., (1984 and 1986), described statistically significant increased incidences of TVMs in male rats at doses of 0.5 and 2.0 mg/kg/day (18.3% and 16.7%, respectively, compared with 5% in controls). Three rats in each of the two groups had extension to the peritoneal cavity. The first occurrence of mesothelioma was noted in a rat in the 0.5 mg/kg/day group that died on day 646. All mesotheliomas were classified as malignant. Reactive mesothelium was noted in rats from all dose groups and the highest incidence (eight rats affected, 13.3%) occurred in rats dosed at 0.01 mg/kg/day.

### Mesothelioma in Male Rats in Two-Year Acrylamide Studies

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<th>Study</th>
<th>Dose (mg/kg/day)</th>
<th>0</th>
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<th>0.5</th>
<th>2.0</th>
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<td>60</td>
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<tr>
<td>Friedman</td>
<td>102</td>
<td>204</td>
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<tr>
<td>Johnson</td>
<td>3¹, 7, 11², 10²</td>
</tr>
<tr>
<td>Friedman</td>
<td>4, 9, 8, 13³</td>
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¹Number of animals affected per dose group.
²Statistically significant difference from control group, mortality adjustment via Mantel-Haenszel procedures (Peto, 1974) α = 0.05
³Statistically significant difference from control group, (Peto et al., 1980) p<0.001.

The lifetime study by Friedman et al., (1995) confirmed the increased occurrence of scrotal mesotheliomas involving the tunica vaginalis at the high dose among male F344 rats exposed to acrylamide in the drinking water at doses of 0, 0.1, 0.5 or 2.0 mg/kg/day. Based on the coincidence of tunica vaginalis mesothelioma tumors and Leydig cell tumors in male F344 rats and the association between hormonal imbalance and Leydig cell tumors in male F344 rats,
these investigators suggested that a hormonal mechanism might also be involved in the pathogenesis of mesothelioma.

Damjanov and Friedman (1998) compared the light and electron microscopic features of 28 tunica vaginalis testis mesothelial cell tumors from F344 rats that received acrylamide in drinking water at 0.1, 0.5 or 2.0 mg/kg/day over a 104-week period to seven tumors that occurred spontaneously in concurrent control males. They observed three distinct patterns (papillary, tubular or solid) and concluded each tumor had papillary features and focal tubular or solid components. Mitotic figures were uncommon, and no metastasis or invasion was observed. By light microscopy and transmission electron microscopy, they found no differences in tumors of acrylamide-treated rats from those in the control group rats. On the bases of their findings they concluded that the mesothelial tumors occurring in acrylamide-exposed rats are more comparable to benign scrotal mesotheliomas and adenomatoid tumors than to malignant diffuse peritoneal mesotheliomas that occur in humans.

Using blocks and slides of tissues with tunica vaginalis testes from 38 Fischer 344 rats from the study by Friedman et al. (1995) (A Lifetime Oncogenicity Study in Rats with Acrylamide), Iatropoulos et al., (1998) characterized the location, extent, severity, pattern and shape of proliferative lesions. Proliferation was quantitatively assessed using PCNA immuno-stained sections, morphometry, and blinded observation. Three types of lesions were identified using diagnostic guidance reported by McConnell et al., (1992): focal mesothelial hyperplasia, hyperplastic adenomatoid mesothelium (benign mesothelioma), and malignant mesothelioma that had three different patterns – plexiform, tubular, and mixed. There was no difference in the pattern of cell proliferation in any of the five dose groups represented in the study (control group 1; control group 2; and low, middle and high doses of acrylamide). The highest cell proliferation values were noted in the low exposure group. These investigators noted that tumors that were classed as benign occurred in testes where Leydig cell tumors occupied less than 50% of the testes and those classified as malignant mesothelioma occurred in testes where Leydig cell tumor occupied more than 75% of the testes. On the basis of this investigation, Iatropoulos and coworkers determined that the occurrence of mesothelioma was unrelated to acrylamide exposure since there were no compound-related increases in cell proliferation, no specific
neoplastic pattern attributable to the compound, and no increase in mesothelial hyperplasia in the high-dose group as might be expected from a progressive neoplastic process.

Crosby et al., (2000) have disclosed anatomical findings and provided explanation for the extension of tunica vaginalis mesotheliomas to the peritoneal cavity that differentiates the behavior of the F344 rat tunica vaginalis mesothelioma from that expected to occur with tunica vaginalis mesotheliomas in humans. Using tissues from a two-year study of potassium bromate administered in drinking water to F344 rats (DeAngelo et al., 1998), they mapped the anatomical distribution of mesothelial lesions associated with the tunica vaginalis of the testes and the spleen. Their data suggest that the testicular and peritoneal mesotheliomas arose from the mesorchium and mesosplenium, respectively. They further noted the comparative difference in the anatomy of the human parietal tunica vaginalis and that of the F344 rat. In contrast to humans in which the visceral and parietal tunica vaginalis is a completely enclosed pouch, the rat has parietal tunica vaginalis that is continuous with peritoneal parietal mesothelium at the inguinal ring and visceral tunica vaginalis that is continuous with the mesorchium. This anatomical difference was concluded to facilitate unimpeded spread of mesothelioma arising in the mesorchium to the peritoneal cavity of F344 rats.

**Overview of Acrylamide Genotoxicity and Toxicology Studies in Rats**

Acrylamide is a white crystalline solid that is produced from acrylonitrile. Acrylamide monomers are readily linked to form polymers. Polyacrylamides are used as flocculents in the treatment and purification of drinking water. This use and industrial use are considered the main sources of release of acrylamide monomers in the environment (WHO, 1985). Acrylamide is readily absorbed following ingestion, inhalation, or dermal exposure. Occupational exposures are considered to occur mainly through dermal and inhalation exposures in acrylamide production plants where workplace sampling has disclosed average levels from personal sampling to be 0.6 mg/m$^3$ with a range of 0.1 to 3.6 mg/m$^3$. More recently there has been concern about oral exposure to unexpectedly high levels of acrylamide in cooked starches, notably French fries, potato chips, and breads (IARC, 1994). Acrylamide’s occurrence in these cooked foods has been traced to the Maillard reactions that occur between amino acids and sugars when heated (Mottram et al., 2002). However, one population-based case control study (Mucci et al., 2003)
has discounted the impact of dietary exposure to high levels of acrylamide in certain cooked foodstuffs (including fried potatoes and baked breads) on excess human risk for bowel, bladder, and kidney cancer. These results for dietary exposure are pertinent as another study of a small cohort of 371 workers exposed to acrylamide via organic dyes had higher cancer-related mortality than expected due to cancers of the digestive and respiratory tract (Sobel et al., 1986). An effect might be expected in the gastrointestinal tract, kidney or urinary bladder since acrylamide is water soluble, readily absorbed by the gastrointestinal tract, and both acrylamide and its metabolite glycidamide are conjugated by glutathione and excreted in urine. A subsequent study of a cohort of 8500 workers found little evidence of overall excess risk of cancer mortality (Marsh et al., 1999), but disclosed highly variable data that suggested an excess of thyroid cancer and a dose-relationship with pancreatic cancer. The study had greater sensitivity to detect an effect on lung cancer incidence and showed minimal excess risk between exposed and unexposed workers.

IARC classified acrylamide as a probable human carcinogen primarily on the basis of toxicity data derived from in vitro models and animal models. The important findings for genotoxicity, acute toxicity, repeated dose toxicity, and chronic toxicity/carcinogenesis are summarized below.

**Genotoxicity**

Acrylamide monomer has been demonstrated to be clastogenic as demonstrated by chromosomal aberrations and sister chromatid exchange data obtained using mouse cell systems (Adler et al., 1988; Backer et al., 1989; Tsuda et al., 1993).

In one study acrylamide monomer was found to induce DNA damage in the spore rec assay on *Bacillus subtilis*, but was not mutagenic in an *Escherichia coli* WP2 uvrA’/ microsome assay, Salmonella/ microsome test systems, and a Chinese hamster V79H3/HPRT system (Tsuda et al., 1993). In clastogenic assays in the same study acrylamide monomer induced chromosomal aberrations (chromatid gaps, breaks and exchanges) and polyploidy in V79H3 cells; increased the frequency of sister chromatid exchanges in V79H3 cells; and induced malignant transformation in BALB/c3T3 mouse cells. On the bases of these results acrylamide monomer was considered to be a typical clastogenic rodent carcinogen without mutagenic potential. In agreement with the classification of acrylamide as a clastogen, malignant transformation of mouse
C3H/10T1/2 and NIH/3T3 cells has also been observed (Banerjee and Segal, 1986). Genotoxicity has been reviewed more extensively by Dearfield et al. (1995), Bolt (2003) and Allen et al. (2004).

**Acute Toxicity**

Short-term, low-level acrylamide doses of 2 and 15 mg/kg/day via gavage for two or seven days resulted in no clinical signs of toxicity or toxicity-related deaths in 7-week-old F344 rats. A slight dose-dependent increase in plasma thyroxine ($T_4$) and thyroid stimulating hormone (TSH) was noted (Khan et al., 1999). Thyroid follicle colloid area was decreased and height of thyroid follicular epithelium was increased. Long Evans rats that were 60 days of age developed lethargy in response to high acute intraperitoneal doses of a acrylamide (up to 150 mg/kg) (Crofton et al., 1996). No histological effects were associated with acute doses.

**Repeated Dose Toxicity**

Neurotoxicity that is manifested clinically and morphologically is a primary feature of repeated dose acrylamide toxicity. Crofton et al., (1996) reported overt signs of peripheral neuropathy (weakness, ataxia, dragging of hindlimbs) three weeks after rats were exposed to an intraperitoneal dose of acrylamide of 20 mg/kg/day. In a 10d, 30d, and 90d study, signs of neurotoxicity were first noted in the 10 mg/kg/day group after eight weeks of daily intraperitoneal dosing. Functional deficits were noted for motor activity after acute and repeated doses, effects on grip strength and acoustic startle response were dependent on the duration of exposure. Recovery of function was independent of duration of dosing, taking three to four weeks, but axonal degeneration seen after 30 and 90 days of exposure did not recover within the three to four week period. A more extensive pathology evaluation of rats exposed to acrylamide at 0, 0.05, 0.2, 1.5, or 20 mg/kg for up to 93 days with a 144-day recovery period (Burek et al., 1980) also disclosed peripheral neuropathy (signs after 92 days); and atrophy of skeletal muscle, testicular atrophy, and distended urinary bladders that were attributed to nerve degeneration. Nerve degeneration was noted via electron microscopy in rats in the 1 mg/kg/day group. The lesion was reversible in this dose group. Both acrylamide and its reactive metabolite, glycidamide, produced circling behavior after dosing, ataxia after nine days, and central and
peripheral neuropathy in male Sprague-Dawley rats that received daily intraperitoneal injections of acrylamide at an acrylamide equivalent dose of 50 mg/kg for 14 days (Abou-Donia et al., 1993).

**Chronic Toxicity - Oncogenicity**

Two similarly designed chronic toxicity/oncogenicity studies of acrylamide have been completed using F344 rats exposed to acrylamide in drinking water at similar doses for two years (Johnson et al., 1986; Friedman et al., 1995). The study by Johnson and colleagues administered acrylamide at doses of 0, 0.1, 0.5, and 2.0 mg/kg/day to both male and female rats. In this study both males and females in the 2.0 mg/kg group developed increased incidences of thyroid follicular cell tumors. Males dosed at 2.0 mg/kg also had increased incidence of adrenal gland pheochromocytomas. Mesothelioma of the tunica vaginalis testis with and without metastasis, was increased at 0.5 and 2.0 mg/kg in males. Females in the 2.0 mg/kg group also had increased incidences of mammary tumors, glial tumors in the central nervous system, oral cavity squamous cell papillomas, uterine adenocarcinomas, clitoral gland adenomas, and pituitary adenomas. An increased incidence and severity of tibial nerve degeneration was seen as early as 12 months of dosing in the 2.0 mg/kg/day group and was more severe after 24 months of dosing in this group. All other acrylamide dose groups were not different from the control group at any necropsy interval.

The study reported by Friedman et al., (1995) used doses of 0, 0.1, 0.5 or 2.0 mg/kg/day for males and 0, 1.0, or 3.0 mg/kg/day for females. Females in the 3.0 mg/kg/day group had an increased incidence of thyroid follicular cell neoplasms. Females in the 1.0 and 3.0 mg/kg/day groups had increased incidences of mammary fibroadenomas and total mammary tumors. In males thyroid follicular cell adenomas and mesotheliomas of the tunica vaginalis testes were increased in the 2.0 mg/kg/day group. In this study nerve degeneration was seen microscopically in males in the 2.0 mg/kg/day group and females in the 3.0 mg/kg/day group, but clinical signs or external lesions suggestive of neuropathy were not observed.
Spontaneous Incidence of Mesothelioma and Reactive Mesothelial Lesions in F344 and Other Rat Strains

Several peer-reviewed articles and government technical reports have documented the incidence of mesothelioma and mesothelial reactive lesions in F344 and other rat strains. In their report of the classification and anatomical distribution of 62 spontaneous mesotheliomas in F344 rats from lifetime studies, Shibuya et al., (1990) confirmed the tendency for increased spontaneous incidence of mesothelioma in F344 male rats and the predilection of mesothelioma for the genital mesothelium of the tunica vaginalis of the testes and epididymis in these rats. These studies included 1910 males and 1910 females. Incidences of mesothelioma were 57 of 1910 (3.0%) in males and five of 1910 (0.3%) in females. In males 17 tumors were confined to the genital mesothelium of the tunica vaginalis testis and epididymis; 27 tumors were in genital mesothelium and had peritoneal extension; six were in pleural, peritoneal, and genital mesothelium; two were in peritoneal mesothelium; one tumor each occurred in the pleural, and the pericardial and pleural mesothelium, respectively; and one tumor involved four areas: pericardial, pleural, peritoneal, and genital mesothelium. These researchers also confirmed that the epithelial type of mesothelioma was the predominant histological type of tumor and was most characteristic of the mesotheliomas in genital mesothelium in F344 rats. Their data disclosed that there were 48 epithelial type tumors in males and three in females; two fibrous type tumors each in males and females, respectively; and seven mixed type tumors, all in males. Only one of the 18 tumors in the male genital mesothelium was a mixed type, all other fibrous or mixed types occurred outside the genital mesothelium. The predominance of the epithelial type of tumor in the F344 male rat genital mesothelium differentiated it from the asbestos-induced mesothelioma that was considered to be a mixed type of tumor that arose from submesothelial connective tissue.

Using 419 Fischer-344 and 304 Fischer X Brown Norway (FBNF₁) male and female rats that were allocated to ad libitum fed and food restricted groups and subgroups according to longevity, Thurman et al., (1995) observed incidences from 0 to 8% in the testes of male rats in the different subgroups. They concluded that the incidence of mesothelioma was low in both genotypes and that incidence was not affected by food restriction.

A historical control report of spontaneous incidence of neoplasia in F344 rats (NTP, 2003) that was generated from the National Toxicology Program (NTP) Toxicology Data Management
System (TDMS) reported incidences of benign and malignant mesothelioma that occurred in control rats in 37 chronic toxicity/carcinogenicity studies of chemicals tested at four separate testing facilities. This report included oral, inhalation, and dermal routes of exposure. Among 1059 male rats there were three with benign mesothelioma and 45 with malignant mesothelioma. One female rat among 1109 that were examined had a malignant mesothelioma.

An updated report of historical control tumor incidence data in the NTP TDMS (Constella Group, Inc., 2004) disclosed 22 occurrences (4.8%) of malignant mesothelioma among 460 Fischer-344 male rats on the NTP 2000 diet that were vehicle controls in studies that used dietary dosing. No occurrences of benign mesothelioma were reported.

In other strains Crl:CD® (SD) rats that had tumor incidence recorded in a compilation of spontaneous neoplasms in male rats from control groups of toxicity studies that were approximately 104 weeks in duration (Giknis and Clifford, 2004), one rat out of 2145 (0.047%) had mesothelioma involving the testes, one rat out of 2145 had malignant mesothelioma involving the salivary gland, and three rats (0.14%) out of 2146 had mesothelioma involving the mesentery. The data in this compilation were from control animals from 30 studies that were conducted at eight different contract or industrial testing facilities in the US, Europe, Canada, and Japan. The routes of dosing used for the 30 studies in the compilation included: dietary, gavage, and subcutaneous. The available data do not allow a separate characterization of mesothelioma incidence by the specific route of dosing used in the study.

A compilation of neoplasms observed in 555 male Wistar Han rats that were from the control groups of 10 studies that were conducted in the US or Europe at four different industrial or contract facilities (Giknis and Clifford, 2003). The studies were 104 weeks in duration and used the dietary, gavage or inhalation routes for dosing. The data does not permit further subgrouping of mesothelioma incidence according to the specific route of dosing that was used. The tabulation of data for the testis for each of the 10 studies showed that the highest incidence of testicular mesothelioma (two occurrences among 55 rats, 3.6%) were reported for the same study that had the highest incidence of interstitial cell adenoma (six occurrences). There were two or less occurrences of interstitial cell adenoma in the other nine studies in the compilation.

The comparison of data for the F344, Wistar Han and Charles River Sprague-Dawley rats indicates that tunica vaginalis mesothelioma much less prevalent than in the F344 rat.
Orally Administered Xenobiotic Associated Mesothelioma or Reactive Mesothelial Lesions in F344 and Other Rat Strains

Herein, information that reviews or discusses chemically-induced mesothelioma or reactive mesothelial lesions focuses on the effects of orally administered chemicals via gavage, drinking water or in feed, as these routes are more relevant to occurrences of mesothelioma in rats exposed to acrylamide via drinking water. Ten chemicals in the NTP TDMS database (NTP, 2005) are known to cause mesothelioma in orally dosed F344 rats in 2-year studies: 2,2-bis (bromomethyl) - 1,3-propanediol; 3, 3’-dimethoxybenzidine dihydrochloride; dimethylbenzidine dihydrochloride; ethyl tellurac; glycidol; methyleugenol; nitrafurazone; o-nitrotoluene; purified pentachlorophenol; and orthotoluidine hydrochloride.
<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>CARCINOGENIC DOSES</th>
<th>MESOTHELIOMA?/OTHER TUMOR SITES?</th>
<th>TREATMENT-RELATED LEYDIG CELL PROLIFERATIVE RESPONSE?</th>
<th>MUTAGENIC?</th>
<th>CLASTOGENIC?</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2-bis (bromomethyl)-1,3-propanediol</td>
<td>≥5000 ppm/day</td>
<td>Yes/urinary bladder, oral cavity, esophagus, forestomach, intestines, lung, thyroid</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>3, 3'-dimethoxybenzidine dihydrochloride</td>
<td>170 &amp; 330 ppm/day</td>
<td>Yes/skin, liver, oral cavity, intestine, Zymbal's Gland</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>dimethylbenzidine dihydrochloride</td>
<td>70 &amp; 150 ppm/day</td>
<td>Yes/skin, liver, oral cavity, intestine, Zymbal's Gland</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>ethyl tellurac</td>
<td>300 &amp; 600 ppm/day</td>
<td>Yes, but equivocal/ Harderian gland</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>glycidol</td>
<td>38 and 75 mg/kg/day</td>
<td>Yes/mammary, brain, skin, intestine, Zymbal's Gland</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>methyleugenol</td>
<td>≥37 mg/kg/day</td>
<td>Yes/liver, glandular stomach, biliary system</td>
<td>No</td>
<td>No</td>
<td>Yes (only SCE¹ +)</td>
</tr>
<tr>
<td>nitrafurzone</td>
<td>310 &amp; 620 mg/kg/day</td>
<td>Yes/prepuce</td>
<td>No, testes degeneration</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>o-nitrotoluene</td>
<td>≥625 ppm/day</td>
<td>Yes/liver, lung</td>
<td>Yes, LCTs decreased with increased dose</td>
<td>No</td>
<td>Yes (only SCE +)</td>
</tr>
<tr>
<td>purified pentachlorophenol</td>
<td>1000 ppm/day</td>
<td>Yes/nose</td>
<td>No</td>
<td>No</td>
<td>No (weak + CA &amp; SCE)</td>
</tr>
<tr>
<td>orthotoluidine hydrochloride</td>
<td>3000 &amp; 6000 ppm/day</td>
<td>Yes/male spleen &amp; female spleen, urinary bladder, mammary</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

¹Abbreviations: CA = chromosomal aberrations; SCE = sister chromatid exchange; LCTs = Leydig cell tumors
MacKenzie and Garner (1973) compared neoplasms in six sources of rats that had received various irradiated food diets and were killed when debilitated or at approximately two years of age. They observed 749 tumors among 2,082 male and female rats that included 258 Sprague-Dawley, 535 Charles River-SD, 268 Holtzman-SD, 217 Diablo-SD, 131 locally bred Osborne-Mendel, and 673 locally bred Oregon rats. One mesothelioma was found in a male Oregon rat that had severe ascites. It covered most abdominal organs and was noted to have invaded the parenchyma of the spleen where it formed nests and cords resembling carcinoma in the deeper tissue.

Conduct of the Reviewing Pathologist's Examination

A reexamination of the hematoxylin and eosin stained histologic slides of testes and epididymides from male F344 rats in the 2-year study of acrylamide administered in drinking water reported by Johnson et al., (1984), was conducted by Dr. Rodney A. Miller, Experimental Pathology Laboratories, Inc. (EPL®) in accordance with guidelines in USEPA Pesticide Regulation (PR) Notice 94-5, August 24, 1994 (USEPA, 1994). The purpose of this peer review was to validate the accuracy and consistency of the initial histopathologic examination of tissues and to employ current histopathologic criteria and nomenclature for proliferative or reactive lesions involving mesothelial lining cells of the tunica vaginalis testes (Hall, 1990) during the reexamination of the slides. The reviewing pathologist reexamined all sections of testes and epididymides that were present from all male rats (generally six sections of testis and six sections of epididymis per rat). The results of the peer review and original diagnoses were used by the PWG Chairperson to determine which slides the PWG panel reviewed.

Conduct of the Pathology Working Group Review

A Pathology Working Group (PWG) Review, consisting of independent consultants with expertise in the evaluation of toxicology study data, interpretation of study results from carcinogenicity bioassays, testicular pathology and mechanisms of testicular toxicity, and mesothelioma in rodents, was held in Raleigh, NC on January 27, 2005. The purpose of the PWG was to review diagnoses of representative mesothelial lining cell proliferative and reactive lesions in the tunica vaginalis testes of rats dosed with acrylamide in drinking water for two years.
and to provide guidance on the biological significance and potential relevance of the rat proliferative lesions to humans exposed to acrylamide.

The PWG Review was chaired by Dr. Henry G. Wall, Experimental Pathology Laboratories, Inc. (EPL®), who organized the meeting and arranged for the expert panelists to participate in the meeting. The Chairperson was also responsible for conducting the meeting, leading the discussion, and preparing a report of the PWG’s findings. Curricula vitae for each of the panel members are included in Appendix B of this report. The members of the expert panel are listed as follows:

- Henry G. Wall, D.V.M., Ph.D., DACVP, DABT
- Chairperson
- Keith A. Johnson, D.V.M., Ph.D., DACVP
- Study Pathologist and Expert Panel Member
- Rodney A. Miller, D.V.M., Ph.D., DACVP
- Peer Review pathologist and Expert Panel Member
- Dianne M. Creasy, Ph.D., FRC Path
- Expert Panel Member
- Chirukandath Gopinath, B.V.Sc., Ph.D.
- Expert Panel Member
- Ernest E. McConnell, D.V.M., MS, DACVP, DABT
- Expert Panel Member

At the outset of the slide examination diagnostic criteria for mesothelial cell proliferative lesions of F344 rats established by Hall (1990) were reviewed and agreed to be applied during the microscopic examination. The PWG examined coded slides consisting of representative sections of the testes and epididymides of 42 rats that had neoplastic or reactive lesions of the tunica vaginalis. They also examined all sections of testes and epididymides from three rats for which there were differences in the diagnoses provided by the Study Pathologist and the Reviewing Pathologist, and examined the testes and epididymides of five rats that were not diagnosed as having a proliferative or reactive lesion of the tunica vaginalis by either the Study or Reviewing Pathologist. The purpose of this reexamination of these lesions was to provide the PWG members with a thorough knowledge of the nature of the changes of concern so they could evaluate potential mechanisms for each lesion diagnosed and to enable them to provide advice regarding the biological significance of the lesions in the F344 rat and their relevance to humans. Each participant recorded his or her diagnoses and comments on a worksheet prepared by the Chairperson. Each lesion was discussed by the group, reexamined if necessary, and the final opinions were recorded on the Chairperson’s worksheets.
The animals and slides examined for each of the diagnoses are tabulated in Appendix A.

RESULTS AND DISCUSSION

Pathology Working Group Slide Review

The Pathology Working Group (PWG) participants light microscopic examination of testes and epididymis sections from 50 rats provided familiarization with lesions representing the full spectrum of morphologic changes associated with tunica vaginalis testes mesothelium in the study reported by Johnson et al., (1986). The PWG detailed results are presented in Appendix A. There was agreement with diagnoses by both the Study Pathologist and Reviewing Pathologist for tissues from 43 rats including the five rats that did not have a mesothelial cell neoplasm or reactive lesion diagnosis assigned by either the Study or Reviewing Pathologist. The Pathology Peer Review disclosed only three animals with different diagnoses for tunica vaginalis mesothelial lining cell effects than those rendered by the Study Pathologist. For two of these three animals where the original diagnoses were “reactive mesothelium” and “mesothelioma”, the PWG panel consensus was “no mesothelial cell lesion observed.” For the other animal, whose original diagnosis was “no mesothelial cell lesion observed,” the consensus diagnosis was “reactive mesothelium.” The PWG panel also determined that “no mesothelial cell lesion observed” was the consensus diagnosis for six rats that were diagnosed by both pathologists as “reactive mesothelium.” These differences would not change the original interpretation of the chronic toxicity or carcinogenicity potential that was originally reported for the study.

Assessment of the Biological Significance of Mesothelial Lining Cell Proliferative or Reactive Lesions Observed in F344 Rats

Upon completion of the examination and discussion of microscopic slides the PWG Panel evaluated and discussed published acrylamide toxicity assessments and toxicology research data and assessed the role of genotoxicity and hormonal imbalance in the induction of mesothelial cell tumors and the relevance of tunica vaginalis mesotheliomas (TVMs) in the F344 rat testes to human health risk assessment.
Genotoxicity

As previously summarized several investigators have concluded that acrylamide is clastogenic on the basis of in vitro animal cell systems (Reviewed by Dearfield, et al., 1995; Allen et al., 2004). Recently in vivo genotoxicity data from several sources were considered and employed in a dose-response modeling case study of acrylamide to determine the application of the methodology to risk assessment (Allen et al., 2004). This report noted that only weak interaction of acrylamide with DNA has been demonstrated and that DNA interactions with acrylamide exposure are probably due to the epoxide, glycidamide. Although the glycidamide pathway is known to exist in rats, its role in the pathogenesis of mesothelioma of the tunica vaginalis testis of the F344 rat has not been established.

Metabolism and Adduct-Formation

Fennell, et al., (2005) have summarized the metabolism of acrylamide and recently characterized hemoglobin adduct formation in humans that received 0.5, 1.0 or 3.0 mg/kg of radiolabeled acrylamide orally, or a 50% solution that was dermally applied. Acrylamide is metabolized by two major pathways: glutathione conjugation and epoxidation to glycidamide. Both acrylamide and its reactive metabolite glycidamide can form adducts with hemoglobin. In rodents cytochrome P450 2E1 catalyzes acrylamide epoxidation. Human metabolism of acrylamide was compared to that of F344 rats that had received acrylamide at 3.0 mg/kg via gavage. Humans were found to metabolize acrylamide to glycidamide more slowly than F344 rats and eliminated acrylamide more slowly than F344 rats. F344 rats metabolized a greater amount of acrylamide by the glycidamide pathway than humans.

Cell Proliferation

Using F344 and Sprague-Dawley rats dosed via drinking water with acrylamide at 0, 2 or 15 mg/kg, for 7, 14 or 28 days, Lafferty et al. (2004) evaluated DNA synthesis, mitosis, and apoptosis in the thyroid follicular epithelium, testicular mesothelium, and adrenal medulla, the target tissues for acrylamide induced neoplasia in F344 rats, as well the non-target tissues liver and adrenal cortex. In a second experiment, DNA synthesis was evaluated in the same tissues using only F344 rats and acrylamide doses of 0 or 2 mg/kg in drinking water to two groups and
the same regimen was applied to another two groups of rats with the addition of 1-aminobenzotriazole (ABT), a cytochrome p450 inhibitor, at 100 mg/kg/day intraperitoneally to each group. Dosing in the second experiment was for seven days. The first study disclosed that there were no effects of acrylamide on mitosis or apoptosis in any of the five tissues that were examined in either rat strain. DNA synthesis was increased in the thyroid of F344 rats at both doses and only at 2 mg/kg in Sprague-Dawley rats. DNA synthesis was increased in the adrenal medulla of both rat strains at both doses and was highest after seven days. DNA synthesis was increased in the testicular mesothelium at all doses and all time points in F344 rats and was only increased in Sprague-Dawley rats dosed at 15 mg/kg. In the second study, liver weight and relative liver weight were increased in rats that received ABT. Acrylamide, co-treatment with ABT, increased DNA synthesis in thyroid follicular cells, but that increase was not different from the increase induced by ABT alone. Compared to untreated controls, ABT alone had no effect on adrenal medulla DNA synthesis and co-treatment with acrylamide prevented acrylamide-induced increased DNA synthesis. Acrylamide had no effect on DNA synthesis in the liver with or without ABT. In contrast ABT alone or with acrylamide increased liver DNA synthesis. In testicular mesothelium, ABT alone did not increase DNA synthesis and did have any influence on acrylamide induced DNA synthesis. These studies demonstrated that neoplasia in the thyroid, adrenal medulla and testicular mesothelium in the F344 rat was correlated with increased DNA synthesis in the target organ, that there were strain differences between Sprague-Dawley and F344 rats that suggest the larger percentage of acrylamide converted to glycidamide in the F344 rat by the action of cytochrome P450 contributed to the induction of thyroid and testicular mesothelial tumors and would also indicate a rationale for the unique sensitivity of F344 rats to develop thyroid and tunica vaginalis mesotheliomas when chronically dosed with acrylamide.

**Hormonal Mechanisms**

O'Shea and Jabara (1971) provided one of the early reports that linked mesothelial cell proliferation in genital tissues to the effect of a sex hormone. They examined multiple organs from intact female, ovariectomized female, and male dogs that were treated with subcutaneously dosed stilbestrol (300 to 750 mg) over a prolonged period (262 to 578 days). Microscopic papillary lesions were noted in the serosa of the testis and epididymis of one male and on the
testicular serosa of another male. Two male dogs were unaffected. Three of five ovariectomized females had mild papillary lesions on uterine serosa extending onto the broad ligament. No papillary lesions were observed on serous surfaces of the uterus or broad ligament in intact females.

Berman and Rice (1979) provided data and one of the earlier interpretations that the F344 rat had increased likelihood to develop mesothelioma of the tunica vaginalis, that the testicular mesothelium had a unique property that was distinct from mesothelium elsewhere, and recognized a coincidence of mesothelial cell tumors and Leydig cell tumors in F344 male rats. Their study provided a comparative assessment of the induction of mesothelioma and proliferative lesions of the testicular mesothelium in F344, Sprague-Dawley and Buffalo rats by a single intraperitoneal injection of methyl (acetoxymethyl) nitrosamine (DMN-Oac), the reactive metabolite of dimethylnitrosamine. In discussing their observations, Berman and Rice presumed that testicular mesotheliomas were produced by the action of DMN-Oac, and suggested that mesothelium might be a target due to a higher rate of cell division.

The KS Crump Company (1999) completed a comprehensive weight of evidence evaluation of the carcinogenic potency of acrylamide and concluded that acrylamide was unlikely to be a carcinogen affecting tunica vaginalis testes of humans. Their report documented the uniqueness of the F344 rat development of mesotheliomas affecting the tunica vaginalis of the testes following chemical exposures. This was done using data from 400 bioassays in rat. They also noted the propensity of male F344 rats to have high incidences of Leydig cell tumors and considered that mesothelioma was linked to Leydig cell tumors in this strain of rats. Their report emphasized a potential hormonal connection based on data indicating that acrylamide reduced serum prolactin via its interaction with dopamine and proposed that this interaction led to Leydig Cell tumors that were metabolically more active, thereby contributing to greater hormonal imbalance in the testicular environment where mesothelioma occurred.

Carcinogenic agents may affect the F344 rat testes via a mechanism that involves dopamine agonist-like activity or directly reduce prolactin, thereby reducing LH receptors and testosterone, and stimulating proliferation of Leydig cell. However, this effect is considered unlikely in humans because decreased prolactin does not reduce the number of LH receptors in human testes (Clegg et al., 1997). Humans would not be expected to develop Leydig cell tumors
or have increased risk for the development of mesothelioma stimulated by hormonal imbalances in the testicular environment.

**Alternative Pathogeneses for Mesothelioma and Reactive Mesothelium in Acrylamide-Exposed Rats**

The report by The KS Crump Company (1999) briefly addressed the potential role of paracrine and autocrine growth factors in the pathogenesis of mesotheliomas. Research in this area is very active. McLaren and Robinson (2002) have reviewed research activity in this area noting the production of growth factors by mesothelial cells, growth factor receptors on mesothelial cells, IL-6 secretion by mesothelial cells, and role of growth factors in the malignant transformation of mesothelial cells. Specifically relevant to F344 rats, Kuwahara et al., (2001) evaluated transforming growth factor β (TGF-β) production in F344 rat derived mesothelioma cell and normal mesothelial cell cultures and found that mesothelioma cells showed higher levels of TGF-β mRNA expression when compared with normal mesothelial cells. Treatment with exogenous TGF-β had no influence on the growth pattern of the mesothelioma cell line, but did slightly induce the normal mesothelial cells. The authors considered their findings in the F344 rat mesothelioma cell line to be consistent with results reported for human and murine mesothelioma cells derived from asbestos-related mesothelioma in both species and that TGF-β may promote aggressive growth of mesothelioma cells through an autocrine mechanism.

The discovery that SV40 produced mesothelial tumors in hamsters led Carbone and her colleagues (2002) to investigate the role of this agent in humans. Using PCR amplified DNA from human mesothelioma samples they demonstrated SV40 large T antigen (tag) in 27 of 48 mesothelioma samples. This finding was of concern due to the demonstrated prevalence of SV40 in human mesotheliomas and because the known ability of SV40 to transform cells. Although the strongest causative link for mesothelioma in humans is asbestos exposure, the presence of an agent that may be active in neoplastic development complicates assessments of mesothelioma causality and pathogenesis in humans. The role of the SV40 polyomavirus has received no attention in studies of tunica vaginalis mesothelioma in F344 rats.
Assessment of the Relative Risk to Humans of the Mesothelial Lining Cell Proliferative or Reactive Lesions Observed in Rats

Mesothelioma in Humans

Epithelioid mesothelioma is the predominant type of mesothelioma that occurs spontaneously and is associated with mesothelioma of the tunica vaginalis testes in F344 rats when attributed to chemical exposures. A variety of types of malignant mesothelioma occur in humans including, epithelioid, biphasic (mixed), and sarcomatoid patterns (Segal et al., 2002). Rare histological variants include small cell, lymphohistiocytoid, deciduoid, and desmoplastic mesotheliomas. Benign mesothelial tumors include well-differentiated papillary mesothelioma, benign multicystic mesothelioma (peritoneal inclusion cyst), and adenomatoid tumor. Epithelial or mixed type tumors tend to have a more favorable prognosis than sarcomatoid tumors.

Few cases of mesothelioma of the tunica vaginalis testis have been reported in humans. A 1998 review (Plas et al., 1998) noted 73 cases having been reported in a 30-year period. Though rare they are considered to occur in all age groups, this contrast with the unusually late onset in male F344 rats. It was noted that 34.2% of the cases in humans had a history indicating exposure to asbestos. There were no clear associations with other causes. Of 74 cases found in the literature, 51 cases had histomorphologic data. Among the 51 cases, 31 were the epithelioid type, and 19 were the biphasic type. Lymphatic or distant metastases were reported for several of the cases.

The F344 Rat as a Predictive Model for Mesothelioma in Humans Exposed to Acrylamide

The findings of several investigations (Berman and Rice, 1979; Kurokawa et al., 1983; Snellings et al., 1984; Kari et al., 1989) indicate that mesotheliomas of the tunica vaginalis testes occur spontaneously at a rather high incidence, that the mesothelial response following chemical exposures is unique to males and has the highest prevalence in the tunica vaginalis testes. The facts that mesotheliomas occur predominantly in male F344 rats, originate in and are usually limited to the serosa of the tunica vaginalis testes; and that characteristics of the tumors associated with acrylamide exposure do not differ from characteristics of tumors that occur spontaneously are data that would indicate this tumor response in rats is unlikely to be related to
genotoxicity of acrylamide and are irrelevant to assessment of human risk associated with acrylamide exposure.

CONCLUSIONS

In consideration of the expertise and all data reviewed, the Pathology Working Group concluded that:

- The Fischer 344 rat is not a good model for identifying chemically-induced testicular related effects because of the very high spontaneous rate of Leydig cell tumors
- The TVMs in this study appear to be rat specific and most likely not relevant to other species including man
- A genotoxic mechanism is not likely involved as:
  - the liver, which is the major site of metabolism, was not a target and no non-scrotal areas of the mesothelium were involved
  - the tumors have a late onset (noted after 92 weeks)
  - the mesothelial tumors are present in only one sex (males) and only affect one mesothelial site (tunica vaginalis)
- Hormonal profile, particularly as relates to prolactin in the Fischer 344 rat is not relevant to man
- Hormonal imbalance (prolactin-related) is the most likely mechanism of tumor formation. Since the hormone profile is not relevant to man, this mechanism is not relevant to man
- Tunica vaginalis testes mesotheliomas are extremely rare in man, but relatively common tumors in the Fischer 344 rat
- Acrylamide-induced TVM tumor morphology was not unique but the same as TVM tumor morphology in control animals

HENRY G. WALL, D.V.M., Ph.D.
Chairperson

Date
REFERENCES:


Berman JJ; Rice JM. 1979. Mesotheliomas and proliferative lesions of the testicular mesothelium produced in Fischer, Sprague-Dawley and Buffalo rats by methyl (acetoxymethyl) nitrosamine (DMN-Oac). Vet Pathol 16:574-582.


APPENDIX A

Study Pathologist’s Diagnosis, Reviewing Pathologist’s Diagnosis
And Pathology Working Group’s Consensus Diagnosis
APPENDIX B

Curricula Vitae