

# NORTH AMERICAN POLYELECTROLYTE PRODUCERS ASSOCIATION

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October 22, 2008

National Center for Environmental Assessment  
US Environmental Thomas Miller  
Designated Federal Officer (DFO)  
EPA SAB Staff Office (1400F)  
U.S. EPA  
1200 Pennsylvania Avenue, NW.  
Washington, DC 20460

**Re:** Induction of Tunica Vaginalis Mesotheliomas in Rats by Xenobiotics

Dear Dr. Miller:

On behalf of the North American Polyelectrolyte Producers Association (NAPPA), I hereby submit the attached manuscript entitled Induction of Tunica Vaginalis Mesotheliomas in Rats by Xenobiotics. This manuscript, which was prepared by a series of prominent researchers lead by Dr. Robert Maronpot of Experimental Pathology Laboratories (EPL), was recently submitted to Critical Reviews in Toxicology. An early draft of this manuscript was provided to the SAB/EPA in July of this year.

NAPPA wishes to bring this new manuscript to the SAB's attention in the context of the Acrylamide Review Panel (ARP) report on acrylamide that will be reviewed on October 28. NAPPA sponsored this effort at EPL primarily to further expound on issues associated with the relevance of tunica vaginalis mesotheliomas (TVM) to human cancer risk, as well as to evaluate the suggestion in the ARP report that all chemicals that cause TVM tumors are mutagenic. As noted in response to question #18:

*The only agents known conclusively to induce tumors of the brain and peritesticular mesothelium in rats are all DNA-reactive, and in fact a single exposure to a direct-acting mutagenic carcinogen has been observed to suffice for tumor induction at either site.*

As discussed in the attached manuscript, compounds that were found to exhibit robust TVM responses tended to be mutagenic in Salmonella but not in all cases. More importantly, only 2 of the 7 compounds with non-significant to marginal TVM responses (which includes acrylamide) were found to be Ames test positive.

Maronpot et al. examined the nature of TVM responses in 21 published rat cancer bioassays. The manuscript also highlights the lack of relevance that these rodent tumors have to man. The assessment explains that TVMs are seen most frequently in F344 male rats, as opposed to other rat strains, and are causally associated with the high background incidence of Leydig cell tumors of the testes of these rats. Hormone imbalance brought about by perturbations of the endocrine system is proposed as a key factor leading to both spontaneous and treatment-associated TVM.

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NAPPA maintains that TVMs in rodents should not be considered germane to a human health risk assessment associated with acrylamide exposure. It is significant to note that the draft IRIS assessment acknowledged this view noting that “there is some evidence to suggest that acrylamide can promote or enhance age-related decreases in serum prolactin and testosterone in older male F344 rats (Friedman et al., 1999b; Khan et al., 1999; Ali et al., 1983; Uphouse et al., 1982) and that this enhancement may lead to the development of tunica vaginalis mesotheliomas due to larger adjacent Leydig cell tumors (Iatropoulos et al., 1998).” However, EPA stated that before concluding that TVM’s are not relevant to man, there was a need for additional information in other animal species. The draft IRIS assessment states:

*Additional support for this proposal, such as the lack of mesotheliomas in other rat strains or other animal species exposed chronically to AA, however, is not available.*

NAPPA believes that the ARP should have more seriously considered these issues in its review of the draft acrylamide IRIS assessment. There is no indication in the draft ARP report that the information and analysis by EPL was considered. NAPPA further maintains that the SAB should be recommending to EPA that the ongoing mouse chronic bioassay being conducted by NCTR should address the limitation highlighted by the Agency by providing information on another animal species.

Please let me know if we can clarify any of this mention. Dr. Al Wiedow, a member of NAPPA will be presenting on this topic at the SAB meeting. If desired, we can arrange for Dr. Maronpot to be available by phone.

Sincerely,  
/SIGNED/  
Robert J. Fensterheim  
Executive Director

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## Induction of Tunica Vaginalis Mesotheliomas in Rats by Xenobiotics

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## Glossary of Abbreviations Used in This Review

Ah – aryl hydrocarbon receptor  
AKT – a family of genes that encode protein kinases  
AP-1 – activator protein 1  
ARNT – aryl hydrocarbon receptor nuclear translocator  
BrdU - bromodeoxyuridine  
CDKN2A/ARF – cyclin dependent kinase inhibitor 2A/ADP ribosylation factor  
EGF – epidermal growth factor  
EGFR – epidermal growth factor receptor  
Flt-1 – a tyrosine-protein kinase  
HGF – hepatocyte growth factor  
IGF2 – insulin-like growth factor 2  
IL-6 – interleukin 6  
KDR – kinase insert domain receptor  
LH – leutinizing hormone  
LHRH – leutinizing hormone releasing hormone  
LOH – loss of heterozygosity  
Mdr1 – multiple drug resistance gene 1  
Nf2 – neurofibromatosis type 2  
NOEL- no observed effect level  
NTP – National Toxicology Program  
p16 – a cyclin-dependent kinase inhibitor gene  
p16(INK4a) – a p16 gene that regulates cell cycle  
p19(ARF) – a tumor suppressor that attenuates degradation of p53  
p38MAPkinase – p38 mitogen-activated protein kinase  
PAS – Periodic Acid Schiff stain  
PDGF – platelet-derived growth factor  
TGF-beta – transforming growth factor beta  
TSG – tumor suppressor gene  
TVM – tunica vaginalis mesothelioma  
VEGF – vascular endothelial growth factor  
Wnt/beta-catenin – wingless-type gene that is the homolog of the mouse int-1 oncogene  
WT-1 – Wilm’s tumor 1

## **ABSTRACT**

To better understand the relevance of tunica vaginalis mesotheliomas (TVM) to human cancer risk, we examined the nature of TVM responses in 21 published rat cancer bioassays against the backdrop of the biology and molecular biology of mesothelium, and of spontaneous and treatment-induced TVM. Although relatively rare in all species including humans, TVM are seen most frequently in F344 male rats, as opposed to other rat strains, and are causally associated with the high background incidence of Leydig cell tumors of the testes of these rats. Hormone imbalance brought about by perturbations of the endocrine system is proposed as a key factor leading to both spontaneous and treatment-associated TVM. Of 21 F344 rat studies with a treatment-associated TVM response, 7 were judged to have a non-significant to marginal response, 11 had a robust TVM response, and 3 were non-informative due to early mortality from other induced tumors. Of the 11 chemicals with robust responses, 8 were directly mutagenic in Salmonella and 3 are known to be mutagenic after metabolism. Only 2 of the 7 with non-significant to marginal responses were Ames test positive. TVM responses are F344 rat-specific, their incidence can be exacerbated by treatment, and their causal association with F344 rat Leydig cell tumors indicates that when this rat bioassay tumor response is not robust, it is not relevant to humans and does not pose a risk for human cancer.

## **INTRODUCTION**

Spontaneously occurring mesotheliomas have been documented in a wide range of animals but are relatively rare. They have been observed in humans, lower vertebrates, domesticated and laboratory reared mammals, avian species, and marsupials, and occur in the thoracic and abdominal cavities (Ilgren, 1993; Crosby, 2000; Crosby et al., 2000) with rare reports of atriocaval mesotheliomas in cardiac chambers (Hoch-Ligeti et al., 1986; Peano et al., 1998; Chandra et al., 1993). Spontaneous mesotheliomas, which occur primarily in the scrotal sac and peritoneal cavity, have been documented in various rat strains, with the highest frequency occurring in male Fischer 344 rats (Solleveld et al., 1984; Deerberg and Rehm, 1981; Pelfrene and Garcia, 1975; Gould, 1977). These peritoneal mesotheliomas occur in rats 20 to 24 months of age or older and arise in the mesothelium investing the testis, epididymis, and scrotal sac, and may extend or seed into the peritoneal cavity.

Mesotheliomas can be induced by a wide variety of agents including various forms of asbestos, other natural and man-made fibers, metals, viruses, synthetic estrogens, and individual chemicals (Ilgren, 1993; Ilgren and Wagner 1991; Pelnar, 1988). Recently, multi-walled nanotubes injected intraperitoneally have been shown to induce peritoneal mesotheliomas in mice (Takagi et al., 2008). Depending upon the route of exposure, induced mesotheliomas also can occur in the thoracic or peritoneal cavity.

While the diagnostic terms for mesotheliomas used in the studies reviewed in this document include testicular mesothelioma, epididymal mesothelioma, peritoneal mesothelioma, and malignant mesothelioma, all are considered to have arisen in the tunica vaginalis mesothelium. Morphologically, tunica vaginalis mesotheliomas are typically less invasive and have fewer stromal components than the more familiar asbestos-induced pleural mesotheliomas. Tunica vaginalis mesotheliomas in rats rarely metastasize, and are confined to the scrotal sac and abdominal cavity.

This review provides a brief overview of basic biology, key events, mode of action, and examples of xenobiotics that have been associated with tunica vaginalis mesotheliomas in F344 rats based on the National Toxicology Program (NTP) database and a search of literature. This review was undertaken to understand and evaluate the relevance of this unique F344 rat tumor to human health risk assessment. Because tumors initiated by direct DNA interaction (genotoxic mechanisms) are regulated in a different fashion from those that arise from non-DNA reactive modes of action, it is important to understand the etiology of these tumors and whether they are relevant to humans. We postulate that the high incidence of Leydig cell tumors in the F344 rat is causally linked to development of tunica vaginalis mesotheliomas.

## **EMBRYOLOGY**

During early embryogenesis the coelom is a common cavity of mesodermal origin that will ultimately form the pleural, peritoneal, and cardiac cavities and mesothelial linings. This mesoderm forms two types of epithelial cells, viz., *mesothelium* which is a

squamous cell that forms from mesoderm and lines body cavities, and *endothelium*, which is a squamous cell that lines vascular and lymphatic channels (Banks, 1993). During development, the septum transversum, which will become the future diaphragm, separates the pleuropericardial membranes from the peritoneal membranes to form the separate pleural and peritoneal cavities (Hall, 1990; Arey, 1954). The peritoneal cavity and its contained abdominal organs are lined by a single layer of flattened mesothelial cells supported by delicate fibrous connective tissue. Peritoneal mesothelium extends into the scrotum and lines the surfaces of the testes, epididymis, and mesorchium where it is referred to as the tunica vaginalis. Since mesothelial linings in the pleural, peritoneal and scrotal cavities all derive embryologically from the same coelomic mesoderm, it is reasonable to expect the biology and pathobiology of thoracic and peritoneal mesothelium to have common attributes. Furthermore, since mesothelium embryologically derives from mesoderm, it is not surprising that new mesothelium can arise from existing adjacent mesenchyme during wound healing in serous cavities (Lewis, 1923).

### **FEATURES OF SPONTANEOUS AND TREATMENT-INDUCED MESOTHELIOMAS OF RATS**

Spontaneously occurring mesotheliomas are rare and, in general, are more commonly seen in males. A comprehensive listing of mesotheliomas in animals and humans can be found in the publications by Ilgren (Ilgren and Wagner, 1991; Ilgren, 1993). The highest background incidences of spontaneous mesotheliomas occurs in rats, and range from 0.2 to 5%. With rare exceptions, rat mesotheliomas occur in aged males, originate in the tunica vaginalis, and may spread by extension or seeding into the peritoneal cavity. Spontaneous mesotheliomas have been seen in Wistar, Sprague-Dawley, and other rat strains (Pelfrene and Garcia, 1975; Deerberg and Rehm, 1981) but most reports and descriptions in the literature are based on examples in Fischer 344 males (Goelz et al., 1993; Shibuya et al., 1993; Shibuya et al., 1990; Tanigawa et al., 1987; Gould, 1977; Hall, 1990; Mitsumori and Elwell, 1988).

Chemical exposure-associated tunica vaginalis mesotheliomas in rats have been identified in several cancer bioassays conducted for safety assessment or hazard identification, as well as in specific research studies. With the exception of reduced latency and an increased tendency to extend into the peritoneal cavity, the pathological features of these treatment-associated tumors are indistinguishable from those in concurrent controls and spontaneous cases reported in the literature.

The abundant literature dealing with pleural mesotheliomas associated with human exposure to asbestos and other fibers will not be covered in detail in this review, other than to compare and contrast the fiber-induced tumors with the chemical-induced tumors, where appropriate. Nonpleural mesotheliomas, including tunica vaginalis mesotheliomas, have been reported in humans (Hassan and Alexander, 2005). Spontaneous tunica vaginalis mesotheliomas are rare in humans, with fewer than 100 cases reported in the literature in the last 36 years (Guney et al., 2007; Winstanley et al., 2006; Carp et al., 1990; Jones et al., 1995; Plas et al., 1998; Gupta et al., 1999; Antman et

al., 1984). In contrast to the rat, the tunica vaginalis in the adult human does not directly connect to the peritoneal cavity. Consequently, tunica vaginalis mesotheliomas in humans are typically confined to the scrotal vaginal tunics, are locally invasive in about 50% of the cases and, when metastatic, typically spread via the lymphatics (Guney et al., 2007). In a review of 74 human cases, lymph node metastases occurred in approximately 15% of the cases of tunica vaginalis mesothelioma (Plas et al., 1998). A correlation of asbestos exposure with some cases of human tunica vaginalis mesothelioma has been suggested (Guney et al., 2007; Plas et al., 1998).

The histomorphology of tunica vaginalis and peritoneal mesotheliomas in rats is similar in spontaneous and chemically induced lesions, and is histologically indistinguishable from tunica vaginalis and peritoneal mesotheliomas in other species, including humans. Mesotheliomas vary from complex papillary to sessile nodular growths with a sarcomatous component. Smaller papillary lesions consist of a fibrovascular stroma lined by a single layer of flattened to cuboidal mesothelial cells, while larger papillary structures may have areas covered by multiple irregular layers of mesothelial cells forming a pavement or stratified pattern. Tumor cells are cuboidal to polygonal with round to oval nuclei and a prominent nucleolus, and may be arranged in solid sheets, nests, or in glandular and tubular structures. They can form cystic structures in which connective tissue cyst walls are lined by flattened to cuboidal mesothelial cells. The tumor cells may occasionally contain iron-positive material, are mucicarmine positive, and are typically positive for hyaluronic acid. Intracellular keratin and vimentin, and WT-1, can be detected by immunohistochemistry. Mesotheliomas in rats can be classified as epitheliomatous, sarcomatous, or mixed. This classification scheme is consistent with classification of mesotheliomas in humans. As in humans, rat peritoneal mesotheliomas arising in the tunica vaginalis may have features of malignancy, including pleomorphism, cytological atypia, and local invasiveness. Ultrastructurally, mesothelioma cells rest on a distinct basal lamina, have microvilli, junctional complexes, abundant cytofilaments, pinocytotic vesicles, dilated RER cisternae and a prominent Golgi apparatus (Damjanov and Friedman, 1998).

As with most well-studied cancers, a spectrum of lesion severity ranging from hyperplasia to benign neoplasia and ultimately to malignant neoplasia is characteristic of tunica vaginalis and peritoneal mesotheliomas in rats. Mesothelial hyperplasia ranges from a focal or multifocal increased density of usually plump to cuboidal mesothelial cells arranged as a single layer lining a serosal surface, to a blunt but small papillary projection lacking a fibrovascular stalk but sometimes associated with a small amount of connective tissue. Benign mesothelioma typically forms as a papillary structure with single and stratified layering of mesothelial cells lining a fibrovascular stalk. Non-papillary growth patterns of stratified mesothelial cells on a fibrous tissue base may also be considered benign. The mesothelial cells in benign mesotheliomas are generally cuboidal to polygonal and uniform. It is easy to appreciate that benign mesothelial lesions represent a morphological continuum with hyperplasia, and differences of opinion relative to diagnoses between the two would not be surprising. Malignant mesotheliomas have a spectrum of easily recognized morphological features including cellular and nuclear atypia, a pleomorphic growth pattern, and invasion through the serosa, and typically involve multiple sites throughout the peritoneal cavity. Because malignant

mesotheliomas can form glandular and tubular structures, they must be distinguished from metastatic adenocarcinoma. The lack of a primary adenocarcinoma elsewhere in the body, plus use of immunohistochemical staining, are used to support a diagnosis of malignant mesothelioma. Organizations such as the NTP do not subclassify mesotheliomas, but rather consider all mesotheliomas to be potentially malignant. In contrast, literature reports often consider mesotheliomas confined to the scrotal tunics, and without localized invasion, to be benign, while those that spread to the peritoneal cavity and are pleomorphic with cellular atypia and invasive features are generally considered malignant.

Distinguishing mesotheliomas from adenocarcinomas is an important consideration in diagnosis of human cases, especially for lesions in the thoracic cavity. Consequently, a large battery of stains, including immunohistochemical stains, has been used to assist in the diagnosis (Ordonez, 2003) (Table 1). While these stains can help in differential diagnosis, they do not distinguish benign from malignant mesotheliomas (Friedman et al., 1996). Several of these staining methods work well with rodent tissues although not all have been applied to rodent mesotheliomas as yet. Before the advent of immunohistochemistry staining batteries, staining for the acid mucopolysaccharide, hyaluronic acid, was commonly used to distinguish mesotheliomas from adenocarcinomas. Hyaluronic acid can be identified by Alcian blue (pH 2.5) staining with and without hyaluronidase. Mesotheliomas are generally Periodic Acid Schiff (PAS) negative after diastase treatment.

## **BIOLOGY AND MOLECULAR BIOLOGY OF THE MESOTHELIUM AND MESOTHELIOMAS**

Mesothelial cells are relatively easy to culture *in vitro* where they can undergo spontaneous as well as treatment-induced transformation and gain malignant phenotypes (Kobliakov et al., 2006). Consequently, much of the literature on the biology of normal and transformed mesothelium derives from *in vitro* studies. Similarly, cells derived from spontaneously occurring and induced mesotheliomas have been studied in *in vitro* test systems. Based on the similar embryological origin of the pleural and peritoneal mesothelium, it is reasonable to assume a similar biology in cell cultures derived from either of these tissue sites.

Normal and spontaneously transformed rat mesothelial cells studied *in vitro* express CYP1A1 and CYP1B1 mRNAs, which are decreased in transformed cells and in asbestos-induced mesothelioma cells from Wistar rats (Kobliakov et al., 2006). P-Glycoprotein, the *mdr1* gene product, was not detected in normal mesothelial cells. Furthermore, mRNA for the Ah receptor and ARNT, proteins that regulate induction of CYP enzymes via signal transduction in the cell nucleus, did not differ among the various cultured cells. The relevance of these *in vitro* findings relates to the biological functions of the studied proteins. The CYP enzymes potentially oxidize xenobiotics in some cases to metabolites which can induce cellular toxicity and carcinogenicity unless eliminated from the organisms by conjugation with glutathione or other cell substances, and in other cases detoxify xenobiotics to polar less toxic substances. P-Glycoprotein is a

transmembrane pump that functions to eliminate xenobiotics from cells. Its absence in mesothelial cells suggests that the cells are not able to eliminate potentially harmful xenobiotics by this specific mechanism.

Insulin-like growth factors (IGFs) are polypeptides that are associated with cell proliferation and differentiation. Cell lines from normal rat mesothelium and from spontaneous rat peritoneal mesotheliomas express RNA transcripts for IGF2, but cell lines from asbestos-induced rat mesotheliomas do not (Rutten et al., 1995). Since all 3 cell types have receptors for IGF2, as well as for IGF1 and insulin, the expression of IGF2 in the normal rat mesothelium and in the spontaneous mesothelioma indicates the probability that IGF2 is functioning as an autocrine growth factor, and suggests that asbestos-induced mesotheliomas arise through a different transformation pathway than do spontaneous mesotheliomas.

The basic immunobiology associated with mesotheliomas is poorly understood. Using a mouse model of malignant mesothelioma, Bielefeldt-Ohmann et al., (1994) showed significant production of the cytokines TGF-beta, interleukin-6 (IL-6), IL1, and tumor necrosis factor (TNF), by the mesothelioma cells. The authors suggested that the elaboration of these factors by the mesothelioma cells is contributory to sabotaging antitumor host defenses, and can induce perturbations in immune surveillance.

#### Oncogenes.

Oncogenes appear to play a minor role in the pathogenesis of mesotheliomas. Nishiyama et al. (1995) found no point mutations in H-, K- or N-*ras* proto-oncogenes, or the *p53* tumor suppressor gene, in three ferric nitrilotriacetate-induced peritoneal mesotheliomas in Wistar rats. In an analysis of 17 human and 22 rat asbestos-induced mesotheliomas, no mutations in exons 12, 13, or 61 of the K-*ras* proto-oncogene were identified by direct DNA sequence analysis (Ni et al., 2000). There is some evidence, however, that the early response gene pathway leading to chronic stimulation of cell proliferation is involved in asbestos-induced rat mesotheliomas. A dose-dependent induction of *c-fos* and *c-jun* mRNA in rat mesothelial cells by asbestos leads to persistent induction of AP-1 transcription factors which drive the cell proliferation process (Heintz et al., 1993). Thus, this early response gene pathway involved in asbestos-induced rat mesotheliomas leads to chronic stimulation of cell proliferation. The fibrous geometry of the particulates appears to be critical in induction of *c-fos* and *c-jun* in rat pleural mesothelial cells, with crocidolite and chrysotile asbestos causing a more dramatic increase in these early response genes than nonfibrous particles (Janssen et al., 1994). There is also some evidence that this induction of *c-fos* and *c-jun* in rat mesothelial cells by asbestos is not directly triggered by active oxygen species generation. The initial response of rat mesothelial cells to active oxygen species is an increase in antioxidant enzymes followed by induction of *c-fos* and *c-jun*, secondary to a redox-sensitive component in the signaling cascade influenced by intracellular thiol (glutathione) levels. (Janssen et al., 1995). Although there have been a number of studies of the role of *c-fos* and *c-jun* in asbestos-induced mesotheliomas, there have been no similar studies of chemically induced tumors.

#### Tumor suppressor genes.

In contrast to oncogenes, tumor suppressor genes (TSG) appear to play a more important role in mesothelial tumorigenesis. Alterations in tumor suppressor genes are characteristic of human malignant mesotheliomas (Apostolou et al., 2005) and are also seen in murine mesothelioma animal models (Kane 2006). In general, TSG are important regulators of cell cycle machinery. In human malignant mesotheliomas there is frequent inactivation of *Nf2* and loss of p16(INK4a) secondary to deletion of the CDKN2A/ARF locus. There are also indications of alterations in p19(ARF), AKT, and WT-1. Genetic alterations in *p16* and *Nf2*, both of which are important regulators of the cell cycle, have been identified in human malignant pleural mesothelioma and in asbestos-exposed, *Nf2*-deficient mice (Jaurand & Fleury-Feith, 2005). These studies show a similar profile of TSG alteration in asbestos-induced mesotheliomas in mice and humans. Inactivation of *Nf2* is typically associated with tumors of neuroectodermal origin. P16/CDKN2A, as a tumor suppressor gene, is an important inhibitory protein that maintains the necessary balance between cyclin activation of cell proliferation and inhibition of the uncontrolled cell division that is characteristic of cancer cells. It is also potentially important in cell motility and invasiveness (Kane, 2006).

In another study, alterations of p16, 85% of which were homozygous deletions, were present in all 40 human malignant mesothelioma cell lines examined, and homozygous deletions were present in 5 of 23 (22%) primary malignant mesotheliomas (Cheng et al., 1994). *Nf2* mutations were detected in 8 of 15 (53%) human malignant mesothelioma cell lines, nearly all of which were confirmed in matched primary tumor DNAs (Bianchi et al., 1995). Asbestos-exposed *Nf2*(+/-) knockout mice had significantly accelerated mesothelioma development compared with similarly exposed wild type littermates (Altomare et al., 2005a). Biallelic inactivation secondary to loss of the wild type allele occurred in all the knockout mice and in 50% of the wild type mice. Alterations in p19/Arf and p15/Cdkn2b were frequent in asbestos-treated mice hemizygous for *Nf2*, with similar alterations in human mesothelioma cell cultures (Lecomte et al., 2005). These same authors also noted loss of heterozygosity for *Nf2*, as was noted by Altomare et al., (2005a).

No *p53* mutations were detected in an analysis of 17 human and 22 rat asbestos-induced mesothelioma tissue samples (Ni et al., 2000), and neither spontaneous rat mesotheliomas nor erionite-induced mesotheliomas in rats were found to have *p53* alterations (Kleymenova et al., 1999). On the other hand, there was a low rate of *p53* mutations in mesothelioma cells from asbestos-treated *Nf2* hemizygous mice (Lecomte et al., 2005). While *p53* does not appear to play a major role in malignant mesotheliomas, there is an accelerated development of asbestos-induced mesotheliomas in heterozygous *p53* +/- mice (Vaslet et al., 2002). As the tumors develop in these mice there is loss of heterozygosity accompanied by genetic instability, decreased apoptosis, and accelerated tumor growth and invasiveness. The murine *Nf2*+/- model of environmental carcinogenesis is remarkably similar to human malignant mesothelioma and recapitulates many molecular features of the human tumor (Altomare et al., 2005b).

The WT-1 suppressor gene is expressed in normal and neoplastic mesothelial cells in rats and humans (Walker et al., 1994), and immunohistochemical staining for

WT-1 is useful in distinguishing mesotheliomas from adenocarcinomas and other neoplasms.

From these findings regarding tumor suppressor genes, it is apparent that there is considerable genetic instability in both human cases and mouse models of mesothelioma, and that multiple TSG are involved in mesothelial tumorigenesis. It is likely that vasmultiple molecular events, interacting either sequentially or in the aggregate, are involved in the development of mesotheliomas.

#### Other molecular factors.

AKT is a protein kinase that is important in mammalian cell signaling. It plays an important role in tumorigenesis and therapeutic resistance and is frequently inactivated in human malignant mesotheliomas, as well as in Nf2(+/-) mice (Altomare et al., 2005a,b).

#### Growth factors and cytokines.

A number of different growth factors associated with proliferation of normal and neoplastic mesothelial cells have been documented and much of what has been learned about these factors was generated from *in vitro* cell culture studies.

Normal rat pleural mesothelial cells exposed *in vitro* to long carcinogenic mineral fibers upregulate epidermal growth factor receptor (EGFR), with increases in EGFR protein occurring 24 hours prior to initiation of the protein kinase mitogenic signaling cascade leading to increased cellular proliferation (Faux et al., 2001). Furthermore, fibers with greater potential to cause mesothelioma induce a more marked upregulation of EGFR than less carcinogenic fibers. The EGFR response is linked to phagocytosis of the mineral fibers by the rat mesothelial cells.

The bioactivity of TGF-beta in two mesothelioma cell lines established from spontaneous rat mesotheliomas was 30 to 70 times higher than in normal rat mesothelium (Kuwahara et al., 2001). Based upon application of exogenous TGF-beta to the mesothelioma cell lines and normal rat mesothelial cells, the authors suggested that rat mesothelioma cells produced TGF-beta through an autocrine mechanism that stimulates their growth.

Using asbestos-induced murine mesothelioma models, it was noted that TGF-beta production by mesothelioma cells may permit their escape from immune surveillance based on down-regulation of lymphocyte surface markers (Bielefeldt-Ohmann et al., 1994). TGF-beta 1 and 2 isoforms are expressed by both human and murine malignant mesothelial cells, and inhibition of TGF-beta by antisense RNA reduces the anchorage-independent growth of malignant mesothelial cells *in vitro* and their tumorigenicity *in vivo* (Fitzpatrick et al., 1994). Inhibition of TGF-beta also led to increased T-lymphocyte infiltration into tumors. Thus, it appears that TGF-beta has tumor enhancing effects in mesothelial tumorigenesis.

Altered expression of platelet-derived growth factor (PDGF) is characteristic of human mesotheliomas. There is no expression of PDGF in asbestos-induced rat mesotheliomas, although the PDGF receptors are highly expressed (Walker et al., 1992).

The species differences between human and rat mesothelioma cells suggest that expression of PDGF may be species-specific, at least for asbestos-induced mesotheliomas.

The growth factors TGF-beta, EGF, and PDGF all independently stimulate a round of cell proliferation in serum-deprived, quiescent, primary normal human mesothelial cells (Gabrielson et al., 1988). When the growth medium is supplemented with chemically denatured serum, these same growth factors can sustain continuous replication of mesothelial cells. Based on the responses to PDGF and TGF-beta, the authors concluded that mesothelial cells have growth regulatory properties similar to connective tissue cells. Normal human mesothelial cells secrete more TGF-beta than mesothelioma cell lines. In contrast mesothelioma cell lines secrete more PDGF than normal human mesothelioma cells (Gerwin et al., 1987).

TGF-alpha is expressed in asbestos-transformed rat mesothelial cells but not in spontaneously transformed mesothelial cells, while both cell types express functional EGF receptors (Walker et al., 1995). Although TGF-alpha inhibits the growth of spontaneously transformed mesothelial cells, it also functions in an autocrine growth control fashion to stimulate growth of asbestos-transformed mesothelial cells (Walker et al., 1995). The implication of this study is that differences in mesothelioma etiology may be responsible for differences in the molecular biology of these neoplasms.

Based upon VEGF expression levels and VEGF blocking by neutralizing antibodies in 4 human malignant mesothelioma cell lines, as well as in biopsies of malignant mesothelioma, VEGF appears to be a key regulator of malignant mesothelioma cell growth (Strizzi et al., 2001). Since malignant mesothelioma cells also express the tyrosine kinase-related VEGF receptors Flt-1 and KDR, VEGF is believed to function as an autocrine growth factor in human malignant mesothelioma.

Cell lines from normal rat mesothelium, as well as spontaneous and asbestos-induced mesothelioma cell lines, all express IGF1, IGF2, and insulin receptors. However, there is ubiquitous expression of IGF2 (important in cell proliferation and differentiation) by normal rat mesothelium and spontaneous mesothelioma cell lines but not by asbestos-induced mesothelioma cell lines (Rutten et al., 1995). Hence, IGF2 appears to function as an autocrine or paracrine growth factor in normal and spontaneously altered rat mesothelial cells. The authors suggested that changes in growth factor expression may be a consequence of different pathways of cell transformation.

Immunostaining of human malignant mesothelioma tissue specimens shows elevated expression of phosphorylated/activated AKT kinases which are protein kinases important in mammalian cell signaling (Altomare et al., 2005b). Hepatocyte growth factor HGF/met receptor signaling in human and murine malignant mesothelioma cell lines is associated with HGF-inducible AKT activity, and suggests that this pathway may be amenable to targeted pharmacological therapy (Altomare et al., 2005b).

In a study of the gene expression profile of rat peritoneal mesotheliomas induced by *o*-nitrotoluene or bromochloroacetic acid, Kim et al., (2006) utilized Ingenuity Analysis Pathway software to identify 169 cancer-related genes. They identified activated

IGF-1, p38 MAPkinase, Wnt/beta-catenin and integrin signaling pathways in these tumors. The authors concluded that the mesotheliomas induced by these two agents were similar to human mesotheliomas with respect to their cellular and molecular features.

In summary, based on several *in vitro* studies effects on cell signaling and cell proliferative responses in normal and transformed mesothelium are influenced by several growth factors and cytokines functioning in an autocrine fashion.

## **EXPERIMENTAL MODELS OF MESOTHELIOMA**

### *In vitro*/cell culture models.

Much of our knowledge of the molecular biology of mesotheliomas has been derived from studies using primary and established cultures of normal and transformed mesothelium, as well as cell lines derived from human and rodent mesotheliomas (see Biology/Molecular Biology section of this review). New cell lines are being continually established and described (e.g., Orengo et al., 1999; Veldwijk et al., 2008; Davis et al., 1992; Marsella et al., 1997; Kane, 2005).

### *In vivo* animal models.

In a recent review, Kane (2006) briefly discussed animal models of mesothelioma, including genetically modified mouse models. Intraperitoneal and intrapleural injections of rodents with asbestos results in malignant mesotheliomas which are similar to human mesotheliomas with regard to latency, patterns of growth, and development of ascites (Engelbrecht and Burger, 1975; Wagner et al., 1973; Adachi et al., 1994; Schurkes et al., 2004, Davis et al., 1992). Lymphatic metastasis and invasion of abdominal adipose tissue and diaphragm muscle resemble cases of diffuse malignant mesothelioma in humans (Altomare et al., 2005a). Murine peritoneal mesotheliomas have histopathological growth patterns and phenotypic markers including cytokeratins, *N*-cadherin, and WT1 which are seen in human diffuse malignant mesotheliomas (Kane, 1998).

While only a minority of human malignant mesotheliomas carry *p53* mutations (Kane, 2006), heterozygous *p53*-deficient mice have accelerated development of asbestos-induced peritoneal mesothelioma (Vaslet, 2002). Heterozygous *Nf2*-deficient mice also show accelerated development and increased invasiveness of peritoneal mesotheliomas following exposure to crocidolite asbestos (Fleury-Feith et al., 2003; Altomare et al., 2005a). The relevance of this model relates to common occurrence of molecular alterations in *Nf2* in human malignant mesothelioma. A subset of asbestos-exposed heterozygous *Nf2* – deficient mice develop mesotheliomas with loss of *p53*, possibly due to the colocalization of *Nf2* and *p53* on mouse chromosome 11 (Kane, 2006). The reported cooperativity between *Nf2* and *p53* would be expected to increase the invasive and metastatic potential of the induced mesotheliomas (McClatchey et al., 1998; McClatchey, 2000). In asbestos-induced murine mesotheliomas in heterozygous *Nf2*-deficient mice, there is constitutive activation of the *Akt* pathway (Altomare et al., 2005b), a pathway frequently upregulated in human mesotheliomas and a key pathway in cell growth and proliferation. It is also noteworthy that the majority of mesotheliomas

induced in heterozygous *Nf2*-deficient mice exhibit codeletion of *p16(Ink4a)* and *p19(arf)* (Kane, 2006), which is frequently observed alterations in human malignant mesotheliomas (Altomare et al., 2005b).

While simian virus 40 has been shown to induce a high incidence of mesotheliomas in hamsters (Cicala et al., 1993), implication of SV40 as a cofactor in asbestos-induced human mesothelioma development is based on identification of SV40 viral sequences in asbestos-associated mesotheliomas, and a causative role for SV40 in human mesotheliomagenesis remains controversial (Gazdar et al., 2002; Toyooka et al., 2002; Klein et al., 2002; Terracini, 2006; Emri et al., 2000). Genetically engineered mice with SV40 T-antigen under control of regulatory elements of the cytokeratin 19 gene develop several epithelial neoplasms in addition to a moderate frequency of mesotheliomas, but due to fertility problems this model is not readily available (Grippo and Sandgren, 2000).

## **TREATMENT-ASSOCIATED TUNICA VAGINALIS MESOTHELIOMAS IN RATS**

### Proposed Modes of Action

Hormone imbalance brought about by perturbations of the endocrine system has been proposed as a key event ultimately leading to both spontaneous and treatment-associated tunica vaginalis mesotheliomas in rats (Turek and Desjardins, 1979; Tanigawa et al., 1987, Shipp et al., 2006). The feasibility of a hormonally driven process was originally appreciated based on the observation that diethylstilbestrol induced mesotheliomas on the genital organs in both sexes of dogs (O'Shea and Jabara, 1971). Decreased testosterone in aging rats leads to Leydig cell hyperplasia and ultimately Leydig cell tumors (Turek and Desjardins, 1979). This aging change is especially dramatic in the F344 rat which has a high spontaneous incidence of Leydig cell tumors (range 88 to 96%), in contrast to other rat stocks used in chronic studies (Boorman et al., 1990; Maekawa and Hayashi, 1992; Takaki et al., 1989; Solleveld et al., 1984). For example, based on Leydig cell hyperplasia, it has been proposed that testicular aging changes seen at 12 months in F344 rats (Kanno et al., 1987) are equivalent to testicular aging changes in 2-year old Wistar rats. The occurrence of Leydig cell tumors, in turn, is causally linked to development of tunica vaginalis mesotheliomas in F344 rats (Turek and Desjardins, 1979).

In the sexually mature rat, both leutinizing hormone (LH) and leutinizing hormone releasing hormone (LHRH) stimulate Leydig cells to produce testosterone (Capen, 1996; Prentice and Meikle, 1995). The testicular LH receptors and the serum testosterone levels decrease in rats between ages 4 and 18 months. In this age range, the testicular LH receptors and testosterone levels are correlated and balanced. As the testosterone levels decline with age, there is a compensatory increase in circulating LH to increase the level of testosterone. The compensatory action results in an increase (hyperplasia) of Leydig cells to increase testosterone levels. Ultimately the compensation is inadequate to maintain youthful levels of testosterone and the testicular-LH interaction strikes a new balance at a lower level (Amador et al., 1985). The ratio of the two is the same as before, but the levels are lower. LH continues to stimulate the Leydig cells to

divide in an attempt to reach youthful levels of testosterone, resulting in progression of the proliferating Leydig cells from hyperplasia to Leydig cell tumors. The testosterone-LH ratio changes once Leydig cell tumors are formed. Leydig cell tumors produce less testosterone than normal Leydig cells. Thus, an age-associated hormonal imbalance persists in older rats bearing Leydig cell tumors. In addition to decreased testosterone, there is an increase in Leydig cell LH receptors, an increase in serum progesterone, decreased prolactin, and decreased LH. In other words, the balance between testicular LH receptor levels and serum testosterone that was present during the 4 to 18 month age interval changes, and the levels of the different hormones become unbalanced in the presence of Leydig cell tumors.

Perturbations in the hypothalamic-pituitary-testis axis lead to Leydig cell proliferation, based on circulating levels of both LH and LHRH and the number of their cognate receptors on Leydig cells. While it may at first seem counter-intuitive, increases as well as decreases in prolactin levels can affect the hypothalamic-pituitary-testis axis and lead to Leydig cell hyperplasia and Leydig cell tumors.

The decrease in testosterone that ultimately leads to Leydig cell proliferation can also be brought about by an age-related increase in prolactin production in rats (Mahoney and Hodgen, 1995; Esquifino et al., 2004; Capen et al., 2002; Turek and Desjardins, 1979). The increased prolactin leads to decreased gonadotrophin releasing hormone (LHRH) as well as decreased LH secretion. Since rat Leydig cells have LHRH receptors that are responsive to LH and LHRH, the hormonal cascade initiated by increased prolactin leads to reduced testosterone production, as is reflected by the decreased serum testosterone levels seen in the aging rat (Mahoney and Hodgen, 1995). It is important to note that while rat Leydig cells have LHRH receptors, human Leydig cells do not (Prentice and Meikle, 1995).

Alternatively, decreased prolactin production may occur secondary to the action of dopamine agonists on the hypothalamus (Prentice and Meikle, 1995; Cook et al., 1999). The decreased prolactin leads to a decrease in LH receptors on the Leydig cells and thereby results in reduced testosterone production. This then causes a compensatory increase in circulating LH and a sustained increase in circulating LH results in Leydig cell hyperplasia and Leydig cell tumors (Prentice and Meikle, 1995).

The proof that age-related hormonal perturbation leads to Leydig cell tumors in the rat is supported by experiments in which Leydig cell hyperplasia and Leydig cell tumors are prevented by testosterone supplementation (Chatani et al., 1990; Fort et al., 1995). Similarly, the hormonal effects leading to Leydig cell tumorigenesis can be mimicked by different classes of chemicals that act through the hypothalamic-pituitary-gonadal axis to ultimately affect LH and testosterone, and lead to Leydig cell hyperplasia and Leydig cell tumors (Shipp et al., 2006). In addition, GnRH receptor agonists cause development of Leydig cell tumors by binding to LHRH receptors on Leydig cells (Prentice and Meikle, 1995; Donaubaue et al., 1987). This latter mechanism is unique to the rat since human Leydig cells do not have LHRH receptors (Prentice and Meikle, 1995).

Leydig cell tumors and their accompanying alterations in systemic hormonal levels have pleiotropic effects on the tissues of the genital system, including decreased spermatogenesis, seminiferous tubule atrophy, and atrophy of seminal vesicles (Kanno et al., 1987; Bartke et al., 1985). Intratesticular androgen levels are significantly higher than circulating levels (Foster, 2007). The alterations in androgen levels that accompany Leydig cell tumors are reflected as a transudate in the interstitial fluid within the testes as well as in the tunica vaginalis fluid compartment. The mesothelium bathed by the tunica vaginalis fluid is exposed to a higher concentration of the altered hormonal levels, probably by diffusion, than would occur following exposure via the circulatory system (Karpe et al., 1982; Gerris and Schoysman, 1984). Exposure of tunical vaginalis mesothelium to altered levels of androgens may trigger mitogenesis via mesothelial cell production of growth hormones that operate in an autocrine fashion, as occurs in other male reproductive system tissues (McKeehan et al., 1984; Kyprianou and Isaacs, 1988). The growth hormones released from the stimulated tunica vaginalis mesothelium include TGF-beta, PDGF, IGF2, and EGF, all of which stimulate mitogenesis. Continued enhanced proliferation of the tunica vaginalis mesothelium will lead to hyperplasia, with a subsequent increased probability for development of genetic damage and subsequent mesotheliomas.

An alternative hypothesis for induction of tunica vaginalis mesotheliomas secondary to Leydig cell tumors in rats relates to the physical pressure or mechanical stress placed on the mesothelial cells lining the scrotal tunics by the enlarged testes (Tanigawa et al., 1987). Based on the idea that pleural mesotheliomas may, in part, be a consequence of physical stimulus from asbestos fibers (Shabad et al., 1974; Stanton and Wrench, 1972), and because of it is known that transformed mesothelium expresses growth factors that stimulate its own mitogenesis (Gerwin et al., 1987; Versnel et al., 1988), it is reasonable to expect that physical pressure from testes enlarged by Leydig cell tumors could lead to transformation and/or growth factor secretion by tunica vaginalis mesothelium. This possible mode of action is further supported by the observation that visceral pleural mesothelial cells release significant levels of the growth factor PDGF in response to mechanical forces (Waters et al., 1997). As is the case with virtually all studies of carcinogenesis, alternative modes of action are not necessarily mutually exclusive, and more than one may act in concert to produce an adverse effect.

While hormone imbalance and mechanical force represent most likely key events for induction of both spontaneous as well as treatment-associated increases in tunica vaginalis mesotheliomas in rats, and especially in the F344 rat, alternative pathways for exacerbation of tumor development from exposure to xenobiotics are certainly plausible. Assuming that a xenobiotic agent or its metabolite can reach the tunica vaginalis mesothelium, both direct genotoxic action or indirect DNA damage via reactive oxygen species could also explain an exacerbation of the low spontaneous background incidence of this tumor. Similarly, enhanced cell proliferation, possibly secondary to irritation, inflammation, or mechanical stress, could contribute to an exacerbation of this low incidence spontaneous tumor. An association between chronic inflammation and both human pleural and rat peritoneal mesothelioma induction has been reported (Hillerdal and Berg, 1985; Grimm et al., 2002).

Evidence for an oxidative stress mode of action is supported by intraperitoneal injection of xenobiotics such as ferric saccharate or ferric nitrilotriacetate (Okada et al., 1989; Nishiyama et al., 1995) as well as by oral exposure to potassium bromate (Kurokawa et al., 1983 ; DeAngelo et al., 1998 ; Wolf et al., 1998) which produce reactive oxygen species (ROS) that can potentially have direct action on tunica vaginalis mesothelium. ROS are also considered important mediators in asbestos-induced mesotheliomas (Attanoos and Gibbs, 1997; Schurkes et al., 2004; Adachi et al., 1994). Alternatively, increases in replicative DNA synthesis in mesothelium that could lead to mesothelioma development either by directly affecting cell cycle machinery or secondary to gene alterations in cell cycle machinery has been shown in testicular mesothelium following subchronic exposure to acrylamide (Lafferty et al., 2004).

From a review of several agents associated with increases in tunica vaginalis mesotheliomas in F344 rats and occasionally in other rat stocks, one or more of the above described key events may be operating in the genesis of tunica vaginalis mesotheliomas. Likely modes of action for mesothelioma induction will be addressed for the specific xenobiotics associated with increases in this tumor and are described in the following sections. Twenty-one substances that were associated with increased incidences of tunica vaginalis mesothelioma in chronic rat carcinogenicity studies were identified in the National Toxicology Program database (Table 2) and in an extensive review of published literature, and their effects are described below.

#### Cancer Bioassays Associated with Increases in Tunica Vaginalis Mesotheliomas in Rats

Most rat cancer bioassays with some evidence of mesothelioma induction reported by NTP or in the literature were conducted using F344 rats. The NTP studies utilized F344 rats from a closed colony, and the other studies used F344 rats from different commercial sources. Consequently, the sensitivity of F344 rats to spontaneous and induced mesotheliomas extends to different colonies of these rats. The specific studies presented below are arranged in order, by route of administration.

##### Specific Chemicals - Intraperitoneal Route of Administration

Various forms of asbestos and a variety of other durable fibers and agents, including ceramic fibers, silicon carbide, stone wool, slag wool, glass wool, erionite, and cellulose, induce peritoneal cavity mesotheliomas in rats by i.p. injection (Wagner et al., 1973; Davis et al., 1986; Mast et al., 1994; McConnell, 1995; Miller et al., 1999; Kamstrup et al., 2002; Kleymenova et al., 1999). These same agents have been shown to induce pleural cavity mesotheliomas in experimental animals injected by the intrapleural route. The various intraperitoneal injection studies have been carried out in different strains such as Osborne-Mendel, Wistar, and F344, sometimes in females rather than males, and typically have used a single intraperitoneal injection. Adhesions and chronic inflammation generally accompanied the induced mesotheliomas which occurred several months after treatment. These studies are not summarized or discussed in detail, below.

Three non-fibrous chemical agents, when introduced into the peritoneal cavity of rats, led to development of tunica vaginalis mesotheliomas. Based on the anatomy of the rat, fluid injected into the abdominal cavity can easily get into the scrotal sac and lead to

exposure of the tunica vaginalis mesothelium. Direct acting carcinogens such as nitrosamines or agents that bring about oxidative stress, either as a primary effect or secondary to peritoneal inflammation, can also cause tunica vaginalis mesothelioma when injected into the peritoneal cavity of rats.

*Methyl(acetoxymethyl)nitrosamine.*

In a comparison of three different rat strains, Berman and Rice, 1979, reported on induction of testicular mesotheliomas following a single intraperitoneal injection of methyl(acetoxymethyl)nitrosamine (DMN-OAc), a short-lived, direct acting carcinogen (Table 3). In addition to the mesotheliomas, atypical mesothelial hyperplasia was noted in rats that didn't develop the tumor. The authors offered the opinion that testicular mesothelium has properties that are distinct from mesothelium elsewhere, and that the ability of mesothelium to respond to chemical carcinogens is an almost exclusive property of testicular mesothelium. In another publication, the authors showed that the spectrum of tumors induced by DMN-OAc in rats is dependent upon the route of administration (Berman et al., 1979).

The average age of death for the treated rats ranged from 14.8 to 16 months, while the average for control rats ranged from 17.2 to 20.9 months. Although DMN-OAc is a direct-acting carcinogen and does not require metabolic activation, it is clear that genetics can influence the susceptibility to mesothelioma formation. Furthermore, the highest incidence of mesothelioma (46%) occurred in the Buffalo rat which did not have any Leydig cell tumors, suggesting that hormonal effects were not driving the response in this particular study. It is noted that even gavage administration of nitrosamines causes peritoneal mesotheliomas (Lijinsky et al., 1985), suggesting that mesothelium may be especially sensitive to nitrosamine carcinogenesis. Methyl(acetoxymethyl)nitrosamine is mutagenic in the Ames test (Table 29).

*Ferric Saccharate.*

Daily intraperitoneal injections of ferric saccharate, which is a colloidal iron, and ferric saccharate plus nitrilotriacetic acid (NTA) for 3 months resulted in a high incidence of mesotheliomas in male Wistar rats (Table 4) (Okada et al., 1989). NTA stabilizes the iron which allows it to more efficiently induce ROS which then promote lipid peroxidation, enhancing the carcinogenic action of iron.

The mesotheliomas were confined to the tunica vaginalis in the ferric saccharate group. Six of the 14 mesotheliomas in the ferric saccharate-NTA group were disseminated throughout the abdominal cavity.

Intramuscular injection site neoplasms have been induced by iron dextran complex (Richmond, 1959) indicating that injected iron can cause cancer at the site of injection. In the Okada et al., 1989 study, the mesotheliomas appeared to arise in the tunica vaginalis, presumably because the injection iron became localized in the testicular sac following intraperitoneal injection. The authors suggest free radical production with localized enhancement of the carcinogenic action of iron by NTA as the likely mode of

action for mesothelioma induction. NTA is not mutagenic in the Ames or mouse lymphoma mutation tests, or produce chromosome damage in mammalian cells in vitro; there are no reported mutagenicity studies of its combination with ferric saccharate.

#### Cytembena.

In an NTP bioassay of cytembena, a cytostatic agent, F344 rats and B6C3F1 mice received intraperitoneal injections 3 times a week for 104 weeks (NTP TR 207). Cytembena produced a strong mesothelioma response and was the only tumor induced in male rats in this study (Table 5). Female rats had an increase only in mammary fibroadenomas, and had 2 malignant abdominal mesotheliomas at the high dose. No induced tumors were seen in mice of either sex.

There was significant, drug-related chronic inflammation in the peritoneal cavity in both sexes of rats, and the inflammation occurred at a greater frequency and severity in the females. While mesotheliomas occurred in 2/50 high dose females, the significantly more robust response was seen in the males. There was no dose response; a maximum response was seen at both doses, and the mesotheliomas were present throughout the abdominal cavity, inclusive of the testis and epididymis. The induction of mesotheliomas in this study is most probably a consequence of inflammation, in combination with the sex predilection for tumor induction in the tunica vaginalis of male F344 rats. The mice in this study received higher doses than the rats, did not have chronic peritoneal inflammation, and did not have mesotheliomas. This observation serves to reinforce the commonly accepted observation that mice in cancer bioassays do not develop mesotheliomas, even following multiple direct intraperitoneal injections for 2 years, and that rats are more sensitive to mesothelial tumorigenesis. Cytembena is mutagenic in the Ames test and produces chromosome damage in cultured mammalian cells, but did not induce chromosome damage in mouse bone marrow cells following i.p. injection. (Table 29).

#### Specific Chemicals - Inhalation Route of Administration

Three inhalation 2-year cancer bioassays resulted in induction of tunica vaginalis and associated peritoneal mesotheliomas in male F344 rats.

#### Ethylene oxide.

Ethylene oxide, a highly reactive alkylating agent used in chemical synthesis, and to a lesser extent for sterilization and fumigation, was tested by inhalation exposure in F344 rats at 10, 33, and 100 ppm (Snellings et al., 1984). At the end of the 2-year study there was an increased incidence of tumors in both sexes with increases in brain tumors in both sexes, mononuclear cell leukemia and mammary gland adenomas and adenocarcinomas in females, and peritoneal mesotheliomas in males (Table 6). There was a high incidence of Leydig cell tumors in all groups of male rats and a variety of endocrine neoplasms in both male and female rats. Snellings et al. (1984) used two equally sized but separate control groups. A different inhalation study at 50 and 100 ppm in male F344 rats also resulted in an increased incidence of peritoneal mesotheliomas (Table 6) (Lynch et al., 1984). This latter study also documented an increase in mixed

cell gliomas in the brain and mononuclear cell leukemia in the ethylene oxide exposed males.

The overall frequency of mesotheliomas in the Snellings et al., (1984) study was not statistically significant by a 2-tailed Fischer's exact test. However, there was a statistically significant trend test and the cumulative percent of rats developing mesothelioma was significantly increased in the 100 ppm group versus the controls, from the 21<sup>st</sup> month to study termination. The late-developing mesotheliomas were probably influenced by the altered hormonal milieu associated with age-associated Leydig cell tumors in F344 rats. In the Lynch et al., (1984) study there was a dose-related increase in mesotheliomas with a statistically significant increase in the 100 ppm exposed rats.

In both studies, treatment-associated mesotheliomas arose in the tunica vaginalis and some spread into the abdominal cavity. They were morphologically similar to spontaneously occurring mesotheliomas. While the mechanism for induction of mesotheliomas by ethylene oxide remains unclear, the spectrum of other lesions in endocrine tissues and testes potentially implicates a hormonal factor in their development. Ethylene oxide is positive in the Ames test (Table 29) and most in vitro and in vivo genetic toxicity tests.

#### 1,2-Dibromoethane.

1,2-Dibromoethane is a multisite, trans-species carcinogen following inhalation exposure, and produces nasal, pulmonary, and mammary tumors, as well as hemangiosarcomas (NTP TR 210). Inhalation of dibromoethane for 2 years produced a strong mesothelioma response in male F344 rats (Table 7). There was an increase in mammary fibroadenomas in female rats. Primary lung tumors, hemangiosarcomas, fibrosarcomas, nasal carcinomas, and mammary adenocarcinomas were induced in B6C3F1 exposed mice (NTP TR 210).

There was a high Leydig cell tumor frequency in the control and exposed groups. 1,2-Dibromoethane caused testicular degeneration that might explain the reduced number of Leydig cell tumors in the high exposure rats. In an older NTP gavage study in Osborne-Mendel rats, increased forestomach and liver tumors, as well as hemangiosarcomas were reported, but no mesotheliomas were present (NTP TR 86).

The mechanism by which 1,2-dibromoethane induced mesotheliomas is unknown. Glutathione conjugation of 1,2-dibromoethane leads to formation of an episulfonium ion that is DNA reactive, suggesting a genotoxic effect. The typically high incidence of Leydig cell tumors in the low exposure group and the known testicular toxicity even at low doses ([www.epa.gov/iris](http://www.epa.gov/iris)) suggest a profound perturbation of hormonal balance that might have contributed to the robust mesothelioma response. 1,2-Dibromoethane is mutagenic in the Ames and mouse lymphoma tests, and produces chromosome damage in mammalian cells in culture and in mouse bone marrow cells (Table 29).

### 1,2-Dichloroethane (DCE).

A low incidence of malignant mesotheliomas in the peritoneal cavity, especially in the scrotal sac, was reported at 160 ppm DCE in an inhalation study using F344 rats (Table 8.) (Nagano et al., 2006). The mesotheliomas at this highest concentration exceeded the historical control, but the incidence was not statistically significantly increased compared to the concurrent control.

Other tumor responses in the Nagano study included subcutaneous fibromas and mammary fibroadenomas in male and female rats, as well as mammary adenomas and adenocarcinomas in the female rats. In an older NCI gavage bioassay in Osborne-Mendel rats, mesotheliomas were not observed (NTP TR 55), and there was no mention of testicular Leydig cell tumors in the study report. DCE was carcinogenic in B6C3F1 mice causing mammary and endometrial tumors in females and lung tumors in both sexes (NTP TR 55). In an older inhalation study in F344 rats, exposure to 50 ppm DCE did not result in a tumor response (Cheever et al., 1990). DCE is mutagenic in the Ames and in vitro cytogenetics tests, but did not induce micronuclei in bone marrow of dosed male or female mice (Table 29).

### Specific Chemicals - Dosed Feed Route of Administration

Ethyl tellurac. A dose feed study of ethyl tellurac in F344 rats produced an equivocal tunica vaginalis mesothelioma response that showed a statistically significant trend, but was not significant by pairwise comparison (Table 9) (NTP TR 152). This was the only tumor response seen in rats in this study, and the chemical was judged to exhibit equivocal evidence of carcinogenicity. There was no mention in the report of Leydig cell tumors.

The judgment to consider the ethyl tellurac bioassay as not positive was based on a non-significant pairwise statistical comparison to the concurrent control, and the historical control incidence (12/416; 2.9%) for the testing laboratory. An increased frequency of Harderian gland adenomas in treated male and female mice was considered equivocal evidence of carcinogenicity. Ethyl tellurac is not mutagenic in the Ames test, mutagenic in the mouse lymphoma test, and produced an equivocal increase in chromosome aberrations in cultured mammalian cells (Table 29).

### *o*-Nitrotoluene.

Two prechronic and one carcinogenicity study on *o*-nitrotoluene have been conducted by the NTP (NTP Tox 23, NTP Tox 44, NTP TR 504). Mesothelial hyperplasia and mesotheliomas involving the tunica vaginalis surface of the epididymis were seen in rats receiving 5000 and 10000 ppm *o*-nitrotoluene in their diet for 13 weeks (Table 10). A follow-up 26-week prechronic study was conducted to compare the tumor responses of *o*-nitrotoluene and *o*-toluidine HCl given at equimolar concentrations in the diet, and to investigate the role of intestinal flora in metabolism of *o*-nitrotoluene (NTP

Tox 44). This 26-week study included a 13-week *o*-nitrotoluene exposure, followed by an additional 13 weeks on control diet (i.e., stop study). Mesothelial hyperplasia and mesotheliomas were seen at the 13-week interim sacrifice, in the stop-exposure group at study conclusion, and in the rats continuously exposed to *o*-nitrotoluene for 26 weeks (Table 11). The 2-year cancer bioassay of *o*-nitrotoluene included dietary doses of 625, 1250, and 2000 ppm, and incorporated a 3-month stop study in which rats were fed diets containing 2000 or 5000 ppm *o*-nitrotoluene followed by undosed feed for the remainder of the two years. All stop-study rats, and all but three of the rats given 1250 ppm, died before the end of the two years. The incidences of mesotheliomas in this study are summarized in Table 12.

In the 2-year study, the mesotheliomas were located in the tunica vaginalis of the testis or epididymis with some cases extending into the abdominal cavity. The majority of the mesotheliomas in treated rats were large and locally invasive. *o*-Nitrotoluene is not mutagenic in the standard Ames test. However its nitro group can be reduced by anaerobic gut flora to ultimately yield a DNA reactive metabolite. The formation of *o*-benzyl glucuronide is a critical step in leading to formation of the DNA-reactive metabolite. Basically, intestinal microflora hydrolyze the glucuronide and reduce the nitro group to form *o*-aminobenzyl alcohol. Upon reabsorption of the *o*-aminobenzyl alcohol, it is sulfated and binds to DNA.

Because reduction of the nitro group of *o*-nitrotoluene by anaerobic gut flora yields *o*-toluidine, which is mutagenic in the Ames test, a 26-week study comparing equimolar doses of *o*-nitrotoluene and *o*-toluidine was conducted. The incidence of mesothelioma was greater, and the latency less, for rats administered *o*-nitrotoluene (NTP Tox 44). Similarly, the liver effects, including cholangiocarcinomas, were greater for *o*-nitrotoluene than for *o*-toluidine. The lower potency of *o*-toluidine compared to *o*-nitrotoluene with respect to liver lesions and mesothelioma induction suggests that the effects of *o*-nitrotoluene involve more than the simple intestinal reduction of the nitro group. *o*-Nitrotoluene produced testicular degeneration in the 26-week toxicity study as well as in the two-year cancer study. This would lead to hormonal perturbations which, in the two-year study, were the likely cause of the reduced Leydig cell tumors in the high-dose males. An associated Leydig cell tumor reduction associated with testicular toxicity has been noted for other chemicals (Boorman et al., 1985). There was clear evidence of carcinogenicity in treated mice based on increased frequencies of hemangiosarcomas, large intestinal carcinomas and hepatocellular neoplasms.

*o*-Nitrotoluene was not mutagenic in the Ames test and did not induce chromosome aberrations in cultured mammalian cells, or micronuclei in mouse bone marrow cells when given in the feed to males and females, or when given i.p. to male mice or male and female rats.

#### *o*-Toluidine HCl.

*o*-Toluidine is a trans-species carcinogen that produced tumors in both sexes of F344 rats and B6C3F1 mice. Tumor types included a variety of splenic and other tissue mesenchymal tumors, urinary bladder transitional cell neoplasms, subcutaneous fibromas, hepatocellular neoplasms, hemangiosarcomas, and mammary gland fibroadenomas. *o*-Toluidine HCl induced a low incidence of epididymis mesotheliomas in F344 rats in a 26-week *o*-nitrotoluene/*o*-toluidine comparative study (NTP Tox 44) (Table 13). An older cancer bioassay had documented a high overall incidence of mesotheliomas involving multiple tissues in the abdominal cavity and the scrotal tunica vaginalis (NTP TR 153) (Table 14). An increase in mammary fibroadenomas was present in female rats.

The mesotheliomas in the 2-year study (Table 14) were morphologically similar to spontaneous and treatment-related mesotheliomas in other studies. A few of the more fibrous mesotheliomas contained foci of osseous metaplasia. In light of the known genotoxicity of *o*-toluidine (Table 29), it is likely that the mode of action for mesothelioma induction involves DNA damage to the tunica vaginalis mesothelium in addition to the contribution of hormonal imbalance associated with aging male F344 rats bearing Leydig cell tumors. Hemangiosarcomas and hepatocellular neoplasms were increased in *o*-toluidine-treated mice. *o*-Toluidine was mutagenic in the Ames and mouse lymphoma cell tests, produced chromosome aberrations in mammalian cells in culture, and contradictory results in two mouse bone marrow micronucleus tests.

#### 2,2-Bis(bromomethyl)-1,3-propanediol.

2,2-Bis(bromomethyl)-1,3-propanediol is a widely used flame retardant. It is genotoxic in a number of test systems. A dosed feed 2-year bioassay in F344 rats, which included a 3-month exposure stop study, produced a multi-site tumor response, including an increased incidence of mammary fibroadenomas in male and female rats (NTP TR 452). There was a strong peritoneal mesothelioma response in the male rats (Table 15). Other tumor responses in rats were seen in the skin, Zymbal's gland, oral cavity, esophagus, forestomach, small and large intestines, urinary bladder, lung, thyroid gland, hematopoietic system, and seminal vesicle. Neoplastic responses were also present in both sexes of B6C3F1 mice.

2,2-Bis(bromomethyl)-1,3-propanediol is one of 14 brominated chemicals studied by the NTP in 2-year rodent carcinogenicity studies. Thirteen of those 14 brominated chemicals were found to be carcinogenic, but only three (1,2-dibromoethane, 2,2-bis(bromomethyl)-1,3-propanediol, and potassium bromate) produced TVM. There are two hypotheses for the carcinogenic activity of brominated chemicals: (1) oxidative damage to DNA and other cellular constituents resulting from the induction of ROS, and (2) formation of DNA adducts when the C-Br bond is broken leaving a carbon-containing electrophilic group. In oral administration studies with potassium bromate [see below], which also produces mesotheliomas in male F344 rats, there is a significant increase in 8-hydroxydeoxyguanosine, which is a biomarker of oxidative damage (Kurokawa et al., 1983; Kasai et al., 1987; Sai et al., 1992). 2,2-Bis(bromomethyl)-1,3-propanediol is mutagenic in the Ames test and produces chromosome aberrations in cultured

mammalian cells, but yielded equivocal results in a mouse bone marrow micronucleus test.

#### Nitrofurazone.

Mesotheliomas were induced in male F344 rats in the dosed feed study of nitrofurazone (NTP TR 337) (Table 16). The mesothelioma response, which was not dose-related, was considered equivocal evidence of carcinogenicity by the peer review panel, arose in the tunica vaginalis, with some mesotheliomas spreading to the peritoneal cavity and invading the underlying soft tissue. There was a treatment-related increase in preputial adenomas and carcinomas, and a significant increase in mammary fibroadenomas in the female rats. Previous studies suggested that nitrofurazone was a mammary gland carcinogen. There was an increase of ovarian cancer in mice. Taken together, the tumor responses indicate the nitrofurazone may act through hormonal effects.

Poor survival of the high dose group is the likely reason for the decrease in mesotheliomas at the 620 ppm dose as compared to the lower dose. There was a dose-related decrease in Leydig cell tumors, also partly a reflection of poor survival in the high dose group. The obligatory role for nitro reduction in nitrofurazone-induced mutagenicity may be related to the widespread tumorigenicity in rats and mice (Kari et al., 1989). Nitrofurazone was mutagenic in the Ames and mouse lymphoma mutation tests and produced chromosome aberrations in cultured mammalian cells, but did not induce micronuclei in bone marrow cells of mice (Table 29).

#### Pentachlorophenol.

Pentachlorophenol is a wood preservative, as well as an herbicide, fungicide, and germicide. In a dosed feed study with pentachlorophenol, an increase in peritoneal mesotheliomas was seen in the stop-study F344 rats but not in the continuously exposed rats (NTP TR 483) (Table 17). A marginal increase in nasal carcinomas (1/50 versus 5/50) was also present in the stop-study males. No other treatment-related neoplasms were present in the males, and no treatment-related neoplasms were present in the female rats. Increases in liver and adrenal tumors and hemangiosarcomas were seen in pentachlorophenol-treated mice (NTP TR 349).

The mesotheliomas arose in the tunica vaginalis and had the histomorphological characteristics of the spontaneous and chemically-induced mesotheliomas seen in other studies. Extension into the peritoneal cavity was evident in 5 of the mesotheliomas in the stop-study group and the 1 mesothelioma in the control. Pentachlorophenol was non-mutagenic in the Ames test, and only weakly positive in an *in vitro* chromosome aberration test in cultured mammalian cells, and did not induce micronuclei in mouse or rat bone marrow cells.

Although pentachlorophenol is not mutagenic in bacterial test systems, one of its major metabolites, tetrachloro-*p*-hydroquinone, is genotoxic, covalently binds to DNA, and can induce oxidative damage to DNA. Oxidative damage, as assessed by 8-

hydroxydeoxyguanosine adducts, has been found in livers of mice exposed to pentachlorophenol, as well as elevated hemoglobin adducts in males and females (NTP TR 483). Thus, it is probable that the mesotheliomas seen in rats exposed to the high dose of pentachlorophenol in the NTP study are a consequence of the oxidative damage to mesothelium of the tunica vaginalis. Given that there was also a high incidence of Leydig cell tumors in the treated rats the altered hormonal milieu associated with the proliferating Leydig cells may also have contributed to the development of tunica vaginalis mesotheliomas.

#### Specific Chemicals - Dosed Water Route of Administration

Tartrazine (FD&C Yellow No. 5). Tartrazine is a food, drug, and cosmetic coloring agent. In a 2-year dosed water study using F344 rats, mesotheliomas were present only at the lower dose (Table 18) of tartrazine (Maekawa et al., 1987). There was a persistent decreased body weight gain in the 2% group starting at experimental week 40. Based on a lower than expected incidence in the control group (historical incidence was 4.1%), absence of a positive trend, and absence of hyperplastic or preneoplastic lesions in the peritoneal cavity, the authors concluded that the occurrence of peritoneal mesotheliomas was not related to treatment. It is mentioned in the publication that the mesotheliomas are similar to those seen spontaneously in the F344 male. The incidence of Leydig cell tumors in this study was greater than 94% in the control and low dose groups and was 100% in the high dose group. There was an increased incidence of endometrial stromal polyps in the low dose female rats that the authors concluded was not treatment-related.

Tartrazine was not mutagenic in the Ames test but produced chromosome aberrations in *cultured mammalian cells* (Table 29).

#### 3,3'-Dimethoxybenzidine hydrochloride

In a chronic dosed water study, terminated at 21 months due to early tumor-induced mortality, there was induction of tumors at multiple tissue sites including a marginal peritoneal mesothelioma response in male F344 rats (Table 19) and a robust mammary gland adenocarcinoma response in female rats (NTP TR 372). 3,3'-Dimethoxybenzidine hydrochloride was considered to have clear evidence of carcinogenicity based on statistically significant increases in tumors at multiple sites. It is a member of the aromatic amine class of chemicals which when metabolically activated induce a variety of tumor types. Activation of *ras* oncogenes was identified in some of the induced epithelial tumors.

The incidences of mesothelioma were not statistically significant by pairwise comparison, although there was a significant positive trend. There was significant early mortality in all treated males and females with greater than 50% mortality by week 86. At study termination (94 weeks) only 8 low dose males were alive among the treated rats. The authors of the technical report suggested that the mesothelioma incidences might have been higher had the rats lived longer. 3,3'-Dimethoxybenzidine is mutagenic in the

Ames and mouse lymphoma mutation tests, but did not induce chromosome aberrations in cultured mammalian cells (Table 29).

*3,3'-Dimethylbenzidine hydrochloride.*

In a 14-month dosed water study in F344 rats, 3,3'-dimethylbenzidine HCl produced a peritoneal mesothelioma response (Table 20) that showed a positive trend and was statistically significant at the highest dose. The authors of the technical report attributed the mesotheliomas to the test chemical and suggested that the incidence of mesothelioma might have been higher except for the reduced survival in the two highest dose groups. 3,3'-Dimethylbenzidine HCl was considered to have clear evidence of carcinogenicity based on robust responses at multiple other tissue sites (NTP TR 390).

3,3'-Dimethylbenzidine is a congener of 3,3-dimethoxybenzidine. Activation of the *H-ras* oncogene was detected in several epithelial neoplasms. 3,3'-Dimethylbenzidine was mutagenic in the Ames and mouse lymphoma mutagenicity tests, and induced chromosome aberrations in cultured mammalian cells (Table 29).

*Potassium bromate.*

Potassium bromate is a rodent carcinogen and is nephrotoxic and neurotoxic in humans. Because potassium bromate is a biproduct of water disinfection by ozonation, there has been interest in testing it for adverse effects by dosing in drinking water. Four drinking water cancer bioassay studies have been conducted in F344 rats and peritoneal mesotheliomas were induced in each study. The incidences of mesothelial responses in these studies are summarized in Tables 21, 22, and 23.

In the 1983 study, the earliest mesothelioma was observed after 72 weeks of treatment. The mesotheliomas were frequently seeded throughout the abdominal cavity and were associated with massive hemorrhagic ascites which, according to the authors, lead to severe anemia and early death.

Mesothelioma responses in the 1986 study were observed at doses of 30 ppm and higher with a statistically significant increase at 500 ppm, but the tumor incidences between 30 and 250 ppm were not dose-related. The occurrence of Leydig cell tumors was 95 to 100% in all groups, including the controls.

The origin of the mesotheliomas in this study was the tunica vaginalis mesothelium with involvement of the vaginal tunic, including the mesotheliomas that were present throughout the abdominal cavity. The TVM tended to be bilateral with some exceptions. Based on the book chapter by Hall (1990) and a pathology peer review of this study, the additional peritoneal sites of mesothelioma are considered neither additional primary tumors nor metastases. TVM in F344 rats typically spread by extension and seeding rather than via vascular or lymphatic routes of metastasis.

The design of this study with interim time points permitted the opportunity to examine the temporal sequences associated with development of treatment-induced

mesotheliomas. While all mesotheliomas were considered malignant by the authors, a single case of mesothelial hyperplasia, and 1 rat with a small mesothelioma confined to the parietal vaginal tunic, were seen at 52 weeks. Spreading to other peritoneal sites was not present until after 78 weeks of treatment. Spreading was by extension or implantation (i.e., seeding) and most commonly involved spleen, gastrointestinal tract, mesentery, and pancreas. This study showed the origin of the mesotheliomas to be in the tunica vaginalis.

An extensive re-examination of the study materials from the Wolf et al., 1998 study was reported by Crosby et al., 2000. Using cross sections of the rat testes to map the TVM, it was concluded that the mesorchium was the major tissue target site for potassium bromate-induced mesotheliomas. The authors discuss several factors that may contribute to TVM development. However, as with other brominated chemicals, oxidative damage (DeAngelo et al., 1998) and formation of oxidative DNA adducts (Kurokawa et al., 1983; Kasai et al., 1987; Sai et al., 1992) are the most likely mode of action for induction of the TVM response. Potassium bromate is mutagenic in the Ames test (Table 29).

#### Acrylamide.

Two separate bioassays in which acrylamide was administered to F344 rats in drinking water have been reported (Johnson et al., 1986; Friedman et al., 1995). Mesotheliomas of the tunica vaginalis were documented in both studies (Table 24). Only some of the mesotheliomas were present in the abdominal cavity, while all were present in the vaginal tunics of the scrotal sac. Neither publication tabulates the incidence of testicular Leydig cell tumors, however, the laboratory study report for the Johnson et al. (1986) study shows that 57 of the 60 males in each group, including the control group, had Leydig cell adenomas. A retrospective examination of study slides from the Friedman et al. (1995) study was conducted by Iatropoulos et al., 1998, who found that the degree of morphological progression of the tunica vaginalis mesotheliomas was correlated with the size of the Leydig cell tumors. The malignant mesotheliomas, as classified by Iatropoulos, were seen only in rats that had 75% or greater of their testicular parenchyma replaced by Leydig cell tumors. The mesothelial tumors that they classified as hyperplasias were present in rats in which the Leydig cell tumors occupied 24% or less of the testicular parenchyma.

Acrylamide is not mutagenic in the Ames test, but produces chromosome aberrations in cultured mammalian cells. It produces chromosome aberrations and micronuclei in mouse, but not rat bone marrow cells, and chromosome damage in male germ cells of rats and mice.

The probable mode(s) of action for induction of TVM associated with exposure to acrylamide has been extensively reviewed (Shipp et al., 2006). Administration of acrylamide to rats produces a dose-related reduction in prolactin and testosterone thought to be centrally mediated via the dopaminergic system (Friedman et al., 1999; Agrawal et al., 1981; Ali et al., 1983, Uphouse et al., 1982). The enhanced dopamine signal, with its

associated decreases in prolactin secretion, would trigger down-regulation of Leydig cell LH receptors (Prentice et al., 1992), reduced testosterone, and a compensatory increase in LH, which in turn stimulates proliferation of Leydig cells (Cook et al., 1999). The altered hormonal milieu is then reflected as a transudate in tunical vaginalis fluid, and the exposed tunica vaginalis mesothelium proliferates via an autocrine response to growth factor production. A physical stimulus affecting tunica vaginalis mesothelium from testes enlarged by Leydig cell tumors may also lead to elaboration of growth factors by the mesothelium and an autocrine-mediated cell proliferative response.

#### Specific Chemicals - Gavage Route of Administration

##### *Methyleugenol.*

The gavage administration of methyleugenol (in 0.5% methylcellulose) to F344 rats resulted in induction of multiple tumor target sites (NTP TR 491) with a strong mesothelioma dose response (Table 25). Fifty of 60 males and females received 300 mg/kg methyleugenol for 52 weeks and then were administered methylcellulose vehicle, alone, for the next 53 weeks.

There was a dose-related increase of Leydig cell tumors in core study rats. Of the five 300 mg/kg treated rats and the five controls examined at 12 months, all had Leydig cell tumors, nine of which were bilateral. Of the 50 remaining stop study rats, five had TVM. Mammary gland fibroadenomas were also increased in dosed male rats. Other induced neoplasms included benign and malignant liver tumors, benign and malignant gastric neuroendocrine tumors, benign kidney tumors, and benign and malignant connective tissue tumors of the skin. Liver and glandular stomach neoplasms were increased in treated mice. While methyleugenol is not mutagenic in the Ames test, or induce chromosome damage in cultured mammalian cells or mouse bone marrow, its metabolism is associated with adduct formation, and beta-catenin mutations have been reported in methyleugenol-induced mouse liver tumors (Devereux et al., 1999).

##### *Benzaldehyde.*

In a 2-year gavage study of benzaldehyde in F344 rats (NTP TR 378) using corn oil as the vehicle, a marginal TVM response (Table 26) was not considered related to treatment, and the chemical was judged not to be a carcinogen in rats. This judgment was influenced by lack of a dose response and the laboratory's mesothelioma historic control incidence of 8% in male F344 rats. The incidences of Leydig cell tumors in the control and low dose groups were greater than 90%, while only 63% of the high dose males had Leydig cell tumors. There was some evidence of treatment-related neoplasia in mice based on forestomach squamous cell papillomas. Benzaldehyde was not mutagenic in the Ames test, but did induce mutations in the mouse lymphoma test, and it did not induce chromosome aberrations in cultured mammalian cells. (Table 29).

##### *Glycidol.*

Exposure to glycidol produces a marked carcinogenic response with tumors at multiple sites in both sexes of F344 rats and B6C3F1 mice (NTP TR 374). Peritoneal mesotheliomas are among the tumor responses in male rats that showed a dramatic increase in a 2-year gavage study in which glycidol was administered in a water vehicle (Table 27).

All mesotheliomas were present in the tunica vaginalis, many with extension into the abdominal cavity. They were classified into benign and malignant neoplasms. Mesotheliomas confined to the vaginal tunics were considered benign and those that spread into the abdominal cavity and/or had cytological features of malignancy were considered malignant. The histomorphological features of the malignant mesotheliomas included pleomorphism, cytological atypia, local invasiveness, and implant metastasis throughout the abdominal cavity. Malignant mesotheliomas were considered rapidly lethal; the first death attributed to mesothelioma occurred in a high dose male at study week 49.

Despite early tumor-associated mortality in the treated males, the control and dosed male groups all had high incidences of Leydig cell tumors. Mammary gland neoplasms were dramatically increased in female rats. Epithelial tumors were increased at multiple sites in treated mice. Glycidol is a direct alkylating agent, forming promutagenic adducts in DNA, and is mutagenic in the Ames test and produces chromosome damage in cultured mammalian cells and mouse bone marrow. The relationship between adduct formation and tumorigenesis is in part attributed to the relative susceptibility of the exposed tissue. The robust mesothelioma response observed in the glycidol study is most probably a consequence of the combined effects of localized genotoxicity and the susceptibility of tunica vaginalis mesothelium to the hormonal imbalance in F344 rats associated with aging and the development of Leydig cell tumors.

#### Specific Chemicals - Topical Application Route of Administration

##### 2,3-Dibromo-1-propanol.

Topical application of 2,3-dibromo-1-propanol produced a marginal mesothelioma response in male rats (Table 28) but clear evidence of carcinogenicity at other sites (NTP TR 400). There was also clear evidence of carcinogenicity in mice based on increased incidences of epithelial neoplasms.

The study was terminated after 51 weeks, because of reduced survival in the high-dose groups resulting from chemically induced neoplasms. Early mortality began at week 45. Major induced tumors involved the nasal cavity, skin, oral cavity, and gastrointestinal tract. The incidence of Leydig cell tumors was low because of early study termination, with the highest incidence of 34% seen in the low-dose group. However, the incidence of Leydig cell hyperplasia was up to 56% in the low dose group suggesting that the paracrine hormonal secretion by the proliferating Leydig cells also contributed to the early appearance of tunica vaginalis mesotheliomas in treated rats. 2,3-Dibromo-1-propanol is mutagenic in the Ames and mouse lymphoma mutation tests and produces chromosome aberrations in cultured mammalian cells, but did not induce micronuclei in mouse bone marrow cells (Table 29).

## **GENOTOXICITY**

Analyses of carcinogenicity and genotoxicity databases (Ashby & Tennant, 1988; Gold et al., 1993, 2001) have shown that some tumor types/locations are associated with genotoxic chemicals and some are associated with non-genotoxic chemicals, although the association appeared to be less strong in the Gold et al. (1993, 2001) compilations, which examined the NTP and other data sources than in Ashby and Tennant (1988). In this latter study that examined only chemicals tested by the NTP (Ashby and Tennant, 1988), there was an association of some tumor sites with mutagenicity. That is, some tumors/tumor sites were responsive primarily to chemicals that were mutagenic in the Salmonella test, some were responsive primarily to chemicals that were not mutagenic in Salmonella, and other sites appeared to be responsive to both mutagenic and non-mutagenic carcinogens.

Genotoxicity in this context is defined as positive results in the Salmonella mutagenicity (Ames) test. A positive response in an *in vitro* mammalian cell chromosome aberration test, by itself, is not considered to be definitive evidence of genetic toxicity because of the predilection of this test to produce positive results as a secondary response to cell toxicity, or to high osmolarity or changes in growth medium pH (Brusick, 1986; Scott et al., 1991; Morita et al., 1992).

Ashby and Tennant identified two chemicals among the NTP database that induced tunica vaginalis mesotheliomas, glycidol (Ashby and Tennant, 1991a) and 1,2-dibromoethane (Ashby and Tennant, 1991b), both of which were mutagenic in Salmonella. The (Gold et al., 1993, 2001) compilations of cancer site and mutagenicity do not distinguish tunica vaginalis mesotheliomas from other testicular tumors, and do not list mesothelioma as a tumor type.

Chemicals reported here to induce tunica vaginalis mesotheliomas (see Table 29) were classified according to the potency of their tumor induction, e.g., robust or nonsignificant-to-marginal, and their genetic toxicity. The criterion for a robust tumor response was that the magnitude of the highest incidence, regardless of dose, was >18%. This criterion was determined by examining the incidence data, the likely mode of action, and/or the final interpretation of the specific cancer bioassays. For 1,2-dichloroethane the 10% (5/50) TVM response was not statistically significant versus the concurrent control (0/50) (Nagano et al., 2006). The 10% TVM response in the low dose animals in the benzaldehyde study was judged to be a non-carcinogenic response by the NTP peer review board (NTP TR 378) because, although it was greater than the concurrent control, it was equivalent to the 8% historical control incidence in the testing lab. The 12% TVM response induced by tartrazine did not exhibit a dose response, and was considered to be not treatment-related by the author (Maekawa et al., 1987). The nitrofurazone TVM response of 14% was seen at the lower dose without evidence of a dose response (NTP TR 337). A 16% TVM response in the ethyl tellurac study, although dose-related was considered equivocal by the NTP peer review board (NTP TR 152). The TVM found in the acrylamide studies are considered centrally mediated and secondary to Leydig cell tumors (Shipp et al., 2006). The 18% pentachlorophenol response was seen only in a stop study where the dose exceeded the maximum tolerated dose (NTP TR 483). The gap between the 18% incidence of TVM in the pentachlorophenol study and a 24%

incidence of TVM for methyl eugenol, prompted selection of 18% as a cut-off incidence for classifying the potency of the TVM response. Latency, as defined by the week to first observed TVM, of less than 60 weeks was a feature of robust responses (Table 29).

Regardless of the conclusions of Gold et al. (1993, 2001) who found that genotoxic and nongenotoxic chemicals produced similar tumor induction patterns, there was a clear distinction between the chemicals that produced robust, and those that produced weak, tunica vaginalis mesothelioma responses. Of the 10 chemicals producing robust responses that had genetic toxicity test results, 8 (80%) were mutagenic in Salmonella. One of the outliers, nitrotoluene, requires anaerobic activation as present in vivo, in contrast to the aerobic conditions present in the Ames test. Where *in vitro* cytogenetics results were available, they supported the Salmonella results. In contrast, only 2 of the 7 chemicals (29%) that produced non-significant-to-marginal responses, were mutagenic in Salmonella. There were an additional three chemicals in this group that were negative in the Salmonella test but positive in the chromosome aberration test, one of which, acrylamide, also produced chromosome damage in the *in vivo* bone marrow test.

Three NTP studies were terminated early, i.e., less than 2 years, because of mortality from other tumors. 2,3-Dibromo-1-propanol, 3,3'-dimethoxybenzidine 2HCl, and 3,3'-dimethylbenzidine 2HCl, which are all mutagenic in Salmonella, had overall TVM frequencies of 8, 10, and 7%, respectively. Because TVM tend to be late occurring neoplasms, especially in controls, early study termination because of other tumor responses would not allow for adequate exposure time to fully assess the magnitude of a potential mesothelioma response in these 3 studies.

The chemicals in Table 29, and their putative metabolites, present a wide range of structures and chemical characteristics. Some, e.g., methyl(acetoxymethyl)nitrosoamine, glycidol, ethylene oxide, can form DNA adducts. Others, e.g., nitrilotriacetic acid, methyl eugenol, potassium bromate, ethyl tellurac, do not appear to have any direct DNA-reactivity, but may induce their damage through the induction of reactive oxygen species. And others, e.g., acrylamide, are known to be both DNA-reactive (through its metabolite, glycidamide) and capable of inducing oxidative stress and hormonal changes.

The one conclusion that is obvious from this compilation is that the induction of tunica vaginalis mesotheliomas is not confined to genotoxic chemicals. A significant fraction of these tumors are induced by chemicals that are considered to be nongenotoxic, presumably acting through a mechanism(s) that do(es) not involve direct DNA interaction.

### **RELEVANCE OF TUNICA VAGINALIS MESOTHELIOMAS IN RATS TO HUMAN HEALTH**

Of the 21 xenobiotics associated with a mesothelioma response in rats that are addressed in this document, 7 are judged to have a non-significant to marginal response, 3 are relatively non-informative with respect to their potency due to early study

termination because of tumors other than mesotheliomas, and 11 exhibited a robust mesothelioma response. Highlights of the findings in these studies are summarized in Table 30, and the categorization of the responses are in Table 29. If one excludes the three robust chemicals that were identified via the intraperitoneal route of exposure, where the xenobiotic would have direct contact with mesothelium, the remaining 18 studies were done using F344 rats. For the 11 chemicals with a robust mesothelioma response, a genotoxic mode of action may be associated with that target tissue response. However, the presence of Leydig cell tumors in the F344 rats, and the evidence linking Leydig cell tumors to tunica vaginalis mesotheliomas, suggests a contributory effect of the Leydig cell tumor burden.

The occurrence of xenobiotic treatment-associated tunica vaginalis mesotheliomas by other than the intraperitoneal route is a feature unique to the male F344 rat. This tumor response is an exacerbation of a well-documented, low spontaneous background rate of tunica vaginalis mesothelioma in these rats. A key event associated with the xenobiotic induction of TVM in the F344 rat is the age-associated and high incidence of testicular Leydig cell tumors. The local hormonal milieu in the tissues adjacent to the Leydig cell tumors is altered and the hormonal imbalance is reflected as a transudate in the tunica vaginalis fluid. This, in turn, leads to an autocrine growth factor response in the tunica vaginalis mesothelium as a primary mode of action, resulting in mesothelial hyperplasia and ultimately mesothelioma. Since it has been shown that mesothelial cells respond to pressure or shearing forces by elaborating autocrine growth factors, the markedly enlarged testes from the Leydig cell tumor burden can also initiate a mitogenic stimulus. Thus, a specific primary mode of action for developing tunica vaginalis mesotheliomas in the F344 rat is dependent upon enhanced mitogenesis caused by autocrine growth factors in the stimulated tunica vaginalis mesothelium. Given the extremely low incidence of Leydig cell tumors in humans, a F344 rat tunica vaginalis mesothelioma response attributed to this primary mode of action is not considered relevant to human cancer induction.

To further understand the factors associated with Leydig cell biology, an expert panel of scientists identified 7 mechanisms that could lead to Leydig cell hyperplasia and adenoma formation (Clegg et al., 1997). Two hormonal modes of action, viz., GnRH agonism and dopamine agonism, were considered not relevant to humans. GnRH agonism is unique to the rat since human as well as monkey and mouse Leydig cells do not express the LHRH receptor (Prentice and Meikle, 1995). Dopamine agonism leads to decreased prolactin secretion by the pituitary which, in turn, leads to down-regulation of Leydig cell LH receptors, decreased testosterone, and a compensatory increased circulating LH to raise testosterone levels (Cook et al., 1999; Prentice et al., 1992). The increased LH leads to Leydig cell proliferation and ultimately to Leydig cell tumors (Cook et al., 1999; Prentice and Meikle 1995). This dopaminergic mode of action is unlikely in humans because the number of LH receptors per Leydig cell is 14 times less than in the rat, and Leydig cell tumors are extremely rare in humans (Prentice and Meikle 1995; Foster, 2007). Five additional hormonal modes of action for Leydig cell tumor induction that are potentially relevant to humans include androgen receptor antagonism, 5-alpha-reductase inhibition, inhibition of testosterone biosynthesis, aromatase inhibition, and estrogen agonism. Rodents have greater sensitivity than humans to these hormonal

effects. The expert panel recommended a margin of exposure (MOE) approach be used when a rodent Leydig cell tumor response is attributable to one of these 5 modes of action. If the compound under investigation was mutagenic, then a case-by-case judgment regarding human health risk was recommended.

There are species and strain differences that indicate a tunica vaginalis mesothelioma response by other than the peritoneal route of exposure is specific to the F344 rat. Examination of the literature indicates that a tunica vaginalis response to xenobiotic exposure is generally not seen in other strains and stocks of rats, even following sustained increased LH levels (Prentice et al., 1992). The aging F344 rat has a more advanced development of testicular changes, including Leydig cell tumors, than other rat stocks (Kanno et al., 1987) and a greater background incidence of testicular mesotheliomas. In several hazard identification cancer bioassays conducted, in parallel, in F344 rats and B6C3F1 mice, a tunica vaginalis mesothelioma response was never seen in mice, nor was a mesothelioma response seen in female rats. Consequently, the male F344 rat specificity of tunica vaginalis mesothelial tumorigenesis is not likely to be relevant to other species or pose a human cancer risk.

Among the xenobiotics reviewed in this report, some are direct alkylating agents with clear genotoxicity and a robust tunica vaginalis mesothelioma response (Tables 30 and 31). Robust TVM responses have been observed in rats exposed to alkylating agents such as glycidol (NTP TR 374) and nitrosamines (Berman and Rice 1979; Lijinsky et al., 1985; Greenblatt and Lijinsky 1972). The relationship between adduct formation and tumorigenesis is, in part, attributed to the relative susceptibility of the exposed tissue. It has been suggested that tunica vaginalis mesothelium, as opposed to mesothelium elsewhere in the body, has unique properties making it more responsive to chemical carcinogens (Berman and Rice, 1979). The robust mesothelioma response observed in the glycidol study is most probably a consequence of the combined effects of localized genotoxicity and the susceptibility of tunica vaginalis mesothelium to the hormonal imbalance in F344 rats associated with aging and the development of Leydig cell tumors.

Another example of a robust tunica vaginalis mesothelioma response occurred following exposure to *o*-nitrotoluene. For this chemical, the formation of *o*-benzyl glucuronide is a critical step in leading to formation of DNA-reactive intermediates. Intestinal microflora hydrolyze the glucuronide and reduce the nitro group to form *o*-aminobenzyl alcohol. Upon reabsorption of the *o*-aminobenzyl alcohol, it is sulfated and binds to DNA. Two brominated chemicals, 2,2-bis(bromomethyl)-1,3-propanediol and potassium bromate, produced a robust mesothelioma response (Table 29). Hypotheses for the carcinogenic activity of brominated chemicals include oxidative damage to DNA and formation of DNA adducts when the carbon-bromine bond is broken (Kurokawa et al., 1983; Kasai et al., 1987; Sai et al., 1992; De Angelo et al., 1998). It is noted, however, that even for genotoxic xenobiotics producing a robust tunica vaginalis mesothelioma responses in male F344 rats, there are no mesotheliomas in female rats or in mice, thereby underscoring the unique sensitivity of the tunica vaginalis mesothelium in male F344 rats.

Some tissue-specific responses are characteristic of epigenetic modes of action in the non-significant to marginal tunica vaginalis mesothelioma responses (Table 29) .

Using acrylamide as an example, the adrenal pheochromocytoma, tunica vaginalis mesothelioma, and thyroid follicular adenoma responses in the male F344 rat (Johnson et al., 1986; Friedman et al., 1995) are consistent with rodent-specific targeting of endocrine-sensitive tissues, and have little relevance to human cancer risk (Cohen 2004). Exposure of F344 and Sprague-Dawley rats to acrylamide has been shown to increase replicative DNA synthesis in these tumor target tissues, but not in non-target tissues (Lafferty et al., 2004). Furthermore, blocking cytochrome P450 activity, and thus the formation of the DNA-reactive metabolite of acrylamide, glycidamide, did not abolish replicative DNA synthesis in the tunica vaginalis mesothelium. From these findings, it is apparent that the tunica mesothelioma response occurred through a mode of action independent of oxidative metabolism of the chemical to a DNA reactive metabolite (Lafferty et al., 2004). Acrylamide also has dopaminergic activity in the F344 rats, which leads to decreased circulating prolactin followed by enhancement of spontaneous, age-associated Leydig cell tumorigenesis (Friedman et al., 1999). As a result, the tunica vaginalis mesothelioma response in acrylamide-treated F344 rats is most likely caused by a hormonally mediated and autocrine growth factor-driven mesothelial mitogenesis mode of action. A similar autocrine growth factor-driven mode of action, although not necessarily amplified by dopamine agonism, is believed to be a primary cause of the observed tunica vaginalis mesothelioma responses seen for other chemicals with a non-significant to marginal response (Table 29). Thus, these xenobiotics with a non-significant to marginal tunica vaginalis mesothelioma response that is unique to the F344 rat do not pose a significant risk for human carcinogenesis (see Table 31).

## CONCLUSIONS

The primary conclusions based upon this review of tunica vaginalis mesotheliomas in rat bioassays are as follows:

- Tunica vaginalis mesotheliomas are low incidence spontaneous neoplasms in rats that can be exacerbated by treatment.
- Tunica vaginalis mesotheliomas in rats originate in the mesothelial lining of the scrotal sac, testes, epididymides, and mesorchium and can spread to the abdominal cavity by extension or seeding since the scrotal sac mesothelium is continuous with the peritoneal cavity mesothelium.
- A majority of chemicals that are associated with a non-significant to marginal tunica vaginalis mesothelioma induction are non-genotoxic based on the Ames test, whereas chemicals producing a robust response tend to be Ames test mutagens.
- The mesothelioma responses to xenobiotic exposure by other than the peritoneal route are male F344 rat-specific. They are never seen in female F344 rats or in either gender of mice in conventional cancer bioassays, and have not been reported in other rat strains used for carcinogenicity testing.
- Spontaneous, as well as several, xenobiotic-associated tunica vaginalis mesotheliomas are causally associated with Leydig cell tumors that lead to an autocrine growth factor-induced mesothelial mitogenesis.

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Table1. Some immunohistochemical stains used to distinguish human mesotheliomas from adenocarcinomas

Protein/peptide target	Mesothelioma	Adenocarcinoma
Cytokeratin 5/6	Positive	Negative
Thrombomodulin	Positive	Negative
Calretinin	Positive	Negative
Epithelial membrane antigen (EMA)	Positive	Most are Negative
Vimentin	Positive	Negative
WT1	Positive	Negative
HBME-1	Positive	Negative
Carcinoembryonic antigen	Negative	Positive
CK20	Negative	Positive
B72.3	Negative	Positive
BerEp[4]	Negative	Positive
BG8	Negative	Positive
TTF-1	Negative	Positive
Leu M1	Negative	Positive

Table 2. Xenobiotics associated with tunica vaginalis mesotheliomas in rat studies  
(arranged alphabetically)

<b>Xenobiotic Agent</b>	<b>CASRN</b>	<b>Reference</b>
Acrylamide	79-06-1	Johnson et al., 1986 Friedman et al., 1995
Benzaldehyde	100-52-7	NTP TR 378
2,2-Bis(bromomethyl)-1,3-propanediol	3296-90-0	NTP TR 452
Cytembena	21739-91-3	NTP TR 207
1,2-Dibromoethane	106-93-4	NTP TR 210
2,3-Dibromo-1-propanol	96-13-9	NTP TR 400
1,2-Dichloroethane	107-06-2	Nagano et al., 2006
3,3'-Dimethoxybenzidine	20325-40-0	NTP TR 372
3,3'-Dimethylbenzidine	612-82-8	NTP TR 390
Ethylene oxide	75-21-8	Snellings et al., 1984 Lynch et al., 1994
Ethyl tellurac	20941-65-5	NTP TR 152
Glycidol	556-52-5	NTP TR 374
Methyl(acetoxymethyl)nitrosoamine	56856-83-8	Berman & Rice 1979
Methyleugenol	93-15-2	NTP TR 491
Nitrilotriacetic acid ± ferric saccharate		Okada et al., 1989
Nitrofurazone	59-87-0	NTP TR 337
<i>o</i> -Nitrotoluene	88-72-2	NTP TR 504 NTP TOX 23 NTP TOX 44
Pentachlorophenol, purified	87-86-5	NTP TR 483
Potassium bromate	7758-01-2	Kurokawa et al., 1983 DeAngelo et al., 1998 Wolf et al., 1998
Tartrazine	1934-21-0	Maekawa et al., 1987
<i>o</i> -Toluidine HCl	636-21-5	NTP TR 153 NTP TOX 44

Table 3. Frequency of proliferative mesothelial lesions and Leydig cell tumors following a single intraperitoneal injection of methyl(acetoxymethyl)nitrosamine in three strains of male rats

Strain	Treatment	Mesothelioma	Mesothelial Hyperplasia	LCT
F344	Control	0/15 (0%)	0/15 (0%)	7/15 (47%)
	DMN-OAc	9/25 (36%)	5/25 (20%)	11/25 (44%)
Sprague-Dawley	Control	1/27 (3.7%)	2/27 (7.4)	4/27 (15%)
	DMN-OAc	4/27 (15%)	4/27 (15%)	1/27 (3.7%)
Buffalo	Control	1/25 (4%)	2/25 (8%)	0/25 (0%)
	DMN-OAc	12/26 (46%)	3/26 (11%)	0/26 (0%)

LCT = Leydig cell tumor

Table 4. Frequency of mesotheliomas following intraperitoneal injection of ferric saccharate in male Wistar rats

Treatment Groups	Mesothelioma
Physiological saline	0/20 (0%)
NTA (83.5 mg/kg/d)	0/20 (0%)
Ferric saccharate (5 mg Fe/kg/d)	9/19 (47%)
Ferric saccharate + NTA	13/19 (68%)

Table 5. Frequency of peritoneal and tunica vaginalis mesotheliomas in male F344 rats given 3 times weekly injections of cytembena for 2 years

Tumor	0 mg/kg (untreated)	0 mg/kg (vehicle)	7 mg/kg	14 mg/kg
Mesothelioma	1/50 (2%)	3/50(6%)	37/50 (74%)	36/50 (72%)

Table 6. Frequency of mesotheliomas in male F344 rats exposed to ethylene oxide vapor for 2 years

Study	0 ppm	10 ppm	33 ppm	50 ppm	100 ppm
Snellings et al., 1984	2/97*(2%)	2/51(4%)	4/39(10%)	-	4/30(13%)
Lynch et al., 1984	3/78(3.8%)	-	-	9/79(11%)	21/79(27%)

\* Combined controls (1/49 & 1/48)

Table 7. Frequency of mesotheliomas in male F344 rats exposed to 1,2-dibromoethane by inhalation for 2 years

Tumor	0 ppm	10 ppm	40 ppm
Mesothelioma	1/50 (2%)	13/50 (26%)	26/50 (52%)
Leydig cell	35/50 (66%)	45/50 90%)	26/50 (52%)

Table 8. Frequency of mesotheliomas in male F344 rats exposed by inhalation of DCE for 2 years (Nagano et al., 2006)

Tumor	0 ppm	10 ppm	40 ppm	160 ppm
Mesothelioma	1/50 (2%)	1/50 (2%)	1/50 (2%)	5/50 (10%)

Table 9. Frequency of mesotheliomas in F344 rats administered ethyl tellurac in the diet for 2 years

Group	0 ppm	300 ppm	600 ppm
Mesothelioma	0/20 (0%)	2/49 (4%)	8/50 (16%)

Table 10. Frequency of epididymal mesothelial lesions in rats receiving *o*-nitrotoluene in the diet for 13 weeks (NTP Tox 23)

Effect	0 ppm	625 ppm	1250 ppm	2500 ppm	5000 ppm	10000 ppm
Mesothelial hyperplasia	0/10	-	-	-	0/10	2/10
Mesothelioma	0/10	-	-	-	3/10	0/10

Table 11. Frequency of epididymal and testicular mesothelial lesions in rats in the 26-week dietary *o*-nitrotoluene study (NTP Tox 44)

<b>13-wk interim</b>	Normal GI flora		Altered GI flora*	
	0 ppm	5000 ppm	0 ppm	5000 ppm
Epididymis mesothelial hyperplasia	0/10	0/20	0/10	2/20
Epididymis mesothelioma	0/10	0/20	0/10	2/20
<b>Stop-exposure</b>	Normal GI flora		Altered GI flora	
	0 ppm	5000 ppm	0 ppm	5000 ppm
Testis mesothelioma	0/10	2/20	0/10	4/20
Epididymis mesothelial hyperplasia	0/10	2/20	0/10	1/20
Epididymis mesothelioma	0/10	4/20	0/10	8/20
<b>26-wk continuous exposure</b>	Normal GI flora			
Testis mesothelioma	0/10	2/20	nd	nd
Epididymis mesothelial hyperplasia	0/10	2/20	nd	nd
Epididymis mesothelioma	0/10	7/20	nd	nd

\* Rats treated with [antibiotic] to alter the intestinal flora

nd - not done

Table 12. Frequency of mesotheliomas in male F344 rats in the 2-year feed study of *o*-nitrotoluene (NTP TR-504)

Group	0 ppm	625 ppm	1250 ppm	2000 ppm	2000 ppm stop exposure	5000 ppm stop exposure
Overall rate	2/60(3.3%)	20/60(33%)	29/60(48%)	44/60(73%)	44/60(73%)	54/60(90%)
Terminal rate*	2/39(5.1%)	5/18(28%)	1/3(33%)	0/0	10/11(91%)	0/0

\* Rates in animals that were alive at 104 weeks.

Table 13. Frequency of mesotheliomas in F344 rats receiving dietary *o*-toluidine HCl for up to 26 weeks (NTP Tox 44)

Diet	0 ppm	5000 ppm
<b>13-Week Interim</b>		
Epididymis mesothelioma	0/10	0/20
<b>Stop-exposure</b>		
Epididymis mesothelioma	0/10	2/20
<b>26-Week continuous exposure</b>		
Epididymis mesothelioma	0/10	0/20*

\* One rat had mesothelial hyperplasia.

Table 14. Frequency of mesotheliomas in F344 rats  
receiving dietary *o*-toluidine for 2-years (NTP TR 153)

Group	0 ppm	3000 ppm	6000 ppm
Mesothelioma	0/20	17/50 (34%)	9/49 (18%)

Table 15. Frequency of mesotheliomas in male F344 rats administered

2,2-bis(bromomethyl)-1,3-propanediol in the diet

Group	0 ppm	2500 ppm	5000 ppm	10000 ppm	20000 ppm Stop study
Overall rate	0/51 (0%)	3/53(5.6%)	8/51(16%)	9/55(16%)	26/60(43%)
Terminal rate*	0/26(0%)	0/20(0%)	4/13(31%)	1/1(100%)	0/0

\* Rates in animals that were alive at 104 weeks.

Table 16. Frequency of mesotheliomas in male F344 rats administered nitrofurazone in the diet for 2 years

Group	0 ppm	310 ppm	620 ppm
Overall rate	0/50 (0%)	7/50 (14%)	2/50 (4%)
Terminal rate*	0/33 (0%)	2/30 (7%)	0/20 (0%)

\* Rates in animals that were alive at 104 weeks.

Table 17. Frequency of mesotheliomas in a 2-year study with continuous exposure and a stop study in which male F344 rats received pentachlorophenol in the diet for 1 year (NTP TR 483)

Group	0 ppm	200 ppm	400 ppm	600 ppm	1000 ppm for 1 year - stop study
Overall rate	1/50 (2%)	0/50 (0%)	2/50 (4%)	0/50 (0%)	9/50 (18%)
Terminal rate	0/12* (0%)	0/16 (0%)	0/21 (0%)	0/31 (0%)	4/27 (15%)

\* Rates in animals that were alive at 104 weeks.

Table 18. Frequency of mesotheliomas in male F344 rats in a 2-year dosed water study of tartrazine (Maekawa et al., 1987)

Group	0%	1%	2%
Mesothelioma	0/48 (0%)	6/49 (12%)	0/49 (0%)

Table 19. Frequency of mesotheliomas in male F344 rats administered

3,3'-dimethoxybenzidine hydrochloride in drinking water for up to 21 months (NTP TR 372)

Group	0 ppm	80 ppm	170 ppm	330 ppm
Overall rate	2/60 (3%)	1/45 (2%)	7/75 (9%)	6/60 (10%)
Terminal rate	1/44 (2%)	0/8	0/0	0/0

Table 20. Frequency of mesotheliomas in male F344 rats administered

3,3'-dimethylbenzidine HCl in drinking water for 14 months (NTP TR 390)

Group	0 ppm	30 ppm	70 ppm	150 ppm
Overall rate	0/60 (0%)	0/45 (0%)	3/75 (4%)	4/60 (7%)
Terminal rate*	0/60 (0%)	0/41 (0%)	3/50 (6%)	0/0

\* Rates in animals that were alive at 104 weeks.

Table 21. Frequency of mesotheliomas in a drinking water study of potassium bromate in F344 rats reported by Kurokawa et al.

Tumor (Study)	0 ppm	15 ppm	30 ppm	60 ppm	125 ppm	250 ppm	500 ppm
Mesothelioma (Kurokawa et al., 1983)	6/53 (11%)	--	--	--	--	17/52 (33%)	28/46 (61%)
Mesothelioma (Kurokawa et al., 1986)	0/19 (0%)	0/19 (0%)	3/20 (15%)	4/20 (20%)	2/24 (8%)	3/20 (15%)	15/20 (75%)

Table 22. Frequency of mesotheliomas in the drinking water study of potassium bromate in F344 rats (DeAngelo et al., 1998)

Group	0 g/L	0.02 g/L	0.1 g/L	0.2 g/L	0.4 g/L
Mesothelioma	0/47 (0%)	4/49 (8%)	5/49 (10%)	10/47 (21%)	27/43 (63%)

Table 23. Frequency of mesotheliomas in the drinking water study of potassium bromate in F344 rats (Wolf et al., 1998)

Group	0g/L	0.02 g/L	0.1 g/L	0.2 g/L	0.4 g/L
Week 12	0/6(0%)	0/6(0%)	0/6(0%)	0/6(0%)	0/6(0%)
Week 26	0/6(0%)	0/6(0%)	0/6(0%)	0/6(0%)	0/6(0%)
Week 52	0/6(0%)	0/6(0%)	0/6(0%)	1/6(17%)	0/6(0%)
Week 78	0/6(0%)	0/6(0%)	0/6(0%)	0/6(0%)	4/6(67%)
Week 100	0	4/49(8%)	5/50(10%)	10/47(21%)	27/43(63%)

Table 24. Frequency of mesotheliomas in F344 rats in two separate studies in which acrylamide was administered in drinking water

Study	Doses in mg/kg/day				
	0	0.01	0.1	0.5	2.0
Johnson et al., 1986	3/60 (5%)	0/60 (0%)	7/60 (12%)	11/60 (1%)	10/60 (17%)
Friedman et al., 1995	8/204* (4%)		9/204 (4%)	8/102 (8%)	13/75 (17%)

\* Pooled control groups (4/102 and 4/102)

Table 25. Frequency of mesotheliomas in male F344 rats gavaged with methyleugenol for two years (NTP TR 491)

Group	0 mg/kg	37 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg 52-week exposure stop study
Overall rate	1/50 (2%)	3/50 (6%)	5/50 (10%)	12/50 (24%)	5/50 (10%)
Terminal rate*	0/20 (0%)	1/16 (6%)	0/15 (0%)	0/0	0/0

\* Rates in animals that were alive at 104 weeks.

Table 26. Frequency of mesotheliomas in male F344 rats administered benzaldehyde by gavage in corn oil (NTP TR 378)

Group	0 mg/kg	200 mg/kg	400 mg/kg
Overall rate	0/50	5/50 (10%)	2/50 (4%)
Terminal rate	0/37	4/29 (14%)	1/21 (5%)

Table 27. Frequency of mesotheliomas in male F344 rats in a 2-year water gavage glycidol study (NTP TR 374)

Group	0 mg/kg	37.5 mg/kg	75 mg/kg
Overall rate	3/49 (6%)	34/50 (68%)	39/47 (83%)
Terminal rate*	0/16	0/0	0/0

\* Rates in animals that were alive at 104 weeks.

Table 28. Frequency of mesotheliomas in male F344 rats treated topically with 2,3-dibromo-1-propanol for 51 weeks (NTP TR 400).

Group	0 mg/kg	188 mg/kg	375 mg/kg
Mesothelioma	0/50 (0%)	1/50 (2%)	4/50 (8%)

**Table 29. Genetic toxicity of chemicals associated with tunica vaginalis mesothelioma (TVM) induction in rats, arranged in order of decreasing maximum tumor frequency**

Carcinogen	CASRN	TVM frequency <sup>a</sup>	Wk to first TVM <sup>b</sup>	Ames test	In vitro cyto	In vivo cyto
<b>Robust TVM induction</b>						
o-Nitrotoluene	88-72-2	90%	13	—*	—	—
Glycidol	556-52-5	78%	49	+	+	+
Cytembena (ip)	21739-91-3	74%	45	+	+	—
Nitrilotriacetic acid ± ferric saccharate (ip)		68%	NA	**		
Potassium bromate	7758-01-2	63%	52	+		
1,2-Dibromoethane	106-93-4	52%	50	+	+	—
Methyl (acetoxymethyl)nitrosoamine (ip)	56856-83-8	46%	NA	+		
2,2-Bis(bromomethyl)-1,3-propanediol	3296-90-0	43%	52	+	+	<b>E</b>
o-Toluidine HCl	636-21-5; 95-53-4 <sup>c</sup>	34%	26	+	+	+/-
Ethylene oxide	75-21-8	27%	NA	+	+	+
Methyleugenol	93-15-2	24%	58	—	—	—
<b>Non-significant-to-marginal TVM induction</b>						
Pentachlorophenol, purified	87-86-5	18%	72	—	<b>w+</b>	—
Acrylamide	79-06-1	17%	66	—	+	<b>w+</b>
Ethyl tellurac	20941-65-5	16%	NA	—	<b>E</b>	
Nitrofurazone	59-87-0	14%	67	+	+	—
Tartrazine	1934-21-0	12%	NA	—	+	
Benzaldehyde	100-52-7	10%	80	—	—	
1,2-Dichloroethane	107-06-2	10%	NA	+	+	—
<b>Not Classifiable</b>						
2,3-Dibromo-1-propanol	96-13-9	***	51	+	+	—
3,3'-Dimethoxybenzidine HCl	20325-40-0	***	48	+	—	
3,3'-Dimethylbenzidine HCl	612-82-8	***	44	+	+	

Ames test, Salmonella mutagenicity result; in vitro cyto, chromosome aberrations in Chinese hamster cells in culture; in vivo cyto, chromosome aberrations or micronuclei in bone marrow cells of treated mice.

\*Nitrotoluene is mutagenic in Salmonella when activated by enteric organisms.

\*\* Nitrilotriacetic acid, by itself, is negative in the Ames test and was not tested in the chromosome aberration test. Nitrilotriacetic acid + ferric saccharate has not been tested in the Ames test or in chromosome aberration tests.

\*\*\* Studies were terminated early due to other tumor formation; cannot be classified as to TVM potency.

a. % of treated animals with TVM; maximum response recorded

b. Study week at which earliest TVM was identified

c. combined results from testing different salts of the same parent chemical

ip, intraperitoneal administration; +, positive response; w+, weakly positive; -, negative; E, equivocal response; +/-, conflicting results; blank, not tested; NA, data not available

Table 30. Summary of chemicals associated with tunica vaginalis mesotheliomas in rats, arranged by route of administration

Agent	Route	Strain	TVM	LCT	Other treatment-associated tumors
Methyl(acetoxymethyl) nitrosamine	IP	F344	36%	++	NR
Methyl(acetoxymethyl) nitrosamine	IP	SD	15%	+	NR
Methyl(acetoxymethyl) nitrosamine	IP	Buf	46%	0	NR
Ferric saccharate	IP	Wist	47%	NR	NR
Ferric saccharate + NTA	IP	Wist	68%	NR	NR
Cytembena NTP TR 207	IP	F344	74%	++	FR – Mammary fibroadenomas
2,3-Dibromo-1-propanol NTP TR 400	Topic	F344	8%	+	MR&FR – Tumors of the nasal cavity, skin, oral cavity, esophagus, forestomach, intestines, liver, kidneys, Zymbal gland MR – Splenic hemangioma/hemangiosarcoma FR – Clitoral gland tumors MM&FM – Skin & forestomach tumors MM – Liver and lung tumors
Ethylene oxide	Inh	F344	26%	++	MR&FR – Brain tumors FR – Mammary adenomas & adenocarcinomas
1,2-Dibromoethane NTP TR 210	Inh	F344	52%	++	MR&FR – Nasal carcinomas FR – A/B tumors; Mammary fibroadenomas MM – A/B tumors FM – Hemangiosarcomas; S/C Fibrosarcomas & Nasal carcinomas
1,2-Dichloroethane	Inh	F344	10%	NR	MR&FR – S/C fibromas and mammary fibroadenomas FR- Mammary adenomas and adenocarcinomas

<b>Agent</b>	<b>Route</b>	<b>Strain</b>	<b>TVM</b>	<b>LCT</b>	<b>Other treatment-associated tumors</b>
Ethyl telluric NTP Tr 152	Diet	F344	16%	++	MM&FM-Harderian gland tumors
o-Nitrotoluene NTP Tox 23; NTP Tox 44 NTP TR 504	Diet	F344	90%	++	MR&FR – S/C tumors & Mammary fibroadenomas MR – Liver tumors (including cholangiocarcinomas) & A/B tumors FR - Liver adenomas MM&FM – Hemangiosarcomas & Cecal carcinomas FM – Liver tumors
o-Toluidine Hydrochloride NTP Tox 44 NTP TR 153	Diet	F344	34%	++	MR&FR-Splenic sarcomas MR – S/C Fibromas FR – U. Bladder carcinoma & Mammary adenomas & adenocarcinomas MM – Hemangiosarcomas FM – Liver adenomas or carcinomas
2,2-bis(bromomethyl)-1,3-propanediol NTP TR 452	Diet	F344	43%	++	MR&FR – Mammary fibroadenomas; oral cavity and esophagus carcinomas; thyroid follicular cell tumors MR – Skin tumors; U. bladder carcinomas; A/B tumors; S/C fibromas; forestomach papillomas; intestinal tumors MM&FM – A/B tumors; Harderian gland tumors MM – Renal adenomas FM – S/C sarcomas
Nitrofurazone NTP TR 337	Diet	F344	14%	+	FR – Mammary fibroadenomas FM – Ovarian tumors
Pentachlorophenol NTP TR 483	Diet	F344	18%	++	MR – Nasal carcinomas MM & FM – Liver & adrenal tumors FM – Hemangiosarcomas
Tartrazine (FD&C Yellow No. 5)	Water	F344	12%	++	FR – Endometrial stromal polyps

Agent	Route	Strain	TVM	LCT	Other treatment-associated tumors
3,3'-Dimethoxybenzidine HCl NTP TR 372	Water	F344	10%	++	MR&FR – Tumors in the oral cavity, large intestine, liver, Zymbal gland and skin MR – Tumors in small intestine and brain FR – Mammary adenocarcinoma; tumors in clitoral gland and uterus
3,3'-Dimethylbenzidine HCl NTP TR 390	Water	F344	7%	+	MR&FR – Tumors of the skin, Zymbal gland, liver, oral cavity, intestines and lung MR – Preputial gland tumors FR – Clitoral gland tumors
Potassium bromate (Multiple published studies)	Water	F344	75%	NR	MR – Kidney and thyroid tumors
Acrylamide (2 published reports)	Water	F344	17%	++	MR&FR – Thyroid follicular tumors FR – Mammary fibroadenomas
Methyleugenol NTP TR 491	Gav	F344	24%	++	MR&FR – Liver tumors; Neuroendocrine stomach tumors MR – Kidney tumors; mammary tumors; S/C tumors MM & FM – Liver tumors MM – Glandular stomach tumors
Benzaldehyde NTP TR 378	Gav	F344	10%	++	MM&FM – Forestomach papillomas
Glycidol NTP TR 374	Gav	F344	83%	++	MR&FR – Brain, forestomach and thyroid tumors MR – Mammary fibroadenomas, Intestinal tumors, skin tumors; Zymbal gland tumors FR – Oral cavity tumors, clitoral gland tumors, leukemia MM&FM – Harderian gland and skin tumors MM – Forestomach, liver and lung tumors FM – Mammary tumors, Uterine tumors, S/C tumors

TVM = highest % incidence tunica vaginalis mesothelioma LCT = Leydig cell tumor response [+ = < 79%; ++ = ≥80%]

MR = Male Rat FR = Female Rat MM = Male Mouse FM = Female Mouse NR = Not reported

SD = Sprague-Dawley Buf = Buffalo Wist = Wistar NTA = Nitrlotriacetate acid

IP = intraperitoneal Tpoic = topical Inh = inhalation Diet = dietary Water = drinking water Gav = gavage

Table 31. Human relevance framework for tunica vaginalis mesothelioma induction in F344 rats secondary to enhanced mesothelial mitogenesis

Alternative Key Events	Degree of Certainty in F344 Rat	Human Relevance
Presence of Leydig cell tumors causally related to tunica vaginalis mesotheliomas	Reasonably certain. Size of Leydig cell tumors correlated with tunica vaginalis mesotheliomas and localized growth factors. Localized peritesticular hormonal imbalance stimulates mitogenic autocrine growth factors from mesothelial cells. (Turek & Desjardins 1979; Gerwin et al., 1987; Karpe et al., 1982; Bartke et al., 1985; Versnel et al., 1988)	Not relevant. Leydig cell tumors are extremely rare in humans. There are significant differences in production and responsiveness between rat and human mesothelial cells. (Clegg et al., 1997; Walker et al., 1995; Walker et al., 1992)
Physical pressure or shearing forces due to enlarged Leydig cell tumor-bearing testes	Good evidence. Evidence for altered growth factor expression in transformed mesothelial cells in vitro. (Tanigawa et al., 1987; Gabrielson et al., 1988; Gerwin et al., 1987; Waters et al., 1997)	Not relevant. Leydig cell tumors are extremely rare in humans. (Clegg et al., 1997; Walker et al., 1995; Walker et al., 1992)
Age-associated increased prolactin leading to decreased circulating testosterone	Certain. Increased prolactin causes decreased LHRH and LH and inhibition of testosterone production. (Mahoney & Hodgen 1995; Capen et al., 2002)	Not relevant. Human Leydig cells do not have LHRH receptors. LH receptors not responsive to prolactin. (Prentice & Meikle 1995)
Decreased prolactin secretion from pituitary via dopamine agonists	Certain for specific chemicals. Serum prolactin levels decrease in rats. Decrease in LH receptors. (Prentice et al., 1992; Prentice & Meikle 1995; Friedman et al., 1999; Uphouse et al., 1982)	Not relevant. Human LH receptors not responsive to prolactin. (Wahlstrom et al., 1983)
Spontaneous age-associated decrease in testosterone and LH receptors and compensatory increase in LH	Certain. Responsible for the high spontaneous incidence of Leydig cell tumors in older F344 rats. (Amador et al., 1985; Maekawa & Hayashi 1992; Takaki et al., 1989; Solleveld et al., 1984; Foster 2007; Tanigawa et al., 1987; Turek & Desjardins 1979; Prentice & Meikle 1995; Capen 1996)	Uncertain. The number of LH receptors is 14 times greater in rats compared to humans. (Prentice and Meikle, 1995)
LHRH receptor agonist induced Leydig cell tumors	Reasonably certain for specific chemicals. Binding to rat LHRH receptors on Leydig cells produces Leydig cell tumors. (Prentice & Meikle 1995)	Not relevant. Human Leydig cells do not have LHRH receptors. (Prentice & Meikle 1995)