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α2u-Globulin Nephropathy and Renal Tumors in National Toxicology Program Studies

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Abstract

Chemically induced renal neoplasms in male rats, developed coincident with α_{2u}-globulin nephropathy, are not considered predictive of risk to humans by the International Agency for Research on Cancer (IARC) and the U.S. Environmental Protection Agency. Criteria have been defined to establish the role of α_{2u}-globulin nephropathy in renal carcinogenesis, based on a proposed mode of action involving sustained tubular cell proliferation resulting from α_{2u}-induced nephropathy, with consequent development of neoplastic lesions. Recent NTP studies demonstrated inconsistencies with this proposed mechanism, including in some cases, far weaker kidney tumor responses than expected based on the extent of α_{2u}-globulin nephropathy. NTP studies with decalin, propylene glycol mono-t-butyl ether and Stoddard solvent IIC included extended evaluations of α_{2u}-related nephropathy, and were thus used in assessing the linkage between key events in 90-day studies with renal tumors in 2-year studies. This review revealed no or at best weak associations of tumor responses with renal α_{2u}-globulin concentrations, indices of cell turnover, or microscopic evidence of α_{2u}-associated nephropathy in prechronic studies. While tumor responses corresponded somewhat with a measure of cumulative α_{2u}-associated nephropathy (linear mineralization of the papilla) at the end of the 2-year studies, the severity of chronic nephropathy was generally in best agreement with the pattern of tumor response. These results suggest that while α_{2u}-globulin nephropathy may contribute to the renal tumor response, the critical component(s) of the nephropathy most closely associated with the development of tumors could not be clearly identified in this review.

Keywords

α_{2u}-globulin nephropathy; male rats; renal tubular cell tumors; pathogenesis

Introduction

Tumors in experimental animal cancer studies that are associated with species-specific mechanisms or modes of action may not be considered predictive of similar risk to humans. If a mode of action can be clearly demonstrated to not operate in humans, experimental animal responses are not considered relevant for cancer risk assessment (Cohen et al., 2003). A framework for human relevance analysis of information on carcinogenic modes of action has been developed and tested (Meek et al., 2003). Inappropriate use of experimental animal data can have adverse public health implications either leading to restriction of the use of a beneficial substance, or exposure of humans and the environment to a hazardous substance. Thus, it is

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critically important that modes of cancer action in rodent studies that are accepted by the scientific and regulatory communities as not relevant for humans, be periodically reexamined for consistency and coherence with data from emerging studies.

The development of kidney tumors in male rats in association with chemically induced α_{2u}-globulin nephropathy is one mechanism not considered a predictor of carcinogenic risk to humans by the IARC and the U.S.EPA (EPA, 1991; Swenberg and Lehman-McKeeman, 1999). The lack of relevance of the α_{2u}-globulin mechanism for the evaluation of carcinogenic risk is based on the absence of production of an analogous protein in humans. Strict scientific criteria have been outlined for establishing the role of α_{2u}-globulin-associated nephropathy in male rat renal carcinogenesis (Table 1).

These criteria were based on an hypothesized mechanism of action involving renal tubular cell death resulting from accumulation of a chemical-α_{2u}-globulin complex resistant to lysosomal degradation, compensatory sustained cell proliferation, and ultimately, development of neoplasms. In part because only very few chemicals have been shown to fulfill every one of the necessary criteria, an alternative mechanism of action of α_{2u}-related kidney tumor formation has been proposed (Melnick, 1992; Kohn and Melnick, 1999; Melnick and Kohn, 1999), and the subject has been the topic of extensive debate (Borghoff et al., 1993; Melnick, 1993; Ashby, 1996; Huff, 1996; Ashby, 1997; de la Iglesia et al., 1997; Dietrich, 1997; Melnick, 1997; Melnick et al., 1997).

A number of structurally diverse chemicals have been shown to induce α_{2u}-globulin nephropathy in the male rat. In addition to increases in the number, size and altered shape of hyaline droplets in the proximal tubules, α_{2u}-globulin nephropathy has been defined to include additional histological alterations (Hard et al., 1993; Swenberg and Lehman-McKeeman, 1999). Lysosomal dysfunction associated with excessive accumulation of α_{2u}-globulin is thought to initiate cell death, degeneration and necrosis of tubular epithelial cells. Cell loss, in turn, produces accumulation of α_{2u}-globulin and cellular debris as granular casts primarily at the cortico-medullary junction, and stimulates regenerative epithelial cell proliferation. Upon continuing exposure, mineralization within the loops of Henle in linear profiles, exacerbation of age-related chronic progressive nephropathy and atypical renal tubular hyperplasia occur after several months of treatment. Although direct evidence for this is lacking, it is thought that atypical hyperplastic foci, in turn, progress to renal adenomas and carcinomas (Hard, 1990).

Recent studies conducted by the National Toxicology Program (NTP) have comprehensively characterized the renal toxicity and carcinogenicity of several α_{2u}-globulin-inducing chemicals. Male rats exposed for 3 months via whole-body inhalation to decalin, propylene glycol mono-*t*-butyl ether (PGMBE) and Stoddard solvent IIC (SS IIC) developed alterations suggestive of α_{2u}-globulin nephropathy, including overall dose-related increases in renal α_{2u}-globulin protein and cell labeling indices (Dill et al., 2003; Doi et al., 2004a; Doi et al., 2004b). Interestingly, the development of renal neoplasms in chronic bioassays of PGMBE and SS IIC was unremarkable, although the magnitude of the α_{2u}-globulin nephropathy appeared equivalent to that previously seen with decalin and in other studies. Our analysis was conducted to systematically review key events in α_{2u}-globulin nephropathy in select NTP studies, and to evaluate the association among these key events and renal tumor outcomes. The purpose of this review was to uncover possible dissimilarities in chemical-related α_{2u}-globulin nephropathy in 3-month studies that might explain the variation in tumor responses subsequently observed in the 2-year studies.

Materials and Methods

Source of Data

The NCI/NTP database was searched for studies with a diagnosis of hyaline droplet accumulation in kidney tubules of rats in prechronic studies. Additional chemicals were identified that were associated with α_{2u}-globulin accumulation in studies reported by others (Swenberg and Lehman-McKeeman, 1999; Melnick and Kohn, 1999; Lock and Hard, 2004) and also studied for cancer by the NCI/NTP. Because the purpose of this evaluation was to examine associations among key events in the proposed α_{2u}-globulin nephropathy mode of tumor development in male rats, the datasets selected for evaluation could include only those for which sufficient information was available to conduct such analysis. Although many chemicals in the NTP database have been shown to induce α_{2u}-globulin in renal tubules (Table 2), only those satisfying all of the following criteria were included in this review: a) Diagnosis of hyaline droplet accumulation in kidney tubules of male rats in 3-month studies; b) Additional pathologies consistent with α_{2u}-globulin nephropathy; c) Development of renal tumors in 2-year studies exclusive to male rats; and d) At least one common exposure/dose level utilized in both the 3-month and 2-year studies. Studies of only 3 contemporary chemicals met these criteria and are included in this review (Table 3). Results from these studies are compared with data available for the prototypical α_{2u}-globulin inducing chemical *d*-limonene.

Selected Studies and Quantitative Endpoints

This retrospective evaluation included only male F344/N rats. Decalin, PGMBE and SS IIC-exposed animals (whole-body inhalation studies, 6 hours per day, 5 days per week, for 14 or 104/105 weeks) were 6 weeks of age at the beginning of the studies. Note for the studies on decalin and PGMBE the top exposure concentration used in the 3-month study was the same as that used in the 2-year study and retrospective histopathology evaluations on rats exposed for 3 months used materials from the original prechronic studies. For SS IIC, the 2-year study top concentration was half that used in the 3-month study, and the retrospective prechronic histopathology analyses included this higher dose. However, measures of cell proliferation and renal α_{2u}-globulin content were performed on a satellite group examined 3-months into the 2-year study at the exposure concentrations employed in that study (0, 138, 550, and 1,100 mg/m^3). Doses or exposure concentrations are shown in Table 3, and more information on experimental design and materials and methods for these studies can be found elsewhere (NTP, 1990;2004;2004a;2005;Dill et al., 2003; Doi et al., 2004a,2004b).

For the studies with *d*-limonene, animals were dosed starting at 7 weeks of age with the chemical in corn oil by gavage once per day, 5 times per week for 3 months or 2 years. Because limonene was the index α_{2u}-globulin nephropathy chemical, characterization of the nephropathy was carried out in a subsequent 14 exposure, 21-day study performed following completion of the 2-year cancer study. Animals in this subsequent study were 18 weeks of age at study start. Animals in all prechronic studies were 19-21 weeks old at the time that measurements were performed. Analyses of renal concentrations of α_{2u}-globulin were performed in kidney homogenates using a competitive indirect ELISA technique (Borghoff et al., 1992). Estimates of renal tubular cell proliferation rates were conducted using Proliferating Cell Nuclear Antigen (PCNA) labeling for decalin and PGMBE (Dill et al., 2003; Doi et al., 2004b), and bromodeoxyuridine (BrdU) labeling for SS IIC (Doi et al., 2004a). This endpoint was not evaluated in the limonene study.

Histopathology Reviews

The diagnoses of pathologies in NTP studies are uniformly consistent in the identification of lesions within a particular study. However, some diagnostic variation can exist among different

studies, particularly in the scoring of severity of lesions because this is in part dependent on the total spectrum of lesions present in a given study. Thus, renal lesions of interest from prechronic studies were retrospectively evaluated for incidence and/or severity by a single pathologist (GH) to allow for direct comparisons across studies. Severity grades ranged from 1-4, with 1 = minimal, 2 = mild, 3 = moderate and 4 = marked.

Entire longitudinal, H&E-stained kidney sections from male rats from prechronic studies (n = 10 unless otherwise specified) were evaluated for the presence of intracytoplasmic hyaline droplets in the proximal tubules, regenerative cortical tubules, and granular casts in the corticomedullary junction. Hyaline droplets were graded as 0 = no protein droplets observed, 1 = occasional small protein droplets observed, 2 = frequent small protein droplets observed, 3 = frequent small to moderately sized protein droplets observed, 4 = frequent large protein droplets observed. Regenerative tubules defined as clusters of basophilic tubules in which epithelial cells had increased nuclear density and occasional mitotic figures were counted throughout the cortex. All granular casts of cellular debris within tubules in the outer medulla/corticomedullary junction were counted. Counts of clusters of tubular regeneration and granular casts are not standard practice, but were performed in the current study as a more quantitative, and hence, less subjective approach to estimate the severity of these changes.

Histopathology slides from 2-year studies were also reevaluated by a single pathologist (JS) for incidence and severity of chronic nephropathy, linear papilla mineralization, and atypical renal tubular hyperplasia, as well as incidence of tubular adenomas, or adenomas and carcinomas combined. A fraction (30/50) of randomly selected single longitudinal kidney sections from 2-year studies was included in this review. Evaluation of only 60% of the animals in each dose group was done because using more than 30 animals per group achieved relatively smaller increases in power to detect endpoint associations. Because slides from only a portion of the animals from the studies were evaluated, the lesion counts do not match those in the original technical reports of these studies.

Nephropathy consisted of a spectrum of lesions including multifocal tubular regeneration, tubular protein casts, thickening of the tubular and glomerular basement membrane, interstitial fibrosis, and chronic inflammatory cell infiltration. The criteria used for grading the severity of nephropathy have been previously published (Rao et al., 2001). Linear mineralization designated regularly arranged basophilic mineralized linear deposits distributed mainly in the lower half of the renal papillae (minimal = involvement of papillae up to 25%; mild = 26-50%; moderate = 51-75%; marked = 76% and above).

Atypical renal tubular hyperplasia consisted of discrete, focal to multifocal proliferative lesions of renal tubules characterized by expansion of a single or several profiles of the same tubule by a multilayered epithelium, containing cells somewhat larger than normal with prominent nucleoli. The cytoplasm of affected cells is faintly basophilic with a homogenous-tinctorial sheen. When present, the tubular basement membrane is often variable in thickness. Although capillaries may be noted in association with hyperplastic lesions, invasion into the lesion is not present, a point of differentiation along with loss of tubular integrity when comparing hyperplasia to small adenomas. Grading of hyperplasia is generally subjective based primarily on the size of the lesion, number of affected tubular profiles present and complexity of the hyperplastic epithelium.

Renal tubular adenomas were larger discrete lesions that ranged from greater than 5 tubule diameters to 1 mm or more in size. They consisted of a solid mass of large, closely packed tubular epithelial cells. Cells were moderately pleomorphic. Renal tubular carcinomas were differentiated from adenomas in that they were usually larger, less discrete, had a prominent vascular supply, and more anaplasia and cellular atypia.

Statistical Methods

Tests of significance included pairwise comparisons of each exposed group with controls. Continuity corrected Poly-3 tests (Bailer and Portier, 1988) were used in the analysis of lesion incidence and reported P values are one sided. Average severity values were analyzed for significance with the Mann-Whitney U-test (Hollander and Wolfe, 1973).

Results

Quantitative Endpoints

Increases in renal concentrations of α_{2u}-globulin, relative to controls, are depicted in Figure 1 and numerical data presented in Table 4. Data for *d*-limonene were expressed as ng/μg total protein and are from the study where the chemical was dosed 14 times over 21 days, while data for decalin, PGMBE, and SS IIC were expressed as ng/μg soluble protein, and were collected at the end of 3-month exposures. α_{2u}-Globulin concentrations were significantly increased following exposure to all doses of *d*-limonene (~100-180%), and generally, to the highest concentrations of decalin (~ 220-320%), PGMBE (~ 90-120%), and SS IIC (~ 80-150%). Relative increases in α_{2u}-globulin concentrations were higher for decalin than for PGMBE or SS IIC. Data are presented in Figure 1 as percent increase over controls because the assays were done at different times using different reagents.

Labeling indices of tubular epithelial cells were calculated by dividing the number of labeled nuclei by the number of total nuclei. Increases in labeling indices relative to controls are shown in Table 5 for decalin and PGMBE. Cell labeling in these studies was measured with PCNA immunostaining at 3 time points during the course of the 13-week studies. For the SS IIC study, cell labeling was accomplished using a 24-hour infusion of BrdU with osmotic minipumps. Thus, the data are not directly comparable with the measures taken from the decalin and PGMBE studies. An analysis of tubular cell labeling index was not performed for the *d*-limonene study. Labeling indices were significantly increased following exposure to all concentrations of decalin, and the top 2 concentrations of PGMBE at all time points and at the 550 and 1,100 mg/m^3 concentrations of SS IIC (~ 60-135%). The highest relative increases in cell labeling with PCNA (approximately 2-fold) were seen with the highest concentrations of PGMBE at 13 weeks and decalin at 6 weeks.

Retrospective Histopathology Evaluation of Prechronic Studies

Hyaline droplet accumulation in renal tubular cells was diagnosed in essentially all animals in all groups of control and treated male rats, however, the severity of the lesion was higher in some dose groups (Figure 2). Hyaline droplet severity was generally dose related for decalin, PGMBE and SS IIC. Regenerating clusters of injured renal tubules were also observed in most animals in the prechronic studies. Incidences and cluster counts are shown in Figure 3. The data for decalin and SS IIC showed quite similar, dose-related increases in cluster counts at concentrations ultimately used in the 2-year studies, whereas the changes in counts for PGMBE were minimal across the exposure concentrations. Figure 4 shows the numbers of granular casts present in the cortical tubules. d-Limonene, decalin and SSIIC showed generally dose or concentration-related increases, while this lesion was rare in the PGMBE study.

Retrospective Histopathology Evaluation of 2-Year Studies

The severity of linear mineralization in the kidneys of male rats at the end of the 2-year studies was increased in exposed animals in all the studies (Figure 5). Although the increases were modest, of the measures taken at 2 years, this endpoint showed the clearest dose response, particularly for decalin and SS IIC when also considering the incidence of the lesion in the groups. The incidence of this lesion in the top exposure group of the PGMBE study was

surprisingly low when compared to the middle exposure concentration. This lesion was noted in only one control rat across the 4 studies.

Chronic nephropathy is a common age-related lesion in male F344 rats, and exposure to 3 chemicals resulted in a moderate increase in the severity of this lesion, with decalin again showing the strongest evidence for a chemically related response when compared to the controls (Figure 6). For SS IIC, the severity of chronic nephropathy was not increased, and as outlined next, this chemical had the fewest renal tumors of the 4 at the conclusion of the 2-year studies. The assessment of tubular hyperplasia is shown in Figure 7. These lesions, which are considered preneoplastic, occurred at a low frequency in the 4 separate 2-year studies and with no clear dose response in either incidence or severity.

Tumor Response

The tumors seen in the animals evaluated in these studies are shown in Table 6. The majority of the tumors were tubular cell adenomas. The incidences in the decalin and PGMBE studies exhibited a dose response, while the top exposure group in the SS IIC study had only 1 tumor in the 30 animals examined (standard sections). Renal tubular cell tumors typically occur in NTP studies with an average incidence of about 0.4%, thus a single tumor in an exposed group may or may not have resulted from the chemical under study. In fact, the tumor responses in the PGMBE and SS IIC studies (based on the entire set of 50 animals) were determined insufficient to be considered related with certainty to the chemical exposure.

Based on a qualitative assessment, none of the nonneoplastic endpoints considered part of the α_{2u}-globulin nephropathy syndrome evaluated in these prechronic or 2-year studies was consistently predictive of the ultimate tumor outcome in the 2-year studies. However, the pattern of increased severity of chronic nephropathy at the end of the 2-year studies was somewhat consistent with the renal tumor response.

Discussion

Renal tubular cell tumors occurring in chemical carcinogenesis studies are often considered not predictive of human hazard when they appear coincident with nonneoplastic changes suggestive of α_{2u}-globulin related nephropathy. In fact, the unique susceptibility of the male rat for tumors arising through this postulated sequence of events has been used to showcase how mode of action can be used in the determination of the potential human relevance of tumors arising in animal cancer studies (Cohen et al., 2003).

The key events in α_{2u}-globulin nephropathy associated tumorigenesis and associated histopathological features have been described in great detail (Swenberg and Lehman-McKeeman, 1999; Meek et al., 2003). Yet it has been our experience in reviewing the results of the studies shown in Table 3 that the syndrome actually presents in a wide variety of ways. The studies of t-butyl alcohol are particularly illustrative in that the involvement of α_{2u}-globulin in the renal pathology caused by this chemical in the male rat has been interpreted differently among pathologists even within the same institution reviewing the same set of slides (Lindamood et al., 1992; NTP, 1995).

The findings from these current studies provide a more systematic basis on which to evaluate quantitative relationships between certain key events in the α_{2u}-globulin hypothesis and renal tubular cell tumors in rats. The results support our earlier impressions of the lack of a strong association of any particular manifestation of the syndrome with the ultimate tumor outcome. It must be pointed out that our evaluations of these events are largely limited to 2 times during the studies, 3 months and 2 years, and only during the prechronic studies is α_{2u}-globulin present in measurable amounts.

For this reason, the retrospective pathology review focused on renal changes that reflected persistent damage from earlier insult, i.e., linear mineralization of the papillae, and chronic nephropathy. To the extent that these measures represent cumulative damage, it could be argued that they might better represent the contribution of the totality of the α_{2u}-globulin related pathology occurring during the 2-year study and that may not be apparent at the 3-month time point.

The initial indication that a chemical may induce α_{2u}-globulin-related nephropathy is often an observation in 14- or 90-day studies of an increase in hyaline droplets in the tubular epithelium. Droplets are typically seen in control male rats, and increases have resulted from exposures to a variety of hydrocarbons and some pharmaceuticals (Gopinath et al., 1987). These droplets are thought to be largely comprised of α_{2u}-globulin, and immuno-assays have been developed to provide a quantitative measure of renal accumulation. Using these methods we found that the largest increases in accumulation of α_{2u}-globulin in relation to controls occurred in the decalin study, which had the most robust renal tumor response. However, we also noted a significant increase in renal α_{2u}-globulin accumulation at the top exposure concentration in the SS IIC studies, which was associated with a much more modest tumor response.

We examined 3 measures of renal tubular damage in prechronic studies, counts of granular casts, clusters of regenerating tubules and cell labeling indices. The accumulation of casts was again most marked in the decalin studies, although quite similar counts were seen at the top exposure concentration with SS IIC as were noted at the tumorigenic 150 mg/kg dose in the limonene study and the 100 ppm exposure concentration in the decalin study. Cluster counts also showed close similarities in the SS IIC studies and counts with limonene and decalin at doses/concentrations that caused tumors in the 2-year studies. The limited direct comparisons that could be made with the cell labeling studies were mentioned earlier, but it is interesting to note the overall pattern of response for PGMBE where an elevation in cell labeling equivalent to that seen with decalin was associated with a moderate number of regenerating tubules, few granular casts, and a much more modest tumor response.

Of the measures evaluated at 2 years, the incidence and severity of linear papilla mineralization and the severity of chronic nephropathy appeared at least somewhat predictive of the tumor outcome. The severity of linear mineralization was greatest with limonene and decalin, but the incidence and severity were also markedly increased with SS IIC in the absence of a significant tumor response. Increases in the severity of chronic nephropathy were noted with limonene, decalin and PGMBE, but not with SS IIC. Chronic nephropathy is a common condition in aging male rats and the severity of this lesion is increased by a wide variety of chemical exposures, including many chemicals that do not induce or bind to α_{2u}-globulin. It is possible that α_{2u}-globulin associated nephropathy may simply contribute to a weak background tumorigenic stimulus provided by age-related chronic progressive nephropathy.

Alternatively, other as yet undetermined factors may better account for the renal tubular cell tumor responses in male rats in chemical carcinogenesis studies. These results suggest that while α_{2u}-globulin nephropathy may contribute to the renal tumor response, the critical component(s) of the nephropathy most closely associated with the development of tumors cannot clearly be identified. Thus reliance on evidence of α_{2u}-globulin associated nephropathy in determining the potential human hazard from chemicals that cause renal tubular cell tumors in rats may need to be reconsidered.

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Abbreviations

NTP, National Toxicology Program; IARC, International Agency for Research on Cancer; US EPA, United States Environmental Protection Agency; NCI, National Cancer Institute; PCNA, Proliferating cell nuclear antigen; BrdU, Bromodeoxyuridine; SS IIC, Stoddard solvent IIC; PGMBE, Propylene glycol monobutyl ether.

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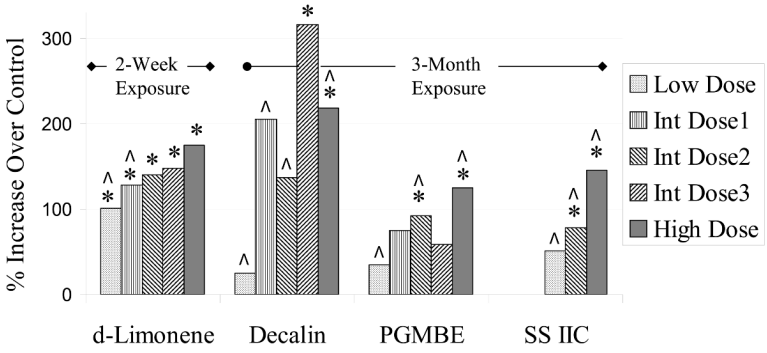

Figure 1.

Relative increases of mean renal α_{2u}-globulin concentrations, following 3-month exposures to decalin, propylene glycol mono-t-butyl ether (PGMBE), and Stoddard solvent IIC (SS IIC) to male rats; *d*-limonene-treated animals were exposed for 2 weeks. All rats (n = 10) were approximately 18 weeks old at the time of measurements. Analysis of renal concentrations of a2u-globulin was performed in kidney homogenates using a competitive indirect ELISA technique. Doses used in the 2-week limonene studies were 75, 150, 300, 600, and 1,200 mg/kg. Doses or exposures in prechronic studies that were common to those in 2-year studies are indicated by ^. Significantly greater than controls, $p \leq 0.05$.

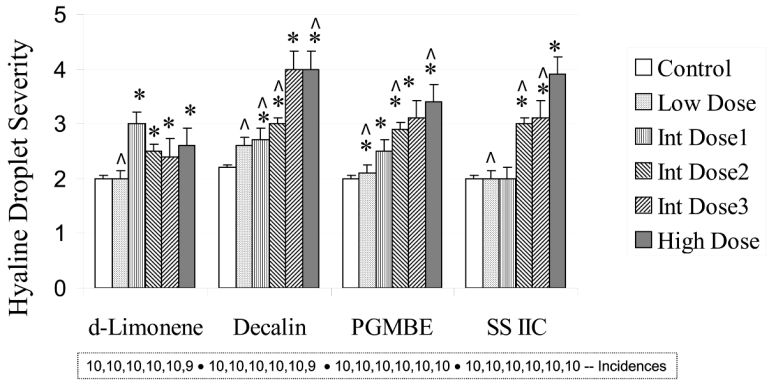

Figure 2.

Severity of hyaline droplet accumulation in renal proximal tubules of male rats (n = 10), following 3-month exposures to *d*-limonene, decalin, propylene glycol mono-t-butyl ether (PGMBE), and Stoddard solvent IIC (SS IIC). Data are presented as mean ± S.E.; *significantly greater than controls, $p \leq 0.05$. Incidences of the lesion are presented in the box insert. Doses or exposures in prechronic studies that were common to those in 2-year studies are indicated by ^.

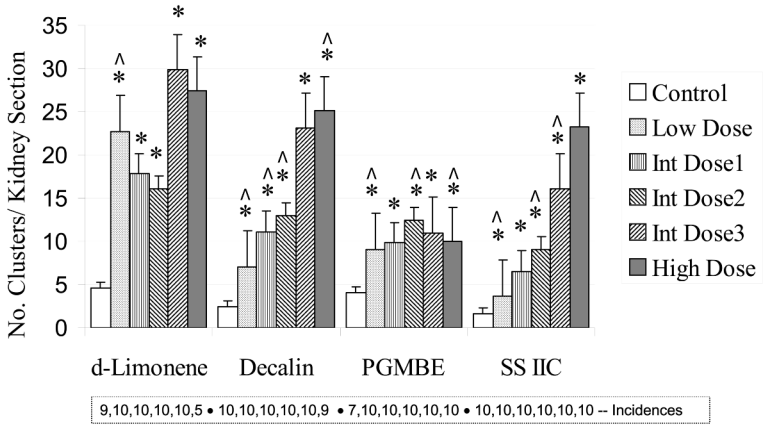

Figure 3.

Tubular regeneration cluster count in a single longitudinal section of the renal cortex of male rats (n = 10), following 3-month exposures to *d*-limonene, decalin, propylene glycol mono-t-butyl ether (PGMBE), and Stoddard solvent IIC (SS IIC). Data are presented as mean ± S.E.; *significantly greater than controls, $p \leq 0.05$. Numbers of animals with the lesion are presented in the box insert. Doses or exposures in prechronic studies that were common to those in 2-year studies are indicated by ^.

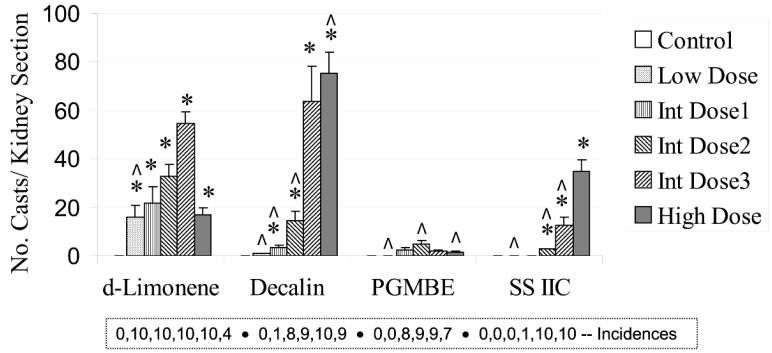

Figure 4.

Granular cast count in a single longitudinal section of the renal outer medulla of male rats (n = 10), following 3-month exposures to *d*-limonene, decalin, propylene glycol mono-t-butyl ether (PGMBE), and Stoddard solvent IIC (SS IIC). Data are presented as mean ± S.E.; *significantly greater than controls, $p \leq 0.05$. Numbers of animals with the lesion are presented in the box insert. Doses or exposures in prechronic studies that were common to those in 2-year studies are indicated by ^.

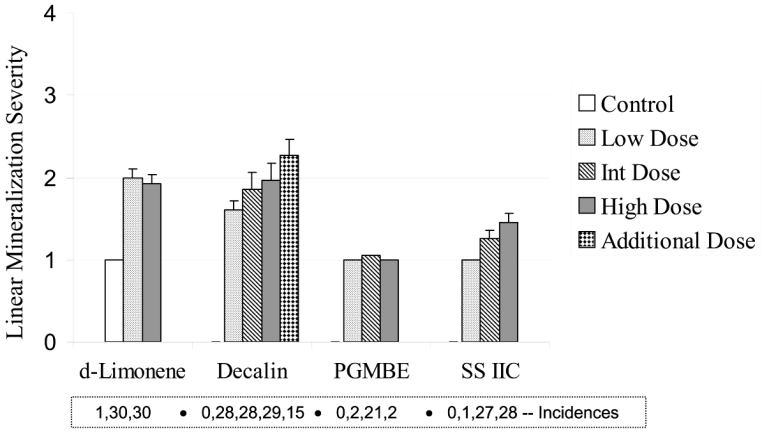

Figure 5.

Severity of linear mineralization in the renal papilla of male rats (n = 30), following 2-year exposures to *d*-limonene, decalin, propylene glycol mono-t-butyl ether (PGMBE), and Stoddard solvent IIC (SS IIC). Additional dose in decalin treatment consisted of 15 male rats. Data are presented as mean ± S.E.; statistical analyses were not performed because of the low or zero incidences in controls. Numbers of animals with the lesion are presented in the box insert.

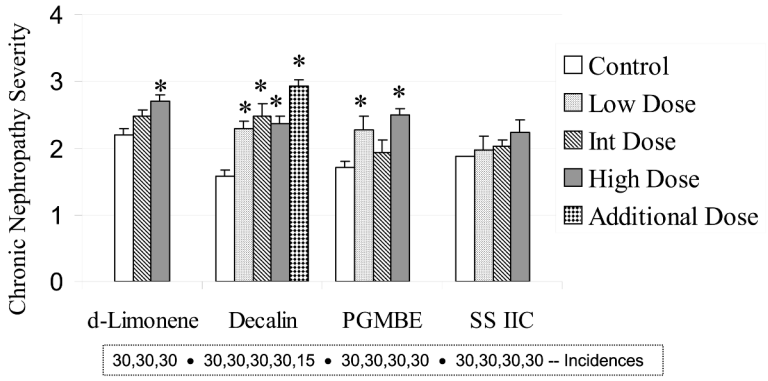

Figure 6.

Severity of chronic nephropathy in male rats (n = 30), following 2-year exposures to *d*-limonene, decalin, propylene glycol mono-t-butyl ether (PGMBE), and Stoddard solvent IIC (SS IIC). Additional dose in decalin treatment consisted of 15 male rats. Data are presented as mean ± S.E.; *significantly greater than controls, $p \leq 0.05$. Numbers of animals with the lesion are presented in the box insert.

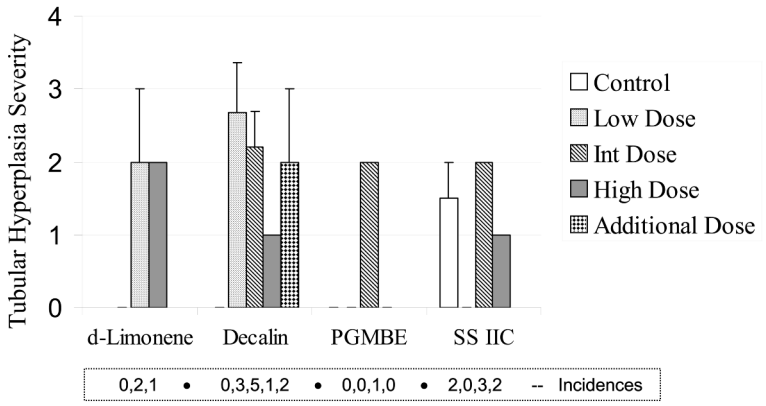

Figure 7.

Severity of renal tubular hyperplasia in male rats (n = 30), following 2-year exposures to *d*-limonene, decalin, propylene glycol mono-t-butyl ether (PGMBE), and Stoddard solvent IIC (SS IIC). Additional dose in decalin treatment consisted of n = 15 male rats. Data are presented as mean ± S.E.; *significantly greater than controls, $p \leq 0.05$. Numbers of animals with the lesion are presented in the box insert.

Table 1

Criteria to establish the role of α_{2u}-globulin nephropathy in renal carcinogenesis.

US EPA (1991)[a]
Increased number and size of hyaline droplets in renal proximal tubule cells of treated male rats
Protein in the hyaline droplets is α_{2u}-globulin Additional pathological sequence of lesions
IARC (1999)[b]
Essential Evidence
Tumors occur only in male rats
Acute exposure exacerbates hyaline droplet formation
α_{2u}-Globulin accumulates in hyaline droplets
Subchronic lesions include granular casts and linear papillary mineralization
Absence of hyaline droplets and other histopathological changes in female rats and mice
Negative for genotoxicity
Additional Supporting Evidence
Reversible binding of chemical to α_{2u}-globulin
Increased sustained cell proliferation in proximal tubule (P2 segment)
Dose-response relationship between hyaline droplet severity and renal tumor incidence

[a] EPA (1991).

[b] Swenberg and Lehman-McKeeman (1999).

Table 2

List of 2-year rat cancer studies with renal tumors from the NCI/NTP database.

Chemical Name	TR[b]	Year	Reason for Excluding Study from Analysis
Pentachloroethane	232	1983	Lack of hyaline droplet diagnosis in 90-day studies
Isophorone	291	1986	Lack of hyaline droplet diagnosis in 90-day studies
Tetrachloroethylene	311	1986	Lack of hyaline droplet diagnosis in 90-day studies
1,4-Dichlorobenzene	319	1987	Kidney sections were transverse and thus, not comparable to other studies
Dimethyl methylphosphonate	323	1987	Transitional cell neoplasms in the kidney
Hexachloroethane	361	1989	No dose was carried over from 90-day to 2-year studies
Hydroquinone	366	1989	Lack of hyaline droplet diagnosis in 90-day studies
α-Methylbenzyl alcohol	369	1990	Lack of hyaline droplet diagnosis in 90-day studies
ADBAQ	383	1996	Renal tumors occurred in male and female rats
t-Butyl alcohol	436	1995	Low survival in all groups in the study
p-Nitrobenzoic acid	442	1994	Renal tumors not considered chemical related
Pyridine	470	1997	No renal tumors in any dose group carried over from 90-day to 2-year studies
Emodin	493	2001	Lack of additional α_{2u}-related pathologies
Anthraquinone	494	1999	Renal tumors occurred in male and female rats
p-Nitrotoluene	498	2002	Lack of additional α_{2u}-related pathologies

Studies are with a diagnosis of hyaline droplet accumulation in kidney tubules of males in 90-day or 2-year studies, or that have been associated with α2u-globulin accumulation in other reports[a], but not considered further in this analysis.

[a] Swenberg and Lehman-McKeeman, 1999; Melnick and Kohn, 1999; Lock and Hard, 2004.

[b] Technical Report Number.

Table 3

List of NTP studies included in the analysis, and evidence of carcinogenicity based on kidney responses in male F344/N rats.

Chemical Name	TR[a]	Year	Route of Administration	Doses/Concentrations		Evidence of Carcinogenicity[b]
				90-Day	2-Year	
d-limonene	347	1990	Gavage	0, 150, 300, 600, 1200, 2400 mg/kg	0, 75, 150 mg/kg	Clear evidence
decalin	513	2005	Inhalation	0, 25, 50, 100, 200, 400 ppm	0, 25, 50, 100, 400[c] ppm	Clear evidence
propylene glycol mono-*t*-butyl ether	515	2004	Inhalation	0, 75, 150, 300, 600, 1200 ppm	0, 75, 300, 1200 ppm	Equivocal evidence
Stoddard solvent IIC	529	2004	Inhalation	0, 138, 275, 550, 1100, 2200 mg/m^3	0, 138, 550, 1100 mg/m^3	Equivocal evidence[d]

[a] Technical Report Number.

[b] Five categories of evidence of carcinogenic activity are used to summarize the strength of the evidence observed in bioassays conducted by the NTP: “Clear Evidence” and “Some Evidence” are used for chemical-related positive studies, “Equivocal Evidence” is used for uncertain findings that may be interpreted as chemical-related; “No Evidence” refers to no observable effects, and “Inadequate Study” is used for experiments that cannot be evaluated because of major flaws.

[c] The 400 ppm exposure concentration in the decalin study was an additional group of 20 animals included because of uncertainty over the possibility that this exposure concentration would be too toxic for animals to survive throughout a two-year study. This was not the case, and data from 15 randomly selected animals from this exposure group are included in Figures 5, 6, and 7.

[d] There was some evidence of carcinogenic activity of Stoddard solvent IIC in male rats based on increased incidences of adrenal medulla neoplasms; the slight increases of renal tubule adenoma may have been related to Stoddard solvent IIC exposure. The Equivocal Evidence call reflects only the kidney response.

Table 4

Concentrations of α_{2u}-globulin in the kidney of male F344/N rats exposed to *d*-limonene, decalin, propylene glycol mono-*t*-butyl ether and Stoddard solvent IIC.

Chemical	**d-limonene**	**decalin**	**propylene glycol mono-*t*-butyl ether**	**Stoddard solvent IIC**
Exposure duration	21 Days	3 Months	3 Months	3 Months
Number of animals	5 ng/ μg Total Protein	5 ng/ μg Soluble Protein	5 ng/μg Soluble Protein	10 ng/μg Soluble Protein
Control	204 ± 14	60 ± 17	113 ± 37	198 ± 46
Low Dose	409 ± 19**	75 ± 18	153 ± 22	300 ± 34
Intermediate Dose 1	465 ± 22**	184 ± 98	198 ± 44	
Intermediate Dose 2	489 ± 14**	142 ± 38	218 ± 25*	353 ± 30*
Intermediate Dose 3	505 ± 27**	251 ± 69**	179 ± 25	488 ± 72**
High Dose	561 ± 18**	192 ± 93*	254 ± 45*	

Notes: Data are presented as mean ± S.E.

* Significantly different ($p \leq 0.05$) from controls by Dunn's or Shirley's test.

** Significantly different ($p \leq 0.05$) from controls by the Kruskal-Wallis multiple comparison test (d-limonene) or Dunn's or Shirley's test (decalin, propylene glycol mono-*t*-butyl ether and Stoddard solvent IIC).

Table 5

Cell labeling indices determined with PCNA immunostaining during the 13-week prechronic inhalation studies with decalin and propylene glycol mono-t-butyl ether.

Propylene glycol mono-t-butyl ether	**Chamber Control**	**75 ppm**	**150 ppm**	**300 ppm**	**600 ppm**	**1,200 ppm**
Labeling index (%) n = 5						
Week 2	3.6 ± 0.1	3.6 ± 0.2	3.9 ± 0.2	3.4 ± 0.1	4.4 ± 0.2*	5.5 ± 0.4**
Week 6	3.6 ± 0.1	3.2 ± 0.1	3.4 ± 0.2	3.9 ± 0.1	4.3 ± 0.0*	4.9 ± 0.3*
Week 13	3.2 ± 0.2	3.8 ± 0.2	4.3 ± 0.2**	4.1 ± 0.2*	5.0 ± 0.4**	6.6 ± 0.4**
Decalin	**Chamber Control**	**25 ppm**	**50 ppm**	**100 ppm**	**200 ppm**	**400 ppm**
Labeling index (%) n = 5						
Week 2	3.3 ± 0.2	4.2 ± 0.1*	3.9 ± 0.3	4.3 ± 0.2**	4.2 ± 0.3*	4.9 ± 0.3**
Week 6	3.2 ± 0.1	4.2 ± 0.1**	4.8 ± 0.1**	4.6 ± 0.3**	5.0 ± 0.2**	5.9 ± 0.3**
Week 13	3.1 ± 0.2	4.5 ± 0.1**	3.7 ± 0.3*	4.1 ± 0.2*	4.5 ± 0.2**	4.5 ± 0.2**

* Significantly different ($p < 0.05$) from the chamber control group.

** $p < 0.01$, data are presented as mean + S.E.

Table 6

Neoplastic lesions in the kidney of male rats (n = 30) following 2-year exposures to *d*-limonene, decalin, propylene glycol mono-t-butyl ether (PGMBE), and Stoddard solvent IIC (SS IIC). Additional dose in decalin treatment consisted of 15 male rats. Neoplastic incidences herein reported represent a review of 30 out of 50 animals from the original NTP studies, and thus, do not reflect NTP 2-year bioassay results for these chemicals.

	d-limonene		Decalin		PGMBE		SS IIC	
	Adenomas	**Adenomas or Carcinomas**	**Adenomas**	**Adenomas or Carcinomas**	**Adenomas**	**Adenomas or Carcinomas**	**Adenomas**	**Adenomas or Carcinomas**
Control	0	0	0	0	0	0	0	0
Low Dose	5 (17%) *	7 (23%) **	1 (3%)	1 (3%)	1 (3%)	1 (3%)	0	0
Intermediate Dose			2 (7%)	2 (7%)	2 (7%)	2 (7%)	0	0
High Dose	3 (10%)	4 (13%)	6 (20%) *	6 (20%) *	3 (10%)	3 (10%)	1 (3%)	1 (3%)
Additional Dose[f]			3 (20%) *	4 (27%) **				

[f] n = 15.

* Significantly greater than controls, $p \leq 0.05$.

** $p \leq 0.01$.

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