

Consideration of Rat Chronic Progressive Nephropathy in Regulatory Evaluations for Carcinogenicity

Gordon C. Hard,^{*1} Marcy I. Banton,[†] Robert S. Bretzlaff,[‡] Wolfgang Dekant,[§] Jefferson R. Fowles,[¶] Anthony K. Mallett,^{||} Douglas B. McGregor,^{|||} Kathleen M. Roberts,^{|||} Robert L. Sielken Jr.,[‡] Ciriaco Valdez-Flores,[‡] and Samuel M. Cohen[#]

**Private Consultant, 203 Paku Drive, Tairua 3508, New Zealand; †LyondellBasell, Corporate HSE/Product Safety, Houston, Texas 77010; ‡Sielken & Associates Consulting, Inc., Bryan, Texas 77802; §Institut fuer Toxicologie, Universität Würzburg, Würzburg 97080, Germany; ¶Tox-Logic, LLC, Petaluma, California 94954; ||Penman Consulting Ltd., Prospect House, Dorchester DT1 3WE, UK; |||Toxicity Evaluation Consultants, Aberdour KY3 0TU, Scotland, UK; |||B&C Consortia Management LLC, Washington, DC 20037; and #Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198-3135*

¹To whom correspondence should be addressed. E-mail: gordonhard@msn.com.

Received October 3, 2012; accepted October 9, 2012

Chronic progressive nephropathy (CPN) is a spontaneous renal disease of rats which can be a serious confounder in toxicology studies. It is a progressive disease with known physiological factors that modify disease progression, such as high dietary protein. The weight of evidence supports an absence of a renal counterpart in humans. There is extensive evidence that advanced CPN, particularly end-stage kidney, is a risk factor for development of a background incidence of atypical tubule hyperplasia and renal tubule tumors (RTT). The likely cause underlying this association with tubule neoplasia is the long-term increased tubule cell proliferation that occurs throughout CPN progression. As a variety of chemicals are able to exacerbate CPN, there is a potential for those exacerbating the severity up to and including end-stage kidney to cause a marginal increase in RTT and their precursor lesions. Extensive statistical analysis of National Toxicology Program studies shows a strong correlation between high-grade CPN, especially end-stage CPN, and renal tumor development. CPN as a mode of action (MOA) for rat RTT has received attention from regulatory authorities only recently. In the absence of toxic effects elsewhere, this does not constitute a carcinogenic effect of the chemical but can be addressed through a proposed MOA approach for regulatory purposes to reach a decision that RTT, developing as a result of CPN exacerbation in rats, have no relevance for human risk assessment. Guidelines are proposed for evaluation of exacerbation of CPN and RTT as a valid MOA for a given chemical.

Key Words: atypical tubule hyperplasia; chronic progressive nephropathy; end-stage renal disease; renal tubule tumor; mode of action; human relevance.

Chronic progressive nephropathy (CPN) is a distinctive lesion in rats occurring in high incidence culminating in end-stage kidney disease. In some instances, it is associated with a small increased incidence of renal tubule tumors (RTT),

Disclaimer: This manuscript expresses the opinions of the authors and does not reflect the views of their institutions or agencies.

occasionally at statistically significant incidences. We present a brief description of the disease and investigations regarding its relationship to RTT in the rat along with an evaluation of the significance of these tumors in human risk assessment for kidney cancer. A brief summary of regulatory evaluations of chemicals involving exacerbation of CPN is also presented.

CHRONIC PROGRESSIVE NEPHROPATHY

CPN is an important spontaneous renal disease in the commonly used strains of laboratory rat, which has the potential to confound interpretation of kidney findings from experimental pathology and toxicology studies; it is also a risk factor for RTT development. The disease is most severe in Sprague Dawley and F344 strains, and there is a distinct male predisposition to CPN in respect of onset, incidence, and severity progression. Particularly in males, the disease can progress to end-stage kidney, which is a prelude to death from chronic renal failure (Gray, 1977; Hard and Khan, 2004).

The earliest stage of CPN in hematoxylin and eosin-stained sections, identifiable at 2–4 months of age in male rats of susceptible strains, is a focal cortical tubule basophilia with crowded nuclei indicative of simple hyperplasia and conspicuously thickened basement membrane. This is shortly followed by the development of an eosinophilic hyaline cast in the descending limb of Henle of the same nephron, usually seen in the inner stripe of outer medulla (Hard and Khan, 2004). The lesion progresses from a single affected nephron to increasingly larger foci involving neighboring nephrons. With further increase in size, the foci begin to coalesce to form a network of diseased tissue through the cortex with increasing hyaline cast formation in the medulla and extension of casts into the cortex. As severity progresses, glomerulosclerosis, tubule atrophy, matrix expansion, and perivascular aggregates of mononuclear

cells become prominent (Abrass, 2000; Gray 1977; Hard and Khan, 2004). End-stage CPN kidneys are enlarged with prominent hyaline casts and characterized by the involvement of virtually all of the kidney parenchyma accompanied by secondary changes in other organs (parathyroid hyperplasia, mineralization in certain tissues, and fibrous osteodystrophy). The main clinical chemistry alterations occurring in CPN include proteinuria, initially albuminuria, reflecting hypoalbuminemia, and hypercholesterolemia. Serum urea nitrogen and creatinine levels are not significantly elevated until advanced stages of disease (Coleman *et al.*, 1977; Salatka *et al.*, 1971).

The primary factors modifying CPN severity are dietary and hormonal. Restriction of caloric intake reduces disease incidence and severity (Bertani *et al.*, 1989; Keenan *et al.*, 2000; Masoro and Yu, 1989), and increasing dietary protein enhances incidence and severity (Rao *et al.*, 1993). CPN is also strongly influenced by androgens, with castration or estrogen administration reducing CPN in male rats but having no effect in female rats (Baylis, 1994). Pituitary hormones may also be involved because hypophysectomy is inhibitory (Everitt *et al.*, 1983), but there is insufficient information available on possible correlations of individual circulating hormone levels with CPN. The glomerulus has long been thought to be the target in this disease process, with hyperfiltration and functional overload leading ultimately to glomerulosclerosis (Brenner, 1985). However, hemodynamic changes in glomeruli do not appear to be involved (Baylis, 1994), and the etiology of CPN and underlying basis for disease progression remain unknown. A possible contributing factor may be the high protein level of the male rat kidney (Lehman-McKeeman and Caudill, 1992; Neuhaus, 1986).

Critical to understanding the pathophysiological basis underlying CPN, and its relentless progression, will be examination using a variety of sophisticated techniques of the earliest lesions observed in young rats. One aspect that might be resolved by electron microscopy concerns the question of whether the precedent changes occur in the tubule or glomerulus. This would probably require large-area sectioning methods of plastic-embedded tissue blocks. However, laser capture microdissection techniques could be used for isolating the earliest lesions in archived, paraffin-embedded kidney tissue for enabling molecular studies involving gene expression and proteomics.

LINKAGE WITH RENAL TUMOR DEVELOPMENT

A recent survey evaluating the renal histopathology of more than 2000 control male or female F344 rats from 23 National Toxicology Program (NTP) 2-year studies (Hard *et al.*, 2012) has demonstrated that advanced CPN is a major factor underlying spontaneous development of atypical tubule hyperplasia (ATH), an obligate precursor of renal tubule adenoma, and RTT. Severity of CPN was graded on a 0–8 scale, where grade 8 was end-stage kidney, and the incidences of foci of ATH and RTT distributed according to CPN severity grade. This expanded

scale compared with the 0–4 scale usually used aids in distinguishing severity of the early and late changes and is useful in assessment of differences in severity between groups. Almost all of the foci of ATH and adenomas occurred in rats with grades 7 and 8 CPN, and statistical analysis showed a close correlation between these proliferative lesions and advanced CPN, particularly end-stage kidney. Across studies, the incidences of ATH and adenomas in end-stage CPN of male and female rats combined were 13 and 11%, respectively. This contrasts with an average of 2.0 and 1.2%, respectively, of all rats in the survey including all grades of CPN.

There is also evidence that exacerbation of CPN by chemicals occurs quite frequently and can be recognized with the 0–8 grading scheme in subchronic studies (Travlos *et al.*, 2011), as well as in 2-year studies. Chemicals exacerbating CPN to advanced grades, including end-stage kidney, can be associated with a marginal increase in ATH and RTT development. This association is subtle and tends to be a high-dose effect. Chemicals exacerbating CPN to end-stage kidney with a resultant increase in ATH and RTT include hydroquinone (Hard *et al.*, 1997), ethyl benzene (Hard, 2002), quercetin (Hard *et al.*, 2007), and coumarin and primidone (Hard, unpublished data). Although there was no statistical difference in ATH and RTT between control groups and those exposed to tetrahydrofuran, these lesions in both control and treated male rats were associated with advanced CPN (Bruner *et al.*, 2010).

This link between CPN and development of neoplastic and preneoplastic tubule lesions can be explained by the fact that through its progression, CPN is both a degenerative and regenerative disease with a high rate of tubule cell turnover as demonstrated by mitotic activity and DNA synthesis labeling (Hard and Khan, 2004; Hard and Seely, 2006; Konishi and Ward, 1989; Short *et al.*, 1989). CPN therefore appears to represent a spontaneous version of sustained tubule cell injury coupled with sustained tubule cell regeneration.

A similar nephropathy has been described in mice (Wolf and Hard, 1996), but factors influencing its occurrence and severity have not been examined adequately, and any relationship with renal tumor formation has not been investigated.

COMPARISON OF RODENT CPN TO HUMAN RENAL DISEASE

A comparison between CPN in rats and human nephropathies was described (Hard *et al.*, 2009) with the conclusion that CPN is a distinctive entity in rats with no counterpart in human disease. This assessment includes differences in the clinical course of the disease, biological behavior, relationship to diet, immunological factors, and clinical parameters. Most distinctively, the presence of the thickened basement membrane around the basophilic, regenerative tubules is an early presentation in CPN. In humans, a thickened basement membrane is seen only in relationship to atrophic tubules, not in relationship to active

degeneration or regeneration. A major difference between end-stage renal disease associated with CPN in rats compared with end-stage renal disease from any cause in humans is the effect on kidney size. A characteristic feature of CPN in the rat is increasing kidney size and weight with time, up to and including end-stage kidney. This is mainly due to the marked dilation of many tubules with hyaline casts. In contrast, end-stage renal disease in humans is associated with marked contraction in the size of the kidney, frequently resulting in kidneys that can be one-tenth or less the size of a normal kidney. Furthermore, hyaline casts are uncommonly such a prominent feature of human end-stage kidney as they are in advanced rat CPN.

EVALUATION OF CPN AS A MODE OF ACTION FOR RENAL TUMORIGENESIS

When CPN reaches the most advanced stages of severity including end-stage in rats, there is an increased risk for the development of ATH and renal tubule adenomas, with occasional carcinomas occurring. As with other animal tumor findings, the ultimate question is whether there is any potential for increased risk to humans. To assess possible human relevance of the CPN-associated renal tubule proliferative lesions, including tumors, utilization of a framework for analysis of mode of action (MOA) and human relevance can be applied. This framework was developed by the International Life Sciences Institute and further elaborated by the International Program on Chemical Safety. It has proved useful in providing a rigorous and transparent method for analysis of the MOA for animal toxicity, including tumors, and for evaluating the potential relevance to human risk (Boobis *et al.*, 2006, 2008; Meek *et al.*, 2003; Seed *et al.*, 2005; Sonich-Mullin *et al.*, 2001). This framework provides guidance for initially evaluating whether sufficient data are available to support an MOA for a given chemical in the animal model system and then a qualitative and quantitative analysis for concordance of the key events in the animal versus the human. Each toxic endpoint in each organ is analyzed separately, even if ultimately the multiple toxicities including tumor formation have the same MOA. The framework involves an explicit statement of key events beginning with exposure to the chemical and culminating in the toxic endpoint. Once a hypothesized MOA has been analyzed, it is also essential that analysis of possible alternative MOAs is considered. The basis for organizing the data available for a given compound for a given toxic endpoint and criteria for accepting a plausible MOA are essential modifications of those presented by Bradford Hill for guidance on evaluating the possible etiologic basis of epidemiologic findings (Sonich-Mullin *et al.*, 2001).

Once an evaluation of the MOA has been completed and information is concluded to be sufficient for establishment of an MOA, a qualitative and quantitative evaluation of the concordance of each key event between the animal model and humans

is presented. Utilization of a concordance table has proved useful for such purposes. A major utility of this framework proved to be the focus on key events involved in an MOA in distinction from a detailed mechanism of action, which ultimately includes detailed molecular changes.

For chemicals with a postulated MOA for renal tumorigenesis involving exacerbation of CPN, it is important to remember that CPN is a spontaneous entity characterized by a high rate of cell turnover. A postulated sequence of key events for this pathway is as follows:

- (1) exposure to chemical (usually at high concentrations);
- (2) metabolic activation (if necessary);
- (3) exacerbated CPN, including increased number of rats with end-stage renal disease;
- (4) increased tubule cell proliferation because more kidney is damaged due to CPN exacerbation;
- (5) hyperplasia;
- (6) adenoma (infrequently carcinoma).

For such an analysis, it is not necessary to know the detailed etiology or underlying mechanism for CPN. It is adequate for an MOA analysis that the key event be identified as increased CPN with its associated increase in tubular cell proliferation. Criteria for documenting a chemical exposure, enhanced CPN, and its relationship to increased renal tumor formation are presented below.

To evaluate the relevance to humans, the relationship of each of these key events is evaluated qualitatively. As indicated above, CPN does not occur in humans; so, on a qualitative basis, this MOA would not be relevant to humans. This is similar to a variety of other MOAs including the well-documented MOA involving binding of chemicals to α_{2u} -globulin leading to an increased incidence of RTT in male rats (Meek *et al.*, 2003). This MOA in male rats was delineated as involving exposure to the chemical, metabolic activation (when necessary), and binding to the protein α_{2u} -globulin. The noncovalent binding of a chemical makes the protein more resistant to degradation by lysosomal enzymes, leading to hyaline droplet accumulation in cortical tubules with consequent tubule cell loss, cell regeneration, hyperplasia, and ultimately tumor formation. Because humans do not have a protein that behaves in a manner comparable to α_{2u} -globulin, again on a qualitative basis, there is not concordance for this particular key event between rats and humans and therefore this MOA is qualitatively not relevant to humans.

For some chemicals, there is the possibility of more than one MOA being operative in producing a given effect, including neoplasia. An example of this situation is provided by *t*-butyl alcohol. A recent re-evaluation of the renal histopathology of male rats in 13-week and 2-year drinking water exposure studies of this chemical found the evidence to be strongly compatible with the dual involvement of α_{2u} -globulin nephropathy and exacerbation of CPN in the increased incidence of RTT ultimately observed in the longer term study (Hard *et al.*, 2011).

STATISTICAL ANALYSIS OF RELATIONSHIP OF CPN TO RAT RENAL TUMORS

A recent publication (Melnick *et al.*, 2012) raised questions regarding the validity of exacerbation of CPN as an MOA for rat renal tumorigenesis. A major part of their argument is that there is no statistical relationship between the degree of severity of CPN and increased incidences of renal tumors in the NTP studies. The statistical analysis in that publication, however, has several serious deficiencies. Some of these are the result of including chemicals that may enhance renal tumorigenesis by MOAs other than, or in addition to CPN, a possibility that is actually recognized by Melnick *et al.* (2012), yet paradoxically stated as a criticism of the proposed MOA. There has never been a statement that all rat proliferative renal lesions are the consequence of exacerbated CPN. Attention was drawn by Melnick *et al.* (2012) to five chemicals in particular that they considered to provide evidence for other MOAs: anthraquinone, benzofuran, *t*-butyl alcohol, ethyl benzene, and tetrafluoroethylene. The evaluation of a single chemical is very largely restricted to that chemical; therefore, properties of such small populations or even individuals need to be addressed, although such procedures may run counter to methods of statistical analysis. Anthraquinone was reviewed by IARC (2012), where significant numbers of publications are cited that provide adequate evidence for genotoxic/mutagenic activity *in vivo*. Benzofuran exposure of rats was associated with 4/50 renal cell adenocarcinomas in high-dose females compared with 0/50 in controls (IARC, 1995). This is a rare tumor in female rats, and there is no proposed hypothesis that it is a consequence of exacerbated CPN; therefore, some other MOA must be considered. The likely dual modes of action of *t*-butyl alcohol have been mentioned above. The Melnick *et al.* (2012) assessment of ethyl benzene suffers from the absence of any examination of the individual rats with end-stage CPN and proliferative renal lesions. This has been done and published (Hard, 2002) and shows that irrespective of dose group the proportion of end-stage kidneys with atypical hyperplasia, adenoma, or carcinoma was never lower than 70%. Of the five compounds highlighted by Melnick *et al.* (2012), only tetrafluoroethylene appears to present any evidence contradicting the CPN-renal tumor association hypothesis, but it should also be recognized that even this case may be only a consequence of depth of study.

Melnick *et al.* (2012) focused on these five chemicals partly because they thought that the results in male and female rats contradicted the relationship between CPN and renal tumors. Melnick *et al.* (2012), however, failed to acknowledge that the correlation between CPN and RTT was positive for all these five chemicals in both male and female rats (correlations for anthraquinone in female rats, ethyl benzene in male and female rats, and tetrafluoroethylene in male rats are statistically significant at the 5% significance level). The correlation coefficients between CPN and RTT incidence and corresponding *p*-values for these five chemicals and for male and female rats

are as follows (note, the numbers in parentheses are correlation, *p*-value):

- (1) anthraquinone: male (0.7564, 0.1218), female (0.9611, 0.0194);
- (2) benzofuran: male (n/a, n/a because no RTTs), female (0.2402, 0.4228);
- (3) *t*-butyl alcohol: male (0.2796, 0.3602); female (n/a, n/a because no RTTs);
- (4) ethyl benzene: male (0.9618, 0.0191), female (0.9416, 0.0292);
- (5) tetrafluoroethylene: male (0.9022, 0.0489), female (0.7622, 0.1189).

In addition to combining results from chemicals with possible disparate MOAs, Melnick *et al.* (2012) have inappropriately combined multiple subsets of studies such as those involving multiple-step sections with those that have routine single sections; studies utilizing different criteria for a semiquantitative assessment of the degree of CPN; and different grading systems, especially not utilizing the specific relationship between end-stage kidney disease due to CPN and renal tumor formation. They also compared CPN and RTT in studies involving utilization of a high-protein diet (NIH-07) compared with a low-protein diet (NTP-2000), implying that, because protein levels influence CPN, there should also be an influence on renal tumor formation. Their statistical approach is incorrect as is their interpretation.

In one of their comparisons, Melnick *et al.* (2012) selectively compared rats with high CPN scores in the high- versus low-protein studies with tumor formation. They claim that there was no statistical relationship; however, this is an inappropriate selection of animals, not only because different grading systems were utilized, but because not all animals were utilized for the evaluation. If all animals in a study regardless of CPN grade are utilized for comparison, statistical significance is shown between CPN grade and tumor formation.

The appropriate statistical method for evaluating whether two variables are linearly related is to use a simple statistical analysis of correlation. By doing so and using all of the male rat studies in Table 1 of Melnick *et al.* (2012), a positive correlation (+ 0.271) is observed between RTT and mean CPN severity score (Fig. 1) that is highly statistically significant ($p = 0.00003$) using a standard Pearson product-moment correlation coefficient (Snedecor and Cochran, 1980). Similar correlations are observed for studies involving only NIH-07 diet (Supplementary fig. 1) or NTP-2000 diet (Supplementary fig. 2), and for evaluation of unexposed male rats (controls) only (Supplementary figs. 3–5).

The greater difficulty is not so much a statistical comparison of these scores, but that the relationship between CPN and renal tumor formation is in the most advanced stages of CPN severity, particularly end-stage kidney rather than those showing severe but not end-stage changes (grades 7 and 8 on an 8-grade scale compared with animals with lower grades, even 5 and 6, which on a 4-grade scale could readily be classified as grade 3 or 4).

Even when there is a statistically significant relationship between CPN score and renal tumor formation, the more relevant question is the number of animals with end-stage kidney disease and tumor development, an analysis that is not evident from a statistical comparison utilizing CPN mean score and tumor incidence.

REGULATORY STATUS

Regulatory/authoritative bodies have generally not specifically addressed the role of CPN in the MOA for renal tumors. Twenty-one substances reported by the U.S. NTP as showing clear or some evidence of renal carcinogenicity (RTT) in male and/or female rats with associated evidence of severe CPN were identified for review by Melnick *et al.* (2012) and for evaluation by the International Agency for Research on Cancer (IARC), U.S. Environmental Protection Agency Integration Risk Information System (EPA IRIS), and The European Union (EU) Classification, Labeling and Packaging (CLP) Regulation (2008) (Annex VI to Regulation [EC] No. 1272/2008). Also reviewed was the European Chemical Agency Classification and Labeling Inventory (<http://echa.europa.eu/web/guest/information-on-chemicals/cl-inventory>), which contains classification and labeling information on EU notified and registered substances received from manufacturers and importers.

Of the 21 renal tumorigenic substances that exacerbated CPN, only 3 (2-amino-4-nitrophenol, α -methylbenzyl alcohol, and quercetin) produced renal tumors exclusively (Supplementary table 1). These substances have limited evaluations by regulatory authorities, and by IARC only for two substances that were classified in group 3 (not classifiable as to carcinogenicity to humans). The IARC monographs on these substances noted findings of nephropathy or CPN, but there were no comments

or conclusions regarding mechanistic aspects for renal tumor development (IARC, 1993, 1999b).

The majority of the reviewed CPN/RTT substances are multisite carcinogens; hence, authority considerations include other tumor types in their conclusions. Six substances have not received significant authority cancer classifications (Supplementary table 2). These substances had limited evaluations by authorities, and only two were considered by IARC (both classified in group 3). The IARC monograph (IARC, 2000b) for coumarin noted increased severity of CPN in male rats in the chronic study but made no comments or conclusions regarding mechanistic aspects of renal tumor induction and development. Nitrofurantoin was noted to have produced increases in uncommon renal tubular neoplasms in rats and renal tubular tumors in female mice. With these renal tumor types, IARC concluded there was limited evidence for carcinogenicity in experimental animals and classified nitrofurantoin in group 3, a significant increase in the incidences of RTT not leading to a more severe classification because they were regarded as benign tumors (IARC, 1990).

Most of the CPN multisite carcinogens reviewed received significant regulatory authority cancer classifications (Supplementary table 3). In addition, all but tetrahydrofuran have IARC evaluations (IARC, 1999a, 2000a,b, 2012) and all but hydroquinone are group 2B carcinogens (possibly carcinogenic to humans). Hydroquinone is classified by IARC in group 3 and as a category 2 carcinogen (suspected human carcinogen) under the EU CLP regulation. There were no specific comments or conclusions given on renal tumor induction mechanisms. Four substances (anthraquinone, benzophenone, methyleugenol, and methyl isobutyl ketone) have recent reviews by IARC and, where available, include tumor mechanistic discussions and conclusions. For the renal tumor mechanistic discussions concerning these substances, the focus was on α_{2u} -globulin

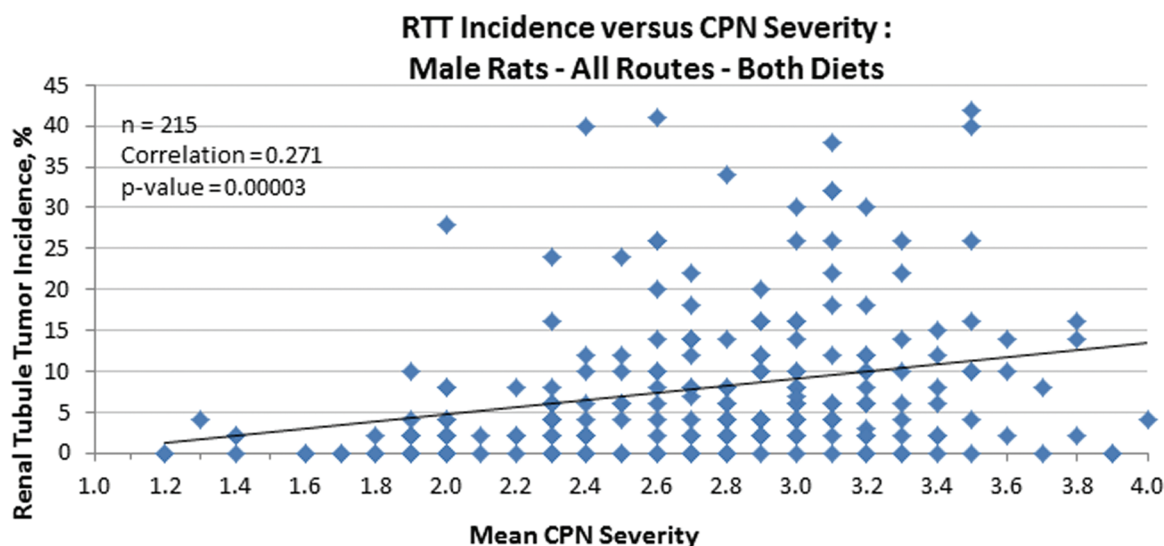


FIG. 1. All male rats: Incidence of RTT determined using the original and multiple section evaluations of the kidneys versus mean CPN severity score in Table 1 of Melnick *et al.* (2012).

nephropathy evaluations. CPN, if mentioned, was not concluded to be a mechanism for renal tumor development. Four substances, chloroprene, ethyl benzene, methyl isobutyl ketone, and tetrahydrofuran, were reviewed by EPA IRIS but only chloroprene and tetrahydrofuran were reviewed after completion of the cancer studies and using EPA's current cancer assessment guidelines (U.S. EPA, 2005). Chloroprene is genotoxic and a multisite carcinogen in rats and mice; hence, rat RTT was likely a minimal consideration in EPA's cancer classification of "likely to be carcinogenic in humans."

Of the reviewed substances, only two reviews by authorities (tetrahydrofuran and ethylbenzene) have explicitly considered CPN as a renal tumor mechanism. The U.S. EPA in its IRIS assessment and the EU Risk Assessment Committee (RAC) recently reviewed tetrahydrofuran carcinogenicity and indicated consideration of CPN for the renal tumors. U.S. EPA (2012) concluded as follows: "There are hypothesized mode(s) of action only for rat kidney tumors and mouse liver tumors. For rat kidney tumors, the hypothesized modes of action include mutagenicity, peroxisome proliferation, α_{2u} -globulin nephropathy, and cytotoxicity not associated with α_{2u} -globulin accumulation.... However, the available evidence is insufficient to support the conclusion that either rat kidney or mouse liver tumors are mediated solely by one of these hypothesized modes of action." Following a proposal by France in 2009 to classify tetrahydrofuran as a category 2 carcinogen (EU CLP), RAC met in 2010 and issued its opinion supporting France's proposal. RAC reportedly considered CPN in their deliberations.

In 2008, Germany prepared an Annex XV transitional report on ethyl benzene as a summary of their evaluation under Regulation EEC No. 793/93 that included an extensive discussion of CPN (BAA, 2008). Their report listed a number of reasons in support of exacerbated CPN as an MOA for the rat kidney tumors and indicated a few reasons counter to this hypothesis. They concluded as follows: "The cons arguments did not represent significant contradictions against the hypothesis. They are judged as some remaining uncertainties on the postulated MOA. Taking all arguments mentioned above, the rapporteur agrees that ethyl benzene carcinogenic action on the rat kidney can be attributed to its mediation

of the CPN. It is suggested that ethyl benzene exacerbates the development of CPN in F344 rats and thereby enhances a more rapid progression to renal tubular tumors." "There is sufficient evidence that kidney tumors in male and female rats are associated with the high strain-specific incidence of CPN that is unknown for humans."

CPN has generally not been considered in regulator/authority classifications. Reasons for this include as follows: (1) many of these chemicals were reviewed more than 10 years ago before MOA, and CPN specifically, were included in as detailed a fashion as now (IARC, 2006) and (2) the presence of only renal tubule adenomas, regardless of mechanism, reduces authority concern as they have been regarded as benign tumors.

There are only two examples (tetrahydrofuran and ethyl benzene), where CPN was considered as an MOA for RTT, but authorities reached different conclusions on human cancer classifications. This could be the result of different RTT and CPN profiles between the substances or the lack of a generally accepted MOA akin to α_{2u} -globulin nephropathy.

PROPOSED CRITERIA FOR EVALUATING A RELATIONSHIP BETWEEN CPN AND RAT RTT DEVELOPMENT

Although the overall conclusions about the relationship between CPN score and incidence of RTTs by Melnick *et al.* appear to be incorrect, they appropriately raise the issue of what criteria are needed for an evaluation of such a relationship. Merely demonstrating an increase in CPN score is not adequate, as described above, because it is the rats that have the most advanced stages of CPN, particularly end-stage kidney that are at risk for developing these tumors and not merely groups of rats with an increased average CPN score. This is likely the explanation for the infrequent correlation between CPN and RTT in female rats. Even though CPN scores can be increased in female as well as in male rats, few female rats actually develop end-stage kidney disease and thus the population at risk would be small and few would be expected to develop RTT. In the Hard *et al.* (2012) survey of control F344 rats, CPN explained the well-known male to female gender difference in RTT incidences in control rats.

Based on extensive analyses of various studies and active discussions, we propose the criteria listed in Table 1 as essential for coming to the conclusion that exacerbation of CPN is a relevant MOA for the increased incidence of RTT in a 2-year bioassay in rats. As described above, there is a possibility that more than one MOA can be involved in the induction of RTT in a given bioassay with a specific chemical, so that the framework requirement for evaluating alternative MOAs is essential.

In summary, CPN is a common renal disorder in rats that frequently progresses to end-stage renal disease. In animals with end-stage renal disease, there is an increased risk for the development of RTT. Because CPN does not have a human analog, if

TABLE 1
Criteria for Considering Exacerbation of CPN as an MOA for RTT in Rats

- | |
|---|
| 1. Lack of genotoxic activity based on overall evaluation of <i>in vitro</i> and <i>in vivo</i> data |
| 2. Tumor incidence is low, usually < 10% |
| 3. Tumors are found toward the end of 2-year studies |
| 4. Lesions are usually ATH or adenomas (carcinomas can occasionally occur) |
| 5. Chemical exacerbates CPN to most advanced stages, including end-stage kidney |
| 6. ATH and tumors occur in rats with advanced CPN and in CPN-affected tissue |
| 7. Absence of cytotoxicity in CPN-affected tubules, in rats with lower grades of CPN, and in subchronic studies |

the MOA for an increased incidence of renal tumors in a 2-year rat bioassay is determined to be due to exacerbation of CPN, it can be concluded that the rat renal tumors are not relevant to human cancer risk.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

FUNDING

Tetrahydrofuran Task Force; the European Chemistry Industry Council (CEFIC) 1,4-Butanedial and Derivatives Sector Group; the CEFIC Fund Oxygenates Association; the CEFIC Styrene Steering Committee; Lyondell Chemical Company; the Toxicology Forum.

ACKNOWLEDGMENTS

This manuscript summarizes presentations and discussions from a session at the Toxicology Forum in Aspen, CO, on July 10, 2012. The authors wish to disclose that G.C.H., S.M.C., K.M.R., D.B.M., J.R.F., and A.K.M. have at times acted as independent consultants to industrial organizations on issues involving rat CPN. M.I.B.'s employer is a chemical company with interests in some chemicals that exacerbate CPN, and R.S.B., R.L.S., and C.V-F. were contracted for the statistical analysis by LyondellBasell.

REFERENCES

- Abrass, C. K. (2000). The nature of chronic progressive nephropathy in aging rats. *Adv. Ren. Replace. Ther.* **7**, 4–10.
- BAA. (2008). *Risk Assessment of Ethylbenzene. Draft*. Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, Anmeldestelle Chemikaliengesetz, Dortmund, Germany.
- Baylis, C. (1994). Age-dependent glomerular damage in the rat. Dissociation between glomerular injury and both glomerular hypertension and hypertrophy. Male gender as a primary risk factor. *J. Clin. Invest.* **94**, 1823–1829.
- Bertani, T., Zoja, C., Abbate, M., Rossini, M., and Remuzzi, G. (1989). Age-related nephropathy and proteinuria in rats with intact kidneys exposed to diets with different protein content. *Lab. Invest.* **60**, 196–204.
- Boobis, A. R., Cohen, S. M., Dellarco, V., McGregor, D., Meek, M. E., Vickers, C., Willcocks, D., and Farland, W. (2006). IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit. Rev. Toxicol.* **36**, 781–792.
- Boobis, A. R., Doe, J. E., Heinrich-Hirsch, B., Meek, M. E., Munn, S., Ruchirawat, M., Schlatter, J., Seed, J., and Vickers, C. (2008). IPCS framework for analyzing the relevance of a noncancer mode of action for humans. *Crit. Rev. Toxicol.* **38**, 87–96.
- Brenner, B. M. (1985). Nephron adaptation to renal injury or ablation. *Am. J. Physiol.* **249**(3 Pt 2), F324–F337.
- Bruner, R. H., Greaves, P., Hard, G. C., Regan, K. S., Ward, J. M., and David, R. M. (2010). Histopathologic changes in the kidneys of male F344 rats from a 2-year inhalation carcinogenicity study of tetrahydrofuran: A pathology working group review and re-evaluation. *Regul. Toxicol. Pharmacol.* **58**, 100–105.
- Coleman, G. L., Barthold, W., Osbaldiston, G. W., Foster, S. J., and Jonas, A. M. (1977). Pathological changes during aging in barrier-reared Fischer 344 male rats. *J. Gerontol.* **32**, 258–278.
- European Union Classification, Labeling and Packaging (CLP) Regulation. (2008). Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Official Journal December 31, 2008.
- Everitt, A. V., Wyndham, J. R., and Barnard, D. L. (1983). The anti-aging action of hypophysectomy in hypothalamic obese rats: Effects on collagen aging, age-associated proteinuria development and renal histopathology. *Mech. Ageing Dev.* **22**, 233–251.
- Gray, J. E. (1977). Chronic progressive nephrosis in the albino rat. *Crit. Rev. Toxicol.* **5**, 115–144.
- Hard, G. C. (2002). Significance of the renal effects of ethyl benzene in rodents for assessing human carcinogenic risk. *Toxicol. Sci.* **69**, 30–41.
- Hard, G. C., Betz, L. J., and Seely, J. C. (2012). Association of advanced chronic progressive nephropathy (CPN) with renal tubule tumors and precursor hyperplasia in control F344 rats from two-year carcinogenicity studies. *Toxicol. Pathol.* **40**, 473–481.
- Hard, G. C., Bruner, R. H., Cohen, S. M., Pletcher, J. M., and Regan, K. S. (2011). Renal histopathology in toxicity and carcinogenicity studies with tert-butyl alcohol administered in drinking water to F344 rats: A pathology working group review and re-evaluation. *Regul. Toxicol. Pharmacol.* **59**, 430–436.
- Hard, G. C., Johnson, K. J., and Cohen, S. M. (2009). A comparison of rat chronic progressive nephropathy with human renal disease—implications for human risk assessment. *Crit. Rev. Toxicol.* **39**, 332–346.
- Hard, G. C., and Khan, K. N. (2004). A contemporary overview of chronic progressive nephropathy in the laboratory rat, and its significance for human risk assessment. *Toxicol. Pathol.* **32**, 171–180.
- Hard, G. C., and Seely, J. C. (2006). Histological investigation of diagnostically challenging tubule profiles in advanced chronic progressive nephropathy (CPN) in the Fischer 344 rat. *Toxicol. Pathol.* **34**, 941–948.
- Hard, G. C., Seely, J. C., Betz, L. J., and Hayashi, S. M. (2007). Re-evaluation of the kidney tumors and renal histopathology occurring in a 2-year rat carcinogenicity bioassay of quercetin. *Food Chem. Toxicol.* **45**, 600–608.
- Hard, G. C., Whysner, J., English, J. C., Zang, E., and Williams, G. M. (1997). Relationship of hydroquinone-associated rat renal tumors with spontaneous chronic progressive nephropathy. *Toxicol. Pathol.* **25**, 132–143.
- IARC. (1990). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 50, Pharmaceutical Drugs*. World Health Organization, International Agency for Research on Cancer, Lyon, France.
- IARC. (1993). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 57, Occupational Exposures of Hairdressers and Barbers and Personal Use of Hair Colourants; Some Hair Dyes, Cosmetic Colourants, Industrial Dyestuffs and Aromatic Amines*. World Health Organization, International Agency for Research on Cancer, Lyon, France.
- IARC. (1995). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 63, Dry Cleaning, Some Chlorinated Solvents and Other Industrial Chemicals*. World Health Organization, International Agency for Research on Cancer, Lyon France.
- IARC. (1999a). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 71, Re-evaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide*. World Health Organization, International Agency for Research on Cancer, Lyon, France.
- IARC. (1999b). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 73, Some Chemicals that Cause Tumours of the Kidney or Urinary Bladder in Rodents and Some Other Substances*. World Health Organization, International Agency for Research on Cancer, Lyon, France.

- IARC. (2000a). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 76, Some Antiviral and Antineoplastic Drugs, and Other Pharmaceutical Agents*. World Health Organization, International Agency for Research on Cancer, Lyon, France.
- IARC. (2000b). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 77, Some Industrial Chemicals*. World Health Organization, International Agency for Research on Cancer, Lyon, France.
- IARC. (2006). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Preamble*. World Health Organization, International Agency for Research on Cancer, Lyon, France.
- IARC. (2012). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 101, A Review of Human Carcinogens: Some Chemicals in Industrial and Consumer Products, Food Contaminants and Flavourings, and Water Chlorination By-products*. World Health Organization, International Agency for Research on Cancer, Lyon, France.
- Keenan, K. P., Coleman, J. B., McCoy, C. L., Hoe, C. M., Soper, K. A., and Laroque, P. (2000). Chronic nephropathy in ad libitum overfed Sprague Dawley rats and its early attenuation by increasing degrees of dietary (caloric) restriction to control growth. *Toxicol. Pathol.* **28**, 788–798.
- Konishi, N., and Ward, J. M. (1989). Increased levels of DNA synthesis in hyperplastic renal tubules of aging nephropathy in female F344/NCr rats. *Vet. Pathol.* **26**, 6–10.
- Lehman-McKeeman, L. D., and Caudill, D. (1992). Biochemical basis for mouse resistance to hyaline droplet nephropathy: Lack of relevance of the alpha 2u-globulin protein superfamily in this male rat-specific syndrome. *Toxicol. Appl. Pharmacol.* **112**, 214–221.
- Masoro, E. J., and Yu, B. P. (1989). Diet and nephropathy. *Lab. Invest.* **60**, 165–167.
- Meek, M. E., Bucher, J. R., Cohen, S. M., Dellarco, V., Hill, R. N., Lehman-McKeeman, L. D., Longfellow, D. G., Pastoor, T., Seed, J., and Patton, D. E. (2003). A framework for human relevance analysis of information on carcinogenic modes of action. *Crit. Rev. Toxicol.* **33**, 591–653.
- Melnick, R. L., Burns, K. M., Ward, J. M., and Huff, J. (2012). Chemically exacerbated chronic progressive nephropathy not associated with renal tubular tumor induction in rats: An evaluation based on 60 carcinogenicity studies by the National Toxicology Program. *Toxicol. Sci.* **128**, 346–356.
- Neuhaus, O. W. (1986). Renal reabsorption of low molecular weight proteins in adult male rats: Alpha 2u-globulin. *Proc. Soc. Exp. Biol. Med.* **182**, 531–539.
- Rao, G. N., Edmondson, J., and Elwell, M. R. (1993). Influence of dietary protein concentration on severity of nephropathy in Fischer-344 (F-344/N) rats. *Toxicol. Pathol.* **21**, 353–361.
- Salatka, K., Kresge, D., Harris, L. Jr, Edelstein, D., and Ove, P. (1971). Rat serum protein changes with age. *Exp. Gerontol.* **6**, 25–36.
- Seed, J., Carney, E. W., Corley, R. A., Crofton, K. M., DeSesso, J. M., Foster, P. M., Kavlock, R., Kimmel, G., Klaunig, J., Meek, M. E., et al. (2005). Overview: Using mode of action and life stage information to evaluate the human relevance of animal toxicity data. *Crit. Rev. Toxicol.* **35**, 664–672.
- Short, B. G., Burnett, V. L., and Swenberg, J. A. (1989). Elevated proliferation of proximal tubule cells and localization of accumulated alpha 2u-globulin in F344 rats during chronic exposure to unleaded gasoline or 2,2,4-trimethylpentane. *Toxicol. Appl. Pharmacol.* **101**, 414–431.
- Snedecor, G. W., and Cochran, W. G. (1980). *Statistical Methods*, 7th ed. The Iowa State University Press, Ames, IA.
- Sonich-Mullin, C., Fielder, R., Wiltse, J., Baetcke, K., Dempsey, J., Fenner-Crisp, P., Grant, D., Hartley, M., Knaap, A., Kroese, D., et al. (2001). IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. *Regul. Toxicol. Pharmacol.* **34**, 146–152.
- Travlos, G. S., Hard, G. C., Betz, L. J., and Kissling, G. E. (2011). Chronic progressive nephropathy in male F344 rats in 90-day toxicity studies: Its occurrence and association with renal tubule tumors in subsequent 2-year bioassays. *Toxicol. Pathol.* **39**, 381–389.
- U.S. EPA. (1991). *Integrated Risk Information Center. Ethylbenzene (CAS RN 100-41-4)*. U.S. Environmental Protection Agency, Washington, DC. Available at: <http://www.epa.gov/iris/subst/0051.htm>
- U.S. EPA. (2003). *Toxicological Review of Methyl Isobutyl Ketone (CAS No. 108-10-1)*. EPA/635/R-03/002. U.S. Environmental Protection Agency, Washington, DC.
- U.S. EPA. (2005). *Guidelines for Carcinogenic Risk Assessment. EPA/630/P-03/001F. Risk Assessment Forum*. U.S. Environmental Protection Agency, Washington, DC.
- U.S. EPA. (2010). *Toxicological Review of Chloroprene (CAS No. 126-99-8)*. EPA/635/R-09/010F. U.S. Environmental Protection Agency, Washington, DC.
- U.S. EPA. (2012). *Toxicological Review of Tetrahydrofuran (CAS No. 109-99-9)*. EPA/635/R-11/006F. U.S. Environmental Protection Agency, Washington, DC.
- Wolf, D. C., and Hard, G. C. (1996). Pathology of the kidneys. In *Pathobiology of the Aging Mouse* (U. Mohr, D. L. Dungworth, C. C. Capen, W. W. Carlton, J. P. Sundberg, and J. M. Ward, Eds.), Vol. 1, pp. 331–344. ILSI Press, Washington DC.