

Doubting Nongenotoxic Mechanisms of Renal Cancer: Comparing Apples and Oranges in the α 2u-Globulin Hypothesis

Doubting accepted mechanisms in biology is not wrong per se, especially as the contrary would be the death blow to any form of advancement in research; however, doubting without good reason will consume precious energy and resources that could be spent on more fruitful and certainly more pressing matters in biological research.

Contrary to good judgment, it appears that some individuals believe that any biological mechanism, however meticulously established and proven with hard data and despite being commonly accepted, should be doubted when the mechanism in question tends to reduce the conception of risk associated with chemicals. This doubt, however, does not appear to stem from hard factual data contradicting the mechanism, but rather from misinterpretation of available data and, in some instances, from the misquotation of the original investigators' conclusions.

One recent example for doubting established and meticulously proven biological mechanisms is the ongoing discussion of the validity of the α 2u-globulin-associated male rat-specific mechanism of renal cancer induction and its ramifications for human health risk assessment. A unique database (certainly in its size and detail when compared to other databases with similar importance for human health concerns) on practically all aspects of this mechanism has been established by numerous scientists from many different unaffiliated laboratories, repeated scientific discussion sessions, several thorough reviews (1–8), and a workshop specifically devised to query all aspects of the data that support the mechanism in question (9). Despite this, some scientists, in an unfathomable quest to differ, choose to ignore the obvious and continue to doubt the validity of this mechanism by throwing up new hypotheses (10) or interpretations of the present data (11). Such maneuvering is all the more reproachable as it is not constructive to risk assessment; it does not help to further the understanding of the currently accepted mechanisms; and foremost, the authors of such devil's-advocate papers have never presented new experimental data to support any of their new insights. In an effort to critically address some of the alternative hypotheses and interpretations presented by James Huff (11) in relation to the α 2u-globulin mechanism, John Ashby, in a letter in

Environmental Health Perspectives (12), asked Huff to relent from generalized statements and to "identify the chemicals to which he referred and present the relevant data for them in a focused paper."

Huff, in a reply in the same issue of *EHP*, rose to the occasion and tried to cement some of his statements and interpretations by specifically naming the chemicals (13). I feel that I must reply to the statements made by Huff in his letter. In order to facilitate the understanding of the following paragraphs, it is necessary for the reader to have insight into the current hypothesis of the etiology of α 2u-globulin nephropathy and associated male rat renal tumors as well as the supporting mechanistic data.

Briefly, male rats as well as female rats, but not mice, hamsters, guinea pigs, rabbits, dogs, nonhuman primates, or humans synthesize low molecular weight proteins (approximately 18,000–20,000 daltons) called α 2u-globulins (also known as A2u in earlier days). Synthesis of α 2u-globulin is reported for female and male rats in the salivary, lachrymal, preputial, meibian, and perianal glands (14,15). The hormonal regulation of α 2u-globulin synthesis in each of these tissues is unique and, most importantly, not sex specific in the lachrymal, salivary, and preputial glands (15,16). These α 2u-globulin isoforms are electrophoretically distinct from the major urinary protein, namely the hepatic form of α 2u-globulin with a molecular weight of 18,700 daltons, which is exclusively synthesized by male rats. The α 2u-globulin forms synthesized in small amounts by female rats are also excreted via the urine. However, these forms of α 2u-globulin show distinct differences to the male rat forms of α 2u-globulin, whether of non-hepatic or hepatic origin, suggesting that they are encoded by different genes (17).

The synthesis and high rate of excretion of the hepatic form of α 2u-globulin, the affinity of some chemicals and/or their metabolites for binding to α 2u-globulin, and the reduced enzymatic breakdown of the chemical- α 2u-globulin complex in lysosomes of the proximal tubule epithelial cells (18) lead to an accumulation of these protein-chemical complexes in the renal cortex of male rats. Subchronic and chronic administration of chemicals was demonstrated to be mandatory for the development of nephrotoxicity and the formation of renal epithelial cell tumors in male rats (2–5). The renal tumors were demonstrated to evolve from a constantly elevated rate of proximal tubule epithelial cell proliferation resulting from the chemical- α 2u-globulin complex-induced cell necrosis and subsequent cell regeneration (19–21). Due to

the species- and sex-specific synthesis of this low molecular weight protein, nephropathy and kidney tumors are found in exposed male rats but not in female rats, α 2u-globulin-deficient NBR male rats (21,22), either sex of mice, guinea pigs, rabbits, dogs, or nonhuman primates (1). Although humans and other species synthesize and excrete low molecular weight proteins via the urine, no interactions of these proteins with chemicals, shown to induce protein-mediated nephrotoxicity in the male rat, are observed (23,24). As a result of a joint effort of basic research conducted in both academic and industrial laboratories, the nongenotoxic mechanism underlying the genesis of these male rat-specific renal epithelial tumors is among the best characterized to date. Based on a 2-year review of this data set, the EPA ruled that the renal tumors detected in male rats following 2-year administration of nongenotoxic nephropathy-inducing compounds, such as unleaded gasoline or the orange juice component *d*-limonene, are of little relevance for human cancer risk assessment.

The EPA ruling on the α 2u-globulin-mechanism and the associated simplifications and better understanding of at least one small part of the otherwise highly enigmatic processes involved in the etiology of cancer has apparently driven James Huff to question the mechanism described above by providing examples of so-called exceptions and discrepancies to the presently accepted mechanism as well as to make unfounded and scientifically unsound statements. For example, to show the weakness of the α 2u-globulin mechanism hypothesis when it comes to extrapolating to the human situation, Huff states the following:

Importantly, two additional chemicals presumed to involve α 2u-kidney tumors (and thus are regarded by some as irrelevant to humans) have now been shown to be associated with kidney cancers in humans: gasoline [(25,26)] and trichloroethylene [(27,28)].

The studies on gasoline that Huff cited in the above context are epidemiological studies. This type of study looks at statistical associations between the increased occurrence of a disease and the exposure to gasoline (leaded gasoline). The weakness of such studies is that causal relationships and mechanisms can rarely be established as would be possible in highly specific animal studies, whereas their advantage lies in the fact that the endpoint of interest, tumors in humans, is inspected directly. However, the mere association of chemical exposure with increased occurrence of disease does not prove that there is a biological relationship. Most of the

epidemiological studies performed on gasoline and the occurrence of human renal cancer have an additional important weakness in that they have not quantitatively assessed the actual exposure to volatile hydrocarbons. In addition, the studies were conducted with reference to leaded gasoline, thus adding the confounding factor of lead exposure, which is known to induce renal cancer in animal studies. Furthermore, in many studies, exposure assessment did not go beyond ascertaining that an individual had been employed by a petroleum company or in a refinery. Two of the studies (25,29) indicate a small increase in relative risk for kidney cancer in subgroups exposed to gasoline. However, in the studies by McLaughlin and co-workers (29,30) the most consistent association with renal cancer was found to be cigarette smoking, while in a subsequent more detailed analysis (31), no overall association was observed between renal cell cancer and employment in a range of occupations with potential exposure to petroleum products.

Siemietycki et al. (25), a study specifically cited by Huff, looked at the risk for renal cancer in persons exposed to gasoline as well as jet fuel. No statistically significant risk for renal cancer was found for gasoline.

The only study reporting an elevated risk and an exposure-response relationship between kidney cancer and exposure to gasoline is the one published recently by Partanen et al. (26). These authors reviewed 672 cases of renal cell adenoma for a case-control study. Owing to poor participation, only 338 sets of cases and controls were ultimately included for analysis, thereby limiting the interpretability of the findings. Lifelong job histories were collected and translated into indicators of industry, occupation, and estimated occupational exposure. An elevated risk for kidney cancer was found to be associated with a history of white-collar occupations; the printing industry; the chemical industry; the manufacture of metal products; mail, telephone, and telegraph services; and iron and metal work. An elevated risk was associated with exposure to gasoline, and an exposure-response relationship was observed for increasing exposure to gasoline. However, a major confounding factor in this risk estimation was that the gasoline used in Finland, especially during the time relevant for exposure of the study group (1920-1968), contained copious amounts of tetraethyl-lead. The exposure to lead was associated with an increased risk for renal epithelial cell cancer, as was also confirmed by animal studies with lead acetate (32).

The association of renal cancer in humans with exposure to gasoline is most likely related to the tetraethyl-lead content of the gasoline and not to the gasoline (volatile

hydrocarbons) itself, as was also clearly pointed out by Partanen et al. (26). Therefore, the statement by Huff, that "gasoline had been shown to be associated with kidney cancer in humans" is a misrepresentation of the scientific facts.

With regard to trichloroethylene, Huff admits that it does not induce the α 2u-globulin-associated nephropathy; therefore, this example is irrelevant and a misrepresentation of facts. Huff tries to back his statement by adding that a chemically similar compound (perchloroethylene), which appears to be linked (no proven causality) with human renal cancer, was demonstrated to induce α 2u-globulin nephropathy and renal tumors in male rats. To substantiate this statement, he quotes Melnick (10), a paper that does not provide new data but selectively quotes Green et al. on perchloroethylene (33). Green et al. (33) clearly state that the tumors observed in the male rat kidney appear to be related to a combination of at least two mechanisms: genotoxicity from the β -lyase pathway and the α 2u-globulin mechanism. However, Green et al. (33) also found that even the genotoxic mechanism is also species and sex specific and is of little relevance to humans. Such synergy between a genotoxic agent (initiator) and a promoting influence (in this case the α 2u-globulin mechanism via enhanced cell proliferation) is well established not only in renal carcinogenesis. That is exactly why the EPA, in its ruling, restricted the relevance of the α 2u-globulin mechanism in conjunction with renal tumors occurring only in male rats to nongenotoxic chemicals. This emphasizes that we cannot define a compound as either being an α 2u-globulin nephropathy inducer or not; we have to understand the etiology of the tumors induced by a given chemical to detect whether only one or several mechanisms are involved in its genesis.

Thus, neither the epidemiological studies on leaded gasoline nor any of the polychlorinated aliphatics cited by Huff lend themselves to questioning the α 2u-globulin mechanism. The available human epidemiological studies and the animal experimental studies were misinterpreted and misrepresented by Huff. This distorts the factual content of the data presented and the ramifications of these facts for human health.

Some of the other chemicals Huff mentioned as being examples that contest the α 2u-globulin mechanism were gabapentin, lindane, decalin, trimethylpentane (unleaded gasoline), dimethyl methylphosphonate, *t*-butyl alcohol, and hexachlorobenzene. I will not discuss all of the chemicals in detail, although none of them are examples that would contest the α 2u-globulin mediated mechanism. Instead, I would like to

point out some important facts that Huff overlooked. The five examples selected for discussion are representative.

As pointed out by Huff, gabapentin induces an accumulation of α 2u-globulin in the proximal tubule epithelial cells of male rats and, after prolonged administration (up to 2, 7, and 14 days) and application of 2,000 mg/kg body weight (bw), even produces some cell necrosis and cell shedding (34). No increase in renal cancer was observed after 2 years of gabapentin administration (34). However, what Huff did not find necessary to point out is that, in all cases where chemicals induced α 2u-globulin nephropathy and renal tumors, increased amounts of proteinaceous intratubular casts, especially at the cortico-medullary junction, and, consequently, increased cell regeneration were observed (20,21,35,36). Indeed, chronic increased cell regeneration is the hallmark of chemically induced renal tumors via the α 2u-globulin mechanism (1). The question then arises whether the prolonged gabapentin administration induced increased numbers of proteinaceous intratubular casts, especially at the cortico-medullary junction, and consequently enhanced rates of cell regeneration. A closer look at the paper of Dominick et al. on gabapentin (34) clearly indicates that no dose response was observed in the number of casts or in the severity of renal tubular regeneration at 13 weeks of exposure (doses up to 3,000 mg/kg bw). This data clearly indicates that the severity of α 2u-globulin accumulation and subsequent cell necrosis and proliferative regeneration was not high enough to allow for formation of renal tumors within the 2 years of gabapentin exposure. Dominick et al. (34) also found foci of atypical hyperplasia in kidneys of male rats treated with 1,000 and 2,000 mg/kg bw at termination of the 2-year bioassay. Atypical tubules and atypical hyperplasia have been recognized as precursors of renal adenoma and carcinoma (19-21,32). The reported number of atypical foci was extremely low in the Dominick et al. study (34). In summary, the present database on gabapentin does not disprove the α 2u-globulin-associated mechanism of renal carcinogenesis, but rather demonstrates that chemicals that cause α 2u-globulin-mediated renal cancer must bind to α 2u-globulin with high affinity to produce chronic elevated cell necrosis and subsequent cell regeneration. Indeed, this view is also shared by Dominick et al. (34) who specifically stated in their paper that

this study suggests that proximal tubular epithelial injury and regeneration that occur in this [α 2u-globulin] nephropathy may need to reach a critical threshold to effectively promote renal neoplasia.

The second compound that Huff mentioned as being one of the compounds that does not induce renal tumors in male rats despite being a typical α 2u-globulin compound is lindane. It is indeed true that lindane and its metabolites can bind to α 2u-globulin and induce the associated nephropathy in male rats during acute exposures (37). Lindane (and/or its metabolites) has been shown in the 2-year carcinogenicity bioassay to increase the incidence of benign and malignant neoplasms in endocrine organs (pituitary, adrenal, and thyroid) of both sexes; however, a high incidence of ovarian carcinoma was also noted (38). Moreover, male rats treated with lindane at 32 and 64 mg/kg bw for 2 years presented with severe testicular atrophy. It is thus quite reasonable to assume that the testosterone level in these rats, as a consequence of testicular atrophy, was low. However, testosterone is extremely important for maintaining a high level of liver-derived α 2u-globulin, as will be outlined below. Indeed the male rat major urinary protein (the hepatic form of α 2u-globulin) is encoded by a family of 25–30 copies of highly homologous (less than 5% divergence in the nucleic acid sequence) genes within the haploid genome, each made up of 7 exons and clustered on chromosome 5 (39,40). The expression of this α 2u-globulin gene family is regulated by a complex interaction of testosterone, glucocorticoids, insulin, growth hormone, and thyroid hormone (4). The synthesis of hepatic α 2u-globulin is under androgenic control, as evidenced by changes in androgen sensitivity and androgenic induction of α 2u-globulin synthesis, which begins at puberty (approximately 40 days in rats), peaks at about 80 days, and then steadily declines in expression level only to cease at senescence (i.e., 750–800 days of age) (41). The maturational rise in α 2u-globulin synthesis and its decline during senescence are associated with corresponding changes in the expression of the androgen receptor gene in the liver and thus with changes in the steady-state level of the α 2u-globulin mRNA (42). Transcriptional activation of the α 2u-globulin gene coincides with its attachment to the nuclear matrix, while conversely, senescent-associated transcriptional inactivation is accompanied by matrix detachment of this gene (40).

Estrogenic steroids are very effective repressors of α 2u-globulin synthesis (43). Indeed, estrogen receptor complexes were shown to bind to the promoter region of the α 2u-globulin genes (44) and were thus able to repress the expression of these genes. It is thus not surprising that female rats, with their inherently high levels of estrogen, do not synthesize hepatic α 2u-globulin or

its corresponding mRNA, despite having the entire complement of hepatic α 2u-globulin genes (16,45,46). Ovariectomized female rats have a very low background level of α 2u-globulin mRNA (47), and ovariectomy in conjunction with androgen treatment of female rats results in an increase of α 2u-globulin mRNA as well as protein product (45,48).

Male rats after puberty, on the other hand, were shown to have high levels of estrogen sulfotransferase, an enzyme able to inactivate estrogen activity and thus maintain androgen sensitivity of the male rat liver during the peak phase of α 2u-globulin synthesis (49).

Based on the assumption that high levels of α 2u-globulin could not have been maintained in male rats treated with high doses of lindane in the 2-year bioassay, due to testicular atrophy and thus a lack of testosterone, it becomes obvious why renal tumors did not develop in lindane-treated male rats. Lindane, therefore, is not a compound that would question the α 2u-globulin-mediated mechanism of male rat renal tumors as Huff believes, but rather it supports the mechanism.

The question that remains is why lindane was reported to induce α 2u-globulin nephropathy. Indeed, α 2u-globulin nephropathy was observed in male rats treated with lindane 10 mg/kg bw day for 5 days (37). The levels of α 2u-globulin and the resistance to lysosomal breakdown of the α 2u-globulin–lindane complex was high enough that this nephropathy could be observed under acute exposure. In contrast, no hyaline droplets, nephropathy, or renal casts were observed in the rats dosed with 32 and 64 mg/kg bw for 2 years in the cancer bioassay (38).

With regard to risk assessment, lindane could have not been regulated under EPA's ruling on α 2u-globulin nephropathy-inducing compounds, based on the fact that no renal tumors were detected in male rats and that tumors in other organs in both male and female rats were induced in the 2-year bioassay. Lindane, however, may well be an example of the problems associated with the maximum-tolerated-dose (MTD) approach in the 2-year cancer bioassays. It is indeed conceivable that male rats would have developed renal tumors in the 2-year bioassay if the chronic dose had been at a concentration that would not have induced testicular atrophy and thus not have impeded testosterone production. In conclusion, lindane does not provide a good example to substantiate theories, but rather it exemplifies how a lack of understanding of mechanisms can lead to misconceptions.

The latter statement also holds true for decalin. Indeed, Huff states that

decalin is probably the first chemical for which no empirical connection could be made between induced hyaline droplet-[α 2u-globulin] nephropathy syndrome and cancer of the renal epithelial cells.

Huff cites Gaworski et al. (50), who quite clearly demonstrate all classical symptoms of an α 2u-globulin-mediated mechanism; this 90-day study could not have promoted the necessary number of preneoplastic lesions that would have progressed to veritable tumors within the 19-month post-exposure observation period. Indeed, the α 2u-globulin-mediated mechanism of renal tumor induction is a pure promotional process. Thus, a chronic stimulus (chronic cell necrosis and subsequent chronic cell regeneration) is necessary for a low induced incidence of renal tumors to be observed at the end of a 2-year bioassay. Furthermore, the promotional effect of the α 2u-globulin mechanism is weak, leading to low renal tumor incidences in 2-year rat bioassays (up to 25%) (51), while other presumably nongenotoxic compounds, e.g., ochratoxin A, lead to high renal tumor incidences (up to 87%) in both sexes of rats (52). Therefore, Huff's statement that

at the end of the post-exposure period, male rats exhibited dose-related chronic progressive necrosis with accentuated tubular degeneration, medullary mineralization, and papillary hyperplasia; however, no tumor of the kidney was reported for male rats or for any other sex–species–exposure group

is incorrect. Indeed, mentioning chronic progressive necrosis and tubular degeneration in conjunction with decalin treatment is misleading in this context. These symptoms are characteristic for aging rats (53), but can be exaggerated in a dose-related fashion following exposure to chemicals (19). However, there is no distinct association or causality between the occurrence lesions of chronic progressive necrosis (also known as chronic progressive nephropathy) and the etiology of α 2u-globulin–chemical complex-mediated renal tumors. Therefore, insinuating that in the case of decalin, despite the presence of chronic progressive necrosis, no tumors related to α 2u-globulin were observed is a misconception that unnecessarily leads to misunderstandings. Unfortunately no 2-year cancer bioassay has been conducted to date on decalin. It is hoped that, as Huff pointed out, the NTP will conduct a 2-year bioassay with decalin using F344 and the α 2u-globulin-deficient NBR rats in the near future. I am quite confident that this study, as many others

before, will substantiate the currently accepted mechanism.

The two last compounds that are worth discussing are trimethylpentane and hexachlorobenzene. Trimethylpentane (TMP) was demonstrated to bind to α 2u-globulin (54–57) and to induce α 2u-globulin nephropathy in male rats (36,58). Prolonged treatment of male rats with TMP exacerbates the nephropathy and increases regenerative cell proliferation in a dose-dependent manner (36,59,60). NBR rats lacking α 2u-globulin do not produce protein accumulation or nephropathy following treatment with TMP (22). TMP was also demonstrated to be a renal tumor promoter (via the α 2u-globulin-mediated mechanism) in a classic initiation–promotion study by Short et al. (19). This fact stands out against Huff's statement that TMP had been demonstrated not to induce renal tumors in a 2-year study. The literature cited, which back the α 2u-globulin-mediated mechanism, appeared in peer-reviewed journals and thus were open for scientific scrutiny. On the contrary, Huff's statement is backed by a personal communication on a bioassay of which the experimental conditions and the actual resulting data are not available; these studies by the Instituto Ramazzini have not been duplicated by another laboratory using a different strain of rats, whereas the studies supporting the α 2u-globulin-mediated mechanism have been reproduced in various laboratories. In this, Huff does not seem to be carefully weighing the evidence.

Hexachlorobenzene (HCB) was shown to induce α 2u-globulin nephropathy in male rats but not in female rats, even when treated for 50 days at the highest dose (61). The statement by Huff that "hexachlorobenzene (HCB) is an example of a chemical that induces the [α 2u-globulin] nephropathy syndrome in male and female Sprague-Dawley rats..." (61) is a misrepresentation of facts. Female rats do not present with the α 2u-globulin nephropathy syndrome in any case so far discussed. Furthermore, female rats did not present with any form of renal pathology, of whatever etiology, in the 50-day study by Bouthillier (61). It is true, however, that both sexes of rats treated with HCB presented with hepatomas (male, 11/56; female, 35/56), hepatocellular carcinomas (male, 4/56; female, 48/56), bile duct adenomas (male, 2/56; female, 29/56), and renal adenomas (male, 42/56; female, 15/56) (62). The incidences of renal adenomas were decisively higher in male than in female rats. The overt increase in liver carcinomas and bile duct tumors in both sexes of Sprague Dawley rats indicates that several

mechanisms are involved in the etiology of liver, bile duct, and renal tumors, one of them most likely being a genotoxic mechanism. Hexachlorobenzene, therefore, is not an example that would contest the α 2u-globulin hypothesis. Huff states that "HCB is the first chemical identified that exhibits the [α 2u-globulin] nephropathy sequelae, and induces renal cell tumors in both genders of rats." This statement is a misconception and misrepresentation of facts. In this context, it is also wrong and misleading of Huff to insinuate that HCB would have been regulated under the EPA ruling discussed here. Indeed, the EPA ruling on α 2u-globulin-inducing compounds clearly concerns compounds only that 1) are nongenotoxic; 2) produce renal tumors in male rats and in no other sex, species, or organ; and 3) induce the α 2u-globulin nephropathy syndrome. Hexachlorobenzene does not fulfill two of the above categories and should therefore not be given as an example in this context.

In conclusion, I would like to emphasize that one needs at least as excellent a database to disprove a currently accepted mechanism as was used to establish the mechanism in the first place. Questioning for queries sake is not helpful, but is rather hindering to those scientists that excel in distinguishing those mechanisms that are relevant to humans and can be predicted via animal experimentation from those that are specific to the surrogate animals only. In his letter in *Environmental Health Perspectives*, James Huff was clearly not able to disprove the currently accepted mechanism; I challenge him to produce some experimental data to substantiate his hypothesis.

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The Relevance of Mechanistic Data to the Interpretation and Extrapolation to Humans of Rodent Carcinogenicity Data

I refer to Huff's response (1) to my letter to *EHP* regarding the adequacy of the α 2u-globulin nephropathy mechanism of tumor induction in male rats (2) and to the response by Dietrich (3) to the points made by Huff (1). Pursuit of the mechanisms by which chemicals are toxic to mammals can be justified in two ways. The first is that the acquisition of such knowledge may increase our ability to prevent or attenuate human diseases. The second is that mechanistic data can contribute significantly to our ability to