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April 20, 2018

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Subject: Information Quality Guidelines (IQG) identifier:
RFC #17003

Additional Information demonstrating that National Toxicology Program Technical Report 494 should not be the basis for provisional screening values presented in Appendix A of "Provisional Peer-Reviewed Toxicity Values for 9,10-Anthraquinone (CASRN 84-65-1)", EPA/690/R-11/007F, Final 2-17-2011 or the screening levels for "Anthraquinone, 9,10-" in EPA's Regional Screening Level Tables

Dear Sir or Madam:

This letter contains additional information to supplement the Request for Correction submitted by Chemical Products Corporation (CPC) on April 6, 2018, assigned Information Quality Guidelines Identifier ***RFC #17003.***

The contents of this letter provide yet another sufficient reason to conclude that the National Toxicology Program (NTP) peer review panel which accepted the conclusions presented in NTP Technical Report 494 (TR-494) were not presented accurate information, thus, the peer review panel was unable to render a sound scientific judgment regarding the conclusions presented in TR-494. The peer review of TR-494 does not meet the "sound and objective scientific practices"

requirement contained in the EPA Guidelines and should not be accepted by EPA as a valid peer review. EPA should not base the provisional screening values presented in Appendix A of "Provisional Peer-Reviewed Toxicity Values for 9,10-Anthraquinone (CASRN 84-65-1)", EPA/690/R-11/007F, Final 2-17-2011 (PPRTV), or the screening levels presented in EPA's RSL tables for the compound "Anthraquinone, 9,10-" on TR-494.

The primary metabolite of 9,10-Anthraquinone (AQ) is incorrectly stated to be 2-Hydroxyanthraquinone in TR-494. Sato et al. (1959)corrected Sato et al. (1956) which is cited in TR-494, but Sato et al. (1959) is not cited in TR-494. Sato et al. (1959) (Attachment 1) reported that only a very small quantity of 2-Hydroxyanthraquinone was found in fresh rat urine.

TR-494 states on page 91, "2-Hydroxyanthraquinone is the major anthraquinone metabolite present in urine regardless of the method of anthraquinone synthesis. Lesser amounts of 1-hydroxyanthraquinone were also present. That 2-hydroxyanthraquinone is a major metabolite of anthraquinone is in agreement with results reported by Sato et al. (1956)...". However, in 1959 Sato et al. Reported, "In a previous study anthraquinone was fed to rats and 2-hydroxyanthraquinone was recovered from the urines (*1*). However, its quantity was found to be very small when freshly voided urine was examined by paper chromatography. This fact suggested that the urine contained a substance which liberated 2-hydroxyanthraquinone on standing. Using S^{35}-sulfate a sulfate conjugate of 2-hydroxyanthraquinone was found."

Sato et al. (1956) was corrected by the authors in Sato et al. (1959) which reports that the primary metabolite of 9,10-anthraquinone found in rat urine is not 2-Hydroxyanthraquinone, but rather the sulfate conjugate of 2-Hydroxyanthraquinone which decomposes on standing to sulfate and 2-Hydroxyanthraquinone. In this context, the term "conjugate" in organic chemistry refers to a compound formed by the joining of two or more chemical compounds. Sato et al. (1959) describes how 2-Hydroxyanthraquinone was reacted with chlorosulfonic acid and chloroform to form the sulfate conjugate of 2-Hydroanthraquinone for use as the lab standard to verify the identity of the compound in fresh rat urine. NTP did not inform peer reviewers of Sato et al. (1959) and it is not cited in TR-494. Instead, NTP presented its positive mutagenicity assay of 2-Hydroxyanthraquinone as documenting mutagenicity in the primary AQ metabolite.

There is a typographical error in "Provisional Peer-Reviewed Toxicity Values for 9,10-Anthraquinone (CASRN 84-65-1)", EPA/690/R-11/007F, Final 2-17-2011 (PPRTV) at page 16 where "2-hydroxyanthracene" is written instead of "2-hydroxyanthraquinone", the purported primary metabolite of AQ in TR-494. The PPRTV states, beginning at the bottom of page 16, "NTP (2005b), however, found that the mutagenicity of 2-hydroxyanthracene, a major metabolite of 9,10-anthraquinone, was 7 times as mutagenic as 2-NA and would be a much more likely candidate for the causative agent, if mutagenicity was involved in the mode of action." Contamination of the TR-494 test article by 9-nitroanthracene (9-NA) is at issue, not contamination by 2-nitroanthracene (2-NA).

There is no scientifically sound basis for considering non-mutagenic 9,10-Anthraquinone (AQ) likely to be carcinogenic to humans. There is

no scientifically sound basis for concluding that non-mutagenic 9,10-Anthraquinone (AQ) caused cancers in the NTP TR494 animal studies. NTP unknowingly conducted animal testing with AQ contaminated by the potent mutagen 9-nitroanthracene.

The 2009 paper, A Data-Based Assessment of Alternative Strategies for Identification of Potential Human Cancer Hazards, by eminent European toxicologist Alan R. Boobis and co-authors is included as Attachment 2. Please refer to page 719, in the first column below Table 2, where the authors state, "The data for anthraquinone are considered suspect because other carcinogenicity studies were negative, and the NTP carcinogenicity study used a batch of anthraquinone contaminated with the potent mutagen 9-nitroanthracene at a level of 1,200 ppm (Butterworth, Mathre, and Ballinger 2001)…."

To sum, in addition to the deficiencies detailed in CPC's April 6, 2018 Request for Correction submission, false information regarding the identity of the primary metabolite of AQ was provided to peer reviewers and incorporated into TR-494. The mutagenicity of the compound NTP incorrectly presented as being the primary metabolite of AQ would have had a significant impact on the peer reviewers' adjudication and renders their acceptance of the conclusions in TR-494 scientifically untenable.

The EPA Guidelines require "influential" scientific information to meet a "higher degree of quality." In particular, EPA has established very rigorous standards for "influential scientific risk assessment information." These stringent quality standards are applicable here.

Conclusion: For the reasons set forth above, CPC respectfully renews its requests that:

(1) Request for Correction, IQG identifier RFC #17003, be granted;

(2) "Provisional Peer-Reviewed Toxicity Values for 9,10-Anthraquinone (CASRN 84-65-1)", EPA/690/R-11/007F, Final 2-17-2011 be immediately withdrawn and revised to provide toxicity values for 9,10-Anthraquinone which are based upon sound science rather than upon NTP Technical Report 494.

(3) "Anthraquinone, 9,10-" be immediately removed from EPA's Regional Screening Level Tables provided on EPA's website at https://www.epa.gov/risk/regional-screening-levels-rsls-generic-tables-november-2017 until such time as "Provisional Peer-Reviewed Toxicity Values for 9,10-Anthraquinone (CASRN 84-65-1)", EPA/690/R-11/007F, Final 2-17-2011 is revised to provide scientifically sound toxicity values for 9,10-Anthraquinone which could justify its inclusion.

Very truly yours,

Jerry A. Cook

Jerry A. Cook
Technical Director

Attachments – 23 pages
Attachment 1 – 3 pages – Sato et al. (1959)
Attachment 2 – 20 pages – Boobis et al. (2009)

cc: ***Via Certified Mail and Electronic Mail***
Dr. Tina Bahadori, Director
EPA National Center for Environmental Assessment
(Bahadori.tina@epa.gov)

ATTACHMENT 1

NOTES

The Journal of Biochemistry, Vol. 46, No. 8, 1959

METABOLISM OF ANTHRAQUINONE

II. SULFATE CONJUGATE OF 2-HYDROXYANTHRAQUINONE

By TOKURO SATO, TAEKO SUZUKI

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AND HARUHISA YOSHIKAWA

(From the Department of Nutrition and Physiological Chemistry, Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, March 11, 1959)

In a previous study anthraquinone was fed to rats and 2-hydroxyanthraquinone was recovered from the urines (*1*). However, its quantity was found to be very small when freshly voided urine was examined by paper chromatography. This fact suggested that the urine contained a substance which liberated 2-hydroxyanthraquinone on standing. Using S^{35}-sulfate a sulfate conjugate of 2-hydroxyanthraquinone was found.

EXPERIMENTAL AND RESULTS

Rats were fed with anthraquinone in the same way as reported previously (*1*). About 100 μc. of radioactive sulfate was injected subcutaneously into the rat and the urine excreted within the next 24 hour period was collected.

Radioautography of the Chromatograms—Ascending chromatography was carried out on Whatman No. 1 paper and the paper was subjected to radioautography. There appeared a spot with R_f value of 0.51 and 0.39 when run in butanol, acetic acid, water mixture (4 : 1 : 1), and in 3 *N* Na_2CO_3, 3 *N* NH_4OH, and butanol (3 : 3 : 9), respectively.

Colour Reaction of the Substance on the Chromatogram—The colour of this spot on the paper was pale yellow and gave no fluorescence under ultraviolet light. The colour did not change by spraying 10 per cent Na_2CO_3 solution followed by diazotized sulfanilic acid. But after the paper was placed in an atmosphere of hydrogen chloride fume for 20 minutes at room temperature, the spot was coloured yellow and turned to red by spraying with alkali. This colour reaction of the hydrolyzed material was the same as that of 2-hydroxyanthraquinone.

The quantity of 2-hydroxyanthraquinone which was contained in this spot was not enough to account for all the concentration of 2-hydroxyanthraquinone found in the urine.

Examination of the Acid Hydrolyzed Product of the Spot—The radioactive spot

was cut out from the paper and eluted with water. Then the eluate was acidified with hydrogen chloride solution to a concentration of 1 *N* and heated at 100° for 1 hour. The solution was condensed by evaporation to a small volume and submitted to paper chromatography using different solvents as described in the previous report (*1*). The R_f values of the obtained spot were in good agreement with those of 2-hydroxyanthraquinone. The spot was cut out and eluted with alcohol. The absorption curve from 210 mμ to 600 mμ coincided with the authentic sample of 2-hydroxyanthraquinon (*1*). Inorganic S^{35}-sulfate was also detected, but no other product was found by fluorescence or colour reaction such as spraying with alkali or diazo reagents.

Stability of the Compound—This compound was labil in water and yielded 2-hydroxyanthraquinone on standing.

An attempt was made to recover the sulfate conjugate in a considerable quantity from rat urine by counter current technique using water and butanol as solvents; but the compound was almost completely decomposed during the manipulation.

Synthesis of the Sulfate Conjugate of 2-Hydroxyanthraquinone—10 mg. of 2-hydroxyanthraquinone was dissolved in 0.75 ml. of pyridine at 0°. To this was added slowly a mixture of 0.05 ml. of chlorosulfonic acid and 0.125 ml. of chloroform. The mixture was stirred for one hour and centrifuged. The supernatant was neutralized with potassium hydroxide solution and subjected to paper chromatography which showed a spot in the same location and with the same reactions and components as were found in the urine of rats fed with anthraquinone. Further purification was abandaned because it was decomposed easily by further manipulation.

Attempts were made, but without success, to conjugate S^{35}-sulfate with 2-hydroxyanthraquinone in rat liver slices or in the supernatant of rat liver homogenates by the method described before (*2*).

DISCUSSION

Rats were fed with anthraquinone and a metabolite which decomposed to sulfate and 2-hydroxyanthraquinone was found in the urine. By paper chromatography and colour reactions this substance was found to be the same with a compound recovered from a procedure to conjugate sulfate with 2-hydroxyanthraquinone. From this evidence the metabolite was concluded to be the sulfate conjugate of 2-hydroxyanthraquinone.

SUMMARY

Urine from rats fed anthraquinone and S^{35}-sulfate was examined by paper chromatography and colour reactions. A metabolite which decomposed tos ulfate and 2-hydroxyanthraquinone was found, and it was concluded to be a sulfate conjugate of 2-hydroxyanthraquinone.

REFERENCES

(*1*) Sato, T., Fukuyama, T., Yamada, M., and Suzuki, T., *J. Biochem.*, **43**, 21 (1956)

(*2*) Sato, T., Suzuki, T., Fukuyama, T., and Yoshikawa, H., *J. Biochem.*, **43**, 421 (1956)

ATTACHMENT 2

Toxicologic Pathology

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A Data-Based Assessment of Alternative Strategies for Identification of Potential Human Cancer Hazards

Alan R. Boobis, Samuel M. Cohen, Nancy G. Doerrer, Sheila M. Galloway, Patrick J. Haley, Gordon C. Hard, Frederick G. Hess, James S. Macdonald, Stéphane Thibault, Douglas C. Wolf and Jayne Wright

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Articles

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A Data-Based Assessment of Alternative Strategies for Identification of Potential Human Cancer Hazards

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Abstract

The two-year cancer bioassay in rodents remains the primary testing strategy for in-life screening of compounds that might pose a potential cancer hazard. Yet experimental evidence shows that cancer is often secondary to a biological precursor effect, the mode of action is sometimes not relevant to humans, and key events leading to cancer in rodents from nongenotoxic agents usually occur well before tumorigenesis and at the same or lower doses than those producing tumors. The International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) hypothesized that the signals of importance for human cancer hazard identification can be detected in shorter-term studies. Using the National Toxicology Program (NTP) database, a retrospective analysis was conducted on sixteen chemicals with liver, lung, or kidney tumors in two-year rodent cancer bioassays, and for which short-term data were also available. For nongenotoxic compounds, results showed that cellular changes indicative of a tumorigenic endpoint can be identified for many, but not all, of the chemicals producing tumors in two-year studies after thirteen weeks utilizing conventional endpoints. Additional endpoints are needed to identify some signals not detected with routine evaluation. This effort defined critical questions that should be explored to improve the predictivity of human carcinogenic risk.

Keywords: carcinogenesis; carcinogenicity testing; DNA reactivity; liver carcinogenesis; kidney carcinogenesis; lung carcinogenesis; immunosuppression; nongenotoxic carcinogens; mode of action.

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This article does not necessarily reflect the opinions or policy of the U.S. Environmental Protection Agency; nor does mention of trade names constitute endorsement. James S. MacDonald's current affiliation is Chrysalis Pharma Consulting, LLC.

Conflict of Interests: The authors have not declared any conflict of interests.

Abbreviations: AAF, 2-acetylaminofluorene; ALT, alanine transaminase; B[*a*]P, benzo[*a*]pyrene; CAR, constitutive androstane receptor; CHIS, Cancer Hazard Identification Strategies (HESI Project Committee); DNA, deoxyribonucleic acid; EPA, U.S. Environmental Protection Agency; HESI, Health and Environmental Sciences Institute; ICH, International Conference on Harmonisation; ILSI, International Life Sciences Institute; IPCS, International Programme on Chemical Safety; MN-NCE, micronucleated normochromatic erythrocytes; MN-PCE, micronucleated polychromatic erythrocytes; MOA, mode of action; NCI, National Cancer Institute; NOS, not otherwise specified; NTP, National Toxicology Program; OECD, Organisation for Economic Cooperation and Development; SCE, sister chromatid exchange; SDH, sorbitol dehydrogenase.

I. Introduction

As part of the hazard identification of regulated chemicals and of those substances nominated to programs such as the National Toxicology Program (NTP), a lifetime bioassay of carcinogenic potential is routinely undertaken in rats and mice. This applies to most drugs, depending on likely duration of treatment, pesticides, veterinary medicines, and food additives. Many industrial chemicals and natural compounds are also subject to such testing. The rodent bioassay used for this purpose was originally developed in the 1940s and 1950s (Berenblum 1969; E. Weisburger 1981, 1983), and the underlying principles of the assay have remained largely unchanged since that time. The bioassay was based on the observation that exposure of experimental animals, as well as humans, to a number of chemicals led to development of cancer. However, at the time, there was little mechanistic understanding of chemical carcinogenesis. Analysis of the results of initial studies led to

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the conclusion that the "majority of all cancer" is caused by chemical or environmental factors (Epstein 1979; Roe 1989). However, it should be noted that, at that time, "environmental" (which simply meant that the etiological factor was extrinsic) was often assumed to mean "chemical." This led to a major focus on identifying chemical carcinogens on the assumption that this would enable the burden of cancer to be substantially reduced.

Inherent in the use of animals for the carcinogenicity bioassay is the assumption that humans and animals behave in a similar way (interspecies extrapolation). In addition, two experimental concepts form the scientific basis on which the bioassay is based.

The first is the empirical relationship developed by Druckrey (1967).

$$d \times t^n = \text{constant},$$

where d is dose; t is time to tumor appearance; and n is a power term, usually 2, 3, or even higher.

The experimental work, mostly with nitrosamines in liver, which led to this relationship, indicated that tumor incidence was directly proportional to dose (dose extrapolation). Thus, incidence could be increased by increasing the dose, or the time to tumor could be decreased, although there was a minimum interval before tumors developed. This approach, however, only worked for genotoxic (DNA reactive) carcinogens. It implied a multistage process for carcinogenesis. A version of this multistage theory derived from epidemiologic data had been previously postulated by Armitage and Doll (1954). However, numerous human tumors, such as Hodgkin lymphoma, breast cancer, osteogenic sarcomas, and childhood tumors, did not show this age relationship.

The second concept is that carcinogenesis comprises multistep stages, which was first demonstrated by the model of tumor initiation and promotion. This was developed to explain the observed data for chemical carcinogenesis in mouse skin by Berenblum and Shubik (1947, 1949). These studies showed that skin carcinogenesis first required a short exposure to certain chemicals, resulting in an irreversible change that was termed "initiation." This had to be followed by prolonged exposure to other chemicals that acted to promote the initiated cells, the effects of which were reversible up to a certain time. This was termed "promotion." In this model, chemicals that act as promoters do not act as initiators. Promotion has to be preceded by initiation; promotion does not need to commence immediately after initiation. It is now recognized that this distinction is not as clear-cut as once believed (see Goodman and Watson 2002).

The model was later shown to apply to a number of other cancer types in rats and mice. It also subsequently acquired a mechanistic interpretation, although the molecular events responsible for the two stages have yet to be completely defined. It is now known that initiation usually involves primary damage to DNA, leading to a critical mutation; while promotion involves proliferation and subsequent steps allowing expression of oncogenicity through acquisition of other changes, which are either genetic or epigenetic (Foulds 1954; Hanahan and Weinberg 2000).

Numerous difficulties were identified with the initiation-promotion model (Cohen 1998b; Cohen and Ellwein 1991). A more definitive model of carcinogenesis, incorporating the concepts of time, genetics, and multiple stages, was postulated by Knudson (1971) based on his investigation of retinoblastoma in children. This model led to the concept of tumor suppressor genes. Utilizing DNA damage and increased cell proliferation (the two fundamental precepts set forth in Knudson's model), Moolgavkar and Knudson (1981) and Greenfield, Ellwein, and Cohen (1984) developed more generalized models based on epidemiologic and animal studies, respectively. J. Weisburger and Williams (1981) also distinguished two classes of carcinogens: genotoxic (more specifically, DNA reactive) and nongenotoxic. Cohen and colleagues have shown that the common factor for the nongenotoxic carcinogens is increased cell proliferation. Although not precisely correct (Cohen and Ellwein 1991), many have used the term "initiator" interchangeably with genotoxic carcinogen and "promoter" with nongenotoxic (non-DNA reactive) carcinogen.

The current carcinogenicity bioassay owes much to lessons learned from the NTP bioassay program originally developed at the National Cancer Institute (NCI). In establishing this program, a key consideration was that because chemically induced tumors are relatively rare, rather than use very large numbers of animals, the maximum dose should be the highest tolerated by the animals (see Haseman 1984), a natural conclusion from the relationship established by Druckrey (1967). The early studies were designed to determine whether industrial chemicals, with structural similarities to established rodent carcinogens such as 2-acetylaminofluorene (2-AAF) and benzo[*a*]pyrene (B[*a*]P), were carcinogenic (E. Weisburger 1983). The majority of such chemicals did prove to be carcinogenic.

From the late 1960s to mid 1970s, on the assumption that most carcinogens were DNA-reactive, considerable effort was spent in developing reliable, short-term tests of genotoxicity. The most significant outcome of this effort was the Salmonella bacterial mutation assay (Ames et al. 1973). It was initially believed that tests such as this could predict most carcinogens. Indeed, as the majority of chemical carcinogens identified up to that time were potent, DNA-reactive compounds, the Ames test was > 90% predictive. The concept was clearly stated in the title of a manuscript by Ames et al. (1973): "Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection." Reflecting the views of a number of scientists at the time, in another paper Ames (1973) stated, "We . . . suggest that the combined bacteria/liver system be used as a simple procedure for carcinogen detection."

As the number and chemical diversity of those chemicals tested within programs such as the NTP increased, a range of chemicals with no structural similarities to known DNA-reactive carcinogens and negative in the Ames assay were found to be carcinogenic, the proportion of these that were positive for carcinogenic activity in rats and mice being similar to that for DNA-reactive compounds. However, the tumor profile obtained with these chemicals differed (Fung, Barrett, and Huff 1995). It subsequently became apparent that many of these

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chemicals caused cancer by mechanisms that did not involve direct reactivity with DNA, and, indeed, they were negative in short-term tests of genotoxicity. It is now known that there are a number of mechanisms by which a chemical can increase tumor incidence in rats and mice in addition to genotoxicity (MacDonald and Scribner 1999).

In general, such nongenotoxic carcinogens act by increasing DNA replications in the pluripotential cells of a tissue, either by increasing cell proliferation and/or by inhibiting apoptosis (Cohen and Ellwein 1990, 1991; Greenfield, Ellwein, and Cohen 1984; Moolgavkar and Knudson 1981). This increases the probability of producing or selecting cells that develop spontaneous errors or of damage induced by primary initiators or secondary mediators such as reactive oxygen species (Ames and Gold 1997; Cohen 1998a). Hence, although many nongenotoxic carcinogens may act through mechanisms that include a DNA damage component (Klein and Klein 1984), a biological threshold for the carcinogenic response to such compounds will exist (Butterworth and Bogdanffy 1999). To induce the production of a secondary genotoxic species, the nongenotoxic carcinogen still has to achieve a threshold concentration to trigger the precipitating biological event, such as cytotoxicity or inflammation (Butterworth and Bogdanffy 1999; Cohen and Ellwein 1991). This contrasts with genotoxic carcinogens for which, at least in theory, there is the potential of a linear, nonthreshold response (U.S. Environmental Protection Agency [EPA] 2005), although a number of groups are strongly challenging this assumption (e.g., Hoshi et al. 2004; Swenberg et al. 2002; Williams, Iatropoulos, and Jeffrey 2004).

The current testing strategy for carcinogenic potential is based on a dual approach:

1. assessment of genotoxic potential and
2. assessment of carcinogenic potential in the lifetime bioassay in rats and mice.

The results from such studies may be supported by other investigations to determine the mode of action (MOA) and its relevance to humans (dose, metabolism, etc.). Such studies have shown that for those compounds that cause cancer by a nongenotoxic MOA, it is usually a secondary consequence of another toxicological perturbation, such as inflammation or cytotoxicity (Cohen et al. 2004; Sonich-Mullin et al. 2001). Indeed, there is evidence that under the right circumstances, almost any agent can cause cancer in experimental animals (Ashby and Purchase 1993; Norton 1981). A key consideration in this respect is that the high doses necessarily used in the cancer bioassay often cause effects unrelated to those observed at lower doses (MacDonald and Scribner 1999). Effects seen under such circumstances often have no relevance to the assessment of human risk.

As knowledge of MOAs of nongenotoxic carcinogens has increased, three concepts have emerged:

1. A number of MOAs for carcinogenicity are rodent-specific.
2. Tumors occur at detectable incidences at the same, and often only at higher, doses than the primary toxicological perturbation.
3. There is a biological threshold for carcinogens with such MOAs.

As a consequence of the above, there is increasing concern that the current cancer bioassay in the rat and mouse in which compounds are tested at up to the maximum tolerated dose is not very predictive of the potential for human carcinogenicity, and in particular that it has a high false positive rate (Alden et al. 1996; Cohen 2004; Ennever and Lave 2003; Gaylor 2005; Rhomberg et al. 2007; Van Oosterhout et al. 1997). Compounds that are carcinogenic as a consequence of direct reactivity with DNA are now identifiable in short-term tests for genotoxicity (Kirkland et al. 2005). Hence, the majority of compounds subject to a cancer bioassay today that give a positive result act by a nongenotoxic mechanism. Many regulatory authorities will permit exposure to compounds, albeit usually for less than a lifetime, that are negative in an adequate range of genotoxicity tests (U.S. Food and Drug Administration [FDA] 1996). This implies that the bioassay is currently required to identify only those compounds that can cause cancer by a nongenotoxic mechanism. In part, the unreliability of the bioassay in rats and mice is due to the existence of rodent-specific mechanisms of carcinogenicity or responsiveness at very high doses via mechanisms not occurring at lower doses.

Advances in both genotoxicity testing and in biomedical sciences justify a critical reevaluation of the need for the cancer bioassay, or even the proposed genetically engineered mouse alternatives (MacDonald et al. 2004), and whether these tests can be replaced by a more systematic, mechanistically based approach. Currently, use of the bioassay results in risk communication problems, requires consumption of significant development costs, and is a difficult system in which to apply advances in biomedical science. Often, these mechanistic research approaches are used only retrospectively to explain false positives, not prospectively to help in evaluation. During product development, it is the elimination of compounds with the potential to cause cancer that is of primary concern, rather than whether they will definitively produce tumors if given for a lifetime. Hence, an important goal is the development of an efficient, reliable, and cost-effective means of assessing human carcinogenic *potential*, rather than carcinogenesis per se, recognizing that some compounds may be abandoned because they are considered potential carcinogens even though this endpoint may not be realized over a lifetime of exposure. However, any such system would have to reliably identify those compounds that would be carcinogenic over a lifetime (low false negative rate), while also having a low false positive rate to ensure cost-effectiveness.

The Health and Environmental Sciences Institute (HESI) established a project to explore the feasibility of such an approach. This was based on developments in the analysis of MOA for chemical carcinogenesis and its human relevance

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primarily by the International Life Sciences Institute (ILSI) and the International Programme on Chemical Safety (IPCS) (Boobis et al. 2006; Meek et al. 2003; Sonich-Mullen et al. 2001). An MOA is characterized by a series of key events, which are the biological processes occurring on the causal path to cancer. Qualitative and quantitative consideration of these key events between experimental animals and humans enables conclusions to be reached about human relevance of the carcinogenic effect of the chemical. The ultimate objective of the HESI initiative was to test a strategy in which compounds are evaluated for carcinogenic potential in rats and mice after exclusion of those that are genotoxic or immunosuppressive as determined in relevant tests routinely undertaken in hazard identification and characterization on the premise that such compounds are known often to possess carcinogenic potential (Cohen 2004). Compounds negative for such effects would be evaluated in subchronic tests (initially thirteen-week studies) for the induction of key events associated with known MOAs for carcinogenicity, which should then be predictive of carcinogenic potential in rats and mice. The human relevance of the MOA would then need to be evaluated.

In this study, the NTP database was evaluated for all compounds that were positive for liver, kidney, or lung tumors over the period 2000 to 2005. This database was selected because it contains comprehensive data on both chronic and thirteen-week studies in rats and mice, is publicly accessible, and is among the most comprehensive available. The period 2000 to 2005 was chosen because, prior to this time, the information available was not comprehensive, precluding full evaluation of the compounds. The target tissues were those most commonly showing a tumorigenic response in rats and mice.

The reliability of short-term tests of genotoxicity to detect genotoxic carcinogens was critically evaluated, that is, it was necessary to establish the confidence that could be placed in a negative response and where the weaknesses were, if any. Precursor effects were sought for nongenotoxic carcinogenicity in subchronic tests (thirteen weeks). As part of this exercise, MOAs for which there were no suitable conventional endpoints would be identified. Potentially suitable endpoints to cover these deficiencies that, if possible, could be assessed in conventional subchronic studies, would be identified. These studies would enable the false negative rate to be determined, that is, those compounds for which no relevant key events could be identified. In a subsequent stage it would be necessary to establish the false positive rate, where the occurrence of key events was not accompanied by a carcinogenic response. Analysis of the key events and carcinogenic response would test the hypothesis that protection against such effects would be adequately protective against carcinogenicity and that by understanding the key events, it would be possible to determine human relevance. Ultimately, it is hoped that the results of such studies will enable the development of a science-based, hierarchical approach to assessing the carcinogenic potential of compounds.

The current study was designed to test the hypothesis that the signals of importance for human cancer hazard identification can be detected in shorter-term studies, rather than routinely relying on data from two-year cancer bioassays in rats and mice.

II. Methods

The HESI Cancer Hazard Identification Strategies (CHIS) Project Committee elected to use the NTP database in this study because it constitutes the most comprehensive, accessible repository of matching subchronic and long-term information on both pathology and other endpoints, for example, clinical chemistry, available to the participants. The Project Committee was greatly assisted in interrogating the database by scientists from the NTP.

The period 2000 to 2005 was evaluated. Prior to 2000, the NTP database does not contain comprehensive information on all aspects of hazard relevant to the project objectives. Hence, only those reports from the beginning of the year 2000 were considered for evaluation. The study was based solely on the tumorigenicity and genotoxicity data available in the NTP database for the chosen compounds, although some slides from male kidneys in the thirteen-week studies were reviewed (see below). Additional literature searches were not conducted.

Compounds in the NTP database were queried for carcinogenic effects in at least one ''cell'' of the bioassay, that is, male or female mice or male or female rats, in liver, kidney, or lung. These tissues/organs were selected for study as they are by far the most common targets for carcinogenicity of chemicals in rats and mice. Sixteen chemicals were identified on this basis. Only studies in Fisher 344 (F344/N) rats and/or B6C3F1 mice were included in the analysis because these two strains were most frequently used by NTP during that period. A customized, defined query tool (Excel spreadsheet) was developed by the CHIS Project Committee to assist with searching and recording the results of subchronic (thirteen weeks) rat and mouse toxicity studies for each of the sixteen carcinogenic compounds identified from the two-year rodent bioassay database. Teams of scientists (the authors) reviewed thirteen-week data and data from mutagenicity assays for the sixteen chemicals, with the objective of identifying early signals of carcinogenic potential, for example, cytotoxicity, hyperplasia, and local irritation. Peer reviewers checked the data recorded. Some peer reviewers were CHIS Project Committee participants; others were not. In all cases, the peer reviews were independent of the query exercise.

Table 1 shows the chemicals identified in the NTP database that were found to produce tumors in one or more of the target organ systems and in one or both of the species tested. To obtain some information on false positives as well as false negatives, the histomorphologic findings from thirteen-week studies were examined for all sixteen compounds for all target tissues, including those without evidence of tumors in two-year studies.

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TABLE 1.—Evidence of genotoxicity or tumors for National Toxicology Program (NTP) chemicals selected for study in the Health and Environmental Sciences Institute (HESI) Cancer Hazard Identification Strategies (CHIS) project.

Chemical	Route	Genotoxic[a]	Tumors[b]		
			Liver	Kidney	Lung
Anthraquinone (NTP 2005a)	Diet	Positive	**Yes** (fm, mm, fr, mr)	**Yes** (fr, mr)	No
Benzophenone (NTP 2006)	Diet	Negative	**Yes** (fm, mm)	**Yes** (mr)	No
Decalin (NTP 2005b)	Inhalation	Equivocal (*in vivo*)	No	**Yes** (mr)	No
Elmiron (sodium pentosan-polysulfate) (NTP 2004a)	Gavage	Negative	**Yes** (hem)[c] (fm, mm)	No	No
Fumonisin B_1 (NTP 2001a)	Diet	Positive (not NTP data)	**Yes** (fm)	**Yes** (fr*, mr)	No
Gallium arsenide (NTP 2000a)	Inhalation	Negative	**Yes**[d] (fr)	No	**Yes** (fr)
Indium phosphide (NTP 2001b)	Inhalation	Equivocal (*in vivo*)	**Yes** (fm, mm, fr, mr)	No	**Yes** (fm, mm, fr, mr)
Methyleugenol (NTP 2000b)	Gavage	Negative	**Yes** (fm, mm, fr, mr)	**Yes** (mr)	No
2-Methylimidazole (NTP 2004d)	Diet	Positive (*in vivo*)	**Yes** (fm, mm, fr**, mr**)	No	No
o-Nitrotoluene (NTP 2002a)	Diet	Equivocal	**Yes** (fm, fr, mr)	No	**Yes** (mr)
Oxymetholone (NTP 1999)	Gavage	Negative	**Yes** (fr)	No	**Yes** (fr)
Propylene glycol mono-t-butyl ether (NTP 2004b)	Inhalation	Negative	**Yes** (mr**)	No	No
Riddelliine (NTP 2003)	Gavage	Positive (*in vitro*)	**Yes** (hem)[b] (mm, fr, mr)	No	**Yes** (fm)
Triethanolamine (NTP 2004c)	Skin	Negative	No	No	No
Urethane (NTP 2004e)	Water	Positive	**Yes** (fm, mm)	No	**Yes** (fm, mm)
Vanadium pentoxide (NTP 2002b)	Inhalation	Negative	No	No	**Yes** (fm, mm, mr)

[a] "*In vivo*" or "*in vitro*" is added when the conclusion is based only on either type of data, not on both.
[b] Species in which tumors appear: fm = female mouse; mm = male mouse; fr = female rat; mr = male rat.
[c] hem = hemangiosarcomas were induced, in addition to hepatocellular tumors.
[d] From Allen et al. (2004).
* Not statistically significant.
** Equivocal.

III. GENOTOXICITY

A. Methods

For the sixteen chemicals in Table 1, genotoxicity data were obtained from the NTP database, summarized, and evaluated. An overall call for genotoxic, not genotoxic, or equivocal was assigned based on all the test data. A summary table (Table 2) indicates potential *in vitro*/*in vivo* genotoxicity ("calls" for interpretation for each test), gives an overall evaluation based on weight of evidence, and includes an analysis for structural alerts for genotoxicity based on criteria published by Ashby and Purchase (1993).

B. Results (Table 2)

In seven cases, data were available for at least three tests (Ames, *in vitro* chromosome aberrations, and *in vivo* micronucleus). More recently, the NTP strategy is to use the Ames test and the *in vivo* micronucleus assay, which are seen as a more definitive assessment of genotoxicity because of the high false positive incidence in the *in vitro* mammalian cell assays (e.g., Kirkland et al. 2005; Matthews et al. 2006). The micronucleus measurement was typically made on blood samples from the three-month study; for five chemicals, there were also data from an "acute" three-day study, usually in bone marrow. Indium phosphide had only *in vivo* micronucleus data. There were no data for Fumonisin B_1 in the NTP database, and a brief literature review was done. (Only NTP data were considered for the others.)

In three cases, the "call" used by NTP for *in vivo* micronucleus data was questioned, that is, the NTP criteria did not appear to take into account historical ranges for micronucleus data:

> Indium phosphide was considered to be negative by NTP; the present authors considered it equivocal because the level of micronucleated polychromatic erythrocytes (MN-PCE) increased from 1.7 in controls to 4.11 in treated females, with a lesser increase in micronucleated normochromatic erythrocytes (MN-NCE).
>
> Propylene glycol mono-t-butyl ether was considered a weak positive in female mice by NTP; the authors of this article considered it negative because the maximum level of micronuclei seen in females was in the range of concurrent (male) and historical controls for the data set examined.
>
> Decalin was considered a weak positive in male and negative in female by NTP; the present authors considered it equivocal or negative overall, again because the maximum level of micronuclei seen was in the range of historical controls for the data set examined.

In some cases, the data from the *in vitro* chromosome aberration test were not considered conclusive because the protocol used had only one "early" sampling time, for example, 10.5 to 13 hours, and it is known that it is more reliable to sample at about 20 hours, as recommended in regulatory guidelines such as those of the Organisation for Economic Cooperation

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TABLE 2.—Summary of structural alerts and findings for genotoxicity tests conducted for sixteen National Toxicology Program (NTP) chemicals with positive tumor findings.

		Genotoxicity findings[a]			
			Mammalian		
Chemical	Structural Alert	Salmonella	*In vitro*	*In vivo*	Comments
Anthraquinone (NTP 2005a)	–	P	No data	P	Genotoxic. Note that the NTP carcinogenicity study used a batch of anthraquinone contaminated with a known mutagen (9-nitroanthracene).
Benzophenone (NTP 2006)	–	N	No data	N	Not genotoxic.
Decalin (NTP 2005b)	–	N	No data	W-P in M; N in F; (E)	Equivocal *in vivo* data; requires more information, as chemical is not genotoxic *in vitro*.
Elmiron (NTP 2004a)	–	N	No data	N	Not genotoxic.
Fumonisin B_1 (NTP 2001a)	–	No data	No data	No data	Genotoxic. Published data: P micronucleus assay *in vitro* and *in vivo*; P SCE and W-P chromosome aberrations *in vitro*; N UDS *in vitro*.
Gallium arsenide (NTP 2000a)	n/a	N	No data	N	Not genotoxic.
Indium phosphide (NTP 2001b)	n/a	No data	No data	N (E)	Minimal data but equivocal *in vivo*.
Methyleugenol (NTP 2000b)	+←	N	N[b]	N	Not genotoxic.
2-Methylimidazole (NTP 2004d)	–	N	No data	P	Positive *in vivo*. Requires more information as the chemical is not genotoxic *in vitro*.
o-Nitrotoluene (NTP 2002a)	+←	N	N[b]	E; E in M; N in F	Equivocal genotoxicity.
Oxymetholone (NTP 1999)	–	N	N	N	Not genotoxic.
Propylene glycol mono-t-butyl ether (NTP 2004b)	–	N	N	W-P in F (N)	Not genotoxic.
Riddelliine (NTP 2003)	+←	P	P	N	Genotoxic.
Triethanolamine (NTP 2004c)	–	N	N[b]	N	Not genotoxic.
Urethane (NTP 2004e)	+←	P	N[b]	P	Genotoxic.
Vanadium pentoxide (NTP 2002b)	n/a	N	No data	N	Not genotoxic.

[a] NTP call; overall call by Health and Environmental Sciences Institute (HESI) committee based on weight of evidence appears in parentheses; P = positive; N = negative; E = equivocal; W-P = weak positive; M = male; F = female; n/a = not available.

[b] *In vitro* aberration assay negative but potentially suboptimal protocol because of harvest time (ten to thirteen hours). *In vitro* sister chromatid exchange (SCE) assay positive.

and Development (OECD) and the International Conference on Harmonisation (ICH) (see also Bean, Armstrong, and Galloway 1992; Galloway et al. 1994). This is not likely to affect the overall conclusions.

The data for anthraquinone are considered suspect because other carcinogenicity studies were negative, and the NTP carcinogenicity study used a batch of anthraquinone contaminated with the potent mutagen 9-nitroanthracene at a level of 1,200 ppm (Butterworth, Mathre, and Ballinger 2001). (A purified sample was negative in the Ames test.) Certainly, it can be said that the material used by the NTP was mutagenic (Doi, Irwin, and Bucher 2005).

C. Discussion/Conclusions

Overall, only three compounds were clearly genotoxic (that is, 2-methylimidazole, riddelliine, and urethane), in addition to anthraquinone. One of these was called positive based on *in vivo* data (i.e., 2-methylimidazole), but was negative in the Ames test and does not contain a structural alert for genetic toxicity. Eight were not genotoxic, three were equivocal, and one had no NTP data (Fumonisin B_1) but has been shown to be positive in a micronucleus assay *in vivo* (Aranda et al. 2000) and *in vitro* (Lerda et al. 2005). The published Ames test data are considered inadequate due to the low dose tested and the use of only three strains of Salmonella. Weak positive results for chromosome aberrations and a positive sister chromatid exchange (SCE) test were reported by Lerda et al. (2005), and a negative *in vitro* unscheduled DNA synthesis (UDS) assay was reported by.

Of the nongenotoxic conclusions, four were based on data from two tests (Ames and *in vivo* micronucleus), and the other four were based on the results of three tests.

Of the equivocal conclusions, three were based on equivocal *in vivo* data. Indium phosphide had no *in vitro* data and equivocal *in vivo* data, and decalin and o-nitrotoluene had negative Ames data and equivocal *in vivo* data. 2-Methylimidazole was also positive *in vivo* but not in the Ames test; *in vitro* cytogenetics data would be of value here.

It is unusual to find positive results *in vivo* when *in vitro* assays are negative. A known confounding factor of *in vivo* micronucleus assays in hematopoietic cells is disturbance of erythropoiesis. Regenerative anemia following bleeding or chemical treatment, stimulation of red blood cell production by erythropoietin, and extramedullary hematopoiesis have been associated with increases in micronuclei in the absence of any treatment with genotoxins (reviewed in Tweats et al. 2007).

An examination of the hematology data from these studies indicates that in some cases regenerative anemia associated

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with altered erythropoiesis may have caused a false positive result, that is, one not associated with genotoxicity. Indium phosphide and 2-methylimidazole both had changes including hematopoietic cell proliferation of the spleen; decalin and o-nitrotoluene did not. It is interesting that even anthraquinone had increases in red blood cell proliferation with large increases in circulating reticulocytes. This might explain why increases in micronuclei were seen only after a three-month treatment and not after three daily doses of anthraquinone and points to the difficulty of interpreting results *in vivo* even with a mutagen. The other chemical that was positive *in vivo*—urethane—had no hematology data reported.

Other possible explanations for *in vivo* micronucleus induction by compounds that do not induce mutations in the Ames test *in vitro* include (1) differences in metabolism *in vivo* versus *in vitro*; and (2) induction of micronuclei that represent chromosome loss and the potential for aneuploidy, that is, the mechanism involves disturbances of chromosome segregation and not DNA reactivity.

For complete transparency, all sixteen compounds, regardless of genotoxicity results, were included in the tabular presentations of data in this study.

IV. IMMUNOSUPPRESSIVE ACTIVITY

A. Methods

The NTP database was searched for clinical and anatomical pathology findings related to disorders of the immune system, including evidence of downregulation (possible immune suppression) and proliferation. The database was also searched for any evidence of neoplasia related to elements of the immune system.

Data were obtained from subchronic studies (thirteen weeks) in F344 rats and B6C3F1 mice for all sixteen compounds derived from the NTP database (Table 1). Findings included changes in hematology (total leukocyte, segmented neutrophil, lymphocyte, and monocyte counts); changes in spleen and/or thymus weights; and histopathological findings in the bone marrow, spleen, thymus, and lymph nodes.

Of the sixteen chemicals evaluated, information for riddelliine, triethanolamine, and Fumonisin B_1 was very limited, and the absence of any effect on the immune system for these three chemicals should be considered with caution.

B. Results (Table 3)

There were ten out of sixteen chemicals with changes in one or more data endpoints related to the immune system. Of these ten chemicals, eight chemicals had changes suggesting down-regulation of the immune system, which, in all cases, were likely secondary to significant stress or illness with release of endogenous glucocorticoids. Two chemicals (o-nitrotoluene and Elmiron [sodium pentosanpolysulfate]) caused a slight increase in lymphocyte counts in male and female rats and the accumulation of vacuolated histiocytes in multiple organs including the lung (see Lung section). It has been suggested that Elmiron may induce a lysosomal disorder that is characterized by histiocytes containing mucins and lipidic material within membrane-bound vacuoles (Nyska et al. 2002).

Gallium arsenide caused contact dermatitis in female mice during a contact hypersensitivity study but no evidence of immunotoxicity in standard toxicity studies. There was an increased incidence of mononuclear cell leukemia in female rats at the end of the two-year carcinogenicity study, with incidences of twenty-two, eighteen, twenty-one, and thirty-three of fifty in the control, low-, mid- and high-dose groups, respectively. This finding was originally considered significant, but this interpretation is debatable. The pathogenesis of this putative increased incidence of mononuclear cell leukemia is uncertain, and it is unlikely related to immunosuppression because the doses used in the two-year studies were significantly (75X) less than the high dose used in the thirteen-week study where there was no evidence of direct immunosuppression (NTP 2000a).

C. Discussion/Conclusions

The interest in evaluating immunosuppressive activity is related to the putative protective role of the immune system in development of cancer. Current ICH guidance lists increased incidence of tumors as one of the five signs of possible immunosuppression in short-term toxicity studies (ICH 2005). The relationship between immunosuppression and cancer is still under investigation, and immunosuppression is currently linked to neoplasia mostly related to infectious agents. In humans, these include Epstein-Barr virus, human herpes virus-8, hepatitis B and C viruses, human papilloma viruses, and *Helicobacter pylori*. Populations most studied for immunosuppression-related neoplasia are HIV/AIDS patients and organ transplant recipients subject to aggressive immunosuppressive therapy and individuals with inherited immunodeficiencies. Interestingly, the types of cancer differ between the two groups, with HIV/AIDS patients more likely to acquire Hodgkin lymphoma, non-Hodgkin lymphoma, Kaposi sarcoma, and anal cancer. Conversely, transplant recipients have much higher standardized incidence ratios than HIV/AIDS patients for several cancers, including malignancies of the vulva and vagina (Grulich et al. 2007; Serraino et al. 2007).

Of the sixteen chemicals reviewed, none caused direct immunosuppression in thirteen-week studies in rats and mice. Many chemicals (eight/sixteen) caused down-regulation of the immune system by one or more standard endpoints in subchronic studies, but in all instances, these were attributed to stress. There were no instances where chemicals that did not show any evidence of immunosuppression in subchronic studies were subsequently tested in a specific immunotoxicity study.

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TABLE 3.—Immunotoxicologic findings in rats and mice following subchronic exposure to National Toxicology Program (NTP) chemicals selected for study in the Health and Environmental Sciences Institute (HESI) Cancer Hazard Identification Strategies (CHIS) project.

Chemical	Species/ gender (M = male; F = female)	Clinical pathology[a]	Organ weights[b]	Histopathology, immune system	Effect on immune system	Immunotoxicity studies
Anthraquinone (NTP 2005a)	Rat M,F Mouse M,F	Negative	Negative	Hematopoietic cell proliferation in spleen; hyperplasia in bone marrow	Negative	Not performed
Benzophenone (NTP 2006)	Rat M,F Mouse M,F	Negative	↓ Thymus	Atrophy in bone marrow (rat); atrophy in thymus (mouse); depletion of cellular lymphoid follicles (mouse)	Stress	Not performed
Decalin (NTP 2005b)	Rat M,F Mouse M,F	Negative	Negative	Negative	Negative	Not performed
Elmiron (NTP 2004a)	Rat M,F Mouse M,F	↑ Lymphocytes ↑ Neutrophils—rats	↑ Spleen	Cellular infiltration of histiocytes in spleen, mandibular, and mesenteric lymph nodes	Inflammation	Not performed
Fumonisin B_1 (NTP 2001a)	Rat M,F Mouse M,F	Information not available	Information not available	Information not available	Information not available	Information not available
Gallium arsenide (NTP 2000a)	Rat M,F Mouse M,F	↑ Neutrophils	Negative	Increased hematopoiesis in bone marrow, spleen; increased cellularity in tracheobronchial, mediastinal lymph node and mandibular lymph node	Inflammation	Contact hypersensitivity (+)
Indium phosphide (NTP 2001b)	Rat M,F Mouse M,F	↑ Neutrophils	↓ Thymus	Hyperplasia in bronchial and mediastinal lymph nodes	Stress; inflammation	Not performed
Methyleugenol (NTP 2000b)	Rat M,F Mouse M,F	Negative	↓ Thymus ↓ Spleen	Negative	Stress	Not performed
2-Methylimidazole (NTP 2004d)	Rat M,F Mouse M,F	↑ Lymphocytes	↓ Thymus ↓ Spleen	Negative	Stress	Not performed
o-Nitrotoluene (NTP 2002a)	Rat M,F	↑ Lymphocytes	Negative	Increased hematopoiesis and hemosiderin in spleen	Stress	Not performed
Oxymetholone (NTP 1999)	Rat M,F Mouse M,F	Negative	↓ Thymus	Negative	Stress	Not performed
Propylene glycol mono-t-butyl ether (NTP 2004b)	Rat M,F Mouse M,F	Negative	Negative	Negative	Negative	Not performed
Riddelliine (NTP 2003)	Rat M,F	Information not available	Information not available	Lymph node congestion and hemosiderin pigments	Negative	Information not available
Triethanolamine (NTP 2004c)	Rat M,F Mouse M,F	Information not available	Information not available	Negative	Negative	Information not available
Urethane (NTP 2004e)	Rat M,F Mouse M,F	↓ Leukocytes ↓ Lymphocytes	↓ Thymus	Lymphoid depletion in spleen, thymus, mandibular and mesenteric lymph nodes; cellular depletion of bone marrow	Stress	Mixed results
Vanadium pentoxide (NTP 2002b)	Rat M,F Mouse M,F	↓ Lymphocytes ↑ Neutrophils	↓ Thymus	Lymphoid depletion in spleen, thymus, mandibular and mesenteric lymph nodes; hypocellularity of bone marrow	Stress; inflammation	Not performed

[a] Clinical pathology parameters evaluated were total leukocytes, segmented neutrophils, and lymphocyte and monocyte counts, and are expressed as increase or decrease compared to concurrent control values (↑ = increase; ↓ = decrease).

[b] Organs weighed included thymus and less frequently spleen. Changes are expressed as increase or decrease compared to concurrent control values (↑ = increase; ↓ = decrease).

Current ICH guidance for chemicals supports the view that specific immunotoxicity investigation is not warranted in these situations (ICH 2005).

There was no clear evidence of neoplasia in elements of the immune system.

For complete transparency, all sixteen compounds, regardless of immunosuppressive activity, were included in the tabular presentations of data in this study.

V. LIVER

A. Methods

In the subchronic (thirteen-week) toxicity studies, the recorded organ weight, clinical pathology, and histopathology data were reviewed for each compound. These included increased relative liver weight, hepatocellular hypertrophy, altered foci, hepatocyte necrosis, hepatocyte vacuolation,

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hepatocyte degeneration, bile duct hyperplasia, increased alanine transaminase (ALT) levels, increased sorbitol dehydrogenase (SDH) levels, and increased bile acid/bilirubin levels. In a similar analysis by Allen et al. (2004), the predictive value of hepatocyte hypertrophy, necrosis, cytomegaly, and increased liver weight in subchronic studies was investigated. In this study, the authors concluded that these four criteria detected 100% of potential liver carcinogens; however, the detection rate included several false positives.

B. Results (Table 4)

For the sixteen chemicals in our evaluation, thirteen were recorded as rodent liver carcinogens (for which increased incidences of hepatocellular adenomas and/or carcinomas occurred, except where footnoted) (either rat [male/female] or mouse [male/female] or both species). For each sex and each species, the tumor outcome, histopathologic changes, significant clinical pathology, and increased relative liver weight are illustrated.

Increased relative liver weight was recorded for at least one sex of one species (rat/mouse) from the thirteen-week NTP toxicity studies in ten of thirteen positive liver carcinogenic compounds. Other single endpoints at thirteen weeks were associated less frequently with tumor outcomes. These included (in at least one sex of one species) hepatocellular hypertrophy or increased bile acids for five of thirteen carcinogens, hepatocellular necrosis or increased ALT levels for four of thirteen carcinogens, hepatocellular vacuolation/degeneration for three of thirteen carcinogens, and altered foci or increased SDH levels for two of thirteen carcinogens.

Association with tumor outcome was strengthened by grouping together thirteen-week toxicological endpoints. Combining the presence of hepatocellular hypertrophy and/or necrosis with increased relative organ weight demonstrated an association with twelve of thirteen liver chemical carcinogens for at least one sex of one species of the NTP bioassay. This increased predictive rate is similar to the results of the previous retrospective study (Allen et al. 2004) (see Liver Discussion section below).

When positive tumor outcomes were collectively considered for both sexes of both species of the cancer bioassay for this particular set of thirteen liver carcinogens, no false positives were recorded. Therefore, if liver-associated changes were observed in any sex/species from the thirteen-week studies, there were always tumors apparent in one of the long-term bioassays.

However, several false positives occurred if single associations are considered between one sex and one species. For example, the male and female rat exposed to benzophenone demonstrated no treatment-related liver tumor response, but in the thirteen-week studies there was increased relative liver weight, an increased incidence of hepatocellular hypertrophy and vacuolation, increased bile acids, and increased SDH levels. Likewise, Elmiron, while inducing increased relative organ weight and hepatocellular vacuolation in the male rat at thirteen weeks, did not induce an increased incidence of liver tumors after two years of treatment.

Similarly, when positive tumor outcomes are collectively considered for both sexes and both species of the cancer bioassay for these thirteen liver carcinogens, only one false negative was apparent. Inhalation exposure to indium phosphide resulted in liver tumors in the male and female mouse long-term bioassay, while there were no changes observed at thirteen weeks.

C. Discussion

Increased relative liver weight, histopathological changes, and increases in clinical pathology parameters in rat and/or mouse thirteen-week subchronic toxicity studies in the NTP database were positively associated with the majority of tumorigenic outcomes. As mentioned above, this concurs with a previous retrospective study using the NTP database (Allen et al. 2004).

Similar to the set of thirteen liver carcinogens examined here, Allen et al. (2004) demonstrated that an increased liver weight was associated with eight of eleven rat liver carcinogens. When considered as separate entities, hepatocellular hypertrophy identified five of eleven carcinogens, and hepatocellular necrosis identified four of eleven carcinogens. Pooling/grouping hepatocyte hypertrophy + necrosis + cytomegaly + increased liver weight identified eleven of eleven liver carcinogens.

Likewise, in another retrospective review of nine nongenotoxic NTP carcinogens, increased relative liver weight was the most highly specific predictor of mouse liver tumors (Elcombe et al. 2002). It has also been noted by the U.S. EPA (2002) that when hepatocellular hypertrophy (and corresponding increased liver size/weight) is accompanied by another more severe toxic change (e.g., clinical pathology changes/other histopathology changes), the combination of these changes may reflect underlying carcinogenic potential in rats and mice.

D. Conclusions

Conventional mammalian toxicological endpoints identified at thirteen weeks are associated with most tumor outcomes as mentioned above, but these indicators produce a number of false positives for compounds tested in the overall NTP database. Conventional endpoints such as increased relative liver weights and corresponding hepatocellular hypertrophy often represent temporal adaptations that demonstrate reversibility upon withdrawal of treatment. In future studies, it will be important to analyze the magnitude and dose response for these effects to determine whether predictivity can be improved. One chemical, indium phosphide, was a false negative on the basis of an absence of any treatment-related, conventional liver changes for male and female mice at thirteen weeks (as similarly reported by Allen et al. 2004). (Note: Supporting evidence for this chemical compound's tumorigenic response in the

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TABLE 4.—Positive hepatocellular tumor outcomes from National Toxicology Program (NTP) two-year rodent bioassays, with preneoplastic liver toxicological endpoints from corresponding subchronic (thirteen-week) toxicity studies in the mouse and rat.

Chemical	Female mouse	Male mouse	Female rat	Male rat
Anthraquinone (NTP 2005a)	↑ Relative organ weight Hypertrophy **Tumors**	↑ Relative organ weight Hypertrophy **Tumors**	↑ Relative organ weight Hypertrophy Bile acid **Tumors**	↑ Relative organ weight Hypertrophy **Equivocal tumors**
Benzophenone (NTP 2006)	↑ Relative organ weight Hypertrophy Bile acid ↑ ALT, ↑ SDH **Tumors**	↑ Relative organ weight Hypertrophy Bile acid ↑ ALT, ↑ SDH **Tumors**	↑ Relative organ weight Hypertrophy Vacuolation Bile acid ↑ SDH	↑ Relative organ weight Hypertrophy Vacuolation Bile acid
Decalin (NTP 2005b)	↑ Relative organ weight	↑ Relative organ weight	↑ Relative organ weight	↑ Relative organ weight
Elmiron (NTP 2004a)	↑ Relative organ weight Vacuolation **Tumors**[a]	↑ Relative organ weight Vacuolation **Tumors**[a]	↑ Relative organ weight	↑ Relative organ weight Vacuolation
Fumonisin B_1 (NTP 2001a)	(28-day study) Hypertrophy Necrosis Bile duct hyperplasia **Tumors**	No treatment-related liver changes	No treatment-related liver changes	(Allen et al. 2004) Degeneration Bile duct hyperplasia (28-day study) **Tumors**
Gallium arsenide (NTP 2000a)	No treatment-related liver changes	No treatment-related liver changes	"Clear evidence of carcinogenicity" (Allen et al. 2004) ↑ Organ weight (Allen et al. 2004) **Tumors**	↑ ALT
Indium phosphide (NTP 2001b)	**Tumors**	**Tumors**	(Allen et al. 2004) Necrosis **Tumors**	(Allen et al. 2004) Necrosis ↑ ALT Bile acid **Tumors**
Methyleugenol (NTP 2000b)	↑ Relative organ weight Necrosis Bile duct hyperplasia **Tumors**	↑ Relative organ weight Necrosis Bile duct hyperplasia **Tumors**	↑ Relative organ weight Hypertrophy Bile duct hyperplasia ↑ ALT Bile acid **Tumors**	↑ Relative organ weight Hypertrophy Bile duct hyperplasia ↑ ALT Bile acid **Tumors**
2-Methlyimidazole (NTP 2004d)	**Tumors**	↑ Relative organ weight **Tumors**	Liver enzyme **Equivocal tumors**	Liver enzyme **Equivocal tumors**
o-Nitrotoluene (NTP 2002a)	↑ Relative organ weight **Tumors**	↑ Relative organ weight	↑ Relative organ weight Bile acid **Tumors**	↑ Relative organ weight Vacuolation ↑ ALT Bile acid **Tumors**
Oxymetholone (NTP 1999)	No treatment-related liver changes	No treatment-related liver changes	↑ Relative organ weight **Tumors**	No treatment-related liver changes
Propylene glycol mono-t-butyl ether (NTP 2004b)	No treatment-related liver changes	No treatment-related liver changes	↑ Relative organ weight	↑ Relative organ weight **Equivocal tumors**
Riddelliine (NTP 2003)	↑ Relative organ weight Hypertrophy	↑ Relative organ weight Hypertrophy **Tumors**[a]	↑ Relative organ weight Hypertrophy Necrosis Altered foci Nodular hyperplasia Bile duct hyperplasia ↑ SDH **Tumors**[a]	Hypertrophy Necrosis Altered foci Bile duct hyperplasia **Tumors**[a]
Triethanolamine (NTP 2004c)	[Inadequate study (Allen et al. 2004)]—Helicobacter infection ↑ Relative organ weight	[Inadequate study (Allen et al. 2004)]—Helicobacter infection ↑ Relative organ weight	No treatment-related liver changes	No treatment-related liver changes
Urethane (NTP 2004e)	Altered foci **Tumors**	Altered foci **Tumors**	↑ Relative organ weight Vacuolation/degeneration Altered foci	↑ Relative organ weight Vacuolation/degeneration Altered foci Bile acid
Vanadium pentoxide (NTP 2002b)	No treatment-related liver changes	No treatment-related liver changes	No treatment-related liver changes	No treatment-related liver changes

[a] Hemangiosarcoma.

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mouse was demonstrated by increased incidences of nonneoplastic eosinophilic foci in a dose-response relationship [for both sexes], as compared to controls, at the two-year time point [as described in the NTP report].)

The authors conclude that conventional liver endpoints currently identified in subchronic (thirteen-week) toxicity studies in rats and mice are not adequate to identify all chemicals with carcinogenic potential.

Additional endpoints may identify other key events that might more accurately predict carcinogenic potential in rats and mice. These key events, in turn, will enhance analysis for defining MOAs to better assess human carcinogenic potential/risk. Specifically, these endpoints include increases in cell proliferation (S-phase response) and induction/inhibition of apoptosis (measurement of labeling indices for both events), constitutive androstane receptor (CAR) nuclear receptor activation (reporter assays), cytochrome P450 induction (direct biochemical measurement), and peroxisome proliferation (measurement of palmitoyl coenzyme A oxidase activity). Such key precursor events could be measured in short-term investigative studies, using three-, seven-, fourteen-, twenty-eight-, and/or ninety-day exposure scenarios.

Further key indicators may be identified from the variety of developing -omics technology platforms, particularly as MOA studies expand into exploring genomic signatures and pathway mapping associated with commonly accepted key events, including CAR activation and peroxisome proliferation.

VI. KIDNEY

The renal tumors referred to in this section are of renal tubular origin. The histologic changes are indicators of tubule injury or change.

A. Methods

Five of the sixteen chemicals identified in the NTP database produced tumors in the rat kidney. No kidney tumors were induced in mice. Four of these chemicals (benzophenone, decalin, Fumonisin B_1, methyleugenol) produced kidney tumors only in the male rat, not in the female. Anthraquinone produced tumors in both the female and male rat.

Initial evaluation for assessment of renal alterations after thirteen weeks of study included parameters that were reported in the histopathology tables by the NTP. The renal alterations and data presented included hyaline droplets, inflammation, chronic progressive nephropathy, and absolute and relative kidney weights. The histopathology evaluation was based on the NTP report, except for Fumonisin B_1, which was based on results of short-term studies that had been previously published (Dragan et al. 2001; Howard et al. 2001; Voss et al. 1995). Kidney weights for Fumonisin B_1 were not available.

Subsequently, and as part of a concurrent evaluation conducted by the NTP, an author of this article (Dr. Gordon Hard) reviewed the slides from male rat kidneys from thirteen-week studies for most of the sixteen chemicals (except Fumonisin B_1), including the additional histopathologic indicators of necrosis/apoptosis, hyperplasia, karyomegaly, vacuolization, tubular basophilia (not associated with chronic progressive nephropathy), and increased mitotic activity. The slides for Fumonisin B_1 were not reexamined during this review because they were not available. However, the slides for Fumonisin B_1 had been reviewed as part of another project (Hard et al. 2001; Bucci et al. 1998).

B. Results (Table 5)

All four chemicals that produced kidney tumors, and for which data were available regarding kidney weight at the thirteen-week time point (anthraquinone, benzophenone, decalin, methyleugenol), had elevated kidney weights (Table 5), both absolute and relative to body weight. For anthraquinone, kidney weight was elevated in both the female and male rats, and both sexes developed renal tumors. Benzophenone treatment increased kidney weight in both female and male rats, but tumors only occurred in the male. Decalin and methyleugenol increased the kidney weights and caused renal tumors only in male rats.

The kidney findings for all of the chemicals are listed in Table 5. The standard histopathologic criteria for evaluating the kidney resulted in a lack of detection of renal alterations after thirteen weeks of treatment with anthraquinone, benzophenone, decalin, or methyleugenol. There was evidence of regeneration associated with benzophenone and decalin treatment. In contrast, Fumonisin B_1 induced extensive apoptosis, and degenerative and regenerative changes at early time points (Dragan et al. 2001; Howard et al. 2001).

Additional targeted analysis that described renal alterations in greater detail than is typical in the standard NTP report demonstrated that there was a significant increase in renal tissue responses with a number of chemicals including the tumorigens. Hyaline droplets were present in female and male rat kidneys following anthraquinone administration, and decalin treatment in male rats only. Regenerative changes were present in the kidneys from male rats treated with benzophenone and decalin. Chronic progressive nephropathy was increased in female rats treated with anthraquinone to a limited extent but significantly in the male rats treated with anthraquinone. Inflammatory changes were also present in male rats treated with benzophenone and decalin. No changes were seen in the mouse kidneys for the five chemicals producing kidney tumors in rats except for nonspecific cellular alterations in male mice administered decalin.

Of the eleven chemicals evaluated in this study that did not produce kidney tumors, five (urethane, oxymetholone, 2-methylimidazole, propylene glycol t-butyl ether, and indium phosphide) produced alterations in the kidneys after thirteen weeks of treatment. Urethane produced nephropathy (not further defined) in male and female mice and male and female rats. Oxymetholone treatment resulted in an increase in kidney weight in the female mouse and in the female and male rat. In addition, there were regenerative changes in kidneys of the female and male rat administered oxymetholone. No renal lesions were seen in the male mouse treated with

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TABLE 5.—Histologic findings in thirteen-week studies with renal tumor outcome in corresponding two-year studies.

Chemical	Female mouse	Male mouse	Female rat	Male rat
Anthraquinone (NTP 2005a)	No renal lesions	No renal lesions	↑ Organ weight[a] Nephropathy Hyaline droplets **Tumors**	↑ Organ weight Nephropathy Hyaline droplets **Tumors**
Benzophenone (NTP 2006)	No renal lesions	No renal lesions	↑ Organ weight Casts Regeneration	↑ Organ weight Casts Regeneration **Tumors**
Decalin (NTP 2005b)	No renal lesions	Cellular alteration	No renal lesions	↑ Organ weight Casts Regeneration Hyaline droplets **Tumors**
Elmiron (NTP 2004a)	No renal lesions	No renal lesions	No renal lesions	No renal lesions
Fumonisin B_1 (NTP 2001a)	No renal lesions	No renal lesions	Apoptosis Regeneration **Tumors** (not statistically significant)	Apoptosis Regeneration **Tumors**
Gallium arsenide (NTP 2000a)	No renal lesions	No renal lesions	No renal lesions	No renal lesions
Indium phosphide (NTP 2001b)	No renal lesions	No renal lesions	Nephropathy	Nephropathy
Methyleugenol (NTP 2000b)	No renal lesions	No renal lesions	No renal lesions	↑ Organ weight **Tumors**
2-Methlyimidazole (NTP 2004d)	Hemosiderin	Hemosiderin	No renal lesions	Nephropathy
o-Nitrotoluene (NTP 2002a)	No renal lesions	No renal lesions	No renal lesions	No renal lesions
Oxymetholone (NTP 1999)	↑ Organ weight Bowman capsule metaplasia	No renal lesions	↑ Organ weight Regeneration Bowman capsule metaplasia	↑ Organ weight Regeneration Mineralization
Propylene glycol mono-t-butyl ether (NTP 2004b)	No renal lesions	No renal lesions	↑ Organ weight Regeneration	↑ Organ weight Regeneration Casts Hyaline droplets
Riddelliine (NTP 2003)	No renal lesions	No renal lesions	No renal lesions	No renal lesions
Triethanolamine (NTP 2004c)	No renal lesions	No renal lesions	No renal lesions	No renal lesions
Urethane (NTP 2004e)	Nephropathy	Nephropathy	Nephropathy	Nephropathy
Vanadium pentoxide (NTP 2002b)	No renal lesions	No renal lesions	No renal lesions	No renal lesions

[a] Increases in organ weights are relative and absolute.

oxymetholone. 2-Methylimidazole treatment resulted in increased nephropathy in the male rat. No renal alterations were present in the female rat, and there were no elevations of kidney weight in either sex of either species. Indium phosphide exposure resulted in chronic progressive nephropathy in both female and male rats. No increase in kidney weight or other kidney findings were found with indium phosphide.

C. Discussion/Conclusions

Based on this limited sample of chemicals that produced kidney tumors in rats in two-year bioassays, all caused detectable alterations after thirteen weeks of treatment. The feature that consistently gave a positive signal was the nonspecific finding of an increase in kidney weight, both absolute and relative. This is similar to what has been reported for rodent liver carcinogens (Allen et al. 2004). Significantly, all exposure groups that had no effects in the kidney after thirteen weeks of treatment had no renal tumors after two years, and all exposure groups that had tumors after two years had renal alterations at thirteen weeks.

In addition to kidney weight, the additional criteria including changes that indicate cell death (necrosis and/or apoptosis) and evidence of regeneration (basophilia, karyomegaly, mitoses) were not consistently diagnosed in the kidneys that were positive for rodent kidney carcinogens in the standard NTP study report. All of the rodent renal carcinogens could be detected in the thirteen-week assays due to diagnosis of hyaline droplets and increased chronic progressive nephropathy, in addition to the above lesions diagnosed on subsequent review. In this set of studies evaluated, there were no false negatives; however, there were false positives in that some exposures caused renal lesions after thirteen weeks, but no renal tumors in two-year bioassays. Thus, utilizing kidney weight and thorough histologic review of the kidneys after thirteen weeks of treatment detected all of the rodent renal tumorigens in this set of studies. For screening purposes, it is essential that false negatives do not occur.

This screening approach does not directly demonstrate mode of toxic or carcinogenic action, nor does it provide definitive information on likelihood of human carcinogenicity. However, the findings in these short-term studies, combined

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with the genotoxicity assessment, can provide helpful clues. For example, for Fumonisin B_1, the MOA appears to include the key events of stimulation of extensive apoptosis with significant regeneration that could lead to kidney tumors (Dragan et al. 2001). Such an MOA potentially could occur in humans. In contrast, the other four chemicals that induced kidney tumors in the two-year bioassay from the current group of chemicals produced kidney tumors by either an increase in chronic progressive nephropathy or by binding to α_{2u}-globulin (as indicated by increased hyaline droplets), leading to tubular cytotoxicity, regeneration, and eventually tumors. These two MOAs are detectable in the thirteen-week screening process. However, neither of these MOAs is considered to be relevant for human cancer risk (Dybing and Sanner 1999; Hard, Johnson, and Cohen 2009; Lock and Hard 2004).

VII. LUNG

A. Methods

This section reviews data from the NTP database obtained for the sixteen compounds in Table 1 and focuses on and focuses on evidence of histomorphologic alterations of the lung identified in thirteen-week studies in two species (B6C3F1 mice and F344 rats) and the presence or absence of lung tumors in these same species from two-year carcinogenicity studies. This evaluation attempts to draw correlations between the occurrences of pulmonary pathology identified in thirteen-week studies with the subsequent emergence of lung tumors. It is important to note that the routes of exposure are variable among the compounds tested and include dosing by drinking water, feed, and inhalation. Therefore, care must be exercised in interpreting the outcomes of localized intrapulmonary high particle burden versus systemic exposure.

As the purpose of this exercise was to identify signals in thirteen-week studies that might predict tumor generation, the data do not take into account the presence or absence of similar signals of inflammation or hyperplasia identified in the two-year bioassay itself. The analysis is only concerned with the presence of those signals at thirteen weeks under the conditions of that particular study. The lack of a lesion, such as inflammation, at thirteen weeks does not nullify a mechanistic association with the emergence of a tumor—only that it was not detected with these routine evaluations at a time point that would allow such signals to be consistent predictors of subsequent tumor formation. Such a lesion might yet occur at a time beyond thirteen weeks and possibly still be associated with the final tumorigenic outcome. Should this be the case, consideration would need to be given as to how it might be taken into account in developing a cancer hazard identification strategy based on the findings of the present study.

The following diagnostic terms for histomorphologic alterations were used by NTP to describe lung lesions in thirteen-week studies: chronic active inflammation, inflammation NOS (not otherwise specified), alveolar epithelial hyperplasia, bronchiolar hyperplasia, proteinosis, fibrosis, histiocytic infiltration, and foreign body. The following diagnostic terms for lung tumors were used by NTP in the two-year bioassay studies: alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma, alveolar/bronchiolar adenoma or carcinoma, and squamous cell carcinoma.

It is important to recognize that the diagnoses described herein are based solely on the written terms presented in various reports and tables in the NTP archives. As per NTP procedures, Pathology Working Groups reviewed the accuracy of lesion diagnoses and descriptive nomenclature at the time each study was conducted. However, examination of histology slides was not repeated for the purposes of this data review.

B. Results (Table 6)

The data in Table 1 show that the correlation of genotoxicity with lung tumor outcome is poor. Two (one equivocal) compounds were genotoxic but failed to induce lung tumors (2-methylimidazole and anthraquinone), three compounds were not genotoxic but did induce lung tumors (oxymetholone, gallium arsenide, vanadium pentoxide), and two compounds were positive for both genotoxicity and lung tumor formation (urethane, riddelliine). It is of value to note that lung tumors were identified in animals given compound by different routes of exposure (Table 6) that include inhalation (gallium arsenide, vanadium pentoxide, indium phosphide), oral gavage (oxymetholone, riddelliine), drinking water (urethane), and diet (o-nitrotoluene), suggesting that direct irritancy that might occur during inhalation is not a prerequisite for initiation of lung tumors, and that other mechanisms of action are also relevant.

As shown in Table 1, seven of the sixteen compounds were identified as inducing lung tumor formation in at least one cell of the two-year bioassay. Four of these seven compounds (urethane with/without 5% ethanol, vanadium pentoxide, indium phosphide, gallium arsenide) also had diagnoses of inflammation and/or hyperplasia at thirteen weeks (Table 6). For animals given urethane (with/without 5% ethanol), inflammation, hyperplasia, and lung tumors were seen only in male and female B6C3F1 mice, but not F344 rats. Riddelliine induced lung tumors only in female B6C3F1 mice without any prior diagnoses of inflammation or hyperplasia at thirteen weeks. Vanadium pentoxide was associated with inflammation, hyperplasia, and lung tumors in male and female B6C3F1 mice and male F344 rats; female F344 rats were without lung tumors. Indium phosphide was associated with inflammation, hyperplasia, proteinosis, fibrosis, foreign body at thirteen weeks, and lung tumors in the two-year studies in male and female B6C3F1 mice and male and female F344 rats. Gallium arsenide was associated with inflammation and hyperplasia in both species in the thirteen-week study, but lung tumors were only identified in female F344 rats in the two-year bioassay.

The presence of inflammation and/or hyperplasia at thirteen weeks without emergence of lung tumors at two years was seen in animals given Elmiron or benzophenone. The lung lesion identified in Elmiron-treated rats was a combination of chronic

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TABLE 6.—Lung tumor outcomes from National Toxicology Program (NTP) two-year rodent bioassays, with preneoplastic lung toxicological endpoints from corresponding subchronic (thirteen-week) toxicity studies in the mouse and rat.

Chemical	Female mouse	Male mouse	Female rat	Male rat	Correlation
Anthraquinone (NTP 2005a; diet)	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	Positive[a]
Benzophenone (NTP 2006; diet)	No inflammation No tumors	No inflammation No tumors	Inflammation No hyperplasia No tumors	Inflammation No hyperplasia No tumors	False positive[b]
Decalin (NTP 2005b; inhalation)	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	Positive
Elmiron (NTP 2004a; oral gavage)	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	Inflammation Histiocyte infiltration No hyperplasia No tumors	Inflammation Histiocyte infiltration No hyperplasia No tumors	False positive
Fumonisin B_1 (NTP 2001a; diet)	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	Positive
Gallium arsenide (NTP 2000a; inhalalation)	No inflammation Hyperplasia Histiocyte infiltration No tumors	No inflammation Hyperplasia Histiocyte infiltration No tumors	No inflammation Histiocyte infiltration No hyperplasia **Tumors**	No inflammation No hyperplasia Histiocyte infiltration No tumors	Positive
Indium phosphide (NTP 2001b; inhalation)	Inflammation Hyperplasia **Tumors**	Inflammation Hyperplasia **Tumors**	Inflammation Hyperplasia **Tumors**	Inflammation Hyperplasia **Tumors**	Positive
Methyleugenol (NTP 2000b; oral gavage)	No inflammation No tumors	No inflammation No tumors	No inflammation No tumors	No inflammation No tumors	Positive
2-Methlyimidazole (NTP 2004d; diet)	No inflammation No tumors	No inflammation No tumors	No inflammation No tumors	No inflammation No tumors	Positive
o-Nitrotoluene (NTP 2002a; diet)	No inflammation No hyperplasia	No inflammation No hyperplasia	No inflammation No hyperplasia	No inflammation No hyperplasia **Tumors**	False negative[c]
Oxymetholone (NTP 1999; oral gavage)	No inflammation No hyperplasia No bioassay	No inflammation No hyperplasia No bioassay	No inflammation No hyperplasia **Tumors**	No inflammation No hyperplasia No tumors	False negative
Propylene glycol mono-t-butyl ether (NTP 2004b; inhalation)	No inflammation No tumors	No inflammation No tumors	No inflammation No tumors	No inflammation No tumors	Positive
Riddelliine (NTP 2003; oral gavage)	No inflammation No hyperplasia **Tumors**	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	False negative
Triethanolamine (NTP 2004c; topical)	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	No inflammation No hyperplasia No tumors	Positive
Urethane (NTP 2004e; drinking water)	Inflammation Hyperplasia **Tumors**	Inflammation Hyperplasia **Tumors**	Inflammation No bioassay	Inflammation No bioassay	Positive
Vanadium pentoxide (NTP 2002b; inhalation)	Inflammation Hyperplasia **Tumors**	Inflammation Hyperplasia **Tumors**	Inflammation Hyperplasia No tumors	Inflammation Hyperplasia **Tumors**	Positive

[a] A positive correlation indicates that the results of the thirteen-week studies accurately predicted either the presence or absence of tumors in a bioassay in at least one species.

[b] A false positive correlation indicates that the data from the thirteen-week studies predicted that lung tumors should have been generated in the bioassay but were absent in all species tested.

[c] A false negative correlation indicates that the data from the thirteen-week studies would not have predicted the generation of tumors in the bioassays, but lung tumors were present in at least one species.

inflammation and infiltration of alveoli by histiocytes, and has been suggested to be a drug-induced lysosomal storage disorder (Nyska et al. 2002). The lesion identified in benzophenone-treated rats was identified only as chronic active inflammation. These data would be considered false positive findings for the thirteen-week studies as predictors for lung tumor formation.

False negative findings were identified for oxymetholone, riddelliine, and o-nitrotoluene based on the absence of lung pathology identified from the thirteen-week studies but

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positive findings of lung tumors in the two-year bioassays. Of these, riddelliine is considered clearly genotoxic. A review of the incidence data for the bioassay studies for each of these compounds clearly supports the identification of compound-induced lung tumors for each.

Seven of sixteen compounds (Fumonisin B_1, triethanolamine, propylene glycol mono-t-butyl ether, methyleugenol, 2-methylimidazole, anthraquinone, and decalin) that were given to males and females of both species had no lung pathology at thirteen weeks and no lung tumors at two years. These results were rated as being a positive correlation between the findings of the thirteen-week studies and the lack of lung tumors in the two-year bioassay.

Carcinogenicity studies were only conducted in a single species for oxymetholone or urethane.

C. Discussion/Conclusions

The presence or absence of inflammation and/or alveolar hyperplasia within the lung following thirteen weeks of exposure appeared to correlate with the presence or absence of lung tumors in eleven of sixteen of the chemicals tested, suggesting an association of events occurring after thirteen weeks of exposure with the ultimate expression of neoplasia. However, there were two false positives in which the identification of inflammation and/or alveolar epithelial hyperplasia did not correctly predict the emergence of tumors in a two-year study. There were two cases of compounds considered nongenotoxic in which lung tumors were identified in a two-year study in the absence of lung pathology in a thirteen-week study in either species tested. It is perhaps not surprising that two of the three compounds administered by inhalation (vanadium pentoxide and indium phosphide) induced the broadest and most consistent degree of pulmonary inflammation and subsequent lung tumors in all species tested. The association of particle burden-induced inflammation in the lung and the occurrence of lung tumors have been well studied (Oberdörster 1995), and the results of the analysis presented are consistent with previous findings.

VIII. SUMMARY AND CONCLUSIONS

Genotoxicity: Four of the sixteen chemicals were considered genotoxic based on NTP data (riddelliine, urethane, 2-methylimidazole, and the anthraquinone preparation). Fumonisin B_1 had some published positive genotoxicity data, and three others were considered equivocal genotoxins (decalin, indium phosphide, and o-nitrotoluene).

Immunosuppressive Activity: None of the sixteen chemicals showed evidence of direct immunosuppression at doses relevant to the bioassay. There was no clear evidence of neoplasia in elements of the immune system.

Liver: Six of the sixteen chemicals evaluated in the HESI CHIS project showed hepatocellular tumors in rats in the two-year bioassay. Of these six, one parameter alone (liver weight) correctly predicted five of six tumor outcomes. Grouping any other precursor with liver weight (i.e., hypertrophy, necrosis, vacuolation, degeneration, liver enzyme) resulted in six of six correct predictions. For mouse liver, nine of the sixteen chemicals showed hepatocellular tumors. Of these nine, liver weight correctly predicted six of nine tumor outcomes. Grouping other precursors with liver weight (i.e., hypertrophy and cellular foci) resulted in eight of nine correct predictions.

Kidney: Five of the sixteen chemicals showed kidney tumors in the rat two-year bioassay, and none caused kidney tumors in mice. All five chemicals caused detectable renal alterations in rats after thirteen weeks of treatment. The feature that consistently gave a positive signal was the nonspecific finding of an increase in kidney weight, both absolute and relative. The combination of kidney weight and a thorough histologic review of the kidneys after thirteen weeks of treatment detected all of the rodent renal tumorigens in this set of studies.

Lung: Seven of the sixteen chemicals produced tumors of the lung in either rats and/or mice. The presence of inflammation and/or alveolar hyperplasia in the lung following thirteen weeks of treatment was observed for four of these sixteen chemicals and for three others, suggesting some degree of a possible correlation between short-term events and the ultimate expression of neoplasia. Two compounds that were not clearly genotoxic produced lung tumors in the absence of any discernible precursor effects in the lung.

Overall Conclusions

> Cellular changes indicative of a tumorigenic endpoint can be identified for most, but not all, of the chemicals producing tumors in two-year studies after thirteen weeks of chemical administration using routine evaluations (see Table 7). Thirteen-week studies utilizing conventional endpoints are currently not adequate to identify all nongenotoxic chemicals that will eventually produce tumors in rats and mice after two years.
>
> Additional endpoints are needed to identify some signals not detected with routine evaluation. Such endpoints might include BrdU labeling and a measure of apoptosis.
>
> Detection of "critical" endpoints, or a critical magnitude of effect, in thirteen-week studies may help distinguish between chemicals that will and will not be tumorigenic after two years (i.e., exclude false positives).

The information obtained in the present study provides a foundation for developing alternative strategies for cancer hazard identification. However, a number of issues were identified that will need to be addressed before such a strategy can be implemented with confidence. A key component of the strategy is the identification of compounds that may be carcinogenic because

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TABLE 7.—Predictivity of carcinogenic effects on the basis of key events observed in thirteen-week studies in rodents (rats and mice).

Tissue	Correct prediction	Predictors
Liver–rat	5/6 (wt only) 6/6 combined predictors	Weight, hypertrophy, necrosis, vacuolation/degeneration, liver enzyme
Liver–mouse	6/9 (wt only) 8/9[a] combined predictors	Weight, hypertrophy, cellular foci
Kidney	5/5	Weight, necrosis/apoptosis, hyperplasia
Lung	4/7 6/7[b] (nongenotoxins)	Inflammation, hyperplasia
Urinary bladder	0/1	Not applicable

[a] The false negative, indium phosphide, was an equivocal genotoxin on the basis of the results of an *in vivo* micronucleus test.
[b] Riddelliine is genotoxic. o-Nitrotoluene was equivocal in an *in vivo* micronucleus test.

of their ability to damage DNA directly. For this purpose, a series of genotoxicity tests is used, that is, *in vitro* tests of mutagenicity and clastogenicity and an *in vivo* micronucleus test. While it was possible to classify some compounds as clearly genotoxic and clearly nongenotoxic, several were considered equivocal on the basis of the results of such tests. Hence, there is a need for a reliable battery of tests to ensure the identification of compounds that are potentially genotoxic carcinogens (Kirkland et al. 2007). Primarily, the emphasis in defining "genotoxic" carcinogens is often on DNA-reactive compounds, but the broader definition of genotoxicity includes a wide range of genomic damage/disturbance, including potential for aneuploidy induction, which is detectable in the micronucleus assay.

The strategy also relies upon the reliable detection of direct immunosuppressive effects of compounds from conventional endpoints measured in short-term studies (e.g., twenty-eight or ninety days). To the extent that it was possible to test this on the basis of the chemicals studied, the approach appears reliable. However, further work is necessary using a range of known positive and negative compounds.

The endpoints assessed in the thirteen-week studies were based on common key events in the MOAs that have been established for nongenotoxic carcinogens (e.g., organ weight as a surrogate for hyperplasia and inflammation). While many of the compounds that were carcinogenic caused signal effects in thirteen-week studies consistent with a nongenotoxic MOA, there were exceptions, particularly in lung and to a lesser extent in liver. However, a known limitation of the study was that the endpoints studied at thirteen weeks did not encompass all of the known key events for potential MOAs of concern. Hence, there was no direct information available on cell proliferation rate, hyperplasia, or apoptosis. For the proposed strategy to succeed, measures of these endpoints will need to be incorporated into conventional study design, or novel biomarkers of these effects will have to be developed and included in some screening level assessment. This could either be in short-term (perhaps even *in vitro*) or in longer-term (e.g., thirteen-week) studies.

The rapid advances in toxicogenomics hold promise of delivering biomarkers that will enable identification of the key biological pathways affected by chemicals. This should provide a basis for defining potential MOAs for these compounds (Frijters et al. 2007).

The present study was designed such that it was possible to evaluate the false negative rate of the proposed strategy. The false positive rate was not determined systematically—it would be necessary to evaluate all of the chemicals in the database over the interval 2000 to 2005. However, even with the limited number of chemicals studied here, it was apparent that the false positive rate in the sixteen that were carcinogenic in at least one of the target organs studied was not inconsiderable. Further work is necessary to determine the basis of this. It is possible that more detailed analysis of the magnitude of the response and the dose-response relationship for carcinogens and noncarcinogens would permit such discrimination. In the longer term, incorporation of some of the novel endpoints discussed above should enable much better discrimination between true and false positives.

The two-year bioassay in rats and mice is, at best, only an indicator of potential hazard. Where the MOA for the (nongenotoxic) carcinogenic response is known, it is apparent that the results of the two-year bioassay are frequently falsely positive with respect to risk of human carcinogenicity (Boobis et al. 2006; Cohen 2004; Holsapple et al. 2006; Meek et al. 2003). This suggests that findings in thirteen-week studies would also be falsely positive with respect to their relevance to cancer in humans. The goal of the proposed strategy is the detection of compounds that are potentially carcinogenic to humans. Hence, rather than having to detect all carcinogens in rats and mice by utilizing histopathologic and other biomarkers of key events for MOAs relevant to humans, such as degeneration, apoptosis, and regeneration, combined with knowledge of the pathways leading to these effects, it would be possible to focus effort on those compounds that are of potential concern. This is an issue that requires critical consideration, since the overall intent of these screening assays, whether two-year bioassays or otherwise, is to detect potential human carcinogens.

The association between MOAs and key events needs to be evaluated in terms of human relevance. Such an evaluation needs to include an understanding of exposure levels in terms of both compound kinetics and dynamics in the rodent and human model (Cohen 2004; Holsapple et al. 2006). It is therefore proposed that there should be a prospective approach to

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define and understand key carcinogenic events with a well-defined dose-response relationship. This information should then be used to determine human relevance in association with human exposure risk assessment (Cohen et al. 2004). This new approach would mitigate routinely relying on data from the two-year bioassay in rats and mice.

The successful development of a strategy such as that proposed here would enable a more mechanistic, science-based approach to the identification of cancer hazard of chemicals. It would provide a systematic means of implementing the insights provided by consideration of MOA and human relevance. Ultimately, the decisions made would be more reliable yet less resource-consuming.

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ABOUT HESI

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