Article

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The "false-positive" conundrum in the NTP 2-year rodent cancer study database

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Abstract

In 1990, Ames and Gold described a conundrum of "too many carcinogens" among chemicals tested in rodent bioassays. Their proposed nongenotoxic carcinogenic mechanism was amplification of the background mutation rate via cytotoxicity induced by high doses of the test chemicals, thereby leading to increases in reparative cellular proliferation rates. Recently, we have statistically and mechanistically analyzed the entire 594-study (470 final reports) NTP 2-year rodent cancer database to better understand the conundrum posed by Ames and Gold. Our analysis provides several lines of evidence that support the contention of Ames and Gold. First, across different routes of administration, relatively phylogenetically similar rats and mice are nonetheless discordant for the development of tumors at similar organ sites. Tumor site concordance across sex within species is higher than tumor site concordance across species. Second, many chemicals negative in the Ames test nonetheless induce tumors in either rats or mice. Third, 11 out of 58 chemicals tested by the inhalation route induce lung tumors in mice and not rats, are negative in the Ames test, and exhibit hyperplasia. In 2017, Tomasetti et al. provided evidence for the clinical relevance in humans of the Ames and Gold mechanism regarding amplification of the background mutation rate by demonstrating that the majority of human tumors result from accumulated mutations due to DNA replication errors.

Keywords

NTP, clinical relevance, background mutation, cellular proliferation, rodent carcinogens, Ames and Gold

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Forward

Prior to beginning the formal Introduction, it might be illustrative to preemptively summarize our conclusions in the name of clarity. First, we have not been able to provide a definitive estimate of the ability of the Ames test to predict the development of tumors in 2-year NTP rodent studies because of the lack of reproducibility of the in vitro genotoxicity assays conducted in different laboratories, at different times, not necessarily using the same protocols. Based on this reproducibility issue, we recommend that rather than relying on a weight-of-the-evidence approach, that the genotoxicity of a chemical under consideration be determined via state-of-the-art testing under Good Laboratory Practice (GLP) conditions, on samples validated for purity documented with a certificate of analysis, using generally accepted protocols conducted in certified laboratories. This philosophical approach is currently accepted by the United States Food and Drug Administration (USFDA). Second, we are not recommending abandonment of the high-dose levels administered to rodents in 2-year bioassays but rather consideration of the mechanism proposed by Ames and Gold for nongenotoxic chemicals that increase cellular proliferation rates as a reparative response to cytotoxicity. This mechanism is currently

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ignored by regulatory authorities. Third, it is not feasible to revisit the genotoxicity of chemicals already tested by NTP, with these recommendations intended for the evaluation of chemicals going forward. Fourth, chemicals of concern that are uniformly negative in state-of-the-art genotoxicity assays might not be appropriate for testing in 2-year rodent bioassays but rather for shorter time periods sufficient for the assessment of noncancer end points.

Introduction

> The Role of the National Toxicology Program is to support the evolution of toxicology from a predominantly observational science at the level of disease-specific models to a predominantly predictive science focused upon a broad inclusion of target-specific, mechanism-based, biological observations.[1]

NTP's intent is to expand the scientific basis for making public health decisions on the potential toxicity of environmental agents. Over the history of the NTP testing program, 594 different 2-year rodent bioassays have been conducted via different routes of exposure including inhalation, feed, gavage, drinking water, dermal, and intraperitoneal injection. These 594 bioassays resulted in successful completion of final NTP reports for 479 chemicals or chemical mixtures, with an additional three chemicals described in two Report on Carcinogens (RoC) reports.

In a series of five earlier publications, we first analyzed the results from 60 2-year inhalation studies conducted by NTP and showed a high level of discordance in tumor formation between rats and mice.[2] Next, we analyzed the results from 212 2-year feed studies conducted in F334/N rats and $B6C3F_1$ mice, and 31 2-year feed studies conducted in Osborne-Mendel rats and $B6C3F_1$ mice. The feed studies showed a higher degree of concordance within either male or female rats, or male and female mice, than between male rats and male mice, or female rats and female mice.[3] In the third study,[4] we analyzed the results from the following: 105 chemicals/chemical mixtures tested in 105 2-year studies conducted by exposing F334/N rats and $B6C3F_1$ mice via gavage; 18 different chemicals tested in 18 2-year gavage studies conducted in Osborne-Mendel rats and $B6C3F_1$ mice; 23 chemicals tested in 21 2-year studies conducted by exposing F334/N rats and $B6C3F_1$ mice via drinking water; 18 chemicals tested in 18 2-year studies conducted by exposing F334/N rats and $B6C3F_1$ mice via dermal application; and 11 chemicals tested in 11 2-year studies conducted by exposing F334/N rats and $B6C3F_1$ mice via intraperitoneal injection. The neoplasticity of each chemical was analyzed for tumor incidence by species–sex category, tumor site concordance across species, and tumor site concordance across sex within species. When available, the Ames *Salmonella* mutagenicity assay results, and any result from a test for genotoxicity other than the Ames test, were correlated with the neoplasticity results. These three studies completed the summary and analysis of the various routes of exposure.

The fourth and fifth studies considered the mechanistic aspects of the various factors potentially influencing tumor induction. In the fourth study,[5] each of the 470 chemicals was categorized from 1 to 48 by the level of "clear" neoplastic evidence in male and female rats, and in male and female mice, and given an ordinal rank from 1 to 135 following additional considerations regarding tumor site concordance and tumor multiplicity. The resultant tumorigenicity category score and ordinal rank score were examined for associations with results in the Ames *Salmonella* mutagenicity assay, presence or absence of structural alerts of carcinogenicity, and three Hansch QSAR parameters, that is, calculated base 10 logarithm of the octanol–water partition coefficient, calculated molar refractivity, and McGowan molecular volume. In the fifth study[6], the Environmental Protection Agency's (EPA) computer program called OncoLogic™ (Oncologic)[7] was used to predict the carcinogenic potential (or degree of concern) for the same chemicals tested in the NTP studies where actual experimental results were obtained. The qualitative Oncologic evaluations described by "degree of concern" were converted to "ordinal rankings of the Oncologic degree of concern" for each chemical in the following descending order: high > moderate–high > moderate > low–moderate > marginal > low–marginal > low. The relationship between the ordinal ranks predicted by Oncologic and the experimental ordinal ranks from the NTP studies on the same chemicals was examined via statistical analysis. In the fifth study, in addition to overall tumors, liver tumor data and median toxic dose (TD_{50}) data on chemicals tested by NTP were obtained from the National Center for Toxicological Research liver cancer database (NCTRlcdb). Of the 470 chemicals for which NTP prepared final reports, liver cancer and TD_{50} data on 128 chemicals were found in the NCTRlcdb. The NCTRlcdb data specifically indicate which rodent species and sex were induced with liver cancer. With this information, an ordinal scale was constructed to rank the concern associated with the liver cancer as follows: high > moderate–high > moderate > low–moderate > marginal > low–marginal > low. The ordinal rankings of the chemicals were used for the statistical analysis of the liver cancer data. Structural alerts data were obtained from information excerpted from Smith et al. (2018).[5]

The intent of this, the sixth paper of the series, is not to provide a comprehensive discussion of the major mechanisms of carcinogenesis. Rather the intent is to elucidate the major carcinogenic mechanism that is currently *not* considered in chemical regulation, that is, the mechanism described by Ames and Gold, whereby the background mutation rate is amplified via cellular proliferation due to cytotoxicity.

All malignant tumors result from the sequential accumulation of specific mutations.[8,9] In chemical carcinogenesis, the source of at least the initial mutation is a chemical

reaction with the cellular DNA.[10] EPA considers this genotoxic mechanism in its regulatory deliberations.[11] EPA also considers the nongenotoxic mechanism relevant to the impact of hormones on cancer risk.[12] Although the source of the cancer-causing mutations can be either exogenous (genotoxic mutagenesis) or endogenous (hormonally induced increase in cellular proliferation or cytotoxicity-induced increase in cellular proliferation), the final development of a malignant tumor is dependent on a specific series of changes to the cellular DNA.[13–15]

NTP considers results from the Ames assay test to be very important in its deliberations as illustrated by the following statement from a recent Report on Carcinogens (RoC)[16]:

> DNA reactivity combined with *Salmonella* mutagenicity is highly correlated with induction of carcinogenicity in multiple species/sexes of rodents and at multiple tissue sites.[17] A positive response in the *Salmonella* test was shown to be the most predictive *in vitro* indicator for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens).[18,19] Additionally, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone . . .

It should also be noted that although in vitro genotoxic assays, such as the Ames test, the micronucleus test, and the chromosomal aberration test, are considered standard tools for investigating chemical mutagenicity, the results of these methods are not necessarily indicative of carcinogenicity.[20]

To eliminate the introduction of selection bias into this analysis, all positive Ames assay *Salmonella* bacterial mutagenicity test results reported in the literature were accepted at face value. Similarly, any positive result in a test of genetic toxicity other than the Ames test was also accepted at face value. NTP's categorization of neoplastic evidence as either "positive" or "clear" was used to determine the tumorigenicity of the tested chemicals.

In the current analysis of the NTP database, we consider the conundrum of the "false-positive" rate most prominently described by Ames and Gold[21] in a classic paper published in 1991 but also noted by many other authors.[22–28] Herein, we provide support for the original hypothesis that the high doses used in 2-year rat and mouse cancer bioassays sometimes induce cellular proliferation via cytotoxicity and repair processes.[29,30] In addition, we suggest possible avenues toward at least a partial disentangling of the "false-positive" conundrum.

Lack of reproducibility in preclinical cancer studies

Prior to discussing the specific aspects and possible solutions to the "false-positive" conundrum in NTP 2-year rodent cancer bioassays, some perspective on the general issue of the lack of reproducibility in preclinical cancer studies might be helpful. At the onset of the process of analyzing the NTP database, our a priori expectation was that the concordance by tumor site between mice and rats was much higher than the analyses demonstrated[2–4] and that the reproducibility of the genotoxicity assays was much tighter.[5,6] After reviewing the results reported by Begley and Ellis[31] described below, we began ideating the interpretability of the NTP database within the larger context of current reproducibility problems in experimental biology. Therefore, although not directly related to this sixth paper of the series, the findings of Begley and Ellis[31] were conceptually helpful to us.

In 2012, Begley and Ellis[31] reported that scientists in the hematology and oncology department at Amgen had over the period of a decade attempted to confirm the published findings related to particular research topics of possible interest to Amgen. Fifty-three papers were deemed "landmark" studies and acknowledged that some of the data might not "holdup" because papers were deliberately selected that described something new, for example, "fresh approaches to targeting cancers or alternative clinical uses for existing therapeutics." These authors described themselves as shocked when scientific findings were confirmed in only 6 (11%) cases. In summary, the state-of-the-art laboratories at Amgen could only repeat 6 of the 53 reportedly landmark preclinical cancer studies. Similarly, it is not surprising that there is a lack of consistency in the tumorigenic potential noted in the NTP studies as compared to analogous tests conducted by others.[32,33] It is also not surprising that there is a serious challenge presented by a false-positive rate yet to be accurately quantitated.

The Ames and Gold hypothesis of "too many rodent carcinogens"

During the 1980s and 1990s, Ames and Gold developed a body of circumstantial evidence suggesting that the current paradigm of conducting 2-year relatively high-dose chemical exposures in rodent bioassays leads to a significant overestimation of the cancer risk to humans. First, they demonstrated a high background mutation rate in rodents.[21] Specifically, a very large oxidative damage rate to DNA occurs as part of normal metabolism with a steady-state adduct formation rate in each rat cell estimated at about 10^6 oxidative adducts and about 10^5 new adducts formed daily. Second, the high background adduct formation rate seen in rodents is related to their high baseline metabolic rate, short life span, and high age-specific cancer rate and stands in contrast with the long life span, lower metabolic rate, and lower age-specific cancer rate seen in humans.[21] Third, due to dietary intake, almost all of the chemicals to which humans are exposed are natural, not synthetic. (This is not to imply that the source rather than the chemical structure of a compound is the relevant factor in its toxicity.) Ames et al.[22] calculated that "99.99% (by weight) of the pesticides in the American diet are chemicals that plants produce to defend themselves." Fourth, at the high-dose levels used in 2-year NTP rodent cancer studies,

Table 1. Percentage of chemicals negative in Ames *Salmonella* mutagenicity test but inducing at least one tumor in rats or mice.

Routes of exposure	Total number of chemicals[a]	Total number of chemicals amenable for evaluation	Chemicals negative in the Ames test	Chemicals negative in the Ames test but induced a tumor either in rats or mice	Percentage of chemicals negative in the Ames test but induced a tumor either in rats or mice
Inhalation studies	60	58	39	34	34/39 (87%)
Feed studies (I)	212	212	132	74	74/132 (55%)
Feed studies (II)	35	35	28	17	17/28 (61%)
Gavage (I)	105	105	79	58	58/79 (73%)
Gavage (II)	18	18	9	6	6/9 (67%)
Drinking water	23	23	9	7	7/9 (78%)
Dermal	18	18	10	7	7/10 (70%)
Intraperitoneal injection	11	11	2	2	2/2 (100%)

I: F334/N rats and $B6C3F_1$ mice; II: Osborne-Mendel rat strain and $B6C3F_1$ mice; RoC: Report on Carcinogens.
[a]Total number of chemicals/chemical mixtures equals 482; 479 come from the 470 NTP reports and 3 come from 2 RoC reports.

mitogenesis increases mutagenesis by amplification of the background mutation rate via increased cell proliferation due to cytotoxicity-induced repair processes.[23]

Recent evidence for the clinical relevance of the Ames and Gold hypothesis

In 2017, Tomasetti et al. provided evidence for the clinical relevance in humans of the Ames and Gold mechanism regarding the amplification of the background mutation rate. These authors sought to determine the relative contribution to human cancers from inherited mutations, mutations induced by environmental factors, or mutations resulting from DNA replication errors (R). They compared the number of normal stem cell divisions with the risk of 17 cancer types occurring in 69 different countries. Tomasetti et al.[13] reported the following results:

> The data revealed a strong correlation (median = 0.80) between cancer incidence and normal stem cell divisions in all countries, regardless of their environment. The major role of R mutations in cancer etiology was supported by an independent approach, based solely on cancer genome sequencing and epidemiological data, which suggested that R mutations are responsible for two-thirds of the mutations in human cancers.

In summary, accumulated mutations due to DNA replication are the driving force behind the majority of human cancers, that is, mitogenesis increases mutagenesis in humans as well as in rodents.

Number of chemicals negative in any Ames Salmonella mutagenicity test that also induced tumors in NTP 2-year studies

Inhalation studies (results for 58 chemicals tested in 60 studies). Fifty-eight of the 60 chemicals ever tested via inhalation in 2-year NTP studies were amenable to evaluation.[2] Of the 58 chemicals tested, 39 were negative in the Ames test. Thirty-four Ames-negative chemicals nonetheless induced at least one tumor in either rats or mice representing a false-negative rate of 34/39 (87%; Table 1).

Feed studies (results for 247 chemicals tested in 243 studies). Of the 212 chemicals tested in 2-year feed studies in F334/N rats and $B6C3F_1$ mice, 132 chemicals were negative in the Ames test. Seventy-four of the 132 Ames-negative chemicals (74/132) showed clear neoplastic activity in at least one species/sex category. For F344/N rats and $B6C3F_1$ mice, the false-negative rate for the Ames test was 74/132 (55%; Table 1).[3]

NTP also conducted 31 2-year feeding studies on 35 different chemicals using the Osborne-Mendel rat strain and $B6C3F_1$.[3] Twenty-eight chemicals were negative in the Ames test. Seventeen Ames-negative chemicals induced at least one tumor in either rats or mice for a false-negative rate of 17/28 (61%; Table 1).

Gavage studies I (results for 108 chemicals tested in 106 studies in F344/N rats and $B6C3F_1$ mice). One hundred and five chemicals were tested via the gavage route in F344/N rats and $B6C3F_1$ mice. Seventy-nine of the 105 chemicals tested were negative in the Ames test. Fifty-eight Ames negative chemicals induced at least one tumor in either rats or mice for a false-negative rate of 58/79 (73%; Table 1).[4]

Gavage studies II (results for 18 chemicals tested in 18 studies in Osborne-Mendel rats and $B6C3F_1$ mice). Eighteen chemicals were tested via the gavage route in Osborne-Mendel rats and $B6C3F_1$ mice. Nine of the 18 chemicals tested were negative in the Ames test. Six Ames negative chemicals induced at least one tumor in either rats or mice for a false-negative rate of 6/9 (67%; Table 1).

Drinking water studies (results for 23 chemicals tested in 21 studies in F344/N rats and $B6C3F_1$ mice). Twenty-three chemicals were tested via the drinking water route in rats and mice. Nine of the 23 chemicals tested via drinking water

were negative in the Ames test. Seven of the nine Ames-negative chemicals induced at least one tumor in either rats or mice for a false-negative rate of 7/9 (78%).

Dermal studies (results for 18 chemicals tested in 18 studies in F344/N rats and B6C3F$_1$ mice). Ten of the 18 chemicals tested negative in the Ames test.[4] Seven of the 10 Ames-negative chemicals induced at least one tumor in either rats or mice representing a false-negative rate of 7/10 (70%; Table 1).

Intraperitoneal injection (results for 11 chemicals tested in 11 studies in F344/N rats and B6C3F$_1$ mice). Eleven chemicals were tested via the intraperitoneal injection route. Two of the 11 chemicals tested were negative in the Ames test. Both of the Ames-negative chemicals induced a tumor in rodents for a false-negative rate of 2/2 (100%; Table 1).

Chemicals negative for structural alerts of carcinogenicity that induced tumors

One hundred thirty-four of the 479 chemicals tested by way of inhalation, feed, gavage, drinking water, dermal administration, or intraperitoneal injection were negative in male and female rats and in male and female mice. Fifty-four of these 134 chemicals ubiquitously negative for neoplasia nonetheless contained a structural alert representing a false-positive rate of 40% (54/134).

There are 330 chemicals that induced at least one tumor. Of these 330 chemicals, 54 chemicals did not possess a structural alert of carcinogenicity resulting in a false-negative rate of 54/330 (16.4%).

Chemicals predicted by Oncologic as carcinogenic or potentially carcinogenic that did not induce tumors

One hundred thirty-four of the 479 chemicals tested by way of inhalation, feed, gavage, drinking water, dermal administration, or intraperitoneal injection were ubiquitously negative in male and female rats and in male and female mice. Fifty-eight of these 134 chemicals ubiquitously negative for neoplasia, nonetheless, had a concern rating from Oncologic of moderate (5 chemicals), low–moderate (31 chemicals), and marginal (22 chemicals) (indicating at least an equivocal carcinogenic activity) representing a false-positive rate of 43% (58/134).

There are 330 chemicals that induce at least one tumor in either rats or mice. The concern ratings from Oncologic are as follows: high (7), moderate–high (38), moderate (83), low–moderate (107), and marginal (45; indicating at least an equivocal carcinogenic activity), low (3; indicating unlikely to be carcinogenic), and inactive (47). Therefore, at least 50 chemicals (low + inactive) of 330 represent a false-negative rate of 50/330 (15.2%). If marginal ratings are included, the false-negative rate is 95/330 (28.8%).

Discussion

The original investigations of workplace-related cancers were initiated by the observations of unusual tumors in association with certain occupations including chimney sweeps,[34,35] mule spinners,[35,36,37] aniline dye workers,[38] clock workers using radium impregnated dye,[39] and chemical workers exposed to benzene.[40] Following detection in humans, the causative agents were later studied in animals.[41–46]

In general, the ongoing efforts to remove carcinogens from the workplace in developed countries have resulted in amelioration of exposure.[47] The paradigm has shifted from explanation to prophylaxis. In the current paradigm, chemicals of sufficient commercial interest or potential hazard as determined by in silico or in vitro methods are tested by the NTP in 2-year studies in rats and mice by the relevant route of administration. Several lines of evidence support the presence of a high "false-positive" rate in these 2-year rodent studies.

In the NTP database, we have identified 180 chemicals whose current genotoxicity test results are negative but that induce at least one tumor in either rats or mice (Table 2). Our examination of the NTP database suggests that it is not possible to retrospectively determine which of these 180 chemicals are definitively nongenotoxic due to questionable data quality. We are not suggesting that high doses should not be used in rodent bioassays but rather that an accurate state-of-the-art determination of the genotoxicity of a chemical should be conducted prior to deciding which chemicals to bioassay in rodents. This conundrum is discussed in Smith and Perfetti.[6]

First, as noted by Ames and Gold,[22] about half of all chemicals tested, whether man-made or naturally occurring, induce tumors in the rodents at the high doses used in these protocols.

As noted previously, sometimes testing nongenotoxic chemicals at high doses induces cytotoxicity which leads to tumor formation via amplification of background mutation rates, e.g. the classic case of bladder cancer induction by saccharin administration to male rats. We neither state nor imply that high doses should not be used in rodent 2-year bioassays, rather that tumors forming from nongenotoxic chemicals should not be assumed to be genotoxic based on tautologically inferred mechanisms such as oxidative stress. To be clear, we are also not saying that oxidative stress is not sometimes clinically relevant, but rather that it tends to be induced in rodents at high doses frequently not relevant to humans experiencing low levels of workplace exposure (see compounds in Table 2).

Second, extensive experimental evidence has demonstrated that increased mitogenesis leads to increased mutagenesis.[30,48] While beyond the scope of this particular article, the interactive effects of mitogenesis and mutagenesis occur across a spectrum rather than in discrete categories.[30] At one end of the spectrum, a completely

Table 2. List of chemicals negative in the Ames assay but inducing at least a single tumor in rats or mice.

Chemical tested (CASRN)	Overall Ames test	Route of administration
Procarbazine hydrochloride (CASRN 366-70 -1)	Negative	Intraperitoneal injection F344/N rats and B6C3F$_1$ mice
Methyleugenol (CASRN 93-15-2)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Tetrafluoroethylene (CASRN 116-14-3)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Chlorinated paraffins (C12, 60% chlorine; CASRN 108171-26-2)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Benzene (CASRN 71-43-2)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Dimethylvinyl chloride (1-chloro-2-methylpropene; CASRN 513-37-1)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
N, *N*-dimethyl-p-toluidine (CASRN 99-97-8)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Furan (CASRN 110-00-9)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
o-Anisidine hydrochloride (CASRN 134-29-2)	Negative	Feed F344/N rats and B6C3F$_1$ mice
A polybrominated biphenyl mixture (Firemaster FF-1; CASRN 67774-32-7) gavage	Negative	Gavage F344/N rats and B6C3F$_1$ mice
A polybrominated biphenyl mixture (firemaster FF-1; CASRN 67774-32-7) feed	Negative	Feed F344/N rats and B6C3F$_1$ mice
Technical grade chlordecone (Kepone; CASRN 143-50-0)	Negative	Feed Osborne Mendel Rats and B6C3F$_1$ Mice
Di(2-ethylhexyl)phthalate (CASRN 117-81-7)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Ethyl acrylate (CASRN 140-88-5)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Nitrilotriacetic acid (NTA) (CASRN 139-13-9) and nitriloacetic acid, trisodium salt (Na_3-NTA-H_2O) (CASRN 18662-53-8)	Negative	Feed F344/N rats and B6C3F$_1$ mice
o-Nitrotoluene (CASRN 88-72-2)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Bromodichloromethane (CASRN 75-27-4)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (CASRN 1746-01-6)	Negative	Gavage Osborne-Mendel rats and B6C3F$_1$ mice
Nitrobenzene (CASRN 98-95-3)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
o-Toluidine hydrochloride (CASRN 636-21-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Anthraquinone (CASRN 84-65 -1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Isoprene (CASRN 78-79-5)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Phenolphthalein (CASRN 77-09-8)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Cumene (CASRN 98-82-8)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Tetrachloroethylene (CASRN 127-18-4)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Nitrofen (CASRN 1836-75-5)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
Chlorendic acid (CASRN 115-28-6)	Negative	Feed F344/N Rats and B6C3F$_1$ Mice
2,4,6-Trichlorophenol (CASRN 88-06-2)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Cinnamyl anthranilate (CASRN 87-29-6)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Nitromethane (CASRN 75-52-5)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Tetrachlorvinphos (CASRN 961-11-5)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
Chloroform (CASRN 67-66-3)	Negative	Gavage Osborne-Mendel Rats and B6C3F$_1$ Mice
1,4-Dichlorobenzene (CASRN 106-46-7)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Pulegone (CASRN 89-82-7)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Reserpine (CASRN 50-55-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Beta-myrcene (CASRN 123-35-3)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
ICRF-159 (CASRN 21416-87-5) 4-[1-(3,5-dioxopiperazin-1-yl)propan-2-yl]piperazine-2,6-dione (Razoxane)	Negative	Intraperitoneal injection F344/N rats and B6C3F$_1$ mice
Antimony trioxide (CASRN 1309-64-4)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Salicylazosulfapyridine (CASRN 599-79 -1)	Negative	Gavage F344/N Rats and B6C3F$_1$ Mice
Pyridine (CASRN 110-86 -1)	Negative	Drinking Water F344/N rats and B6C3F$_1$ mice
Vanadium pentoxide (CASRN 1314-62 -1)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Benzofuran (CASRN 271-89-6)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
C.I. Direct Blue 218 (CASRN 28407-37-6)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Goldenseal root powder (Hydrastis canadensis) (CASRN 84603-60 -1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Naphthalene (CASRN 91-20-3)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
5-Chloro-*o*-toluidine (CASRN 95-79-4)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Androstenedione (CASRN 63-05-8)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Chlorobenzilate (CASRN 510-15-6)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice

(continued)

Table 2. (continued)

Chemical tested (CASRN)	Overall Ames test	Route of administration
Trim® VX	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
TRCP (CASRN 115-96-8)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Heptachlor (CASRN 76-44-8)	Negative	Feed Osborn-Mendel rats and B6C3F$_1$ mice
4-Methylimidazole (CASRN 822-36-6)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Pentachloroethane (CASRN 76-01-7)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
1,1,2,2-Tetrachloroethane (CASRN 79-34-5)	Negative	Gavage Osborne-Mendel rats and B6C3F$_1$ mice
Nalidixic acid (CASRN 389-08-2)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Allyl isovalerate (CASRN 2835-39-4)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
1,1,2-Trichloroethane (CASRN 79-00-5)	Negative	Gavage Osborne-Mendel rats and B6C3F$_1$ mice
N-methylolacrylamide (CASRN 924-42-5)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Diethanolamine (CASRN 111-42-2)	Negative	Dermal F344/N rats and B6C3F$_1$ mice
Aniline hydrochloride (CASRN 142-04-1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Coconut oil acid diethanolamine condensate (CASRN 68603-42-9)	Negative	Dermal F344/N rats and B6C3F$_1$ mice
Malonaldehyde, sodium salt (3-hydroxy-2-propenal, sodium salt; CAS No. 24382-04-5)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Zearalenone (CASRN 17924-92-4)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Chlordane (CASRN 57-74-9)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
Chlorothalonil (CASRN 1897-45-6)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
C.I. solvent yellow 14 (CASRN 842-07-9)	Negative	Feed F344/N rats and B6C3F$_1$ mice
N, *N*′-diethylthiourea (CASRN 105-55-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Hexachloroethane (CASRN 67-72-1)	Negative	Gavage Osborne-Mendel rats and B6C3F$_1$ mice
Methyl carbamate (CASRN 598-55-0)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Nitrofen (CASRN 1836-75-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
p, *p*′-DDE (CASRN 72-55-9)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
Fumonisin B1 (CASRN 116355-83-0)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Ethylbenzene (CASRN 100-41-4)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
2-Methylimidazole (CASRN 693-98-1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Furfural (CASRN 98-01-1)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
β-Picoline (CASRN 108-99-6)	Negative	Drinking water F344/N rats and B6C3F$_1$ mice
Talc containing no asbestos fibers (CASRN 14807-96-6)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
DEHP (CASRN 103-23-1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Tetrahydrofuran (CASRN 109-99-9)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Trimethylphosphate (CASRN 512-56-1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
1,1,3-Trimethyl-2-thiourea (CASRN 2489-77-2)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Ald (CASRN 309-00-2) and Die (CASRN 60-57 -1)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
Chlorinated paraffins (C23, 43% chlorine; CASRN 108171-27-3)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Isoeugenol (CASRN 97-54-1)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
11-Aminoundecanoic acid (CASRN 2432-99-7)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Daminozide (CASRN 1596-84-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Dimethyl hydrogen phosphite (CASRN 868-85-9)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Alpha-methylstyrene (CASRN 98-83-9)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Decalin (CASRN 91-17-8)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
5,5-Diphenylhydantoin (CASRN 57-41-0)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Formamide (CASRN 75-12-7)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Gallium arsenide (CASRN 1303-00-0)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ ice
D & C Red No. 9 (CASRN 5160-02-1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Dapsone (CASRN 80-08-0)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Monuron (CASRN 150-68-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
C.I. Vat Yellow 4 (CASRN 128-66-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Dicofol (CASRN 115-32-2)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
d-Limonene (CASRN 5989-27-5)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Melamine (CASRN 108-78 -1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Piperonyl sulfoxide (CASRN 120-62-7)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Tetralin (CASRN 119-64-2)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Benzophenone (CASRN 119-61-9)	Negative	Feed F344/N rats and B6C3F$_1$ mice

(continued)

Table 2. (continued)

Chemical tested (CASRN)	Overall Ames test	Route of administration
Methyl isobutyl ketone (CASRN 108-10-1)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Hydroquinone (CASRN 123-31-9)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Benzyl acetate (CASRN 140-11-4)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
2-Butoxyethanol (CASRN 111-76-2)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
t-Butyl alcohol (CASRN 75-65-0)	Negative	Drinking water F344/N rats and B6C3F$_1$ mice
Decabromodiphenyl oxide (CASRN 1163-19-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
1,2-Dichloropropane (propylene dichloride; CASRN 78-87-5)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Furfuryl alcohol (CASRN 98-00-0)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
2-Mercaptobenzothiazole (CASRN 149-30-4)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Molybdenum trioxide (CASRN 1313-27-5)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Nickel oxide (CASRN 1313-99-1)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Phenylbutazone (CASRN 50-33-9)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Sodium chlorate (CASRN 7775-09-9)	Negative	Drinking water F344/N rats and B6C3F$_1$ mice
Triamterene (CASRN 396-01-0)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Methylphenidate hydrochloride (CASRN 298-59-9)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Elmiron® (CASRN 37319-17-8)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
3,4-Dihydrocoumarin (CASRN 119-84-6)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Benzaldehyde (CASRN 100-52-7)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
DMMPA (CASRN 597-25 -1)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
CIMSTAR 3800	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Mercuric chloride (CASRN 7487-94-7)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
o-Benzyl-*p*-chlorophenol (CASRN 120-32-1)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Chlorodibromomethane (CASRN 124-48-1)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Furosemide (CASRN 54-31-9)	Negative	Feed F344/N rats and B6C3F$_1$ mice
N, *N*-Dimethylaniline (CASRN 121-69-7)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Isophorone (CASRN 78-59-1)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Stoddard solvent IIC (CASRN 64742-88-7)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
TMPTA (technical grade; CASRN 15625-89-5)	Negative	Dermal F344/N rats and B6C3F$_1$ mice
Tris(2-ethylhexyl)phosphate (CASRN 78-42-2)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
BBP (CASRN 85-68-7)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Dimethyl methylphosphonate (CASRN 756-79-6)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Chlorobenzene (CASRN 108-90-7)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
1,2-Dihydro-2,2,4-trimethylquinoline (CASRN 147-47-7)	Negative	Dermal F344/N rats and B6C3F$_1$ mice
Fenthion (CASRN 55-38-9)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Isobutene (CASRN 115-11-7)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Lauric acid diethanolamine condensate (CASRN 120-40-1)	Negative	Dermal F344/N rats and B6C3F$_1$ mice
α-Methylbenzyl alcohol (CASRN 98-85 -1)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Probenecid (CASRN 57-66-9)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
α,β-Thujone (CAS No. 76231-76-0)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Turmeric oleoresin (CASRN 8024-37-1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Diphenhydramine hydrochloride (CASRN 147-24-0)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Divinylbenzene HP (CASRN 1321-74-0)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ ice
Ethyl tellurac (CASRN 20941-65-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Eugenol (CASRN 97-53-0)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Manganese (II) sulfate monohydrate (CASRN 10034-96-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Oxytetracycline hydrochloride (CASRN 2058-46-0)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Parathion (CASRN 56-38-2)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
PETN (CASRN 78-11-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Phosphamidon (CASRN 13171-21-6)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
Rhodamine 6G (C.I. basic red 1) (CASRN 989-38-8)	Negative	Feed F344/N rats and B6C3F$_1$ mice
THC (CASRN 1972-08-3)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Acetaminophen (CASRN 103-90-2)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Acetonitrile (CASRN 75-05-8)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Alpha-methyldopa sesquihydrate (CASRN 41372-08-1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Ampicillin trihydrate (CASRN 7177-48-2)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Azinphosmethyl (CASRN 86-50-0)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice

(continued)

Table 2. (continued)

Chemical tested (CASRN)	Overall Ames test	Route of administration
2-Chloroacetophenone (CASRN 532-27-4)	Negative	Inhalation F344/N rats (exceptions noted) and B6C3F$_1$ mice
Chromium picolinate monohydrate (CASRN 27882-76-4)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Citral (microencapsulated; CASRN 5392-40-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
DCDD (CASRN 33857-26-0)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
Ald (CASRN 309-00-2) and Die (CASRN 60-57 -1)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
Dimethyl terephthalate (CASRN 120-61-6)	Negative	Feed F344/N rats and B6C3F$_1$ mice
2,5-Dithiobiurea (CASRN 142-46-1)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Fluometuron (CASRN 2164-17-2)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Gamma-butyrolactone (CASRN 96-48-0)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
4-Hexylresorcinol (CASRN 136-77-6)	Negative	Gavage F344/N rats and B6C3F$_1$ mice
Hydrochlorothiazide (CASRN 58-93-5)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Roxarsone (CASRN 121-19-7)	Negative	Feed F344/N rats and B6C3F$_1$ mice
N-Phenyl-2-naphthylamine (CASRN 135-88-6)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Picloram (CASRN 1918-02-1)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice
Polysorbate 80 (CASRN 9005-65-6)	Negative	Feed F344/N rats and B6C3F$_1$ mice
PG (CASRN 121-79-9)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Rotenone (CASRN 83-79-4)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Sodium fluoride (CASRN 7681-49-4)	Negative	Drinking water F344/N rats and B6C3F$_1$ mice
Dibutyltin diacetate (CASRN 1067-33-0)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Pyrazinamide (CASRN 98-96-4)	Negative	Feed F344/N rats and B6C3F$_1$ mice
Pyrimethamine (CASRN 58-14-0)	Negative	Feed F344/N rats and B6C3F$_1$ mice
TDE (CASRN 72-54-8)	Negative	Feed Osborne-Mendel rats and B6C3F$_1$ mice

TRCP: tris(2-chloroethyl) phosphate; DEHP: di(2-ethylhexyl)adipate; Ald: aldrin; Die: dieldrin; DMMPA: dimethyl morpholinophosphoramidate; TMPTA: trimethylolpropane triacrylate; BBP: butyl benzyl phthalate; PETN; pentaerythritol tetranitrate; THC: 1-trans-delta[9]-tetrahydrocannabinol; DCDD: 2,7-dichlorodibenzo-*p*-dioxin; PG: propyl gallate.

nongenotoxic but cytotoxic chemical can induce tumors. However, many chemicals are both genotoxic and cytotoxic to some degree and the impact on tumorigenicity will be some nonlinear combination based on the quantity and qualitative characteristics of the induced mutations and the robustness of the proliferative response.[30]

Third, mice and rats can produce very different responses to chemicals despite their phylogenetic similarities.[63–66] Mice and rats separated along the evolutionary tree sometime between 12 and 24 million years ago. In contrast, humans separated from rodents approximately 80 million years ago.[49] Differential responses in rats and mice to chemicals can be influenced by idiosyncratic P450 metabolism, immune system surveillance, DNA repair mechanisms, macroanatomical differences (e.g. airways), and microanatomical differences (e.g. predominance of Clara cells lining mouse lung epithelium).[50–54] Rats and mice in the NTP database are highly discordant in both the development of tumors and the site of tumor formation, despite their relative phylogenetic closeness. This high discordance begs the question of the potential degree of tumor discordance between humans and rodents.

Fourth, of the 482 chemicals tested by NTP that resulted in the writing of a final report or were described in the two additional RoC reports, 331 were negative in the Ames *Salmonella* mutagenicity test. Two hundred and four of these 331 Ames-negative chemicals induced tumors in either rats or mice (204/331, 62%). If NTP's contention that no battery of in vitro mutagenicity tests is better than just the Ames *Salmonella* mutagenicity assay alone in predicting tumorigenicity in rats and mice,[18,19,55] then the high level of tumor induction by Ames-negative chemicals is consistent with a significant rate of nongenotoxic cytotoxicity-induced cellular proliferation leading to tumor formation. In addition to the large number of Ames-negative chemicals that nonetheless induce at least one rodent tumor, of the 331 chemicals that induced at least one tumor, 54 chemicals did not possess a structural alert of carcinogenicity resulting in a false-negative rate of 54/331 (16.3%). Similarly, Oncologic predicts that 50 chemicals predict either low or inactive carcinogenic potential for a false-negative rate of 50/331 (15.1%). If marginal ratings are included, the false-negative rate predicted by Oncologic is 95/331 (28.7%). However, it should be noted that the relatively low false-negative rates predicted by structural alerts of carcinogenicity and Oncologic are depressed by the high false-positive rates of 40% for structural alerts and 43% for Oncologic.

Chemicals that have achieved the status of becoming candidates for NTP 2-year studies usually fill an important market niche and have outcompeted other chemicals on efficacy, price, safety, ease of manufacture, handling, shipping, or disposal. (One of the factors in the decision to prioritize a chemical for testing is the potential for exposure of workers, the general population, and the environment. Chemicals, especially high-volume chemicals in general

commerce, are more likely to be associated with the above noted exposures.) Replacing such an important chemical due to artifactual rodent carcinogen results with another chemical could be disadvantageous economically and also from a human and environmental health perspective. Given the presence of a significant but hard to quantify induction of tumors by nongenotoxic chemicals, there are several actionable steps that can be taken to better address the artifactual "false-positive" rate of 2-year rodent bioassays. First, although the carcinogenic risk to humans cannot be determined from rodent bioassays, the relative tumorigenicity of NTP-tested chemicals can be compared with one another by either ordinal or percentile ranking. Second, across the NTP database, the prediction accuracy of both positive and negative Ames test results was very low when any single positive or negative Ames test result is accepted at face value. The low prediction accuracy of historical Ames test results suggests that expert panels in collaboration with regulatory agencies would be better served by relying on recently conducted in vitro genetic toxicology test batteries performed under GLP conditions. Finally, small subject number, noninvasive, human evaluations can be conducted on workers potentially exposed to inhaled agents to determine whether the real-world exposures are eliciting pulmonary inflammation that could lead to increased epithelial cell proliferation.[56–60]

Conclusions

Circumstantial evidence from statistical analysis of the entire NTP database strongly suggests that each of the predictive methods for estimating the potential carcinogenicity of chemicals possesses some degree of overestimation of the actual carcinogenic effect. In summary, historical Ames test values, structural alerts of carcinogenicity, and Oncologic, all overpredict the carcinogenicity of chemicals in rodents supporting the hypothesis of Ames and Gold that positive rodent carcinogen bioassays include tumors formed from both mutations induced by the test agent and mitogenic amplification of the background mutation rate. According to Ames and Gold, rodent tumors induced as a result of high doses of the test agent inducing mitogenesis in the absence of exogenous mutation do not represent a comparable risk to humans exposed at noncytotoxic levels.

A voluminous body of evidence demonstrates that mitogenesis increases mutagenesis in both rodents and humans. Current practice by regulatory agencies is to consider any tumor induced in 2-year rodent cancer bioassays to have been produced by genotoxic mechanisms other than in a few specialized cases. This supposition significantly overestimates the risk of cancer to humans from real-world low-dose exposure to chemicals. To prevent a large number of important chemicals from being deselected from the marketplace, chemicals that induce tumors in 2-year NTP-type rodent cancer bioassays should be given consideration as potentially occurring due to increased mitogenesis if a relevant battery of in vitro and in vivo genotoxicity assays conducted in a certified laboratory under GLP conditions and using a highly purified test sample are negative. This consideration of a potential mitogenic mechanism should be given additional weight if the chemical does not possess structural alerts of carcinogenicity or a significant degree of concern in Oncologic, especially given the high false-positive rate for these two predictive methods. Bronchioloalveolar carcinomas in mice represent an initial candidate for a rodent tumor being induced due to mitogenesis rather than agent-induced genotoxicity.[2]

Rather than being consistent with the Precautionary Principle, ignoring the voluminous literature on the important role of mitogenesis in carcinogenesis, and the recent demonstration of the critical role played by DNA replication errors in human carcinogenesis, is inconsistent with NTP's stated goal "... to support the evolution of toxicology from a predominantly observational science at the level of disease-specific models to a predominantly predictive science focused upon a broad inclusion of target-specific, mechanism-based, biological observations." A "best science" approach should follow the law of parsimony and accept that chemicals testing negative for genotoxicity in state-of-the-art, appropriately selected, and well-conducted test batteries are in fact not genotoxic. Further, when chemicals clearly shown not to be genotoxic also induce only a modest or limited tumorigenic response in rodents as determined in comparison with the entire NTP database, the Ames–Gold mitogenic mechanism should be readily considered, an example of this is di(2-ethylhexyl)phthalate.[61]

The authors propose the following:

- First, an accurate state-of-the-art determination of genotoxicity should replace the current expert panel evaluation that uses a weight-of-the-evidence approach that intermingles results of indeterminate validity.
- Second, nongenotoxic chemicals should not be tested in 2-year rodent bioassays. These chemicals should be subjected to shorter duration bioassays to examine noncancer effects.[62]
- Third, a much greater emphasis should be placed on the role of pulmonary inflammation in the development of human disease. In most modern workplaces, dermal and oral exposure is easier to control for (and eliminate) than is exposure to small amounts of inhaled chemicals. Noninvasive determinations of the inflammatory status of exposed workers are much more relevant to human risk assessment than are the results seen in the high-dose arms of rodent bioassays conducted on nongenotoxic but cytotoxic chemicals.[58]
- Fourth, the current paradigm of using high doses in the carcinogen testing of genotoxic chemicals does not currently have an in vitro or in silico replacement. The United States Environmental Protection

Agency (USEPA) has recently issued a draft document on the replacement of animal testing by non-animal methods, but this work is in its initial phases (https://www.fda.gov/Drugs/DevelopmentApprovalProcess/SmallBusinessAssistance/ucm069906.htm). We do not mean to imply that this is problematic but rather it is the specific case of nongenotoxic chemicals inducing tumors due to cytotoxicity that presents a challenge to interpretability.

In summary, if one major improvement could be made in the current system, it would be to rapidly and definitively make an accurate determination of the genotoxicity of a given chemical prior to entering the decision tree of whether and how to bioassay in rodents. The pre-Investigational New Drug (IND) meeting system currently employed by USFDA serves as a model of this type of collaboration between industry and government. The current reliance on historical and recent genotoxicity testing clouds rather than clarifies the situation by introducing a high degree of unnecessary subjectivity into the process.

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