In Vivo Transgenic Bioassays and Assessment of the Carcinogenic Potential of Pharmaceuticals

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There is general agreement in the scientific community on the need to improve carcinogenicity testing and the assessment of human carcinogenic risk and to incorporate more information on mechanisms and modes of action into the risk assessment process. Advances in molecular biology have identified a growing number of genes such as protooncogenes and tumor-suppressor genes that are highly conserved across species and are associated with a wide variety of human and animal cancers. In vivo transgenic rodent models incorporating such mechanisms are used to identify mechanisms involved in tumor formation and as selective tests for carcinogens. Transgenic methods can be considered an extension of genetic manipulation by selective breeding, which long has been employed in science and agriculture. The use of two rodent species in carcinogenicity testing is especially important for identifying transspecies carcinogens. The capacity of a substance to induce neoplasia across species suggests that the mechanism(s) involved in the induction of the neoplasia are conserved and therefore may have significance for humans. Based on available information there is sufficient experience with some in vivo transgenic rodent carcinogenicity models to support their application as complementary second species studies in conjunction with a single 2-year rodent carcinogenicity study. The optional substitution of a second 2-year rodent carcinogenicity study with an alternative study such as an in vivo transgenic carcinogenicity study is part of the International Conference on Harmonization guidance S1B: Testing for Carcinogenicity of Pharmaceuticals. This guidance is intended to be flexible enough to accommodate a wide range of possible carcinogenicity assessment models currently under consideration or models that may be developed in the future. The use of an in vivo transgenic mouse model in place of a second 2-year mouse study will improve the assessment of carcinogenic risk by contributing insights into the mechanisms of tumorigenesis and potential human relevance not available from a standard 2-year bioassay. It is envisioned that this will stimulate the further development of more efficient and relevant methods for identifying and assessing potential human carcinogenic risk, which will benefit public health. — Environ Health Perspect 106(Suppl 1):71–80 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/suppl-1-71-80contrera/abstract.html

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Introduction

Application of new approaches for assessment of the carcinogenic potential of pharmaceuticals such as in vivo transgenic rodent models is a major challenge for the scientific community, industry, and regulatory agencies in part because of the complexity of the carcinogenic process. Cancer is a multistage process associated with changes in the integrity or expression of genomic DNA (1–5). Advances in molecular biology have identified a growing number of genes associated with cancer that include those operationally classified as protooncogenes and tumor-suppressor genes. Many of these genes are highly conserved across species and are associated with a wide variety of human and animal cancers including cancer of the colon, lung, breast, esophagus, stomach, skin, prostate, and the hematopoietic and central nervous systems (6–13).

Transgenic rodents have yielded important new insights into carcinogenic mechanisms and some transgenic models also have potential application for identifying carcinogens (14,15). Transgenic methods can be considered an extension of genetic manipulation by selective breeding that has long been employed in science and agriculture to produce animals with desired phenotypic characteristics and relatively uniform genotype. Transgenic animals useful as cancer models have been developed that contain regulated transgenes, unexpressed reporter genes, or knocked-out alleles of tumor-suppressor genes. Information derived from in vivo transgenic carcinogenicity models can contribute additional insights (16,17) into the mechanism of carcinogenesis and possible human risk that may be of greater value and potential relevance to humans than information from a second conventional 2-year rodent study. Transgenic rodent models incorporating appropriate human protooncogenes or altered tumor-suppressor genes may be better animal surrogates for human cancer assessment than wild-type rodents currently used in carcinogenicity studies.

Application of new approaches such as transgenic rodent carcinogenicity models should advance toxicology and regulatory science and ultimately benefit public health by applying more efficient and more relevant methods for evaluating potential human carcinogenic risk. Promising transgenic rodent carcinogenicity models are currently being extensively characterized and many more will undoubtedly be developed in the future. Regulatory authorities can play an important role in fostering this process by demonstrating a willingness to apply new scientifically acceptable methods through more flexible policies that accommodate improved, innovative approaches for assessing potential human carcinogenic risk.

For human pharmaceuticals, clinical considerations determine the need for...
carcinogenicity studies and influence the benefit-risk assessment. Clinical considerations include the expected duration of treatment, severity of the disease or disorder, nature of the patient population (e.g., children, elderly), extent of human exposure, the availability of other therapies, and toxicity profiles of other available therapies. A weight-of-evidence approach is used to assess the potential carcinogenicity of human pharmaceuticals. This approach includes the evaluation of human and animal metabolic and pharmacokinetic data. The availability of human systemic exposure and metabolism data from clinical trials and the controlled medical use of human prescription drugs are advantages that often are not readily available for evaluating potential human risk in other regulatory areas (e.g., environmental or industrial chemicals). This information is used to assess the adequacy of the rodent models for extrapolation to humans and for estimating the relevant human risk of pharmaceuticals.

**Principal Elements of the Weight-of-evidence Assessment of Carcinogenic Potential**

Evaluation of the results of carcinogenicity studies (including alternative models) and genotoxicity studies include:

- Magnitude of compound-related tumor findings compared to those for the concurrent control
- Evidence of compound-related tumors in two species (transspecies effect)
- Evidence of tumors at multiple sites
- Evidence of tumors in males and females
- Evidence of a compound-related increase in rare tumors
- Evidence of an increased incidence of compound-related fatal tumors and/or tumors with short latencies (appearing before terminal sacrifice)
- Evidence of dose-related tumor findings
- Evidence of a significant increase in the incidence of a tumor or tumors that are histopathologically analogous to human tumors
- Evidence of tumors at common sites in two species
- Evidence suggesting that carcinogenic response elements in the test species are similar to those known to be involved in human cancer (common mechanism)
- Evidence of genotoxicity
- Relative significance of carcinogenicity findings in the context of historical data

Examples of assessment factors in the consideration of the adequacy of the test model include:

- Adequacy of doses used and suitability of the route of administration
- Bioavailability of the test compound in the rodent test model
- Pharmacokinetic profile of the test compound in rodent models and humans
- Metabolic profile of the test compound in rodent test models and humans
- Pharmacodynamic comparability of the test model to humans
- Appropriate application of International Conference on Harmonization (ICH) guidelines

Risk–benefit considerations include:

- Comparison of the clinical systemic exposure at the estimated maximum daily recommended dose to the systemic exposure in the rodent test models
- If systemic exposure information is lacking, the estimated exposure ratio should be based on the surface area (mg/m²) estimation of dose in the test species and the clinical maximum daily recommended dose
- Severity of the disease or disorder and the availability and relative efficacy of alternative therapies
- Toxicity profile of available alternative therapies
- Size and composition of the patient population and the frequency and extent of exposure

**Utility of Two Rodent Species in the Assessment of Carcinogenic Potential**

The rodent carcinogenicity databases for pharmaceuticals from the United States, the European Union, and Japan were evaluated by the ICH safety expert working group to assess the utility of two rodent species for carcinogenicity testing. This analysis supported a more flexible approach to the current practice of 2-year carcinogenicity studies in two rodent species. The U.S. Food and Drug Administration (U.S. FDA) carcinogenicity study database used in this analysis contained 282 pharmaceuticals, with carcinogenic studies in both rats and mice (28). There is a significant (74%) concordance in the results of rat and mouse carcinogenicity studies in the U.S. FDA database and 78% of all compounds with positive tumor findings were identified in a single rat study (rat positive predictivity). This is in accord with the results of the databases of the other ICH regions and the rodent carcinogenicity database of U.S. National Toxicology Program (U.S. NTP) (19,20) and with the Carcinogenic Potency Database (CPD) (21–23). Mouse carcinogenicity studies have a relatively lower overall positive predictivity than rat studies, identifying only 64% of all compounds with positive tumor findings. For compounds that produced tumors only in mice, 71% were single-site tumors. Of the compounds that produced tumors only in rats, 78% were single-site tumors.

In considering the contribution of the second rodent species carcinogenicity study, it was recognized that identifying compounds with transspecies tumorigenic effects is an important component of the weight of evidence for assessing human carcinogenic potential. Compounds that produce transspecies tumors are considered to pose a relatively higher risk to humans than single-species positive compounds (24,25). It is postulated that the capacity of a drug to induce a similar neoplasia across species suggests that the mechanism(s) involved in the induction of the neoplasia are conserved and therefore may have significance for humans. Thus, compounds that produce transspecies tumors generally are considered potentially more hazardous than compounds with single-species, single-site tumor findings. There are, however, circumstances for which single-species findings can have regulatory implications. The strength of the finding, nature of the tumor findings (e.g., rare tumors, especially those histologically similar to human tumors), evidence of genotoxicity, and the degree of similarity in drug metabolism and pharmacokinetics compared to these findings for humans all contribute to the weight-of-evidence approach used to assess the potential human carcinogenic risk. The severity and morbidity associated with the clinical indication and the availability and toxicity of alternative therapies are also important considerations that influence the overall assessment of acceptable risk for human pharmaceuticals.

A major regulatory concern in relying on the results of a single carcinogenicity study in a rodent species is that although all transspecies carcinogens would be detected using a single-species study, it would be impossible to identify transpecies carcinogens. In the U.S. FDA database (18) 52 of 125 drugs (42%) with tumor findings are transspecies positive. Within this group there is a relatively high proportion of unmarketed drugs, older drugs, and drugs marketed with restricted
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clinical use related to carcinogenicity findings. Transspecies rodent tumor findings also have contributed significantly to regulatory decisions that prevented the marketing of drugs or resulted in the removal of drugs from the market.

Single-rodent species tumor findings can also influence the weight of evidence in the assessment of potential cancer risk. Benzene is carcinogenic in the mouse but not the rat and there is sufficient evidence in humans to consider it a human carcinogen. Methapyrilene, an antihistamine, was removed from the market in the United States primarily because of hepatocarcinogenicity with short latency in rats (26). The International Agency for Research on Cancer (IARC) classified the tranquilizer oxazepam as a possible human carcinogen (IARC classification 2B) mainly on the strength of mouse liver findings (27).

Site concordance is not as important as might be anticipated for pharmaceuticals except, for example, when specific receptor-mediated mechanisms of carcinogenesis are implicated. The tumor site concordance of rats and mice is relatively poor. In the U.S. FDA, U.S. NTP, and CPD databases only 22, 26, and 33%, respectively, of all compounds with positive findings produced tumor findings in at least one common site in the rat and mouse (18,19,22). The lack of site specificity has been cited as part of the rationale for a reduced 2-year study protocol using only male rats and female mice (28,29). If tumor site cannot be reliably predicted between rodent species, extrapolation of rodent tumor sites to humans is also questionable (30). There appears to be better transspecies tumor-site concordance across species for a relatively small select subset of IARC 1, 2A, and 2B classified pharmaceuticals (31). This apparent concordance may be related to the genotoxic nature of a majority of the non-hormonal compounds in this group (32); even here concordance is not as drug development.

Elimination of the second rodent species carcinogenicity study would significantly reduce the evidence available for regulatory decisions regarding potential carcinogenic risk and would not improve the assessment of potential human carcinogenic risk or advance the state of regulatory science. In the absence of a second species study, regulatory decisions would be necessary to be based solely on the results of a conventional 2-year carcinogenicity study in one species, with important regulatory consequences. In this situation more reliance would be placed on positive tumor findings in a single study. In the absence of information to the contrary, these findings could effectively be regarded as equivalent to positive findings in two rodent species.

Use of an in Vivo Transgenic Rodent Model in Place of a Second 2-Year Rodent Carcinogenicity Study

A variety of carcinogenic agents may exert their effects by a common mechanism such as the activation of protooncogenes or inactivation of tumor-suppressor genes. In vivo transgenic rodent carcinogenicity models can be used to identify mechanisms involved in tumor formation and as selective tests for carcinogens (33). The optional substitution of a second 2-year rodent carcinogenicity study with an alternative study such as an in vivo transgenic carcinogenicity study is an approach that would contribute new insights into the mechanisms of tumorigenesis and potential human relevance. This option is part of the ICH guidance S1B: Testing for Carcinogenicity of Pharmaceuticals (34), which is intended to be flexible enough to accommodate a wide range of possible carcinogenicity assessment models that may be developed in the future as well as to address test models currently under consideration. Test models noted in the ICH guidance currently under consideration include initiation–promotion models, the neonatal mouse tumorigenicity assay, and in vivo transgenic rodent models. In the ICH guidance it is proposed that a single 2-year carcinogenicity study be carried out in the rodent species that best models human metabolic, pharmacokinetic, and pharmacodynamic parameters. If the rat and mouse appear equivalent in these respects, the rat likely would be considered the standard rodent model because of the greater amounts of information generally available from supporting pharmacology and toxicology studies carried out during drug development.

There is general agreement in the scientific community on the need to improve assessment of carcinogenic risk and incorporate more information on mechanisms and mode of action into the risk-assessment process. These views were expressed by the U.S. NTP Board of Scientific Counselors (35) and IARC (36). Principles for the application of mechanisms of carcinogenesis in risk assessment were presented by IARC and include evidence of genotoxicity, i.e., structural changes at the gene level and evidence of effects on the expression of relevant genes, which would include alterations in the structure or quantity of the product of a protooncogene or tumor-suppressor gene. Evidence that similar mechanisms are acting in humans is considered important in evaluating the relevance of animal findings. For these reasons, positive findings in transgenic models could contribute more to the weight-of-evidence assessment than equivocal tumor findings in a second standard 2-year rodent study.

Transgenic models have been developed that contain regulated transgenes, unexpressed reporter genes, or knocked-out alleles of tumor-suppressor genes. Because of the relative specificity of transgenic carcinogenicity models or other factors that may influence assay sensitivity (e.g., treatment schedule, route of administration, differences in metabolism, and systemic exposure), negative findings in a single transgenic model do not rule out carcinogenic potential in the rodent. They may, however, eliminate some possible mechanisms of tumor formation. Similarly, in a standard 2-year rodent study a negative response in one strain of mice does not necessarily rule out the possibility of a significant positive finding in another mouse strain or in the rat. The significance of negative findings in any in vivo carcinogenicity bioassay is also influenced by the toxicity of the test compound relative to the minimal carcinogenic dose. For toxic compounds the maximum tolerated dose for a 2-year study could be less than the dose necessary to produce a significant tumor response and could also result in a lower systemic exposure than that attained in humans at the maximum therapeutic dose (37). The shorter duration of exposure to test compounds for alternative carcinogenicity models (e.g., 26 weeks for transgenic models) may allow test animals to tolerate relatively higher exposures to toxic compounds than would be feasible in a 2-year carcinogenicity study.

Regulatory Application of Transgenic Mouse Models

Although not all of the mechanisms involved in the induction of cancer are known, there is a large and growing body of evidence on the critical role tumor-suppressor genes and protooncogenes play in the induction of cancer in animals and humans (38–40). The p53 gene is an example of a major tumor-suppressor gene that is highly conserved across species and is involved in a high proportion of human cancers (41,42). The p53 gene product is involved in regulation of the fundamental
processes of apoptosis and DNA repair, which play important roles in cancer formation (43,44). Humans with Li Fraumeni syndrome are at greater risk of cancer because of a deficient p53 allele (45), and aflatoxin-induced hepatocarcinogenesis in humans has been associated with p53 and ras oncogene mutations (46).

Transgenic mice that are deficient in one or both p53 gene alleles have been developed. Heterozygous p53-deficient mice have accelerated tumor responses and low spontaneous tumor rates over a large portion of their lifespan, which allows the use of a smaller number of animals (15-20/group vs 50 or more/group) and shorter treatment periods (24 vs 104 weeks) than a standard 2-year mouse carcinogenicity study. In contrast to heterozygous p53-deficient mice, homozygous mice have relatively short lifespans and a high spontaneous incidence of lymphoma and leukemia, which makes them less desirable test models for carcinogenicity. The p53-deficient mouse is particularly sensitive to genotoxic carcinogens (47). An operational definition of genotoxicity is applied for pharmaceuticals in which a compound that is positive in one or more of the standard genotoxicity test batteries may be considered genotoxic. The standard genotoxicity test battery for pharmaceuticals includes a test for bacterial mutation, a test for chromosomal damage in mammalian cells, and an in vivo test for chromosomal damage in rodent bone marrow [ICH guidance S2A and S2B, available on the internet (34)]. Although pharmaceuticals are screened for genotoxicity and strongly positive compounds generally are eliminated early in drug development, compounds often are developed that have weak or equivocal results in one or more genotoxicity tests that comprise the standard test battery or in genotoxicity tests that are not part of the battery. These compounds would be especially suited for testing in carcinogenicity assays responsive to genotoxic compounds such as the p53-deficient heterozygous mouse. A critical question addressed by this approach is the possible relevance of any genotoxicity findings to any carcinogenic response.

In studies with compounds tested in 2-year mouse studies and the p53-deficient mouse, there is evidence that the tumor site in p53-deficient heterozygous mice was also found in the 2-year mouse study, although not all sites found in the 2-year studies were represented in transgenic studies (15,47). Information derived from transgenic or other alternative models can also be used to elucidate the significance of tumors in a standard 2-year mouse study.

Some potential applications of an alternative test method for carcinogenicity follow:

- As an alternative to a second 2-year rodent carcinogenicity study for pharmaceuticals
- As a complementary confirmatory study for drugs with equivocal carcinogenicity findings in 2-year rodent studies when such findings would adversely influence risk–benefit considerations
- As a preliminary carcinogenic screen to set priorities for full carcinogenicity testing (e.g., retrospective testing of products approved before rodent carcinogenicity studies were routinely required)
- As an alternative to repeating a 2-year rodent carcinogenicity study. A 2-year rodent study may not be adequate for a variety of reasons such as inadequate test animal survival, inappropriate dosing or route of administration, or other factors related to a change in clinical indication or human exposure
- To assess the carcinogenic potential of genotoxic contaminants or degradants that were not present in a drug product when evaluated in 2-year rodent carcinogenicity studies

For example, phenolphthalein was positive in a U.S. NTP 2-year rat and mouse carcinogenicity study; it produced thymic lymphoma, histiocytic sarcoma, and ovarian tumors in the mouse and renal and adrenal tumors in the rat (48). There was also some evidence of genotoxicity from studies including those not generally considered part of the standard genotoxicity battery of tests for pharmaceuticals. Concern for the carcinogenic potential of phenolphthalein was based largely on the mouse hematopoietic tumor findings. To gain further insight into these findings and their potential human significance, the U.S. FDA tested phenolphthalein in the p53-deficient heterozygous mouse in addition to the further studies of the pharmacokinetics and comparative metabolism of phenolphthalein in relation to humans. Phenolphthalein produced dose-related thymic lymphomas in the p53-deficient heterozygous mouse similar to what was observed in B6C3F1 mice in the 2-year study and at similar doses (49). The thymic tumors of phenolphthalein-treated p53-deficient mice were analyzed and found to have lost their wild-type p53 allele (loss of heterozygosity). Thymic lymphoma tumor tissues from the 2-year mouse study were subsequently analyzed for alterations in the level of p53 protein and there was evidence of a increase in nuclear p53 protein (49). The latter finding was considered further confirmation of a treatment-related effect. Alterations in the level of p53 protein have been observed in human tumors such as adenosquamous carcinoma of the pancreas (50). The results from the phenolphthalein p53-deficient transgenic mouse study and the known shared tumor-suppressor function of the p53 gene in rodents and humans contributed information for the weight-of-evidence assessment of the potential human relevance of the rodent tumor findings. This example also demonstrates the value of genetic analysis of tumor tissues from 2-year rodent carcinogenicity studies when there is information from transgenic models or other sources suggesting a specific carcinogenic mechanism that can be appropriately evaluated.

In a second example of the potential value of transgenic models, a low incidence of hepatoblastoma, a rare liver tumor type in mice that also occurs in children, was found in a U.S. NTP mouse carcinogenicity study of methylphenidate. This finding resulted in a notification letter to physicians. To further evaluate the carcinogenic mechanisms associated with this finding, the suitability of the mouse for human extrapolation, and the margin of safety of methylphenidate, a p53-deficient mouse study and a neonatal mouse study were carried out in addition to comparative metabolism and pharmacokinetic studies. No evidence of a carcinogenic response was observed in these studies, which contributed to the weight of evidence that suggested that the risk to humans is minimal.

Protooncogenes can be activated by processes such as point mutations, gene amplification, chromosomal translocation, and retroviral activation. Cellular oncogene products can be nuclear proteins (e.g., myc), cell membrane proteins (e.g., ras), growth factors (e.g., sis), or growth factor receptors (38). Major ras oncogenes include the Harvey (Ha-ras) associated with human epithelial tumors and rodent tumors, the Kirsten (Ki-ras) linked to mesenchymal tumors, and the neoblastoma (N-ras) (51–53). Ras oncogenes represent a large family of related genes linked to cell proliferation and known to function in various human and animal cancers. In mice, ras mutations are found in spontaneous tumors and chemically induced tumors. The type and frequency of ras
mutations in chemically induced tumors often differs from the ras mutations observed in spontaneous tumors (40, 54). Promising in vivo transgenic rodent models employing ras oncogenes include the TG.AC v-Ha-ras oncogene-based mouse model (15,47) and the human c-Ha-ras transgene-based mouse model (55, 56). A transgenic mouse model incorporating the human c-Ha-ras gene is under extensive evaluation in Japan and preliminary results indicate that it can identify transspecies genotoxic carcinogens and may also detect nongenotoxic carcinogens (Table 1) (56).

The xerodermia pigmentosa (XPA)-deficient mouse is another transgenic mouse currently being characterized. In humans the XPA gene is involved in DNA nucleotide excision repair and an XPA-gene deficient mouse model has been developed (57). Mutation or deletion of the XPA gene is associated with enhanced ultraviolet light in B frequency (UV-B)-induced skin tumors and may also be associated with an increased incidence of a wide range of systemic tumors in humans (58). Transgenic XPA-deficient mice are analogous to XPA-gene deficient humans and are hypersensitive to UV-B-induced skin tumors and also appear sensitive to chemically induced tumors. Although promising, the human c-Ha-ras transgenic mouse model and XPA-deficient mouse model have not yet been applied in a regulatory setting.

In the TG.AC transgenic mouse car cinogenicity model, the test compound is topically applied to the shaved skin of mice daily for 20 to 26 weeks and skin papillomas are produced in response to a carcinogen. The skin of the mice in this model behaves in a manner analogous to genetically preinitiated skin (59). The TG.AC model is believed to be sensitive to both genotoxic and nongenotoxic carcinogens (15,47) and may be particularly well suited for the carcinogenicity testing of topically applied and dermal products. It may also have application for assessing photocarcinogenicity, although the utility of this application has not yet been adequately evaluated.

The class 1 IARC carcinogen benzene and the class 2A IARC carcinogen 1,3-butadiene have been used as example compounds to support the utility of standard 2-year mouse carcinogenicity studies for risk assessment. Carcinogens are classified by IARC on the basis of the strength of human, animal, and mechanistic evidence supporting potential human carcinogenic risk. For IARC class 1 compounds there is sufficient human evidence that the compound is carcinogenic. Compounds considered probable human carcinogens are classified in IARC 2A and those considered possibly human carcinogens are classified in 2B. Benzene, which is carcinogenic in a 2-year mouse study and negative in a 2-year rat study, was positive in the TG.AC transgenic mouse and the p53-deficient mouse (Table 1) (15). 1,3-Butadiene is genotoxic and carcinogenic in the 2-year mouse assay. Therefore it is likely that this substance would be positive in the p53-deficient mouse, which is particularly sensitive to genotoxic compounds. Other mouse-only carcinogens in the U.S. NTP database such as chloroethene and nitrofurantoin are genotoxic and contain structural alerts for mutagenicity that make them also likely to be positive in the transgenic mouse models discussed above.

Table 1. Identification of transspecies-positive compounds a or known, probable, or possible human carcinogens (IARC groups 1, 2A, or 2B) by transgenic mouse models.

| Compound                      | TG.AC mouse | p53-deficient mouse | Ras H2 mouse |
|-------------------------------|-------------|---------------------|--------------|
| Sodium arsenite²              | Positive    | NT                  | NT           |
| IARC group 1 human carcinogen| Positive    | Positive            | NT           |
| Negative rat and mouse studies| Nonmutagenic|                    |              |
| Benzone                       | Positive    | NT                  | NT           |
| IARC group 1 human carcinogen| Positive    | Positive            | NT           |
| Nonmutagenic; genotoxic       |             |                     |              |
| p- Cresidine                  | Positive    | Positive            | NT           |
| IARC group 2B possible human  | Positive    | NT                  | NT           |
| carcinogen                    | Mutagenic   |                    |              |
| Mutagenic; weakly genotoxic   |             |                     |              |
| 1-Chloro-2-methylpropene      | Positive    | NT                  | NT           |
| Mutagenic                     |             |                     |              |
| Cyclophosphamide              | NT          | NT                  | Positive     |
| IARC group 1 human carcinogen| NT          | NT                  | NT           |
| Nonmutagenic                  |             |                     |              |
| N-N-Diethylnitosamine         | NT          | NT                  | Positive     |
| Mutagenic                     |             |                     |              |
| 7,12-Dimethylbenz[a]anthracene| Positive    | NT                  | NT           |
| Mutagenic                     |             |                     |              |
| Estradiol                     | NT          | NT                  | NT           |
| IARC group 1 human carcinogen| Positive    | NT                  | NT           |
| Nonmutagenic                  |             |                     |              |
| Ethyl acrylate                | Negative    | NT                  | NT           |
| Nonmutagenic; genotoxic       |             |                     |              |
| Ethylene thiourea             | NT          | NT                  | Positive     |
| Nonmutagenic                  |             |                     |              |
| M-Methyl-N-nitro-N-nitosoguanidine| NT    | NT                  | Positive     |
| Mutagenic                     |             |                     |              |
| M-Methyl-N-nitrosourea        | NT          | NT                  | Positive     |
| Mutagenic                     |             |                     |              |
| Methylazoxymethanol           | NT          | NT                  | Positive     |
| Mutagenic                     |             |                     |              |
| Mirex                         | Positive    | NT                  | NT           |
| IARC group 2B possible human  | NT          | NT                  | Positive     |
| carcinogen                    | Nonmutagenic; nongenotoxic |          |              |
| 4-Nitroquinoline-1-oxide      | NT          | NT                  | Positive     |
| Mutagenic                     |             |                     |              |
| Phenolphthalein²              | NT          | Positive            | NT           |
| Nonmutagenic; weakly genotoxic|            |                     |              |
| Urethane³                     | Positive    | NT                  | NT           |
| IARC group 2B possible human  | NT          | NT                  | Positive     |
| carcinogen                    | Mutagenic   |                     |              |
| Vinyl carbamate               | NT          | NT                  | Positive     |
| Mutagenic                     |             |                     |              |
| Vinyl-1-cyclohexene diepoxide  | NT          | NT                  | Positive     |
| Mutagenic; genotoxic          |             |                     |              |

Abbreviations: Genotoxic, findings generally related to clastogenicity; mutagenic, positive in vitro mutagenicity tests (e.g., Ames/Salmonella test); NT, not tested or test results not available. *Four-cell compounds are those that were tested in 2-year rat and mouse carcinogenicity studies and found to produce tumors in male and female rats and mice. °Data from Tennant et al. (15,47) and Yamamoto et al. (56). °Tennant RW, personal communication. *Data from Dunnick et al. (49).
In U.S. NTP/National Institute of Environmental Health Sciences (NIEHS) studies of the TG.AC mouse, which used 26 compounds tested in 2-year rat and mouse studies representing 0-, 1-, 2-, and 4-study cell-positive compounds (study cells = rat, mouse, male, female), there was concordance between the TG.AC mouse and the results of 2-year bioassays. Essentially all of the 4-cell positive compounds (transspecies in 2-year studies) were positive in the TG.AC transgenic mouse (Table 1). The IARC 2B compound Mirex is positive in a 2-year rat study (mouse not tested) and the TG.AC mouse and the IARC 2B carcinogen p-cresidine is positive in 2-year rat and mouse studies and also in the TG.AC and p53-deficient mouse (15,47).

The selection of an alternative carcinogenicity study is expected to be scientifically justified and should include how the study can contribute additional mechanistic information that may be useful for interspecies extrapolation and the weight of evidence assessment of carcinogenic potential. Issues related to the appropriateness of the model, such as route of administration, operational characteristics of the model, level of characterization and degree of experience with the model and any relevant toxicologic issues associated with the particular pharmaceutical (e.g., genotoxicity) should be addressed. This approach would not significantly reduce—and may even enhance—the weight of evidence available for assessing potential human carcinogenic risk. Alternatively, a sponsor may conduct a second 2-year rodent carcinogenicity study.

In the United States all proposals for the use of any alternative model are reviewed by the U.S. FDA Center for Drug Evaluation Carcinogenicity Assessment Committee (CAC) prior to their initiation when requested by the sponsor. The CAC reviews the justification for the use of a particular model, the appropriateness of the model, protocol design, and dosing and offers concurrence. Concurrence on the acceptability of alternative studies can be granted only when they are formally reviewed and approved by the CAC.

Interspecies Extrapolation

Both human and animal metabolism and pharmacokinetic information are available for human pharmaceuticals and are critical components of the risk-assessment process for both conventional rodent studies and transgenic studies. In considering the results of 2-year rat and mouse studies or transgenic rodent studies, emphasis is placed on findings from the rodent model that best approximate human metabolic and pharmacokinetic parameters. This approach was not possible until the relatively recent advent of improved chemical analytical methods for measuring the concentration of drug and major metabolites in the blood of humans and experimental animals. Comparative pharmacokinetic and metabolism information is also important for dose and test model selection for carcinogenicity studies. An evaluation of the relative systemic exposures of drugs tested at the maximum tolerated dose compared to human systemic exposure at the therapeutic dose indicated that the comparison of dose as a function of body weight generally significantly overestimates the actual relative systemic exposure of the test animal by 6- to 10-fold. For the estimation of comparative systemic exposure, dose expressed as a function of body surface area (mg/m²) is superior to dose expressed as body weight (mg/kg) (37). The ratio of the plasma area under the concentration–time curve for parent drug and major metabolites in rodents and humans is used to estimate the relative systemic exposure of human pharmaceuticals ("Principal Elements of the Weight-of-evidence Assessment of Carcinogenic Potential"). These approaches are applied to rodents in 2-year carcinogenicity studies and transgenic animals as was the case for phenolphthalein.

Validation of Carcinogenicity Bioassays

For regulatory application it is important to use bioassays that have been sufficiently characterized. In a discussion of the application of transgenic rodents in carcinogenicity testing, it is necessary to objectively consider the relative strengths and deficiencies of transgenic models in the context of the strengths and deficiencies of the standard 2-year rodent carcinogenicity study. The acceptability of the 2-year rodent carcinogenicity study was based on the reasonable assumption of a relationship between a biological outcome in animals (neoplasia) and potential human carcinogenic risk. Study protocols evolved over time until a relatively standard protocol developed, influenced by practical considerations of statistical power, assay sensitivity, and economic and resource considerations. The current study gained acceptance by the scientific and regulatory community after accumulation of a sufficient body of experience and demonstrated ability to identify compounds reasonably expected to be carcinogens based on human and other data. Regulatory agencies began to apply the results of rodent carcinogenicity studies on the basis of relatively limited experience with these assays. Although concern has been raised about the application of transgenic models for quantitative risk assessment, the 2-year rodent carcinogenicity study was originally intended to be a qualitative screen for potential carcinogens and may be poorly suited for quantitative risk assessment (60).

Results of transgenic and 2-year studies will not always agree, especially for weaker (single species) carcinogens associated with species-specific mechanisms that may be less significant for human extrapolation. Lack of concordance between methods can also be attributable to spurious findings related to assay variability. In the assessment of potential human carcinogenic risk based on animal studies, first priority should be given to transspecies carcinogens that may be more likely to represent human risk. A critical basic parameter for acceptability of any carcinogenicity test should be the test's demonstrated ability to detect known human carcinogens. There are relatively few (approximately 21) pharmaceuticals identified as group 1 human carcinogens by IARC. The relatively small number of known human carcinogens may be due partly to the limitations of epidemiologic approaches for identifying cancer-causing agents. Compounds that are negative in a 2-year rat and mouse study are considered noncarcinogenic, although factors such as the statistical power of the bioassay, choice of rodent strain, species, or route of administration (61), or the extent of exposure achieved can influence the outcome. In addition it is essentially impossible to demonstrate that compounds that are negative in 2-year studies are noncarcinogenic in humans.

Pharmaceuticals evaluated by IARC and considered human carcinogens are mainly genotoxic substances, immunosuppressants, or hormonal compounds. Most of the nonhormonal organic compounds are mutagens and most of those tested in two rodent species are transspecies carcinogens (25). It often is stated in support of rodent carcinogenicity studies that all known human carcinogens are rodent carcinogens; however, it also should be stated that many of these compounds were not identified in a standard 2-year rodent carcinogenicity protocol. Approximately half the IARC group 1, 2A, and 2B human carcinogens with animal studies were tested
by the intravenous or intraperitoneal route for less than 2 years and in a variety of rodent strains and study protocols (31,62). The positive findings of these studies are considered valid, but equivalent negative studies generally would not be considered adequate evidence of no carcinogenic potential. Although many of the IARC group 1 and 2 compounds were not tested in standard 2-year rodent study protocols, most are expected to be positive in the 2-year rodent bioassay based on their genotoxicity and carcinogenic potency in nonstandard tests. A similar case can be made for transgenic rodent carciogenesis models and it is expected that most of the IARC group 1 and 2 compounds will be positive in many of the transgenic rodent models currently under consideration.

Evaluation of new approaches for assessing carcinogenic potential must be put into proper perspective. General criteria for validation of toxicology assays endorsed by the NIEHS Interagency Coordinating Committee for the Validation of Alternative Methods include assessment of the repeatability and reproducibility of the method at multiple laboratory sites. False positive and false negative rates should be evaluated using appropriate reference compounds in a coded blinded fashion (63). The current 2-year rodent carcinogenicity study was never validated in this manner and there is limited evidence supporting the repeatability and reproducibility of the results of the current rodent carcinogenicity study. Two-year rodent carcinogenicity studies for pharmaceuticals rarely are repeated because of their considerable cost in time and resources. When they are repeated it is generally because of failure of the original study or protocol deficiencies and therefore the results are rarely comparable because of protocol differences between studies (e.g., dose or strain differences). The reproducibility of control findings in the same study, however, can be estimated in carcinogenicity studies that incorporated a protocol with separate duplicate control groups. The U.S. NTP compiled and analyzed the results of carcinogenicity studies of 18 dyes conducted by three trade associations in the same strain and source of rats and mice and using a protocol that included separate duplicate control groups. In 12 of 18 of the studies, statistically significant differences were found in a comparison of the tumor findings in otherwise identical control groups (64). These findings suggest that 2-year rodent carcinogenicity studies may have poor reproducibility because of relatively high levels of variability in the spontaneous tumor incidence rate within studies. This supports the need for a second species carcinogenicity study as a confirmatory study and is in agreement with our experience with pharmaceutical carcinogenicity studies using dual identical controls. An increasing number of carciogenicity studies for pharmaceuticals incorporate two identical but separately housed and analyzed control groups to assess variations in the spontaneous tumor incidence rate within a study.

Genetically inbred rodent strains are used in 2-year rodent carcinogenicity bioassays to minimize variability in tumor responses attributable to genetic differences. Rodent strains currently used in carcinogenicity studies were not selected on the basis of their suitability as human surrogates but for pragmatic reasons such as reasonable sensitivity to carcinogens and acceptable spontaneous tumor rate, lifespan, and animal size. The large accumulated historical record and experience with currently used rodent strains is a major reason for their continued use, although this has also retarded development of improved rodent strains. Although inbred strains are used in 2-year studies to reduce genetic diversity and variability, genetic drift is still a concern. For many rodent strains this has resulted in progressively larger, more obese animals with higher spontaneous tumor rates and reduced lifespans. This genetic drift has compromised assay sensitivity and the usefulness of some rodent strains in carcinogenicity testing and has diminished the value of the historical tumor record for these strains (65-69).

The 2-year rodent carcinogenicity study represents a pragmatic compromise, balancing factors such as test animal sensitivity, spontaneous tumor rate, lifespan, and cost. The relative sensitivity of most rodent strains commonly used in 2-year carciogenicity studies has not been fully evaluated by using identical study protocols and a reference set of known human carcinogens. Positive controls of known carcinogens are not commonly used in the standard 2-year rodent carcinogenicity study protocols but are the rule in transgenic studies. Evidence suggests that there are similarities and significant variations among different rodent strains in their sensitivities to various carcinogens and differences in the nature and incidence of spontaneous tumors (70-78). Many of these differences may be due to species- and strain-related (genetic) differences in metabolism and pharmacokinetics. A wide variability in spontaneous tumor incidence can also occur within a rodent strain (79,80). In addition it is now apparent that uncontrolled ad libitum feeding and body weight in rodent carcinogenicity studies has been an uncontrolled variable that significantly influenced the sensitivity of the bioassay to carcinogens (81). The tumorigenic response to a potent carcinogen such as aflatoxin can be significantly altered by varying the caloric intake and body weight of rodents (82). Assay sensitivity is equally important in the characterization of transgenic rodent models. In transgenic study protocols currently under evaluation, a reference carcinogen at one or two dose levels is used in addition to untreated and vehicle controls to help gauge the sensitivity of the model and variations in sensitivity due to protocol or other interlaboratory study differences.

The sensitivity and variability of transgenic rodent carcinogenicity models should be evaluated in the context of the known variability in sensitivity to carcinogens and variability in spontaneous tumor responses of rodent strains used in standard 2-year carcinogenicity studies. In practice, there is flexibility in the choice of rodent test strains for 2-year carcinogenicity studies and the choice generally is left to the individual study director. Similar flexibility can be applied to transgenic rodent models if the transgenic model chosen is scientifically justified and contributes to the evaluation of human safety.

In part because of the relatively high (75%) concordance in tumor findings in rat and mouse 2-year carcinogenicity studies, the degree of experience and characterization necessary for the regulatory application of a new carcinogenicity test model to be used as a complementary study in conjunction with a single traditional 2-year rodent study could be less extensive than would be expected for a replacement of both 2-year rodent carcinogenicity studies. To encourage innovation, new emerging methods should not necessarily be expected to attain unrealistic levels of validation—levels that were never attained by current 2-year carcinogenicity studies—before they are considered for regulatory application. The extent of validation required for application of new approaches should be considered in the context of the degree of validation available for the current 2-year rodent carcinogenicity study. It should be noted that the ICH guidance on carcinogenicity testing has resulted in increased
interest in the development of transgenic rodent carcinogenicity models, fostered by the willingness of international regulatory agencies to consider new testing models. Because of the relatively short duration of transgenic studies, the smaller number of animals used, and their lower cost, it is anticipated that transgenic models will soon be available that are more extensively characterized than the current 2-year rodent carcinogenicity study.

Conclusions

Transgenic rodent models represent a different and complementary approach to carcinogenicity testing compared to the standard 2-year rodent study. Transgenic models have been developed that contain regulated transgenes, unexpressed reporter genes, or knocked out alleles of tumor-suppressor genes. Although transgenic models incorporating these relatively specific genetic mechanisms may not be responsive to all compounds that tested positive in the 2-year mouse or rat study, transgenic models can and have been developed that incorporate carcinogenic response elements known to be present and functioning similarly in humans. Positive findings in such transgenic models can offer valuable insights into the potential relevance and applicability of tumor findings to humans that are not readily available from standard 2-year rodent studies. Transgenic models incorporating human protooncogenes may also be the only means of identifying carcinogens that act by specific mechanisms not present in conventional rodent strains. Ironically, the relative specificity of transgenic models is viewed as a liability by some who consider the standard 2-year rodent study more widely applicable because it does not depend on any knowledge of tumorigenic mechanisms or mode of action for the assessment of human risk. It must be acknowledged, however, that the absence of such knowledge has been used to question the validity of extrapolating rodent tumor findings to humans. In addition to their application as an alternative to a second 2-year rodent carcinogenicity study, there are other circumstances where a transgenic model may be warranted. There are occasions when a 2-year rodent study must be repeated because of inadequate survival of mice or rats, inadequate dose selection, or the need to evaluate another route of administration because of a change in the clinical indication for a drug. In such circumstances a transgenic rodent model may be a possible alternative to repeating a 2-year carcinogenicity study. In addition the relatively short duration of the study and the reduced numbers of animals necessary for transgenic rodent studies compared to 2-year rodent studies enhance their possible usefulness as carcinogenicity screening studies for decision support early in the drug development process or in the compound selection phase.

Based on available information there is sufficient experience with some in vivo transgenic rodent carcinogenicity models to support their application as complementary second species studies in conjunction with a single 2-year rodent carcinogenicity study when appropriately justified. A properly selected transgenic mouse carcinogenicity study combined with one 2-year rat study can also adequately identify transspecies tumorigens, which are considered most relevant for the assessment of human risk. This approach will stimulate innovation and the development and application of methods that may improve assessment of potential human carcinogenic risk, which in turn will benefit public health.

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