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Alternative Models for Carcinogenicity Testing

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The International Conference on Harmonisation Expert Working Group on Safety suggested that under certain circumstances, data from alternative assays could be used in safety evaluation in place of a second bioassay. Several alternatives were discussed. Six of these models were evaluated in a collaborative effort under the auspices of the Health and Environmental Sciences Institute (HESI) branch of the International Life Sciences Institute (ILSI). Standard protocols, pathology review, and statistical evaluations were developed. Twenty-one chemicals were evaluated, including genotoxic, nongenotoxic, carcinogenic, and noncarcinogenic chemicals. The models that were evaluated included the p53$^{+/-}$ heterozygous knockout mouse, the rasH2 transgenic mouse, the TgAC transgenic mouse (dermal and oral administration), the homozygous XPA knockout and the XPA/p53 knockout mouse models. Also evaluated were the neonatal mouse models and the Syrian Hamster Embryo (SHE) transformation assay. The results of this comprehensive study suggest that some of these models might be useful in hazard identification if used in conjunction with information from other sources in a weight of evidence, integrated analysis approach to risk assessment.

Key Words: **carcinogenesis testing; hazard identification; risk assessment; rodent bioassay; transgenic models.**

As the International Conference on Harmonization (ICH) Expert Working Group on Safety was conducting discussions involving how best to assess potential human cancer risk of pharmaceutical agents, important questions were raised regarding the added value of the second species in the long-term rodent carcinogenicity bioassay. Careful retrospective evaluations of several databases suggested that there were few cases where the data from the second species (usually the mouse) led to definitive decisions on the regulatory fate of the compound (Contrera *et al.*, 1997; Davies and Monro, 1995; Van Oosterhout *et al.*, 1997). It was suggested that, under certain circumstances, data from other alternative assays may prove of equal or greater value to the second bioassay and these were proposed as possible alternatives in the guidance document (ICH, 1995).

While a number of these alternatives had received considerable evaluation with a variety of chemicals (e.g., initiation-promotion models, neonatal mouse), it was clear that there was very little experience with most of the newer models (transgenic or knockout mice) and almost no experience with pharmaceuticals. In an effort to rapidly gain a broader understanding of these newer models, a collaborative effort was initiated under the auspices of the Health and Environmental Sciences Institute (HESI) branch of the International Life Sciences Institute (ILSI). As the focus of the ICH discussions centered on pharmaceuticals, the collaborative effort with ILSI-HESI dealt primarily with this class of agents.

The ILSI-HESI Alternatives to Carcinogenicity Testing initiative was begun in 1996 with the specific purpose of facilitating a focused, systematic evaluation of several of the new alternatives proposed within the ICH guidance (Appendix 1). Participation in this research collaboration was global in scope, encompassing institutions in Europe, Japan, and the United States. To assure representation of a variety of viewpoints and to create a robust process, individuals from academia, government, and industry were asked to join the effort. From the outset, the process was kept as transparent as possible, involving a broad spectrum of interests at all critical steps. A total of 53 sponsors committed to conduct assays and report the results in this consortium, representing a financial commitment of approximately US $33 million.

Oversight of this collaboration was directed by a panel of scientific advisors (Appendix 2) and by a steering committee (Appendix 3). Several key steps led to the development of the database: (1) compound selection; (2) development of common protocols for each assay; (3) establishment of Assay Working Groups to oversee conduct of the assays, review the data, and

The evaluations discussed here are those of the authors, and are not meant to imply an official determination by ILSI-HESI, nor by any of the sponsoring organizations.

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coordinate data analyses; and (4) establishment of uniform criteria for evaluation of responses in these assays.

The compound selection process was guided by the desire to include chemicals that were representative of a broad range of mechanisms, including chemicals that were known human carcinogenic hazards as well as those that were clearly not considered human carcinogens. In addition, a large class of compounds that had produced tumors in rodents by various mechanisms but were not generally considered human hazards was included. Each compound selected had a relatively comprehensive existing database of toxicology information. The categories and lists of the chemicals used are shown in Appendix 4. A restriction in this process was that these chemicals be nonproprietary and multisourced; this necessarily limited the choices of chemicals. The background data available on each compound and the rationale for compound selection were collected and summarized by compound coordinators who also assured the availability of commonly sourced material for duplicate assays (for further details, go to the ILSI web site at http://hesi.ilsi.org/activities/actslist.cfm?pubentityid=8&pubactivityid=26).

It was imperative that a set of well-characterized protocols be established to assure the ability to compare the results of these various assays across laboratories. Incorporated in this process was the careful consideration of dose-level selection for these assays. The protocols and dose selection criteria are available on the ILSI web site (see paragraph above for address). For the mouse models, the oral route of administration (diet or gavage) was generally used, including adaptation of the neonatal mouse model for gavage administration. The TgAC mouse model was usually evaluated by the oral and dermal routes of administration. In most studies, the highest dose evaluated was the maximum tolerated dose (MTD) based on a dose range finding study.

After the development of the protocols and selection of test chemicals, the Assay Working Groups (AWG) became the focus for the effort, serving as the primary contact for questions regarding each assay as well as the vehicle to assure a common approach to data evaluation. These groups were aided in this effort by a pathology subcommittee that provided guidance to assure uniform tissue evaluation and diagnostic nomenclature. Prior to release for entry into the database, the data from each assay were reviewed by the AWG to ensure adherence to the study protocol (including a common approach to dose selection) and a consistent application of evaluation criteria.

From the outset of this initiative, it was agreed that this effort should not be directed toward determination of whether the alternative assay replicated the results obtained in the rodent bioassay, but, rather, whether data from these assays could add value to the process of human risk assessment. While a simple positive or negative determination was not the goal, it was necessary to develop an appropriate means of communicating the results of the studies. Thus a considerable amount of attention was paid to the assessment of the response in each of the respective studies. Each study was evaluated individually and the response criteria established based on the responses in each system. An approach to statistical evaluation was also agreed upon before data evaluation, although this was not established as the sole criterion of a positive or negative response (Popp, 2001). The detailed summaries of the results of these investigations are presented elsewhere (to be published as a collection of articles in *Toxicologic Pathology* in 2001) and include evaluations of the specific models and evaluations across models.

Results of the Assays by Class of Chemical

Nongenotoxic Noncarcinogens

Ampicillin, d-mannitol, and sulfisoxazole are accepted as nongenotoxic (non-DNA reactive) chemicals that are noncarcinogenic in animal models. Although not specifically investigated epidemiologically, they are considered noncarcinogenic for humans. They were negative in all of the *in vivo* models evaluated in the ILSI-HESI project. A major concern was that the specific models being evaluated might be overly sensitive as a consequence of the types of genes that were either inserted or deleted. This was a potentially valid concern since these genetic constructs were specifically designed to increase susceptibility to carcinogenicity in these animals. However, utilizing the protocols developed for this project, these 3 nongenotoxic, noncarcinogenic chemicals were negative in all of the models, providing reassurance regarding model specificity, as would be required for any broad usage of the models for screening purposes.

Genotoxic Carcinogens

Three genotoxic chemicals known to be carcinogenic in animal models and in humans were evaluated, including the cancer chemotherapeutic agents cyclophosphamide and melphalan, and the analgesic phenacetin. Cyclophosphamide and melphalan gave equivocal results in the rasH2 and the dermal TgAC mouse model, and cyclophosphamide gave equivocal results in the rasH2 model. These compounds were positive in all of the other models. In contrast, phenacetin gave variable results, being positive in the rasH2 mouse model, but negative in the p53, XPA, XPA/p53, TgAC, and the neonatal mouse models.

Phenacetin, like other aromatic amines and amides, is considered genotoxic since it is weakly positive in the standard Ames assay, and more strongly positive when hamster microsomes were used for the S9 activation fraction. Phenacetin has been weakly positive as a carcinogen in mouse and rat long-term bioassays. However, it is clearly carcinogenic in humans, producing urothelial carcinomas, primarily in the renal pelvis, but also in the ureters and urinary bladder. In humans, phenacetin-containing analgesics are carcinogenic only after long

periods of exposure, at high doses, and in patients in whom renal papillary necrosis has been produced, suggesting that regenerative processes associated with the papillary necrosis are a major contributor to the carcinogenicity of phenacetin (Johansson *et al.*, 1974). The lack of a positive response in most of the models evaluated in this project is a significant exception to the correlation of genotoxicity and tumorigenicity in these models. This is not considered to diminish the value of the models, but certainly suggests some constraints on how results with these models are interpreted. However, as discussed below, if information from these models is used in conjunction with information from other sources, such as the rat bioassay, Ames assay, and compound chemistry, a reasonable weight of evidence evaluation of potential risk to humans can be achieved.

Immunosuppressants and Hormonal Carcinogens

Cyclosporin A is a pharmaceutical used clinically as an immunosuppressant; it is nongenotoxic and was negative in the 2-year rat bioassay. It gave equivocal results in the chronic mouse bioassay. In humans, cyclosporin A immunosuppression is associated with an increase in the development of certain types of tumors, namely B-cell lymphomas and squamous cell carcinomas, particularly of the cervix.

Cyclosporin gave varying results in the different assays. It was positive in the p53 mouse model when administered in the diet, and it was also positive in the XPA and XPA/p53 mouse models and the dermal TgAC assay. It gave equivocal results in the oral TgAC mouse model and rasH2 model and was negative in the neonatal mouse model. Clearly, this nongenotoxic chemical was positive in some of these assays, demonstrating that these models are not specific for genotoxic (DNA reactive) carcinogens.

In general, the usefulness of animal models in evaluating strongly immunosuppressive chemicals is doubtful. Clinically significant immunosuppression can be produced by administration of specific chemical agents used for organ transplantation or other therapeutic purposes, by inheritance of specific genetic immunodeficiencies, or as a result of acquired immune deficiency syndrome (AIDS). Regardless of how the immunodeficiency is produced, it is associated with an increased risk of certain cancers, namely B-cell lymphomas, usually associated with Epstein-Barr virus (EBV), squamous cell carcinomas associated with human papilloma virus (HPV), particularly of the cervix, and Kaposi's sarcoma associated with herpes virus 8 (HHV-8) in patients with AIDS (Cohen, 1999). These tumors are predominantly associated with viral infections that cannot be kept under control because of the immunodeficiency. It is unlikely that the chemical agents themselves are directly carcinogenic, per se. The carcinogenic stimulus is more likely due to induction of immunosuppression that leads to the specific viral-associated tumors (Cohen, 1999).

The 2 estrogenic compounds evaluated were diethylstilbestrol (DES) and estradiol. DES is carcinogenic in animals and in humans, acting primarily through stimulation of increased cell proliferation by binding to estrogen receptors. However, there is some suggestion that DES forms DNA adducts to a limited extent (Carmichael *et al*., 2001). Similarly, estradiol is known to increase the risk of cancer in animal models and in humans, acting primarily through the stimulation of cell proliferation by binding to estrogen receptors in target tissues. However, there is also some evidence that estradiol is weakly genotoxic and might form DNA adducts (Carmichael *et al*., 2001).

DES was positive in all of the *in vivo* mouse models except the oral TgAC and neonatal mouse models. Estradiol was positive in the TgAC mouse model when administered on the skin, and it was positive in the XPA/p53 mouse model. However, it was equivocal in the p53 and negative in the rasH2, XPA, and oral TgAC mouse models. In the neonatal mouse model, estradiol was tested in 3 different laboratories. Two gave clearly negative results whereas 1 was positive. The reasons for this difference in results is not clear at the present time. DES and estradiol appear to act predominantly by nongenotoxic modes of action through interaction with the estrogen receptor and stimulation of cell proliferation. This is complicated by the DNA-reactive potential of some metabolites. Again, these results do not invalidate the models but do provide guidance as to the difficulties in interpreting genotoxicity merely on the basis of these alternative testing models. As with other screening bioassays, additional information is necessary to rationally develop an assessment of risk of carcinogenic potential in humans.

Nongenotoxic Rodent Carcinogens, Putative Human Noncarcinogens

Several of the pharmaceuticals that were tested in this project were considered to be nongenotoxic chemicals, but were found to be carcinogenic in 1 or more long-term rodent bioassays. However, based either on epidemiological evaluations or mechanistic considerations, these chemicals are not believed to pose a carcinogenic hazard in humans. The background for this statement is provided in the compound selection discussed above and in more detail in the extended publications from this project and at the ILSI web site (see Introduction for address). The chemicals in this category include phenobarbital, methapyrilene, reserpine, dieldrin, haloperidol, chlorpromazine, chloroform, metaproterenol, and sulfamethoxazole. These chemicals were negative in all of the bioassays evaluated except for an equivocal result for chloroform in the p53 mouse model. These results suggest that not only are these models not overly sensitive to noncarcinogens as defined in rodent bioassays, they might actually be more specific in identifying chemicals that are carcinogenic to humans. Since the ultimate goal of these screening assays is predictivity of carcinogenicity in humans, it is reassuring that all of these chemicals were negative in these assays, despite the fact that they have been positive in 2-year rodent bioassays.

Clofibrate, diethylhexylphthalate (DEHP), and WY-14643 are also considered nongenotoxic chemicals that produce tumors in rodent bioassays and are putatively not carcinogenic in humans. All 3 are considered in the class of chemicals known as peroxisome proliferators (Cattley *et al.,* 1998). In contrast to the previous list of nongenotoxic rodent carcinogens that are putative noncarcinogens for humans, the peroxisome proliferators gave widely variable results in the different models. Each of these 3 peroxisome proliferators produced positive or equivocal results in 1 or more of the models, and each of the models, except for the neonatal mouse model, gave positive or equivocal results with 1 or more of these 3 chemicals. Clofibrate was positive in the rasH2 and dermal TgAC mouse models. DEHP was positive in the rasH2 mouse model and equivocal in the p53 mouse model. WY-14643 was positive in the XPA mouse model and equivocal in the oral TgAC mouse model. The reason for the variable results with the peroxisome proliferators is unknown, but again, the results indicate that there is not a complete correlation between direct DNA reactivity (genotoxicity) and positivity in these specific models.

The Syrian Hamster Embryo (SHE) Assay

The above analysis pertained to the *in vivo* models that were evaluated in this project. In addition, the SHE assay, an *in vitro* cell transformation assay, which has been suggested as a carcinogen screening assay, was evaluated as part of this project. Chloroform could not be evaluated because of volatility. Methapyrilene, reserpine, and metaproterenol were not evaluated. The SHE assay gave positive results for nearly all of the chemicals evaluated as part of this project except for phenacetin, d-mannitol, and sulfisoxazole, which were negative. Thus, it does not appear that the SHE assay is able to discriminate between genotoxic versus nongenotoxic chemicals or between rodent carcinogens versus noncarcinogens. It is not predictive of human carcinogenicity or human noncarcinogenicity. Based on this analysis, it would appear that this assay does not provide a significant advantage over the 2-year bioassay approach to hazard identification.

Evaluation of the Models

The neonatal mouse bioassay has been used for more than 40 years (Flammang *et al.,* 1997). It appears to have limited usefulness, not only based on the results of the present chemicals that were tested in this project but also on previously published results. It appears to detect chemicals that are moderately to strongly genotoxic carcinogens. However, it does not detect weak or equivocal genotoxic compounds such as phenacetin or diethylstilbestrol. The conflicting results between the 3 studies on estradiol raise issues of interpretation of the results with this model. The most appropriate application of this assay appears to be to distinguish between definite genotoxicity and weak to nongenotoxic potential. However, such information is usually readily available from other determinations, such as the results of the Ames mutagenesis assay and structure activity relationships based on the chemistry of the compound and its metabolites.

The TgAC mouse model also poses some difficulties with respect to interpretation. It is responsive to DNA-reactive and non-DNA-reactive chemicals that are carcinogenic in rodents as well as many that are known to be carcinogenic in humans. Melphalan and cyclophosphamide were positive by the oral route but gave equivocal results by dermal application. However, several other genotoxic carcinogens have been positive by the dermal route (Tennant *et al.,* 1995). Given the potential for absorption from either site with consequent systemic distribution of the chemical, it is not surprising that positive results for some of the chemicals can be obtained by both routes of administration. Nevertheless, the primary phenotypic marker is the induction of skin tumors. As a screening bioassay for potential human carcinogens, this limited detection system does not pose a difficulty. The model appears to have some usefulness as a screen for potential carcinogenic hazard, but it appears to have limitations with respect to extrapolation of dose and organ specificity to the human situation. However, these limitations are common to the other genetically modified models, even when administered by the oral route, although to a somewhat more limited extent than in the TgAC mouse model.

The p53, rasH2, and the XPA or XPA/p53 mouse models appear to give similar results. Although these genetic constructs are designed to increase sensitivity to carcinogenesis, it is interesting that these models are actually less sensitive to the development of a positive result than the traditional 2-year bioassay in mice or rats. This may be, in part, a reflection of the 6-month exposure duration and the smaller test group size. They gave negative results for nongenotoxic noncarcinogens and were generally positive for the genotoxic carcinogens, with the exception of phenacetin. Interestingly, they were generally negative for nongenotoxic rodent carcinogens that are putatively not human carcinogens. In that respect, these models and the TgAC mouse model appear to have greater specificity with regard to human carcinogenicity than the standard 2-year bioassay in either mice or rats. That is not too surprising, given that the 2-year bioassay has evolved to emphasize sensitivity in hazard identification at the cost of specificity (Boorman *et al.,* 1994; Rall, 2000).

Potential Usefulness of the Models

Definitive statements regarding the potential usefulness of these models in the hazard identification and risk assessment processes are limited by the still relatively small numbers of chemicals evaluated in these different models. Nevertheless, generalizations are beginning to evolve that point to the usefulness of these models in an overall risk assessment process (Gulezian *et al.,* 2000; Tennant *et al.,* 1995). It is clear that by themselves, these models are not definitive determinants of

potential human cancer risk. They serve as hazard identification models similar to the role of the 2-year bioassay. Based on the results so far, it appears that the p53, rasH2, XPA, XPA/p53, and TgAC mouse models, could serve in place of the 2-year mouse bioassay. They appear to have less sensitivity to positives in rodent models that are generally considered not to be carcinogenic in humans, a difficulty that has been widely discussed regarding the standard 2-year bioassay (Boorman *et al.,* 1994; Gulezian *et al.,* 2000; IARC, 1999; Purchase *et al.,* 1998; Tennant *et al.,* 1995). In that respect, these newer models appear to have greater specificity with respect to a correlation to potential human carcinogenicity, and are not overly sensitive as had been feared when the models were originally developed.

Although these models appear to have usefulness in hazard identification, they have limitations with respect to other aspects of risk assessment. The organ specificity of these models does not appear to correlate well with potential carcinogenicity at specific sites in humans. Of the known human carcinogens tested in this project, there is little correlation with target organs in the mice that were positive with these chemicals compared to the organs in which tumors develop in humans. This will significantly limit the insight that can be developed with respect to mechanism of action for these compounds, particularly for chemicals that are not genotoxic.

For many of the chemicals determined as positive in these different assays, the decision was based frequently on an increased incidence of tumors that occur commonly as spontaneous tumors in these mouse strains, as well as in the wild type strains from which these genetically modified mice were developed (i.e., thymic lymphomas and subcutaneous sarcomas). With respect to the sarcomas, it is important to distinguish between those occurring at the site of transponder implantation (used for identification) versus those that arise at other sites. Those related to transponders may be more likely related to foreign body sarcomagenesis rather than being chemically related.

Because of the relatively small number of animals per group (usually 15) compared to the 50 or 60 animals per group in the standard 2-year bioassay, the positive response was frequently seen in these models only at the highest dose. This will limit the applicability of these models for quantitative risk assessment and determination of a dose response. Again, this emphasizes the role of these models as hazard identification tools rather than in applications for quantitative assessment or other components of the risk assessment process. Information for quantitative risk assessment could still be available from the 2-year bioassay in rats and from information from shorter term exposures evaluating intermediate markers of malignancy and from mechanistic research.

Since the genetic constructs developed for these mouse models are frequently focused on DNA damage and DNA repair, these models were presumed to be particularly sensitive to genotoxic carcinogens. However, as seen with the results of the chemicals tested in this project as well as in results published elsewhere, these models are not specific for genotoxic chemicals. Known genotoxic carcinogens, such as phenacetin, are negative in many of these models, and conversely, known nongenotoxic chemicals are positive. For some nongenotoxic chemicals, the corresponding wild-type strain also gave positive results suggesting that the carcinogenic process is independent of the transgene or gene knockout. Regardless, the chemical would give a positive result in the screening assay. Performing the study in both the transgenic and the wild-type strains could provide useful information for interpreting possible mechanisms.

These models can be used as screens for hazard identification. However, like the standard 2-year bioassay, mechanisms will need to be determined from additional research either in the models themselves or from other types of investigations. By themselves, these models do not definitively prove a mechanism or mode of action. Additional mechanistic research will be required to determine relevance of a positive result in these models for human carcinogenicity.

Considerable discussion took place in the development of the specific protocols to be used in the ILSI-HESI project. Future modification of these protocols may be necessary, such as with respect to determination of numbers of animals per group, dosages to be evaluated, and the length of time to be evaluated in determining positive versus negative results. However, it is important that future alterations in protocols are made on a rational basis and not randomly.

In summary, these models appear to have usefulness as hazard identification screening models as part of an initial phase of the risk assessment process. However, they are not definitive proof of potential human carcinogenicity, and they are not proof of a specific mechanism of action. It appears that they could readily serve in place of, rather than merely in addition to, the mouse 2-year bioassay. However, like the 2-year bioassay, the results from tests in these models need to be incorporated into an overall integrated, weight of evidence evaluation for a given compound that takes into account genotoxicity, particularly DNA reactivity, structure activity relationships, results from other bioassays, and the results of other mechanistic investigations including toxicokinetics, metabolism, and mechanistic information.

APPENDIX 1

List of Models Evaluated

- rasH2 transgenic mouse model (Central Institute for Experimental Animals, Japan)
- TgAC transgenic mouse model (Taconic Farms)
- $p53^{+/-}$ knockout mouse model (Taconic Farms)
- $XPA^{-/-}$ knockout mouse model (National Institute of Public Health and Environment, The Netherlands)
- $XPA^{-/-}/p53^{+/-}$ knockout mouse model (National Institute of Public Health and Environment, The Netherlands)
- Neonatal mouse assay (CD-1 or B6C3F1 mice)
- Syrian Hamster Embryo (SHE) assay

APPENDIX 2

HESI Alternatives to Carcinogenicity Testing (ACT) Committee Scientific Advisors

Dr. Dan Casciano, FDA/NCTR
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APPENDIX 3

HESI Alternatives to Carcinogenicity Testing (ACT) Steering Committee

Dr. Sid Aaron, Pharmacia, Inc.
Dr. Gerald Long, Eli Lilly and Company
Dr. R. Michael McClain, Consultant
Dr. James MacDonald, Schering-Plough Corp.
Dr. Alastair Monro, Pfizer (retired)
Dr. James Popp, DuPont Pharmaceuticals
Dr. Denise Robinson, ILSI-HESI
Dr. Ray Stoll, Boehringer Ingelheim
Dr. Richard Storer, Merck & Company, Inc.

APPENDIX 4

Compounds under Assessment through HESI ACT Committee's Collaborative Research Program

Class	Compound
Genotoxic human carcinogens	Cyclophosphamide, melphalan, phenacetin
Immunosuppressant human carcinogen	Cyclosporin A
Hormonal human carcinogens	Diethylstilbestrol, estradiol
Rodent nongenotoxic carcinogens/ putative human noncarcinogens (based on human data)	Phenobarbital, clofibrate, reserpine, dieldrin, methapyrilene
Rodent nongenotoxic carcinogens/ putative human noncarcinogens (based on mechanism)	Haloperidol, chlorpromazine, chloroform, metaproterenol,WY-14643, DEHP, sulfamethoxazole
Nongenotoxic noncarcinogens	Ampicillin, d-Mannitol, sulfisoxazole

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