Animal Carcinogenicity Studies: Alternatives to the Bioassay

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Abstract

Traditional animal carcinogenicity tests take around three years to design, conduct and interpret. Consequently, only a tiny fraction of the thousands of industrial chemicals in use have yet been tested for carcinogenicity. Despite the cost of hundreds of millions of dollars, millions of skilled personnel hours, and millions of animal lives, several investigations have revealed animal carcinogenicity data to be lacking in human specificity (ability to distinguish human from animal carcinogens, where different), which severely limits its human predictivity. Causes include documented scientific inadequacies of the majority of carcinogenicity bioassays, and numerous serious biological and mathematical obstacles, which render attempts to accurately extrapolate human carcinogenicity assessments from animal data profoundly difficult, if not impossible. Proposed modifications have included the elimination of mice, the use of genetically-altered or neonatal mice, decreased timeframes, initiation-promotion models, greater incorporation of toxicokinetic and toxicodynamic assessments, quantitative structure-activity relationship (computerized) expert systems, *in vitro* assays, cDNA microarrays for detecting genetic expression changes, limited human clinical trials, and epidemiological research. Advantages of non-animal assays when compared to bioassays include superior human specificity results, greatly reduced timeframes, and greatly reduced demands on financial, personnel and animal resources. Inexplicably, however, regulatory agencies have been frustratingly slow to adopt alternative protocols. In order to minimize cancer losses to society, a substantial redirection of resources away from excessively slow and resource-intensive rodent bioassays, into the further development and implementation of non-animal assays, is strongly justified and urgently required.

Introduction

The regulation of exposures to potential human carcinogens has traditionally relied heavily on animal carcinogenicity studies. However, several investigations have revealed animal carcinogenicity data to be lacking in human specificity (ability to distinguish human from animal carcinogens, where different), and hence, human predictivity. An investigation of alternative assays is therefore warranted.

Methods

We comprehensively searched the 'Medline' biomedical bibliographic database to locate studies describing existing and developing bioassay alternatives.

Results and discussion

Proposed modifications to the traditional rodent bioassay have included the elimination of mice in favor of rats, the use of genetically-altered or neonatal mice, decreased timeframes, initiation-promotion models, and greater incorporation of toxicokinetic and toxicodynamic assessments. The most promising alternatives, however, are non-animal assays, including quantitative structure-activity relationship (QSAR) expert systems, *in vitro* assays, the use of cDNA microarrays to detect genetic expression changes, human clinical trials and epidemiological research.

Quantitative structure-activity relationships (QSARs)

Computerized structural analyses predict biological activities based on chemical structure. Despite initial disappointments, more recent QSAR databases have shown high utility in predicting carcinogenicity. Matthews and Contrera (1998) described the beta-test evaluation of a QSAR expert system that demonstrated 97% sensitivity for carcinogens and 98% specificity for non-carcinogens. QSAR expert system analysis also has the very strong advantages of being relatively cheap and instantaneous.

In vitro assays

In vitro assays such as bacterial, yeast, protozoal, mammalian and human cell cultures, may all contribute information towards a weight-of-evidence characterization sufficient to render the rodent bioassay unnecessary. Their very short timeframes (hours to days), large financial savings, and tiny quantities (micrograms to nanograms) of test chemical required, all offer strong advantages over traditional rodent bioassays.

Correlations of around 90% and 86% between *in vitro* microbial mutagenesis and mammalian carcinogenesis have been demonstrated for a large array of chemicals. The Syrian Hamster Embryo (SHE) cell transformation assay detects morphological cell transformation—the earliest phenotypically identifiable stage in carcinogenesis—and is probably the most predictive short-term assay. Pienta *et al.* (1977) showed a 90.8% correlation between morphological transformation of SHE cells, despite prior cryopreservation, and the reported carcinogenic activity of a large number of carcinogenic and non-carcinogenic chemicals. The particular advantage of the SHE assay in comparison to other *in vitro* assays is its ability to detect nongenotoxic, as well as genotoxic, carcinogens.

Despite their obvious advantages, the use of *in vitro* cell cultures is limited by concerns that they do not adequately mimic the response of *in vivo* cells at the target site within humans. Such concerns can be minimized by using human primary cell lines, and complex organotypic culture systems, with cofactors and metabolic supplements added to increase longevity and maintain cellular differentiation.

The possibilities for *in vitro* testing will continue to expand with future research. Lichtenberg-Frate *et al.* (2003) demonstrated the genotoxic and cytotoxic sensitivity of a genetically modified yeast (*Saccharomyces cerevisiae*) assay, which used a yeast optimized version of the green fluorescent protein (GFP) fused to the RAD54 yeast promoter, which is activated upon DNA damage. The result was green fluorescence in the presence of several genotoxic test compounds. Thereafter known as the 'GreenScreen,' this assay allows high throughput using minimal quantities of test substances.

The spectrum of compounds detected by the GreenScreen is somewhat different to that detected by bacterial genotoxicity assays; hence, as Cahill *et al.* (2004) propose, this assay, together with a high throughput bacterial screen, and an *in silico* QSAR screen, would provide an effective battery of carcinogenicity screening tests for regulatory purposes.

cDNA microarrays

cDNA microarrays, containing hundreds or thousands of microscopic spots of complementary DNA transcripts (cDNA) of mRNA templates (from which the non-coding intron sequences of the original DNA have been excised), hold particular promise for detecting changes in gene expression caused by carcinogens or other toxins (toxicogenomics), long before more invasive endpoints are reached. Although the use of cDNA microarrays in carcinogen detection is new, early studies have yielded promising results. Particularly exciting, given the present scarcity of alternative models for nongenotoxic carcinogens are the ability of cDNA microarrays to detect them. However, microarray technology remains in its infancy, and several existing limitations would benefit from further research and development.

Epidemiological research

Increased epidemiological research linking cancer incidences with exposure factors in human populations would identify more human carcinogens and presumed non-carcinogens, thereby increasing the data set available for validation studies and QSAR predictive systems. Presently, the human carcinogenicity or non-carcinogenicity of too few chemicals is known. Furthermore, most epidemiologic studies for carcinogens are presently performed on substances already known to be human carcinogens (retrospective studies).

Cancer Centers should be funded to establish tumor registries aimed at identifying new lifestyle, occupational, environmental and medical carcinogens. Post-marketing surveillance should also be required for all pharmaceuticals, with mandatory reporting of adverse side effects.

Data sharing and evaluation

All existing data about a test substance should be collated and examined in a critical and unbiased fashion to determine which, if any, remaining tests are scientifically justified, before those tests are conducted. Contrary to the public interest, much existing data remains excluded from the public domain within pharmaceutical and chemical company files, for commercial reasons.

A combination protocol

The traditional rodent bioassay takes upwards of two years to produce results of poor human specificity, and consequently, predictivity, and is excessively costly in terms of finances, skilled personnel hours, and animal lives. We propose its replacement with the following protocol:

1. **Before any assay is conducted, all existing information about the test compound should be collated and reviewed in a critical and unbiased fashion to determine which tests are scientifically justified.**
2. **Initial screens should include Quantitative Structure-Activity Relationship (QSAR) computerised systems, cell or tissue cultures, and cDNA microarrays, where possible. QSAR expert systems should be used to identify and estimate the toxic effects of specific chemical groups. Ames *Salmonella,* Syrian Hamster Embryo cell transformation, *Saccharomyces* GreenScreen, human basal and target organ cell or tissue culture assays, and other appropriate *in vitro* screening assays, should be fully utilized to seek evidence of cytotoxicity, mutagenicity and genotoxicity. Well chosen and conducted cDNA microarray assays of geno- and nongenotoxicity should be analyzed for changes in genetic expression.**
3. **Following these initial screens, human toxicological studies using biomarkers, barrier models and biological simulations should be appropriately selected to model toxicokinetics and estimate target organ concentrations.**
4. **Thereafter, phase I and II human clinical trials should be carefully conducted to seek evidence of genotoxicity, immunosuppression, hormonal activity or chronic irritation/inflammation.**

A modernized carcinogenicity testing protocol

Properly collating and analyzing this more targeted data is likely to yield a weight of evidence characterization of carcinogenic risk of substantially superior human predictivity than that offered by the traditional rodent bioassay. Additional advantages include the likelihood of greater insights into carcinogenic mechanisms, and substantial savings of financial, human and animal resources.

Further research

A substantial redirection of resources away from the resource-intensive traditional rodent bioassay, into the further development and implementation of the following alternative assays, is clearly warranted:

1. QSAR expert systems, particularly for initial screening, should be further developed and expanded from their traditional reliance on chemical analogues to include information on the structural properties of cellular receptors facilitating toxicity, as this becomes available. Toxicity testing data should be used retrospectively to enlarge QSAR databases.

2. Cell and tissue assays, particularly those using human cell lines, the SHE cell transformation assay, others sensitive to nongenotoxic carcinogens, and the *Saccharomyces* GreenScreen assay, should be further developed and implemented. The availability of human cells and tissues for toxicity testing should be increased.

3. Research into improving cDNA microarray data reproducibility and interpretation should continue.

4. Predictive biomarkers of toxicity should be identified through genomic, proteonomic and clinical research, thereby allowing speedier generation of results, well prior to more invasive endpoints, and facilitating increased understanding of carcinogenesis mechanisms.

5. Increased human epidemiological research is also required, in order to identify more known human carcinogens and presumed non-carcinogens, thereby increasing the data set available for validation studies and QSAR predictive systems. Cancer Centers should be financially supported to establish tumor registries focused on identifying new human carcinogens, and post-marketing surveillance should be required for all pharmaceuticals, with mandatory reporting of adverse side effects.

Regulatory validation and adoption

Despite the 1997 recommendations of the International Conference on the Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use, and the criticisms of numerous additional authors, modernization of bioassay protocols has been painfully slow. Although a slowly increasing number of alternative protocols are being submitted to regulatory agencies, for the most part fear of lack of acceptance of alternatives by regulatory agencies is discouraging the use of alternative assays. Consequently the traditional two-year four-sex-species groups rodent bioassay persists, despite extensive criticism centered around its very poor human specificity, and its subsequent inability to meet the stringent human validation standards required of alternative protocols.

Clearly, regulatory agencies should be required to consider data from promising existing and new alternative testing methodologies, including QSAR expert systems, appropriate *in vitro* assays, cDNA microarrays, human toxicological studies and clinical trials, and biological simulations, alongside traditional rodent bioassay data. They should be required to make science-based decisions on the use of various test methods according to the human sensitivity and specificity data of each, rather than continuing to rely upon cultural testing traditions.

A closely related problem is the cumbersome validation process required of alternative assays, made more difficult by attempts to match outcomes to the variable and inconsistent results of animal bioassays. With the half-lives of new assays likely to be substantially shorter than the time required for traditional validation, the streamlining of validation processes by regulators must become a high priority.

Finally, it is of fundamental importance that harmonization of testing requirements be achieved between regulatory agencies, as has been achieved under the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, which has significantly reduced the need for pharmaceutical testing.

Conclusions

Traditional animal carcinogenicity tests take around three years to design, conduct and interpret. Unsurprisingly, by 1998 only about 2,000 (2.6%) of the 75,000 industrial chemicals in use and listed in the EPA's Toxic Substances Control Act inventory, had been tested for carcinogenicity. The cost of testing just these 2.6% of industrial chemicals in use was hundreds of millions of dollars, millions of skilled personnel hours, and millions of animal lives.

Despite this enormous investment of resources, the poor human specificity, and hence predictivity, of animal carcinogenicity data, has been documented by several investigators. The reasons for this are numerous. When subjected to careful scrutiny by the IARC, the majority of animal carcinogenicity studies have been found to be scientifically inadequate. Additionally, several serious biological and mathematical obstacles render attempts to accurately extrapolate human carcinogenicity assessments from animal data profoundly difficult, if not impossible.

Several alternatives to the traditional rodent bioassay have been proposed, of which the most promising are non-animal assays such as quantitative structure-activity relationship expert systems, *in vitro* assays, the use of cDNA microarrays to detect genetic expression changes, human clinical trials, and epidemiological research. Existing data, much of which remains unavailable within pharmaceutical and chemical company files, should also be better shared.

In contrast with animal bioassays, both the human specificity and sensitivity of alternatives such as QSAR expert systems and *in vitro* assays are very promising. Results are available nearly instantaneously, in the case of QSAR expert systems, or in as little as six hours in the case of enhanced SHE *in vitro* protocols, compared with two years for traditional rodent bioassays. Other advantages include enormous financial and personnel savings, substantial replacement of animal use, and tiny quantities of test chemical required.

Inexplicably, however, regulatory agencies have been frustratingly slow to adopt alternative protocols, preferring to cling to cultural bioassay traditions. In order to minimize cancer losses to society, a substantial redirection of resources away from very slow and resource-intensive rodent bioassays, into the further development and implementation of non-animal alternative assays, is strongly justified, and urgently required.

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References

Available on request.