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REVIEW ARTICLE

Developments in Carcinogenicity testing

Sanyam Gandhi[1], Ajay Sharma[2]

[1]Senior Regulatory Affairs Specialist, GW Pharmaceuticals, Sittingbourne, Kent, ME9 8AG, United Kingdom

[2]Research Assistant. Baylor College of Medicine, 1 Baylor plaza, Houston, USA

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ABSTRACT

This review discusses developments during the last 60 years in field of carcinogenecity testing based on the use of laboratory animals. The first such study involved applications of Coal tar to the ear of rabbits. Improvements that have occurred in the quality of animals and in the way in which tests are conducted are considered, along with the importance of distinguishing between "Genotoxic" and "Nongenotoxic" carcinogens. Various Pharmaceuticals have been detected to give a positive result in the standard "Chronic Bioassay" which consist of and 18 to 24 months daily administration of test compound in the mice and rat. The high incidence of apparently false-positive result in the chronic bioassay may be due some deficiencies as oversensitivity and difference in mechanism of pharmacological action between rodents and human, but, these deficiencies provided ground to develop some alternative models for testing. On the increased knowledge of cellular and molecular mechanism involved in carcinogenicity, extensive discussion have recently taken place between regulatory agencies and industry associations at the occasions of the International Conference Harmonization (ICH). For future prospects, a necessity for a better understanding of how disturbance of physiological and/or hormonal status can predispose to tumor development and for more comparative metabolism studies is stressed.

Key words: ICH, Genotoxic

INTRODUCTION:

Cancer is a dread disease worldwide. Mortality of individuals suffering from cancer is high, despite the current improved methods of precocious detection, surgery and therapy. Although carcinogens have been known for causing cancer, but awareness of human carcinogenicity caused by chemicals is a phenomenon of 20th century. Subsequently from 1960s various models have been developed for carcinogenicity testing. These tests done with rodents, most frequently new born mice. These studies involve feeding or injecting the substance being tested and subsequent examination of the animal for tumor formation. Many deficiencies have been detected in Chronic Bioassay; oversensitivity is the major one. These deficiencies provided means to evolve alternative models and enforce legislation for testing as well. In this era more mechanistic and molecular knowledge has been joined in regards to human carcinogenicity concept, including genotoxic versus epigetic carcinogens and organized regulatory guidelines.

In this review an attempt has been made to discuss the (i) Practical approach for carcinogenesis testing procedures with history, (ii) the scenario of guidelines, and the (iii) new technologies/alternative test models in the assessment of carcinogenic drugs with possible improvements.

History of carcinogenicity testing:

Experimental induction of cancer by chemicals was first reported in detail by Yamagiwa and Ichikawa in 1918, when repeated application of coal tar to the ear of rabbits resulted in skin carcinomas[1]. Over the next few years, Kennaway and leitch confirmed this finding and demonstrated similar effects in mice and rabbits from application of root extracts, other type of tar, and some heated mineral oils. These researchers also observed sometimes skin irritation and hypothesized that cancer stems from the interaction of a chemical with a sensitive site in the cell. Further reading on the history and concepts in cancer is provided by Van Cauteren et al.[2], Roe[3] and Grano et al[4].

When backlite discs, fibrosacromoas, implanted in rats a tumor developed which was first indication that factor other than chemical interactions with specific intracellular sites could be responsible for tumor induction. As same wrapping piece of cellophase film around the kidneys in rat resulted large tumor around the film. In 1960s implementation of various metals such as gold, silver or

*Corresponding author: Sanyam Gandhi

platinum leads to development of sarcomas, subsequent investigations revealed that some food coloring evoked local sarcomas in rats and mice after repeated subcutaneous administration. Further examples of chemical and solid state carcinogens that lead to tumor development in rodents are provided by Grano et al. [4] and Weinburger[5].

During the 1970s Ames postulated that most human carcinogens were "Genotoxic". Genotoxic compounds may react, either directly or indirectly (after metabolism activation) with DNA, which leads to alteration of genetic material, and mostly to carcinogenicity in rodent and humans. On the other hand, many carcinogens act though a different mechanism called "non genotoxic" or "Epigenetic".

Guidelines for Carcinogenicity testing:

The Past of Guidelines: In 1970s and 1980s, the U.S. European and Japanese registration authorities established guidelines for carcinogenicity testing in animals for various chemicals characterised by possible long term intake by man. These chemical included food and color additives, agrochemicals, industrial chemicals, solvents, human pharmaceuticals, and vetenory products.

The guidelines were based upon chronic bioassay of the NTP (National Toxicological Programme) and have indications for rout and frequency of dosing, dose levels, group sizes, duration of study, and observations during the study. A summary of various guidelines is provided in Table 1.

Table 1: Establishment of guidelines for carcinogenicity testing in animals.

Region	Division	Governing body, Year of approval
General	Chemicals	OECD, 1981
EC	Pharmaceuticals Chemicals	EC directives, 1983 EC directives, 1988
US	Food and color Additives Agrochemicals Chemicals	FDA, 1982 EPA- FIFRA, 1984 EPA-TSCA, 1985
Japan	Agrochemicals Vetenary Products Pharmaceutics	MAFF 1985 MAFF 1988 MAFF 1989

EPA: Environmental Protection Agency; FDA: Food and Drug Administration; FIFRA: Federal Insecticide Fungicide and Rodenticide Act; MAFF: Ministry Agriculture Forestry on fisheries; MHW: Ministry for Health and Welfare; OECD: Organisation for Economics Co-operation and Development; TCSA: Toxic Substance Control Act.

Presently Effective Guidelines: Most of the countries are having the specific guidelines for testing of pharmaceuticals for carcinogenicity. In India, Schedule 'Y' of Drugs and Cosmetics Rules 1988 issued by the New Drugs Division of Central Drug Standard Control Organisation (CDSCO), Directorate General of Health Services (DGHS), Ministry of Health and Family Welfare, Govt. of India, deals with the prerequisites to carry out the clinical trials of a new drug before its marketing, depending upon the status of the drug in other countries. It is currently being under revision and expected to be released shortly. As per the regulatory requirements, carcinogenicity testing is needed, when a compound or its metabolite is structurally related to a known carcinogen, or when the nature and action of the drug(s) suggest a mutagenic/carcinogenic potential.[6] The International Agency for Research on Cancer (IARC) regularly reviews and evaluates the most commonly used drugs for carcinogenicity on the basis of genotoxicological and epidemiological data in humans[7, 8]. It provides a reasonably comprehensive catalog of chemicals at various stages of testing that should be consulted before undertaking time-consuming and costly long-term carcinogenesis experiments. Additionally the Center for Drug Evaluation and Research of US FDA in May 2002, has issued guidance on "Carcinogenicity Protocol submissions". This is intended to inform drug candidate sponsor of the types of information the FDA relies on when evaluating study protocols for animal carcinogenicity studies.

Later on, in 1996 an extensive collaborative international research program was initiated and coordinated by the Health and Environmental Sciences Institute (HESI) branch of the International Life Sciences Institute (ILSI) to harmonize and provide the framework for an adequate and systemic evaluation.[9] As the focus of the ICH discussion centered on pharmaceuticals, the collaborative efforts with ILSI-HESI also dealt with pharmaceuticals. The main objective of the collaboration was to generate the scientific data that is needed to understand the benefits and limitations of new carcinogenicity-testing methods, and to assess their ability to add relevant information for predicting human cancer risks. Several chemicals which are genotoxic, non-genotoxic, carcinogenic, and noncarcinogenic were evaluated using the animal models that included the p53+/- knockout mouse, the rasH2 transgenic mouse, Tg.AC transgenic mouse (dermal and oral administration), the homozygous XPA knockout, and the neonatal mouse models. [10, 11,12,13,14]

The following three ICH guidelines on carcinogenicity studies of pharmaceuticals have been issued:

S1A: Guideline on the need for carcinogenicity studies of pharmaceuticals, March 1996. This guideline, in summary, defines the need and conditions under which carcinogenicity studies should be conducted to avoid the unnecessary use of animals in testing.[15]

S1B: Testing for carcinogenicity of pharmaceuticals, July 1997. This guideline provides methods of experimental approaches for the evaluation of carcinogenic potential of pharmaceuticals. This also obviates the necessity for the routine conduct of two long-term rodent carcinogenicity bioassays; whether the use of rats or mice alone would result in the loss of information on carcinogenicity relevant to human risk assessment has been addressed in this guidance.[16]
S1C: Dose selection for carcinogenicity studies of pharmaceuticals, March 1995. *S1C(R):* Addendum to dose selection for carcinogenicity studies of pharmaceuticals: Addition of a limit dose and related notes, July 1997.

Carcinogenicity Testing: Practical Approach

Objectives

Carcinogenicity studies are generally conducted to identify a tumorgenic potential of the drug in animals and to assess the relevant risk in humans. Carcinogenesis is a complex process and no single experimental approach can be expected to predict accurately the carcinogenic potential of a chemical in humans. The basic scheme comprises one long-term (life span) rodent carcinogenicity study plus one other long or short-term study, which provides additional information that is not readily available from the first long-term study. There are several reasons for choosing a short- or medium-term test system as supplement to the 2-year bioassay. These models can provide better answers for the differences in the range of susceptible target tissues in which tumour develop, and the knowledge of compound's ADME (Absorption, Distribution, Metabolism and Excretion)

profile for human risk assessment.

Factors to consider

a.) Drug candidates: Pharmaceuticals, which are expected for continuous use or at least for 6-months clinical use, should be considered for carcinogenicity testing. For pharmaceuticals used repeatedly in an intermittent manner in the treatment of chronic or recurrent conditions, carcinogenicity studies are generally needed. It may be noted that most of the pharmaceuticals indicated for 3-months treatment would also likely be used for 6 months or more. Pharmaceuticals administered infrequently or for short duration of exposure (e.g., anesthetics, critical care medicines, radiolabeled imaging agents, and diagnostic aids) do not need carcinogenicity studies unless there is cause for concern.[17]
Compounds for which carcinogenicity studies are needed should be completed before filing an application (e.g., NDA) for marketing approval. However, completed rodent carcinogenicity studies are not needed in advance of the conduct of large-scale clinical trials, unless there is a special concern for the patient population.[18] For pharmaceuticals intended to treat life-threatening or severely debilitating diseases (e.g., AIDS), carcinogenicity testing need not be conducted before market approval, and studies should be conducted post-approval. If the life expectancy in the indicated population is short (i.e., less than 2-3 years) no long- term carcinogenicity studies may be required. If the therapeutic agent is clinically successful and can be used in non-cancer situations, and life is considerably increased after the treatment, then carcinogenicity studies are generally needed.[17] Certain formulations or delivery systems, which may result in prolonged exposures, need carcinogenicity testing. Carcinogenicity studies are not generally needed for endogenous substances. Endogenous peptides, proteins and their analogs produced by chemical synthesis, by extraction/purification from an animal/human source or by biotechnological methods such as recombinant DNA technology may require special consideration. Although not usually necessary, under certain circumstances such as the treatment duration, clinical indication, or patient population long-term carcinogenicity studies in rodent species should be considered.[19]

b.) Cause for concern: Some pharmaceuticals should be tested for carcinogenicity if there is concern about their carcinogenic potential. Several factors which could be considered may include: (i) previous demonstration of carcinogenic potential in the product class which is relevant to human (class alert); (ii) structure-activity relation suggesting carcinogenic risk; (iii) evidence of pre-neoplastic lesions in repeated dose toxicity studies; and (iv) long-term tissue retention of parent compound or metabolite(s) resulting in local tissue reactions or other pathophysiological responses.[20]

c.) Genotoxicity: Pharmaceutical databases are being used to determine the association between genetic toxicology test findings and rodent carcinogenicity outcomes. Sometimes chemical-induced carcinogenesis may involve a non-genotoxic mechanism. So it is difficult to determine how well genetic toxicology assays predict carcinogenic potential. Indomethacin tested negative for *in vivo* cytogenetic assays in the regulatory tests, but was reported positive for the induction of DNA adducts in the literature. Halothane and pyrazinamide were also *in vivo* positive for comet test in human lymphocytes and induction of sperm head abnormalities in mice, respectively, which are considered non-regulatory tests.[21] The importance of these positive findings in the relatively insensitive rodent cytogenetic assays is unclear. All the intrinsic values and limitations of different test systems should be taken into account to reduce the risk of false-negative results for compounds with genotoxic potential.[22] At the same time, a

single positive result in any assay for genotoxicity does not necessarily mean that the test compound poses a genotoxic hazard to humans.[23] In such a case, mechanistic investigations can result in further details that will aid in taking a regulatory decision.

d.) Experimental design: Two carcinogenicity studies have been recognized: (i) one long-term carcinogenicity study in the rat, a species of choice; (ii) an additional *in vivo* study that supplements standard 2-year rat study. This is either a long-term carcinogenicity study in a second rodent species, mouse, or short- or medium-term *in vivo* rodent test systems. The later is discussed in section "Short or medium-term (alternative) models in carcinogenicity bioassay".

e.) Role of study protocol in designing carcinogenicity studies: The study protocol for a definitive study is largely based on scientific reasoning, experience, and consensus gained over many studies. Parameters such as the number of animals, the adequacy of dose, duration and the gross histopathological examination are considered for a particular study. Several important factors such as species, strain, sex, dose levels, test substance purity, route of administration, and statistical methods are considered in designing a 2-year carcinogenicity study. Other factors to consider are pharmacology, toxicology and metabolism of the drug, and systemic exposure (e.g., as measured by AUC) achieved in the test species as multiples of maximum recommended human dose (MRHD). On the basis of a number of considerations and the extensive database available on tumors, it is considered that the rat is the most preferred species for the standard 2- year carcinogenicity bioassay.[24]

Because a large number of animals and length of time is involved, it is essential that these studies be planned well. Inadequate dosing will result in improper results and may lead to the necessity of repeating these studies. To avoid such problems, the US FDA strongly recommends the sponsor to conduct a 90-day dose range-finding study and submit the results along with the protocol for the carcinogenicity study to the referred division for comment. The intention of this protocol is to inform the drug candidate sponsors about the types of information the agency relies on when evaluating protocols for animal carcinogenicity studies[25]

f.) Species and strain selection: The lifetime (2 year) carcinogenic bioassays are generally conducted in both sexes of rats and/or mice. In most studies, inbred rodent strains are used in order to reduce experimental variables and to enhance the interpretability of the results. The control of experimental variables and the large experience with common laboratory strains of rats and mice represent great advantages for risk assessment. Background data such as food intake, body weight, growth rate, longevity, clinical pathology, and histopathology can facilitate the interpretation of experimental results. Historical data on tumour incidence is considered useful in the interpretation of long-term rodent carcinogenicity bioassays, especially to assess the occurrence of marginally increased tumour incidence and species differences.[26] These data are helpful to avoid the high incidence of background tumors and aging lesions that arise spontaneously in different organs in different strains of rats and mice. As these data are compared with the treated animals, the concurrent control group is considered to be the most critical parameter.[27]

To draw a definite conclusion whether a test compound can be evaluated on a particular species, information on genetic toxicology, tumour incidence, strain of animal, route and dosage regimen, pharmacological or therapeutic activity, development and/or regulatory status, and relevant reason for termination of development are generally considered. Compounds that induce tumors only in a single species are approximately double in number in rats as compared to mice.[28] In a simplistic sense, this implies that the rat is more "sensitive" than the mouse. There are a few instances identified in which mouse tumors are considered as the sole reason for the regulatory action concerning disapproval for marketing of a pharmaceutical.[18] Rodent liver is highly susceptible for the induction of tumors by non-genotoxic chemicals. These tumors are not always relevant to carcinogenic risk in humans and mislead the use of rodent for carcinogenic risk estimation. The rat carcinogenicity study is widely accepted because these results can correlate up to 70% with that of human data.29 Non-genotoxic chemicals induce carcinogenicity in rodents, which is highly dependent on species, strain, and target organ. The induction of carcinogenicity depends on the threshold dose phenomenon and it varies from species to species. Mechanistic studies have permitted the distinction between effects that are specific to the rodent model and those that are likely to have relevance for humans. The specific role of tissue specificity of receptors and their subtypes has increased our understanding of the mechanistic basis of carcinogenesis.[30]

On the basis of metabolic pathways, neither rats nor mice are considered suitable for the conduct of the bioassay.[31] Much attention is now being paid to the pharmacokinetic-pharmacodynamic relation. Rapid progress is being made on the specific role of cytochrome P450 isozymes mediated biotransformation of drugs. Because most of the research activity is confined to rats and humans, the data obtained from mice would be less likely to provide useful metabolic information in mechanistic studies.[32] Owing to the size of the animal, the mouse model is not considered

suitable when it comes to the collection of serial blood samples, microsurgery/catheterization, and organ weights. Animal sacrifice is required to collect blood samples; hence more numbers are required per investigation.[29]

On the basis of the availability of historical control database on tumors, the following strains are commonly used. Rats: Fischer 344, Sprague-Dawley, Wistar. Mouse: B6C3F1, ICR Swiss (CD-1), BALB/c. In a recent study, Britton *et al* (2004) reports that of the three rat strains studied (Harlan Hsd:Sprague-Dawley SD, Harlan Wistar Hsd:BrlHan:WIST, Charles River Crl:CD), Harlan Wistar strain survived in much greater numbers in 104-week carcinogenicity study.[33] The improved survival rate, according to the authors, appeared to be independent of body weight and food consumption and is reflected in the spontaneous pathology profile.

It is now well recognized that the diet fed *ad libitum* can directly enhance weight gain resulting in obesity, increased background tumour development, and non-neoplastic lesions. Owing to these undesirable trends, the longevity of the animals decline, resulting in 50% survival level for some strains of rats before the 24-months target point. Alternative diets and feeding regimens are being evaluated and some results have already been published.[34] There are many options available for *ad libitum* feeding and changes in the dietary content need to be controlled within a study for proper biological and statistical assessment of neoplastic and non-neoplastic lesions.

g.) Group number and size: Generally, for biological and statistical determinations, three treated groups are recommended for establishing any dose-related effects. The conventional number of animals per sex per group is 50 to 100. In many studies, 50% or more survivors, over 18 months for mice and 24 months for rats are observed. This survival rate is considered acceptable for detecting age-related, non-lethal conditions and for statistical analysis.[35, 36] The scientific reason for using more animals per group is to increase the statistical power of the study.[12] Sometimes the control group is doubled to include two concurrent groups with 50 animals per sex per group that generates a large histopathological database. Two control groups are used in cases such as dietary administration where control 1 group is given diet/vehicle *ad libitum*, and in control 2 group, diet is restricted to the amount consumed by the high-dose group. In some studies, 3 control groups are used: 2 diet controls (each kept in two different rooms) and 1 vehicle control. For dose range-finding study, 10 to 30 animals per sex per group are used. Satellite groups each composed of 10 animals per sex per group (at least 2 animals/sex/time point) are used for toxicokinetic measurements.

h.) Route of administration: The route of exposure in animals should be the same as the intended clinical route. If similar metabolism and systemic exposure can be observed through different routes of administration, then carcinogenicity studies should be conducted by using a single route. The target organ for the clinical route should be adequately exposed to the test material. The evidence of adequate target-organ exposure should be obtained from toxicokinetic data.[37] The conventional route of administration is oral by gavage or dietary administration.

Pharmaceuticals applied topically with poor systemic absorption may not need studies by the oral route to assess the carcinogenic effect to internal organs. However, in case of photocarcinogens, dermal application (generally in mice) may be needed. For different salts of the same base, where prior carcinogenicity studies are available, evidence of changes in pharmacokinetics, pharmacodynamics, or toxicity should be provided. When changes in exposure and consequently toxicity are noted with a change in salt or combination with another drug molecule (e.g., fixed dose combination), then additional studies are recommended to determine the carcinogenic potential of the test substance under investigation.[38]

I.) Duration of study: There is a general agreement upon the exposure time of 18 to 24 months for mice and at least 24 months for rats in lifetime carcinogenesis bioassay. Both the time frames agree well for inducing tumors because of continuous administration of test chemical. Extending the study period beyond this time limit, especially for a negative result and using the 50% survivor rule for termination of the study will further increase confidence in test results obtained.[17] The duration for dose range-finding study is 90 days if it is intended to support dose selection for a standard 2-year study. The duration will be 4 week in a wild-type strain if it is intended to support a short-term study.

j.) Issues for the conduct of dose-ranging studies: The 90-day dose range-finding study is initiated to select the high dose (as well as middle and low doses) for the definitive 2-year carcinogenesis bioassay. The metabolic profiles of the drug in humans and the animal species under investigation are provided for the assessment of its carcinogenic potential. It is unnecessary to include the maximum feasible doses in the design of a range-finding study, when a dose lower than the maximum feasible dose administered by the same route of administration is not tolerated or exceed other acceptable dose selection endpoints. In the absence of this information, it may be prudent to include a maximum feasible dose in the design of a range-finding experiment. The maximum dose selected should not result in death or induce any significant toxicity in the tested animals.

k.) Dose selection: Owing to the diverse nature of substances used in pharmaceuticals and many non-genotoxic mechanisms involved in carcinogenesis, a flexible approach is needed for dose selection. However, in the process of defining such a flexible approach, it is recognized that the fundamental mechanisms of carcinogenesis are only poorly understood at the present time. The plasma concentration of drug substances represents an important parameter in selecting a particular dose and improving the design of the rodent bioassay. Consideration of other relevant animal toxicity data and integration with available human data is paramount in selecting the high dose for the carcinogenicity study.[39] The selection of appropriate dose levels is of paramount importance for the successful outcome of any study. Dose selection requires an in-depth knowledge of a compound's pharmacology, repeat dose toxicology, toxicokinetics in both test species, and pharmacokinetics in humans. It is generally agreed upon by all regulatory authorities to set a high dose, which induces some type of toxicity, which is not life threatening for the study period. The best known example is a 10% reduction in body weight gain relative to control. In case of a test substance, which is nontoxic or has a low bioavailability, multi-dose toxicokinetic measurements through different routes should permit in the selection of a route and a dose for maximum exposure. Another alternative but still an arbitrary approach is to determine high multiples of the human dose (30 to 100 times) expressed as an area under the curve (AUC). Such high doses may not be achievable for toxic compounds in rodents.[40]

Several methods are being used in the selection of doses for range-finding experiments. There is no uniformity among regulatory agencies around the world. For example, the maximum tolerated dose (MTD) has been used in the United States, whereas Europe and Japan normally select the high dose on the basis of toxicity endpoints. Some countries resort to high multiples of the MRHD. The chosen doses should clearly elicit effects that can be used as endpoints as recommended in the ICH guidance. Additionally, the study should also include a dose that is without significant toxicity.[41] There are as many as six different methods for selection of the high dose for range-finding experiments.

a) Toxicity-based endpoints: The ICH Expert Working Group on Safety has agreed to use the maximum tolerated dose (MTD) as an acceptable toxicity-based endpoint for selection of the high dose for carcinogenicity studies.[42] The MTD is predicted to produce a minimum toxic effect or the maximum dose that is being tolerated over the course of the study. This type of effect can be predicted from a 90-day dose range-finding study in which minimal toxicity can be observed. Factors such as alteration in physiological function, no more than 10% decrease in body weight gain relative to control, target-organ toxicity, and significant alterations in clinical pathological parameters are considered for high dose selection. It is important that the maximum dose selected should allow an adequate margin of safety, not to significantly disturb physiological function of the animal and finally good survival till the end of the dosing period.

b) Pharmacokinetic endpoints: The dose administered to different species may not correspond to tissue concentrations because of different metabolic and excretory patterns. The unbound drug in plasma is thought to be the most relevant indirect measure of tissue concentrations of the drug. The AUC is considered to be the most comprehensive pharmacokinetic endpoint, because it takes into account the plasma concentration of the test substance and the *in vivo* retention time.[43] For non-genotoxic pharmaceuticals, a systemic exposure representing large multiples of the human AUC (at the maximum recommended daily dose) may be an appropriate endpoint for dose selection. The selection of a high dose for carcinogenicity studies, which represents systemic exposure ratio of 25-fold rodent to human plasma AUC of parent compound and/or metabolites, is considered pragmatic. For example, systemic exposure ratio of 25-fold, rat to human MRHD based on body surface area (mg/m2) would be 25 × 37 (human) / 6 (rat) = approximately 150-fold mg/kg ratio. Therefore, a human dose of 500 mg/day or less could be tested in rats at 1500 mg/kg/day as the high dose.[39]

c) Saturation of absorption: The measurement of saturation of absorption of drug-related substances from systemic circulation are an acceptable method in high dose selection. The low and mid doses are selected on the basis of the saturation of metabolic and elimination pathways of the compound under investigation.[44]

d) Pharmacodynamic endpoints: Pharmacodynamic endpoints for high dose selection are compound specific and are considered for a particular study on the basis of scientific merits. The high dose selected should produce a pharmacodynamic response in animals of such a magnitude that would preclude further dose escalation. Meanwhile, this dose should not disturb normal physiology or homeostasis of animals (e.g., causing hypotension).[45]

e) Maximum feasible dose: The use of pharmacokinetic endpoints (AUC ratio) in dose selection for low-toxicity pharmaceuticals can considerably decrease the need for selecting high doses on the basis of feasibility criteria. However in certain conditions, neither toxicity-based nor a pharmacodynamic-based dose selection can be achieved, and determination of pharmacokinetic endpoints (the 25-fold mg/m[46] ratio of rodent to human AUC or saturation of

absorption) is also not feasible. For non-genotoxic pharmaceuticals in a long-term carcinogenicity testing, a limited dose of up to a maximum of 1500 mg/kg/day in rats is considered acceptable where the MRHD is approximately 500 mg/day. The maximum feasible dose by dietary administration is considered 5% of total diet. In addition to dietary administration, when other routes are followed the high dose will be limited and considered on the basis of practicality and local tolerance.[47]

f) Additional endpoints: Additional endpoints can be used on high-dose selection for rodents on the basis of scientific rationale, which has not been defined in the above guidelines. Such designs are evaluated on the basis of their individual merits for a specific pharmaceutical compound. Mechanisms such as cell proliferation, induction of apoptosis, and epigenetic factors that play a crucial role in the induction of carcinogenesis, should be considered in high-dose selection for carcinogenicity studies.[48]

The mid and low doses provide information that would assist in assessing the relevance of study findings to humans. These doses are selected following integration of rodent and human pharmacokinetics, pharmacodynamic, and toxicity data. Some of the factors to be considered while selecting middle and low doses are: linearity of pharmacokinetics and saturation of metabolic pathways, human exposure and therapeutic dose, pharmacodynamic response in rodents, mechanistic information and potential for threshold effects, and alterations in normal rodent physiology.[49]

Observations and Measurements

All animals should be observed for clinical signs and mortality daily throughout the study. Palpation to examine for nodule formation is conducted frequently. Body weight and food consumption are recorded weekly. Hematology and clinical chemistry parameters are determined once, preferably at the end of the study. However, most of the investigators consider them optional. For toxicokinetics study, blood samples are collected from the caudal vein in unanaesthetized state from satellite animals (5 to 10/ sex/group) at various intervals such as 4, 12, 26, 52 and 103 weeks (morning and afternoon). The satellite animals are usually sacrificed after final sampling without further investigation. At the end, all surviving animals should be subjected to detailed necropsy that includes weighing and histopathological examination of several organs and tissues from control and high dose groups, and for any animal that died or was moribund and sacrificed.

The organs evaluated for histopathology are very exhaustive and are in general, and those organs that are most likely to develop cancer should be evaluated for all animals in all groups. The frequently recommended organs are liver, kidney, adrenal, uterus, and GI tract. Additional organs can be added to monitor a potential target-organ effect. When high-dose effects are observed in the target organs a limited number of tissues are recommended. The interpretation of results from the study will be dependent upon the list of tissues evaluated and the supportive data obtained from repeat dose toxicology studies. Any organ that does not have a human counter part, for example, zymbal glands, should generally be excluded from the study. The type of pathological changes could be either positive neoplastic or non-neoplastic lesions evolved due to either known secondary mechanisms or may be caused by study factors such as dietary intake.

Additional investigations are generally encouraged in all guidelines for better interpretation of the results. It has been recommended that blood smears and organ weights (e.g., adrenals, brain, heart, kidneys, liver, lungs, ovaries, pituitary, spleen, testes, and uterus) should be carried out at the time of termination. Smears of adequate quality, usually from terminal animals, are used in confirming specific granulocytic leukemia in Sprague Dawley rats that have histopathological evidence of such lesions. The organ weights are questionable for organs from a few animals that are distorted in weight due to lesions or tumors. In such a case, they should be excluded from group means.

Future aspects for Carcinogenicity Testing:

With regards to lessons for the future, there is a particular need for molecules that, are responsive to chemicals, exhibit a nongenotoxic mode of action. Owing to several inherent limitations in lifetime rodent carcinogenesis bioassay, extrapolation of the results to humans is difficult. In order to reduce the uncertainties and to render the extrapolation (of animal findings to man) more reliable, various mechanistic end-points have to be explored for a particular study. It is necessary to combine the results of these assays with the results from short-term tests, toxicokinetics, pharmacodynamics, genetic variation, and structure-activity relation. The introduction of new short-term models and the understanding of mechanistic aspects can greatly facilitate not only cancer research but also regulatory aspects of drug safety evaluation.

Alternative models for Carcinogenicity testing

There are various short to medium term carcinogenicity models are available in combination with single two year carcinogenicity testing. Following various alternative short-term models is described.

Transgenic mice: Transgenic mice may provide advantageous in developing a more specific model and reducing the number of animals and time required for bioassay. Some transgenic animals over express (Proto)oncogens such as "TG-AC" and "Tg-rasH_2" express "V-ras and "c-Ha-tas" proto-oncogens respectively.

Neonatal Mice: Neonatal mice have metabolic activity at developing stage which delays excretion of chemicals and this prolongation would have grater chance of changing normal cells into tumor cells.

Initiation-promotion models (in vivo): To detect "Initiator", substance is administered in a single dose or over a period of several days or week. After several week of washout, a "Promotor" is administered. The numbers of preneoplastic or neoplastic changes are examined. To examine weather as agent acts as a "Promoter", the procedure is reversed.

The NTP is also evaluating several lines of genetically altered mice for possible use in identifying and assaying carcinogens. A number if comments and concern are there for offering some thoughts on future directions for this line of research as well as for possible ways in which genetically altered mice might be integrated into a comprehensive testing strategy.[50]

Other Future opportunities

The early changes before a tumor develops ranges from altered hormone levels impaired ion balance and organ enlargement to specific and marked histopathological changes.[51] These Findings may be used for early detection of nongenotoxic carcinogens, and may also be extremely valuable for designing protocols for long tem bioassay. Furthermore, these indicators will lead to elucidation of specific mechanism involved in carcinogenesis. Based upon the above rational following approaches can refinement and reduction in testing.

1. First approach postulates that means of a new drug without a bioassay is usually available by the end of the first clinical studies.

2. Second approaches has been proposed with five stages that focus on chemical structure, DNA reactivity, epigenetic effects, limited bioassay, and finally, the application of "accelerated bioassay". This may require 40 weeks and would have he potential to replace the chronic bioassay in rodents in some circumstances and could serve alternative to a chronic bioassay in a second species. [52, 53]

Conclusion:

In 20th century the concert of carcinogenicity testing and carcinogenesis has evolved enormously, although the standard chronic bioassay still contains many of deficiencies. New carcinogenicity testing strategies, however, are to be expected also. Validation results with regards to the alternative carcinogenicity models will become available and lead new insights in the most appropriate short term carcinogenicity studies. In addition, communication between industry and regulatory authorities will be formulated early in order to allow the most optimal scientific approach.

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