

Design strategies, results and evaluations of long-term chemical carcinogenesis studies

by James Huff, PhD¹

HUFF JE. Design strategies, results and an evaluations of long-term chemical carcinogenesis studies. *Scand J Work Environ Health* 1992;18 Suppl 1:31-7. Long-term toxicology and chemical carcinogenesis experiments typically involve both sexes of two species of rodents divided randomly into sets of 50-60 animals per control and exposure groups. Ordinarily three exposure concentrations are graduated down from a top level likely to show some chemically associated toxicity, but which should not compromise the normal well-being or growth and survival patterns of the animals unduly. Duration of exposure is generally two years. Single, intermittent, or varied exposures are used to mimic specific occupational or environmental situations. Exposures are started prior to conception, during gestation and lactation, or at specified times thereafter. Extensive gross observations and microscopic pathology are performed on each animal, and incidences of neoplastic and nonneoplastic lesions are evaluated in age-adjusted statistical comparisons. The collated findings are then interpreted, evaluated, and presented for scientific peer review in public meetings, the aim being to identify qualitatively those environmental and occupational exposures that may likely induce cancer in humans.

Key terms: B6C3F1 mice, carcinogenesis, chemical carcinogenicity experiments, Fischer rats, identifying chemical carcinogens, two-year bioassays.

For several decades the principal approach used to identify chemical carcinogenic hazards to humans has been to expose laboratory animals to those agents thought to present the most potential for harm. Early experiments often involved only five to ten animals of a single sex and species exposed to a single concentration of a chemical for a duration of one year, with a limited (a single organ or two) pathology evaluation. With time and experience, control groups were added; group sizes were enlarged; two, three, or more chemical exposure levels became routine; and more of the major organs and tissues were examined histopathologically. Chemical agents were selected for study almost exclusively on the basis of the scientific presumption that they would be carcinogenic (eg, structurally related to known carcinogens), and studies were conducted largely not only to determine the magnitude of the hazard, but also to test and verify the prediction. Today, natural and synthetic chemicals or mixtures of chemicals are often chosen on the basis of the size and extent of the populations exposed and on the strength of the available toxicologic evidence used to forecast carcinogenicity.

Nowadays, the size, the number, and the types of experiments being done to prove unequivocally the car-

cinogenicity or noncarcinogenicity of a chemical, and then to identify potential hazards and to estimate risks of a similar occurrence in exposed humans, has become not only demandingly arduous, time-consuming, and controversial, but also exceedingly expensive and at times acrimonious. The societal disputes occur when economically important chemicals are shown convincingly to induce cancer in laboratory animals, and there are no human data to support or deny an association. Differences in scientific opinion, where they occur, lay not on the actual experimental findings, but on the interpretational aspects of the data, on the experimental model used to produce the data, on the person or organization conducting the studies, and perhaps mainly on the eventual personal, economic, and regulatory impacts these findings will cause. As long as these data are used in the risk assessment and regulatory processes, the political and public debates will continue, at times despite the scientific facts. Nonetheless, this panoply of opinion often, but not always, results in better decision making for the shared goal of protecting public health.

Presented in this paper are the basic protocols used for long-term studies, a viable "reduced design" alternative, summarized results from studies on nearly 400 chemicals involving almost 1400 sex-species experiments, and a summary containing a series of conclusions based on the accumulated knowledge gained over the 25 years since the chemical carcinogenesis program began at the National Cancer Institute and later within the National Toxicology Program. A summary comparison is made between carcinogenicity results from rodents and mutagenicity findings from bacteria.

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Strategies, results and values

The source and content description of the data used for this paper are as follows: (i) originated in the late 1960s by the United States National Cancer Institute, (ii) data continued and made more comprehensive since 1978 by the United States National Toxicology Program (NTP), (iii) extensive files contain detailed pathology data on more than 400 long-term (24-month) chemical carcinogenesis studies, (iv) 1600 individual experiments (male rat, female rat, male mice, female mice), (v) 4800 control and exposure groups, (vi) 240 000 laboratory rodents, (vii) 10 million tissue sections evaluated for toxicity and carcinogenicity, and (viii) all specimens, slides, and materials in archives for inspection. The most often practiced design for these long-term chemical carcinogenesis studies can be summarized as follows: (i) purpose: to determine the chronic toxicity and carcinogenic effects of chemicals; (ii) animals: Fischer 344 hybrid rats and B6C3F1 inbred mice (C3H $\times$ C57Bl6); (iii) groups and group sizes: males and females of each species, 50— $\geq$ 60 animals per sex-species group, and concurrent controls and two to four exposure groups; (iv) exposure levels: chosen to show some minimal yet obvious chemically associated toxicity of a degree not to compromise normal well-being or growth and survival; (v) duration of exposure: 24 months (two-thirds of life-span); (vi) route of exposure: priority to human exposure, historically, via feed (60%), oral intubation (27%), inhalation (5%), skin application (3%), intraperitoneal injection (3%), drinking water (2%); (vii) pathology: all animals, complete gross and histological. This design is one that fulfills most national and international scientific requirements established to conduct these studies, as well as for public health and occupational regulatory purposes.

Special studies added to the core or typical study design might include metabolism/pharmacokinetics, reproductive and developmental toxicology, immunotoxicology, stop exposure experimental groups to follow the progression or regression of lesions, cell proliferation measurements to determine possible influence on the carcinogenesis process, interim evaluations (at 6, 12, 15, 18, or 21 months) to observe toxic and early preneoplastic or neoplastic lesions, analyses of oncogene and suppressor gene expressions for mechanistic information, quantifying deoxyribonucleic acid (DNA) damage or repair and DNA adduct formation, and other paradigms as identified. Such supplemental studies help to define better the long-neglected influences of age and stress on the processes of metabolism, cell proliferation or cell turnover, tumor progression, or tumor regression and also to provide greater insight into potential mechanisms or an understanding of the various species, strain, sex, and organ differences in response to chemical carcinogens.

Thus the seemingly endless amount of scientific (and engineering) knowledge and social impact information required to make the most informed decisions about even a single chemical has caused an apparent shift in attention and resources. This change leads away from evaluating as many chemicals as possible for carcinogenicity and towards acquiring extensive data for fewer yet economically important chemicals. Much of this motivation comes naturally from a desire to know more about mechanisms of chemically induced cancers and thus gain better predictability, while some motivation comes from what appears to be prompted more by attempts to show that results from studies on animals are less relevant to potential human risk. The prodigious numbers and types of chemicals available for study, and the length and cost of doing adequate and acceptable carcinogenesis experiments with rodents, makes more difficult and more important the effort to nominate, select, and characterize toxicologically those chemicals that will give the most decision-making knowledge to protect public health better. In an attempt to satisfy both of these needs (a plethora of data about single chemicals or classes of chemicals versus "enough" cancer data on many chemicals), a protocol has been proposed that might help to evaluate more chemicals (1).

Retrospective analyses of the 313 chemical carcinogenesis studies considered to have been conducted adequately in each of four sex-species experiments permit the conclusion that using only the male rat and female mouse portions of these studies would have given the "same" conclusion regarding the carcinogenicity for 298 of 313 chemicals. For the 15 chemicals that would not have been identified correctly using this alternative protocol, two gave positive results for female rats and male mice, four were positive for female rats only, and nine were positive for male mice only. The two chemicals exhibiting carcinogenicity for both species are nithiazide (tumors of the mammary gland in female rats and of the liver in male mice) and phenylbutazone (tumors of the kidney in female rats and of the liver in male mice). Importantly, however, supportive but marginal increases in tumors were observed in the liver of female mice for the former chemical and in the kidney of male rats for the latter chemical. However, these results may have been fully discounted in the absence of the other responses.

Collecting these sex-species data into categories based on the number of positive or no evidence responses reveals that 43 chemicals (13.7%) caused cancer in each of the four sex-species experimental units, 25 chemicals (8%) induced cancer in three of the four units, 56 chemicals (17.9%) produced tumors in two of the four experiments, 38 chemicals (12.1%) were single-sex and single-species carcinogens, and 151 chemicals (48.2%) did not cause any tumors at any of the exposure concentrations in any of the four experimental cells. These qualitative separations should prove of value for selecting those exposures that should

be pursued epidemiologically; that is, a high priority should be given to the 68 chemicals causing cancers in three or four of the four sex-species units evaluated, an attempt being made to locate relevant human cohorts for study. Evaluating concordance for carcinogenic responses in rats and mice shows an overall correspondence of 74% (table 1) (1, 2). The highest concordances are between sexes in each species. Results on the 1394 sex-species experimental units are given in table 2.

The organs or systems most frequently observed in rats and mice (combined) with chemically induced site-specific neoplasms are (in decreasing order): liver (27%); lung, hematopoietic system, and kidney (8% each); mammary glands (7%); forestomach (6%); thyroid glands and Zymbal glands (5%); urinary bladder, skin, and uterus and cervix (4%); and circulatory system and adrenal glands (3%). Comparing this order to the frequency of the most common site-specific tumor types found in humans in the United States population reveals a remarkable similarity, except for the liver (ranked number eight in humans worldwide), adrenal gland tumors, and an organ in rodents (Zymbal glands in the ear) not having an obvious counterpart in humans. For comparative purposes, the forestomach of rodents was taken as associating with the esophagus of humans.

Another set of correlation data comes from comparing tumor incidence rates between those site-specific tumors that occur in unexposed (or control) groups and those that are considered to be caused by chemicals. For F344 rats, there appears to be little or no correla-

tion between the tumor rates in control animals and the frequency of site-specific chemically induced carcinogenic effects. Of the top 10 sites of chemically induced carcinogenicity in male rats, all but two (thyroid gland and hematopoietic system) involve tumors with background rates below 5%, and for most sites (forestomach, intestines, kidney, nasal cavity, and urinary bladder) the control rate is less than 1%. Three of the four most frequently occurring tumors in control animals (pheochromocytomas of the adrenal gland, adenomas of the pituitary gland, and interstitial cell tumors of the testes) are not among the frequent sites of chemical carcinogenicity. For female rats the results are similar. The most frequent tumor observed in control groups is adenoma of the pituitary gland, which has been considered to show chemically related effects with only a single chemical, 2-mercaptobenzothiazole. In contrast, chemically induced increases are frequently observed for uncommonly occurring tumors in the forestomach, kidney, and urinary bladder.

For B6C3F1 mice the results are different. The four most common sites for observed tumors in control male mice [liver, hematopoietic system (lymphoma), lung, and circulatory system (hemangioma/hemangiosarcoma)] are among the five most frequent sites of chemical carcinogenicity. Female mice show a similar pattern, although the relatively commonly occurring tumors of the pituitary gland are not among the top 10 tumor sites of chemical carcinogenesis. The reasons for these differences are not fully known, yet hormonal influences are important factors. Nevertheless, chemical carcinogenicity is often observed in mice at

Table 1. Intra- and interspecies concordance in the carcinogenic responses of rodents in 379 chemical carcinogenicity studies.^a

Comparison	Observed response				Percentage of concordant (+ + or - -) responses ^b
	+ +	+ -	- +	- -	
Male rats versus female rats	88	38	16	207	84.5 (295/349)
Male rats versus male mice	54	54	40	169	70.3 (223/317)
Male rats versus female mice	70	41	41	169	74.5 (239/321)
Female rats versus male mice	53	38	42	185	74.8 (238/318)
Female rats versus female mice	65	28	47	183	76.8 (248/323)
Male mice versus female mice	88	14	31	206	86.7 (294/339)
Rats versus mice	82	40	40	151	74.4 (233/313)

^a Note: equivocal evidence (or marginal) results are considered to be between a positive response and no evidence of a response; these results were placed into the no evidence category.

^b In parentheses the numerators are the numbers of combined + + and - - responses and the denominators are the total numbers of responses (+ +, + -, - +, - -). Not all 379 chemicals were evaluated in each of the four sex-species experiments, hence the differences in numbers.

Table 2. Summary results of 379 carcinogenicity studies on rodents.

Study result	By experiment					By chemical		
	Male rats	Female rats	Male mice	Female mice	Total	Rats	Mice	Overall
Positive	127	107	102	123	459	146	137	198
Equivocal	41	36	35	20	132	48	35	53
No evidence	183	211	204	205	803	162	178	128
Total	351	354	341	348	1394	356	350	379

sites having low background rates, most notably for the forestomach and ovary.

As has been long known, the liver is the most frequent site of chemically caused cancer in laboratory animals. In studies of the NTP 27% (104 of 379) of the chemicals evaluated caused liver tumors, or in 15% (203 of 1394) of the individual experiments (3, 4). However, what may not be fully appreciated is the fact that the liver is the most common site of chemically induced cancer for rats, as well as mice, an important factor when the biological significance of these neoplasms is being evaluated. That is, chemically related liver tumor effects in B6C3F1 mice have been discounted by some investigators on the basis, in large measure, of the relatively high background rate of these neoplasms (31% in male mice and 8% in female mice). Moreover, there appears to be little biological evidence to support this view. Chemically induced liver tumors are more common in female mice than in male mice despite the lower control rate for female mice. Furthermore, liver cancers are relatively uncommon in Fischer rats (< 1%); yet liver cancer remains the most frequent site of chemical carcinogenicity in both species. Thus the liver is an important site of chemical carcinogenesis in rodents, independent of background rates, and should continue to be recognized as an important signal of potential risk to humans. Simply the fact that the reason for the high background rates of this tumor in (male B6C3F1) mice is not fully understood should not be taken to mean that chemically induced tumors in this organ are irrelevant for hazard identification.

Comparing the correspondence between tumors of the liver with tumors of other organs reveals that a significant association exists for liver tumors in mice and "any other site" tumors in rats. For example, if a chemical produced liver cancer in mice, then that chemical was twice as likely to be a carcinogen in the rat (49 of 78, 63%) when compared with a chemical not causing liver tumors in mice (73 of 235, 31%). However, other site-specific tumors in mice are even more predictive of chemical carcinogenesis in the rat. If one divides the chemicals into three groups — those chemicals not causing any carcinogenic effects in mice, those chemicals causing carcinogenic responses in mice only for the liver, and those chemicals causing carcinogenic effects in mice at sites other than the liver — then a clear gradient of predictability becomes obvious: only 21% (40 of 191) of the chemicals not causing any tumors in mice were carcinogenic in rats; 54% (26 of 48) of the chemicals causing only liver tumors in mice were also carcinogenic in rats, and 76% (56 of 74) of the chemicals causing cancer in mice at sites other than the liver were likewise carcinogenic in rats. Again, these facts obviously show a more accurate rendition of the biological implications between these two species and strains than what one reads as data-lacking opinions.

The influence of the background control rate "inherent" within a particular organ or system has little or no biological impact on the actual "inherent" car-

cinogenicity of a particular chemical substance. As noted, a "high" background rate does not correspond with a high frequency of chemically related carcinogenic effects. For organs with very high background rates, however, a chemically induced carcinogenic response is difficult or impossible to detect because of the variable range of "control" data and because the statistical and biological limits may have been reached. For instance, the 64–98% control tumor incidence for interstitial cell tumors of the testes in Fischer rats essentially precludes this organ as a potential site for detecting a carcinogenic effect. In some situations there could be an earlier onset of these tumors that may indicate a chemical effect. Yet in no case has a chemical been judged in experiments of the NTP to induce this tumor type in male F344 rats.

Until now little attention has been given to the impact of various single or multiple parameters (induced cell turnover, organ toxicity, metabolic overload, pharmacokinetics) on the long-term process of chemical carcinogenesis. Much more needs to be learned about the possible influences these parameters may have on the multistage process. Although the findings from these experiments are indeed important and valuable for identifying potential long-term health hazards to humans, one would be better able to predict possible effects on humans, and perhaps to even select better animal models, if, for example, an extensive data base or catalogue existed on normal and exogenously induced cell turnover and cell proliferation patterns, as well as chemically specific pharmacokinetic data to allow better evaluation of scientifically based hypotheses.

Summary

In my judgment, the data from these long-term chemical carcinogenesis studies, together with my experience over the years, permit me to draw the following scientific and public health-oriented conclusions. The abbreviated statements have been taken from various publications, and they should be used for locating the rationales and the details that support the scientifically based interpretations:

1. Carcinogenicity findings from experiments on laboratory animals are logical and scientifically reasonable for identifying and predicting potential carcinogenic effects to humans (1, 5–11).
2. Most chemicals are not considered to be potentially carcinogenic to humans (5, 12).
3. Chemicals considered to be carcinogens may differ with regard to the overall strength of the induced carcinogenic response and, for practical purposes, can be divided into qualitative groupings based on empirical indicators of "potency" (7, 13) (examples: exposure concentrations, number of positive experiments, magnitude of induced tumor incidence rates, dose-response patterns, multiplicity of tumor sites, numbers of tumors per site,

tumor correspondence across sexes and species, common versus uncommon tumors, latency and metastases, preneoplastic lesions).

4. Malignant and benign tumors of the same site or cell type or both should be combined for interpretation (14, 15), but combining biologically unrelated tumors for statistical evaluation should be avoided (14, 16).
5. Benign tumors induced by chemicals are relevant for judging carcinogenicity, and few chemicals produce only benign tumors (15).
6. Site-specific tumor analyses should be used for determining chemically induced carcinogenicity (4, 16).
7. Liver tumors are appropriate and valid for identifying chemical carcinogens and for determining potential cancer hazard to humans (3, 7).
8. Cellular toxicity and resultant sequelae are not uniformly associated with chemically induced carcinogenicity (17–19) in that most chemicals produce carcinogenic responses in organs showing no evidence of toxicity and conversely toxicity occurs frequently in organs having no chemically related carcinogenic effects (19).
9. Most chemical carcinogens do not cause cancer only at the highest exposures used (17, 19, 20), and for those few that do there are no scientific reasons that these should not be considered relevant for human hazard identification.
10. Mechanisms of action are not yet considered to be understood well enough to become significant factors in the evaluations of chemically induced carcinogenesis (21), but certainly all relevant and available biological information, including mechanistic information (22), should be considered when the overall evidence of the carcinogenic responses (and for extrapolative purposes among mammalian species) is decided on.
11. Cell replication (proliferation, mitogenesis) is an important factor in chemical carcinogenesis; yet available scientific data do not sustain the hypothesis that enhanced cellular proliferation in a particular organ associates consistently with an increased induction of neoplasia (19, 23, 24).
12. The route of exposure has little or no effect on the inherent carcinogenicity of a chemical, although the specific sites of carcinogenicity may vary with the route of administration (25).
13. Corn oil used as a vehicle for chemical administration has little or no influence on chemical carcinogenicity (26, 27).
14. Interspecies concordance in carcinogenic response is good; yet at least two species should in most cases continue to be used for identifying chemical carcinogens (1, 2, 28).
15. Short-term in vitro assays (mutagenicity in *Salmonella* or mouse lymphoma cells, sister chromatid exchanges, and chromosomal aberrations in Chinese hamster ovary cells, eg) have a rela-

Table 3. Patterns of short-term in vitro test results and long-term in vivo carcinogenesis results for 114 chemicals. [SAL = *Salmonella typhimurium*, ABS = chromosome aberrations (Chinese hamster ovary cells), SCE = sister chromatid exchanges (Chinese hamster ovary cells), MLA = mouse lymphoma]

Short-term in vitro test and results (SAL, ABS, SCE, MLA)	Rodent carcinogenicity results	
	Positive	Negative
Positive in 4 of 4 assays	22	3
Positive in 3 of 4 assays	14	7
Positive in 2 of 4 assays	11	15
Positive in 1 of 4 assays	9	8
Positive in 0 of 4 assays	11	14
Total (114 chemicals)	67	47

tively low concordance with rodent carcinogenicity (29–31) (see table 3).

16. Caging and cage location have no important impact on chemical carcinogenicity (32, 33).
17. Chemical structure alone and in general does not allow for cancer prediction of carcinogenic effects on rodents or humans (34).
18. False-positive rates associated with evaluating chemically induced carcinogenic effects in rodents are low (35, 36). Unfortunately, little is known about the more public health-important false-negative rate.

For those chemicals or mixtures of chemicals for which there is evidence of carcinogenicity in humans and which have been studied adequately in experimental animals, all have been shown to cause cancer in at least one animal species, and in every case there is one or more common tumor sites (6, 8, 37). For several chemicals the evidence of carcinogenicity in experimental animals preceded evidence obtained from epidemiologic studies or case reports (38–40).

Extrapolating chemical carcinogenesis results from one species to another, and in particular from animals to humans, continues as a major public health goal of these experimental efforts. In my view, long-term experiments that have been designed and conducted adequately and are interpreted in public peer review as showing either “evidence of carcinogenicity” or “no evidence of carcinogenicity” (13) should be used to forecast similar results in humans exposed to the same chemical or chemicals (5).

Acknowledgments

I thank Dr R Melnick and Dr J Bucher for their insightful and perpetual debates on the concepts and value of long-term carcinogenesis studies, as well as for their reading of and meaningful and evocative comments on this particular manuscript. Ms D Mayer nicely verified the citations and references.

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