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Nonlinearity Of Dose–Response Functions For Carcinogenicity

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Carcinogenesis data for 315 chemicals were obtained from the National Cancer Institute–National Toxicology Program (NCI-NTP) bioassay programs and were analyzed to examine the shape of carcinogenesis dose–response curves. Tumor site data were more often consistent with a quadratic response than with a linear response, suggesting that the routine use of linear dose–response models will often overestimate risk. Information from *in vivo* short-term mutagenicity and genotoxicity assays was also obtained for most of these rodent bioassays. It was found that there were no clear relationships between the shape of the carcinogenesis dose–response curve and the result of the short-term test. These observations argue against the concept that carcinogens that are positive in a short-term assay be regulated using a linear dose–response curve and those that are negative be regulated using a sublinear dose–response curve or a safety factor approach.

Introduction

One of the more controversial issues in chemical carcinogenesis is that of estimating low-dose effects (*1–7*). Because of sample size considerations, experimental data must be obtained at relatively high exposure levels and then necessarily extrapolated to relatively low human exposure levels (*5–7*). Although the basic mechanisms of carcinogenicity are not well understood, it is believed that the process is multistage (*2,8–13*). If this is the case, a dose–response curve for a carcinogen would expectably depend on the transition between particular stages or the clonal expansion of cells in a particular stage that the chemical affects (*1,2,4,11–16*). For example, the first stage is thought to involve mutational changes in the DNA as a result of what is believed to be a linear-in-dose genotoxic effect of the chemical (*1,2,4,11,12,15*). Chemical promoters can enhance the expansion of these mutated cells by a selective growth process that is generally assumed to be nonlinear (*1,2,4,11,12,15*). Later steps, such as progression, can also be chemically dependent with both linear and nonlinear dose–response relationships (*12,15,17*). Note also that the chemical itself can undergo metabolic changes that may lead to a nonlinear relationship between the administered chemical and the effective metabolite that induces carcinogenesis (*3,18*).

Accurate determination of the shape of the dose–response curve is critical to predicting low-dose risks. Some dose–response models have the property that they are "low-dose linear," or "linear" for short (*4*). A linear dose–response model is a model in which the slope of the dose–response curve evaluated at dose zero is positive and proportional to dose. One such model is the one-hit/one-stage model. Other models exhibit curvature for which the slope of the dose–response curve is equal to zero at dose zero. The two-hit/one-stage or quadratic model is an example of such a model. Finally, some models exhibit curvature that is greater than linear in the low-dose range. A model that typifies this type of response is the square-root model. The mathematical descriptions of these models are given in the Methods section. These three simple models (square-root, linear, and quadratic) include the three types of qualitative behavior most frequently considered in carcinogenesis studies (e.g., supralinear dose response, linear dose response, and sublinear dose response).

In examining experimental carcinogenesis data from a public health standpoint, we find that a dose–response function that is linear in low doses will typically fit the observed data and will usually overestimate the observed carcinogenic risk at the low-dose level. Thus, the linear model is considered to be a conservative model. However, it is important to determine the actual degree to which the linear model is conservative. One approach to evaluating linearity versus nonlinearity is to examine the available animal data. In this paper we focus on two questions: *a*) What is the usual shape of the dose–response curve for carcinogenic response to environmental agents? and *b*) Do short-term *in vitro* genotoxicity assays predict the shape of these dose-response curves? To answer these questions, we have analyzed data from the NCI/NTP 2-year rodent carcinogenesis studies and from *in vitro* assays for mutagenicity and genotoxicity.

Data and Statistical Methods

In any analysis of carcinogenesis data, one is faced with the difficulties of dealing with small sample sizes. The carcinogenesis data generated from these small samples are often consistent with many different dose–response functions, including a linear

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Table 1. Combinations of primary tumor sites.

All squamous cell and basal cell papillomas, adenomas, and carcinomas of the skin
All fibroma and fibrosarcoma of the subcutaneous tissue
Alveolar bronchiolar adenomas and carcinomas of the lung
All nasal cavity tumors
All hematopoietic system tumors
All circulatory system tumors
Adenomas/nodules and carcinomas of the liver
Adenomas and adenocarcinomas of the tubular cells of the kidney
Papillomas and carcinomas of the transitional cells of the kidney
All urinary bladder tumors
All pituitary tumors
All pheochromocytomas of the adrenal gland
Adenomas and carcinomas of the adrenal cortex
Adenomas and carcinomas of the thyroid C-cells
Adenomas and carcinomas of the thyroid follicular cells
All tumors of the parathyroid
Adenomas and carcinomas of the pancreas islet cells
Squamous cell papilloma and carcinoma of the forestomach
All tumors of the mammary gland
Interstitial cell tumors of the testis
Endometrial stromal polyps and sarcomas of the uterus
All tumors of the zymbal gland
All mesothelioma

dose–response function. However, one could just as easily test to see whether the data are consistent with a particular nonlinear hypothesis, such as a quadratic dose–response function. Because of the small sample sizes, one finds that the data will also be consistent with several nonlinear dose–response functions (*19*). We are interested in the degree to which carcinogenicity data are actually consistent with linearity and whether other possible dose–response functions may be more appropriate.

Our analysis is based on tumorigenicity data from 344 rodent bioassays on 315 chemicals studied by the NCI and the NTP (*20,21*). For each bioassay, cancer sites were identified. The term "site" refers to a specific tumor or class of tumors in one sex/species group for one chemical. Thus, because the NTP feeding study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*22*) recorded significant increases in both thyroid follicular-cell tumors and liver tumors, there would be at least two sites listed for the animals in this study. The tumor Standard Nomenclature of Pathology (SNOP) codes recorded by the NCI/NTP pathologists were not analyzed individually, but instead were grouped as given in Table 1. If tumor SNOP codes were recorded that did not agree with any of the classifications shown in Table 1, they were grouped by tissue site and analyzed separately. Finally, we also considered a grouping of all tumor-bearing animals (excluding interstitial cell tumors of the testes in male Fischer 344 rats). In the 344 rodent bioassays we investigated, there were 21,463 sites. Many of these sites are not related to exposure to the chemical and were excluded from the analysis. We considered two subsets of the full set of 21,463 sites; we analyzed all sites that were statistically significant at $\alpha = 0.01$ using the adjusted quantal response test (*23,24*) and all sites with $\alpha = 0.05$.

When testing at $\alpha = 0.01$, our overall agreement with the calls made by the NCI/NTP review boards was good. For example, of the 298 experiments that included male mice, we found 68 of the 83 NCI/NTP positives and 153 of the 174 negatives (most of the inadequate studies and equivocal results were found to be negative: 29/41). The difference between our results and the NCI/NTP calls is due to *a*) our inability to recognize a significant rare tumor, *b*) our use of a survival-adjusted test for early NCI experiments, and *c*) the use of all tumor-bearing animals, a grouping not used by the NCI/NTP. The general results we observed for $\alpha = 0.05$ were similar to those observed for $\alpha = 0.01$, and thus we only present the results for $\alpha = 0.01$.

For each sex-by-species-by-chemical-by-route combination, there may be several sites exhibiting a significantly increased cancer risk. Historically, risk assessments have been done using either the most potent tumor site or a combination of tumor sites. To mimic what is done in practice, our first analysis presents results for the site with the steepest dose–response curve (most potent site) among all significant sites ($\alpha = 0.01$) for each sex-by-species-by-chemical-by-route combination.

A model of the form

$$P(d) = 1 - e^{-\gamma_1 - \gamma_2 d^{\beta}} \quad (1)$$

was fit to all cancer sites using the survival-adjusted quantal response (*23,24*), where β was fixed at one of three values, $\beta=0.5$ (a square-root model), $\beta=1$ (a linear model), $\beta=2$ (a quadratic model). For each cancer site we determined whether the estimated dose–response relationship was consistent with a linear, quadratic, or square-root model using a standard test of goodness of fit (*25*), testing at $\alpha=0.10$. We were also able to determine if the observed data had more or less curvature than the fitted model (e.g., in the case of a linear model, more curvature would correspond to a quadratic or higher [$\beta > 2$] response, less curvature to a square root or less response [$\beta < ½$]) by evaluating the partial derivative of the log-likelihood with respect to the shape (β) of the dose–response curve.

Results

In Vivo Dose–Response Shape

Table 2 summarizes the results of our analysis of the routine shapes of dose–response curves. In Table 2 (part A) we see that 67% (260/390) of the experimental results are consistent with all three of the models and 84% (326/390) are consistent with two or more models. It is clear that the linear model fits most of the data (327/390) and that all three models fit much of it. If we look at the model that best fits the experimental data without regard to the adequacy of this fit (Table 2, part B), we find that 33% of the sites fit the square-root model best, 20% fit the linear model best, and 47% fit the quadratic model best. Thus, a quadratic dose–response function provides a best fit to the observed experimental results more frequently than does a linear curve or a square-root curve.

Another way to characterize dose–response shape is to assign a shape to each chemical using all positive sites instead of only the most potent site. This can only be done reliably for chemicals for which all significant sites have the same shape. The results show that 58% of the chemicals with at least one significant tumor site have a mixture of dose–response shapes (Table 2, part C). More notably, of the 75 chemicals for which all significant sites had only one shape, most (81%) have solely quadratic dose–response data. The number of sites for each of these 75 chemicals differed according to the shape of the dose–response curve. Eight of the 9 linear chemicals and 2 of the 5 square-root chemicals had only 1 significant site. The 1 remaining linear chemical and the 3

Table 2. Consistency of carcinogenesis data with square root, linear, and quadratic dose-response models.

Part A: Testing for consistency with various models[a]

L,Sr, Q	L,SR, Not Q	L,Q not SR	SR only	L only	Q only	None but <SR	None but >Q
260	36	30	7	1	35	1	20

Part B: Best fits[a]

SR	L	Q	Total
129 (33[b])	77 (20)	184 (47)	390

Part C: Classifying chemicals[c]

Case[d]	SR	L	Q	Mixed	Total
A	5 (2.8)	9 (5.1)	61 (34.5)	102 (57.6)	177
B	50 (12.8)	45 (11.5)	160 (41.0)	135 (34.6)	390

Abbreviations: SR, square root; L, linear; Q, quadratic.

[a]For each sex/species/route/chemical combination examined, the cases reported are for the tissue site with the maximum slope for the dose-response curve under the linear model.

[b]Numbers in parentheses are percents.

[c]Entries are the number of data sets for which all significant tumor sites were best fit by square root, linear, etc.

[d]The cases are (A) each chemical (multiple routes and technical reports are treated as different chemicals) forms one data set tested at the 0.01 level; (B) each sex-by-species-by-chemical combination forms one data set, tested at the 0.01 level.

remaining square-root chemicals had only 2 significant sites. On the other hand, for the quadratic chemicals, 3 had 6 or more sites, 1 had 5 sites, 6 had 4 sites, 4 had 3 sites, 13 had 2 sites, and 34 had only 1 significant site. Thus, the evidence for nonlinear dose-response is stronger for quadratic-only chemicals than it is for square-root-only or linear-only chemicals. If, instead of all sites for each chemical, we consider all significant sites for each sex-by-species-by-chemical combination (case B), there are fewer cases where the results are a mixture of models (only 35%). Again, quadratic models dominate with 63% (160/255) of the unequivocal calls.

To illustrate these findings for specific chemicals, consider the list of 10 examples provided by Bailer et al. (*26,27*), of sites that exhibited square-root dose response from animal carcinogenesis experiments, eight of which were also in our database (1,4-methylene dianiline 2HCl, 1,2-dibromoethane, 1,4-dioxane, 1,3-butadiene, dimethylvinyl chloride, cytembena, 1,5-naphthalene diamine and iodinated glycerol). In all eight cases, we find that the square-root model best fits these data for the specific site chosen by Bailer. However we also find that all eight chemicals produced tumors at numerous sites, and in no case were all of the significant sites square-root in shape. In addition, we find that for the tumor sites chosen by Bailer, the data also fit the quadratic model for four of the chemicals (1,2-dibromoethane, 1,4-dioxane, dimethylvinyl chloride, and 1,5-napthaline diamine) and the linear model for seven of the chemicals (all except iodinated glycerol). For one chemical (iodinated glycerol), none of the three models adequately fit the data (although these data are clearly more extreme than square-root). In five of the eight cases, the survival-adjusted quantal response for the two dose groups exceeded 75%, providing little information on curvature. For two of these chemicals (1,2-dibromoethane and dimethylvinyl chloride), the response, in fact, exceeded 95% for both doses, which provides no information on curvature. Thus, looking at the best-fitting curve for one site for a chemical does not necessarily portray the range of shapes for that chemical.

Prediction of *In Vivo* Dose-Response Shape from *In Vitro* Data

Many authors have suggested that genotoxic compounds are likely to result in linear dose-response relationships (*28–31*) and that nongenotoxic compounds will result in threshold or nonlinear dose response. The belief that genotoxic agents induce linear dose response stems from theoretical arguments about one molecule of a genotoxic compound interacting with DNA resulting in a "single hit." The probability of cancer is then assumed to be proportional to the number of "hits" resulting in a linear, no-threshold dose-response model. The nonlinear shape for nongenotoxic compounds is based on mechanistic arguments concerning cytotoxicity, and promotion, mechanisms that are generally thought to be threshold mediated or nonlinear. We are interested in whether the carcinogenicity data support this theory. Although it would be difficult to reject or accept it on the basis of bioassay data, with a data base this large, we should at least see shape patterns that conform to this theory.

To evaluate the relationship between genotoxicity and dose-response shape, we repeated the analysis of the previous section, stratifying chemicals into those that are mutagenic using the Ames Salmonella assay and those that are not. A chemical was labeled as a positive mutagen if it was positive in any of the various Salmonella assays conducted by the NTP (*32*). Table 3 illustrates the results. There were 367 sex-by-species-by-chemical groups (Table 3, case A) for which the carcinogenesis response was significant at the 1% level ($p<0.01$); 230 (63%) were positive in the Salmonella assay, and 137 (37%) were negative. Most of the sites adequately fit all three of the dose-response models, with 65% (150/230) of the mutagenic compounds fitting all three models, and 67% (92/137) fitting all

Table 3. Consistency of carcinogenesis data with square root, linear, and quadratic dose-response models categorized by mutagenicity in Salmonella.

Part A: Testing for consistency with various models[a]

Salmonella test	L,SR, Q	L,SR, not Q	L,Q not SR	SR only	L only	Q only	None but <SR	None but >Q
+	150	18	18	5	1	21	1	16
−	92	16	10	2	0	13	0	4

Part B: Best fits[a]

Salmonella test	SR	L	Q	Total
+	78 (33.9[b])	43 (18.7)	109 (47.4)	230
−	41 (29.9)	27 (19.7)	69 (50.4)	137

Part C: Classifying chemicals[c]

Case[c]	Salmonella test	SR	L	Q	Mixed	Total
A	+	0 (0.0)	1 (1.1)	28 (31.5)	60 (67.4)	89
	−	5 (6.6)	8 (10.5)	31 (40.8)	32 (42.1)	76
B	+	25 (10.9)	21 (9.1)	91 (36.6)	93 (40.4)	230
	−	23 (16.8)	19 (13.9)	63 (46.0)	32 (23.4)	137

Abbreviations: SR, square root; L, linear; Q, quadratic.

[a]For each sex/species/route/chemical combination examined, the cases reported are for the tissue site with the maximum slope for the dose-response curve under the linear model.

[b]Numbers in parentheses are percents.

[c]Entries are the number of data sets for which all significant tumor sites were best fit by square root, linear, etc.

[c]The cases are (A) each chemical (multiple routes and technical reports are treated as different chemicals) forms one data set tested at the 0.01 level; (B) each sex-by-species-by-chemical combination forms one data set, tested at the 0.01 level.

three models for sites from nonmutagenic compounds. If we look at the best fitting dose–response model (Table 2, part B), there were no significant differences found by dividing the sites into those for mutagenic compounds and those for nonmutagenic compounds. As before, analyses by sex and/or species yielded similar results.

In Table 3, part 3, the results are grouped by chemicals in the same manner as Table 2, part 3. Looking at chemicals alone (Table 3, case A), 97% (28/29) of the Salmonella positives with only one shape were quadratic, and only 70% (31/44) of the Salmonella negatives were quadratic. The same pattern is observed for the sex-by-species-by-chemical classification (Table 3, case B). Thus, we see a pattern of dose–resronse shapes that is exactly opposite to the theoretical patterns suggested for genotoxic and nongenotoxic compounds. Caution must be exercised in interpreting the overall shape of chemicals that are Salmonella positive versus those that are Salmonella negative because chemicals that are Salmonella positive are more likely to have multiple significant tumor sites than are Salmonella negative chemicals (78% of the positives had 3 or more significant tumor sites as compared to 61% of the negatives). Thus, it is easier to label a Salmonella-negative chemical as having a single shape (this is obvious when you look at the "mixed" column of Table 3, part C). For the chemicals with quadratic shape, there is only a slight difference in the number of significant sites for Salmonella-positive versus Salmonella-negative chemicals (i.e., of the 14 quadratic chemicals with 3 or more significant sites, 6 were Salmonella positive and 8 were negative).

From these data, there is no evidence to support the assumption that a positive finding of mutagenicity in Salmonella is predictive of a linear dose–response relationship for carcinogenicity, nor is there evidence that a nonpositive mutagenicity finding is predictive of a nonlinear dose–response relationship.

This analysis was repeated for other short-term assays of genotoxicity including the analysis of sex-linked recessive lethals and reciprocal translocations in Drosophila, chromosomal aberrations and sister chromatid exchanges in Chinese hamster ovary cells, and the mouse lymphoma (LS1784) cell mutagenesis assay. There were fewer chemicals tested in these assays relative to Salmonella. No significant differences were noted between positives and negatives in these assays, and the prediction of dose–response shape was similar (or even less informative) to what was observed for Salmonella.

Discussion

The analysis in this study suggests that the carcinogenic dose response observed in animal studies is often nonlinear, the cancer risk increasing with dose at a rate greater than what would be expected if the effect were proportional to dose. Because the linear model would overestimate risk at the lowest experimental dose in these cases, it is likely that this model would also overestimate the cancer risk at the low doses typical of human exposure levels. The magnitude of this overestimation is unknown, and depends on how far from the experimental range one wishes to estimate effects. It has been shown previously that by considering simple hypothetical kinetic models (*2,3,14,18*), one could overestimate low-dose risk by a factor upwards of 500 without assuming any threshold behavior but by simply letting cancer incidence be proportional to a biologically effective dose. In other models, such as in human cancers resulting from radiation exposure, where one may choose between a linear dose response and a purely quadratic one with exponential cell killing, the risk estimation easily provides a difference of a factor of 100 in the exposure range of 1 rad (*33*). On the other hand, there are examples (*34*), albeit uncommon, of the square-root type response that can result in the underestimation of risk using linear models. These require careful analysis.

Bailer et al. (*26*) analyzed the carcinogenesis bioassay data for 308 chemicals studied by the NCI and the NTP. Fitting the one-hit model (Eq. 1) to these data, they concluded that "the one-hit formula. . .often underestimates lifetime cancer risks in the observable range." This conclusion results from observing that a sizeable portion of the experiments with only two dose groups had response at the middle-dose group higher than what would be predicted by a line drawn from the control response to the high-dose response.

Bailer et al.'s (*26*) analysis differed from ours in several key aspects. The three major differences are in the choice of data sets, the statistical methods, and the restrictions employed to obtain subsets of the data for analysis. Bailer et al. (*26*) concentrated their analysis on all sex/species/tumor sites in the data set with a brief description of a few restrictive analyses concerning significant tumor response and goodness of fit. In our analysis, we considered only data sets for which the modified trend test was significant because it is unlikely that acceptable exposure levels would be estimated for tumor sites without significant tumor risks. They concentrated on curvature without regard to the adequacy of the fitted model, whereas we looked into how often each dose-response shape fit or did not fit the bioassay data. Thus, although they observed a large number of square-root-shaped data sets, the majority of these agree with the linear model. Of the 390 experiments with a significant tumor risk at the 1% level (Table 2, case A), only 8 can be labeled as clearly square-root models. In addition, if truth were the one-hit model, it could be expected that, of those models for which the linear was not the best fit, 50% would be square-root shaped and 50% would be quadratic shaped. Instead, we find that only 34% (202/594) are square-root shaped, suggesting the general tendency of these data is toward quadratic curvature, not square-root curvature. This should not be construed as invalidating the findings of Bailer et al. (*26*). They basically pointed out that the linear model is not the most conservative model to use for many of these data. We agree with this finding. It is not clear, however, whether upper bounds on risk based on the linear model are sufficiently conservative to protect against a square-root model. What we have shown is that, for a rather large percentage of the data, there is a general tendency toward quadratic dose response, suggesting that the linear model will be extremely conservative in many cases.

We have also found that the oft-held belief that genotoxic compounds typically follow a linear dose–response pattern and that nongenotoxic compounds follow a nonlinear or threshold dose–response pattern is not supported by the data. In fact, we find the opposite, with genotoxic compounds differing from linearity more often than nongenotoxic compounds. Metabolic processes (e.g., activation, deactivation, detoxification), biochemical processes (e.g., DNA damage, repair), other cellular processes (e.g., mitosis), the competence of the immune system, etc., all

play an important role in carcinogenic response to chemicals. Because each of these systems or processes can be altered by the presence of a chemical and each will have its own shape for response to varying chemical dose, the dose-response shape for tumor incidence will be a complicated collection of all of these shapes. Thus, as has been stated previously (*35,36*), to presume that knowledge of the presence or absence of chemical effects on one process (DNA damage) is sufficient to explain dose-response shape is naive.

All of this is not to say that linear low-dose extrapolation is not the best policy from a public health standpoint. However, the study results imply that using linear risk estimation may lead to risks that often are overestimated based the experimental data. Although the carcinogenesis data suggest nonlinearity more often than linearity, they make no statement about the presence or absence of threshold levels. With simple, nonlinear kinetic models, one still obtains linearity of response at low-dose levels (*3*). For purposes of low-dose risk estimation and determination of dose-response relationships, dose-response information from other systems such as DNA adduct formation, toxicokinetics, and cellular proliferation should be coupled with the carcinogenesis data for low-dose risk estimation. This approach to carcinogenic risk estimation would incorporate biologically pertinent and measurable parameters into the uncertain and politically volatile business of public health management of chemical carcinogens.

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