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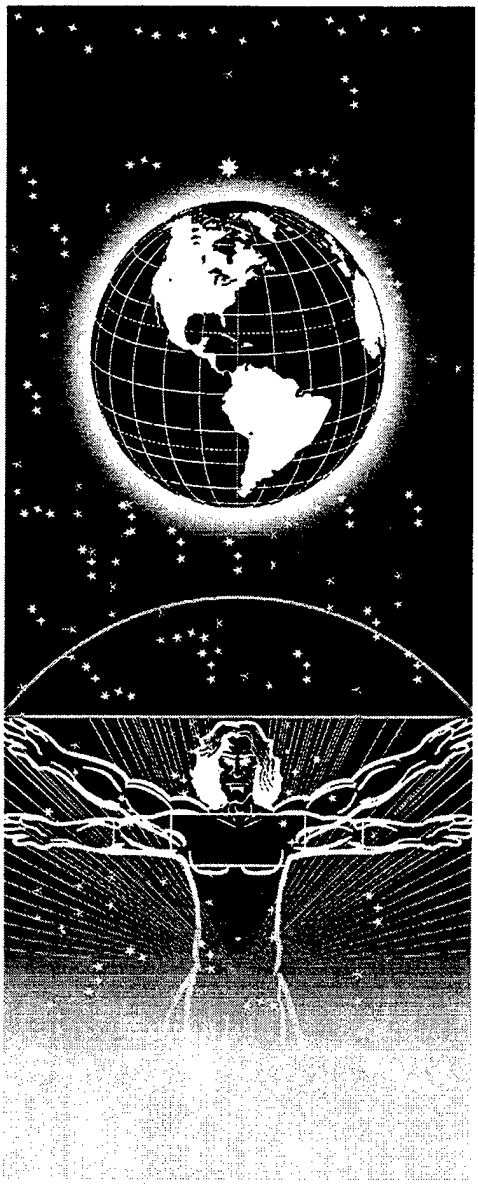

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

Simulation Studies Examining Possible Mechanisms of Trichloroethylene Carcinogenicity

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There has been much recent interest regarding methods of evaluating potential human cancer risks associated with trichloroethylene (TCE). Stochastic biologically based dose-response (BBDR) models offer possible means of quantifying these risks. BBDR models have been used to evaluate the effects of cancer initiators and promoters at a cellular level. This paper examines the sensitivity of the Moolgavkar-Venzon-Knudson (MVK) 2-stage model to variations in model parameters, and the ability of the model to distinguish between initiating and promoting activity of TCE. Maximum-likelihood estimation is used to fit parameters to simulated data sets assuming different carcinogenic mechanisms and low-dose response for TCE. Monte Carlo simulations are used to simulate experimental variability and mechanistic assumptions. Changes in experimental design are evaluated that allow carcinogenic mechanisms to be more clearly distinguished. These analyses provide information regarding uncertainties associated with carcinogenic mechanisms of TCE, and provide possible guidance for laboratory-based toxicological evaluations.

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EXECUTIVE SUMMARY

Objective

There has been much recent interest regarding methods of evaluating potential human cancer risks associated with trichloroethylene (TCE). Stochastic biologically based dose-response (BBDR) models, along with physiologically based toxicokinetic (PBTK) models, offer possible means of reducing uncertainties associated with these risks. BBDR models have been used to evaluate the effects of cancer initiators and promoters at a cellular level. This report examines the sensitivity of the Moolgavkar-Venzon-Knudson (MVK) 2-stage model to variations in model parameters, and the ability of the model to distinguish between initiating and promoting activity of TCE. Maximum-likelihood estimation is used to fit parameters to simulated data sets assuming different carcinogenic mechanisms for TCE, and assuming different dose-response functions for net cell proliferation. Monte Carlo simulations are used to simulate experimental variability. These analyses provide information regarding uncertainties associated with carcinogenic mechanisms of TCE, and provide possible hypotheses for laboratory-based toxicological evaluations.

Summary of Results

- This simulation exercise demonstrates the range of uncertainties that result from different model assumptions applied to rodent toxicity data and demonstrates critical data needs.
- Promotion may be a more important contributor to tumor hazard than genotoxicity over a lifetime of chronic exposure in mice. In these simulations, a doubling of initiation rate resulted in a 5-fold increase in tumor hazard at day 730, whereas a doubling in promotion rate resulted in a 15-fold increase in tumor hazard at day 730.
- The MVK 2-mutation model fit to TCE data is highly sensitive to net cell proliferation rate (α–β).
- The model was not able to discriminate well between the respective contribution to tumor risk from initiating vs. promoting action using standard published rodent data applied to a chronic exposure scenario.

- Improvements in discrimination resulted from simulating early sacrifices (56-500 days), as indicated by decreased correlation coefficients between initiation and promotion parameters.
- Use of a linear model results in implausible parameter fits and overestimates of risk (~3 orders of magnitude at the median) if the true dose-response relationship in cell proliferation is quadratic.
- Reductions in uncertainty would likely result from collection of intermediate foci data and other mechanistic information, as well as incorporating toxicokinetic and metabolite toxicity information into the TCE model.

INTRODUCTION

This work was performed in support of development of risk based human health standards to be applied to trichloroethylene (TCE) under the United States Air Force and GeoCenters Inc Contract N00014-95-D-0048, DO 0003 and Subcontract GC-2994-03-96-004. The focus of this work was to evaluate uncertainties associated with possible carcinogenic dose-response of TCE. The Statement of Work associated with this contract (as of 11/7/96) includes the following analyses:

1. Evaluation of existing TCE toxicity data (discussed under TCE Toxicology);
2. Evaluation of physiologically based toxicokinetic (PBTK) models for TCE (discussed under TCE Toxicokinetics);
3. Quantitative uncertainty analysis and sensitivity analysis to identify important data gaps within PBTK and biologically based dose-response (BBDR) models (discussed under Simulations);
4. Comparisons of appropriate BBDR models (discussed under Simulations).

BACKGROUND

Biologically-Based Cancer Models

Carcinogenic potencies of xenobiotics are estimated by modeling data from epidemiological studies, animal toxicology experiments, and *in vitro* investigations. There are a number of

models that have been used for this purpose; ranging from simple statistical data fits to mechanistic biologically based dose-response (BBDR) models. Existing BBDR models have evolved from the original multistage model of Armitage and Doll (1957) (AD model). Sophisticated variants of the AD model (Kopp-Schneider & Portier 1991), as well as the Moolgavkar-Venzon-Knudson (MVK) 2-mutation clonal expansion model (Moolgavkar & Luebeck 1990) allow incorporation of cellular-level mechanistic events such as dose-response in cell proliferation, and therefore allow exploration of carcinogenic mechanisms. The MVK model is explored here as the basis for simulations.

Figure 1 is a graphical depiction of the MVK model. Briefly, the MVK model simulates cancer as a 2-mutation stochastic process, incorporating rates of mutation and cell proliferation. The MVK model has been applied to a number of experiments designed to investigate carcinogenic mechanisms. For example, the model has been applied to experimental data describing the growth kinetics of enzyme-altered liver foci in rats treated with phenobarbital and α-hexachlorocyclohexane (Luebeck et al. 1995), as well as data describing initiation with diethylnitrosamine and subsequent promotion with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (Moolgavkar et al. 1996). The model can be applied to appropriate human data; for example, interactions between tobacco smoking and radon exposures in humans have been explored in an analysis of the Colorado Uranium miners cohort (Moolgavkar et al. 1993).

Simulation exercises using variants of the AD and MVK models have been conducted in order to explore the behavior of these models. For instance, Kopp-Schneider and Portier (1991) found that the ability to discriminate between different models applied to actual and simulated tumor incidence data is limited. Portier and Edler (1990) found that two-mutation model simulations were unable to clearly distinguish between promotion and initiation mechanisms at low doses. These and other simulation exercises have pointed out the problematic nature of using information from current experimental designs in mechanistic models. However, simulations are useful in terms of positing hypotheses for improved experimental design and data collection.

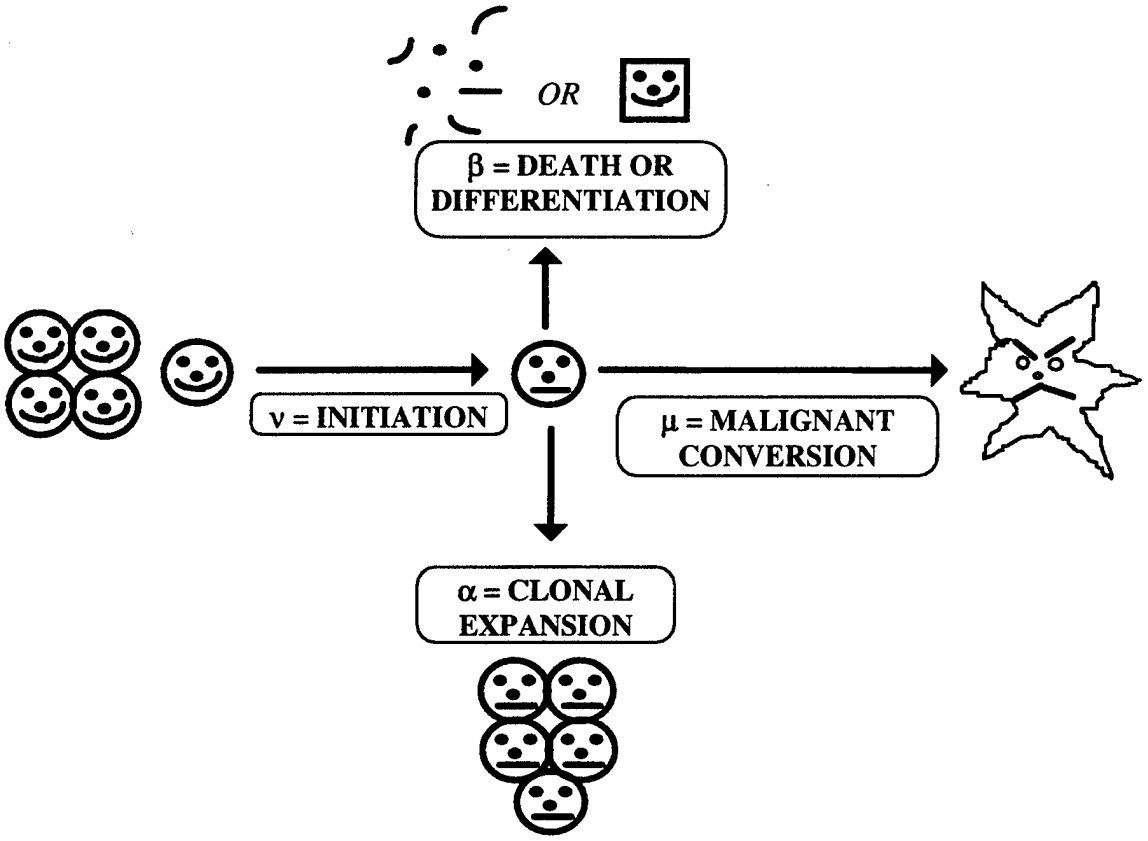

Figure 1: Moolgavkar-Venzon-Knudson (MVK) 2-mutation clonal expansion model. This is a simplification of the model form, which can be found in Moolgavkar and Luebeck (1990).

TCE Toxicology

There is debate regarding the specific mechanisms of action of TCE that may relate to human cancer risk (IARC 1995). TCE has a wide range of toxic effects in humans, mainly manifested at high doses. Exposures to TCE at concentrations above about 200 ppm can cause intoxication, headaches, and neurological problems. Long-term chronic health effects to humans from lower-dose exposures are uncertain. TCE is currently considered by the International Agency for Research on Cancer as "probably carcinogenic to humans" (classification 2A), based on "limited" evidence in humans and "sufficient" evidence from rodent carcinogenicity assays (IARC 1995),

but it is by no means certain that this endpoint is appropriate in terms of risk assessment and management. EPA is currently reviewing its position on TCE's possible carcinogenicity.

TCE (1000 mg/kg by gavage, lifetime exposure) appears to cause liver cancer in mice, but not in rats; and perhaps kidney tumors in rats, but not in mice (NTP 1988, 1990). Increases in lung tumors have been seen in mice inhaling 300 and 600 ppm chronically for a lifetime (Maltoni 1986, 1988). The weight-of-evidence suggests that TCE's hepatic and perhaps renal carcinogenicity is largely attributable to two metabolites: dichloroacetic acid (DCA) and trichloroacetic acid (TCA). Differences in metabolism likely explain inconsistencies in tumor risk across species (IARC 1995). Humans appear to metabolize TCE in a manner more akin to rats than mice; furthermore, peroxisomal proliferation does not appear to occur in human hepatocytes as opposed to rodent hepatocytes upon TCA exposure (Elcombe 1985, Green 1990). The weight-of-evidence suggests that TCE (including metabolites) may act as a mitogen in rodent liver, causing increased cell proliferation; and that genotoxicity is a minor or nonexistent mode of action (Klaunig et al. 1991, Dees & Travis 1993). The appendix (page 36) summarizes the current state-of-knowledge regarding TCE, DCA, and TCA mechanisms of toxicity. Based on this information, it is difficult to draw conclusions as to the human carcinogenicity of TCE at levels likely to be found in the occupational or general environment. A number of investigators have used toxicokinetic modeling approaches to perform interspecies extrapolations for the purpose of risk assessment of TCE (e.g. Bogen & Gold 1997, Cronin et al. 1995), however, these studies have not explored cellular-level mechanistic considerations.

TCE Toxicokinetics

TCE is metabolized by microsomal P450 enzymes (CYP 2E1) to chloral hydrate (CH) and by cytosolic enzymes to dichloroacetic acid (DCA) and other minor metabolites. CH is then oxidized to trichloroacetic acid (TCA) and trichloroethanol. These metabolites are then subject to further degradation and oxidative/reductive metabolism, as well as glutathione and glucuronide conjugation. Minor intermediate metabolites include possible TCE epoxides, dichlorovinylcysteine (DCVC), dichloroacetyl chloride, chloroform, and DCA. The major metabolic urinary excretion products of TCE across species are TCA and glucuronide-conjugated

trichloroethanol (TCOG), although there are species differences in fractional amounts. Mice show higher rates of biotransformation compared to rats. Additionally, mice appear to metabolize TCA to DCA, which appears as 1-2% of total urinary metabolite; whereas rats produce less DCA. Humans have lower rates of metabolism than mice or rats. DCA has not been detected in appreciable amounts as a human excretion product. TCA and DCA appear to be the metabolites that are most important in terms of mammalian liver toxicity (Daniel 1963, Dekant et al. 1986, Green & Prout 1985, Larson & Bull 1992a,b, Templin et al. 1993).

The earliest published multi compartment physiologically based toxicokinetic (PBTK) models for TCE were relatively simple, first-order models using three to five compartments (Fernandez et al. 1977, Sato et al. 1977, Andersen et al. 1987). The Andersen (1987) model was structurally based on Gargas' generic five-compartment PBPK model (1986), and fit to Fischer 344 rat experimental data. One major goal of that initial work was to describe the overall rate of TCE metabolism. A number of subsequent publications included modifications and applications of this model (Fisher et al. 1989, Fisher et al. 1991, Allen and Fisher 1993, Fisher and Allen, 1993). One such modification extended the model to include simple one-compartment models for the metabolite TCA, in order to track the body burden of this compound. Alternate TCE models were also proposed, such as a four-compartment model (Bogen 1988) with saturable Michaelis-Menten metabolism, which was based on an earlier model for styrene (Ramsey and Andersen 1984).

All TCE models published after 1993 appear to be modifications or extensions of Fisher and Allen's work published in that year. The work by Bogen and Gold (1997) relies on steady-state and pseudo-steady state (for regularly repeated bolus doses such as dietary exposure) solutions to the Fisher-Allen 1993 PBPK model, and demonstrates the application of these solution to cancer risk assessment. This model is advantageous in that it proposes a simplified form of the model that only requires minimal computational effort. However, the assumption of steady state exposure is only applicable to a narrow set of possible exposure scenarios, such as proposed maximum chronic doses. Most authors have taken the opposite approach, expanding the Fisher-Allen model to increase its flexibility. The expansions include work by a number of authors

(Clewell et al. 1994, Clewell et al. 1995, Clewell 1996, Cronin et al. 1995, Fisher 1997). Cronin et al.'s model is essentially identical to the Fisher-Allen 1993 model, although it is unique in terms of the ability to propagate parameter variability by means of Monte Carlo simulation.

Substantial differences exist between the current model forms. Fisher's model, for example, utilizes multiple compartments to model the circulation of each of TCE's metabolites, while Clewell chooses single-compartment models for all metabolites except TCOG. The use of more compartments (i.e., Fisher's model) is likely more realistic, and allows the model greater flexibility in simulating observed data;, however, it requires more computational time which may not be necessary or fully justified by the available amount of data. There are also numerous differences between the models in terms of rate constants, physiologic parameter values, and metabolism pathways. These differences reflect the impact of experimental variation and incomplete knowledge on model development, and are not easy to reconcile. A comparison of predicted concentration vs. time curves between models under a variety of exposure conditions would prove useful for evaluating the impact of these model differences on toxicokinetic outcomes of interest.

Our current efforts are targeted toward linking one of the PBTK models with the MVK model in order to provide dose- and time- dependent estimates of risks posed by TCE metabolites. It may be possible to model the effects of key TCE metabolites and their interactions on cellular initiation and promotion rates in the MVK model. While this information is not currently available, hypothesized models (e.g., constant, linear, quadratic) and interaction effects (e.g., additive, multiplicative) of the impact of metabolite doses could be used, and then tested against existing TCE dose-response data. This exercise should help identify likely mechanisms of TCE carcinogenicity. Continuing efforts will also identify the value of incorporating TCE metabolite mechanistic information into the MVK model. A summary of this information appears in Appendix.

METHODS

Hypothetical Experimental Design

Data are limited regarding oral liver carcinogenicity of TCE. Furthermore, tumor incidence data are not ideal for mechanistic modeling. However, it is still possible to explore mechanistic possibilities using the MVK model for the purpose of hypothesis generation. Therefore, simulation exercises were conducted using the MVK model applied to an existing TCE animal toxicology experiment (NTP 1990). This study followed standard National Toxicology Program lifetime carcinogenicity bioassay protocols. The species of interest was the male B6C3F1 mouse (female mice did not have a strong tumor response in this experiment). The organ of interest was the liver. Hepatocellular carcinoma was the endpoint of interest. Extrapolations to the human species would require integration of toxicokinetic differences, and were not evaluated here.

Model Form

The mathematical and biological bases of the MVK model are well-described elsewhere (Moolgavkar & Luebeck 1990, Heidenreich et al. 1997), and for brevity's sake are not repeated here. A semi-stochastic version of the model was used in which the growth rate of normal cells is assumed to be constant, and the growth rate of altered cells is assumed to be stochastic. Figure 1 is a graphical depiction of the model and basic parameters. The Kolmogorov equation-derived exact solution for piecewise constant parameters was used to calculate the tumor hazard function in a variety of scenarios.

Fitting parameters of the MVK model from experimental data is problematic in that not all biological model parameters can be determined from tumor incidence data. Additional information, such as cell kinetic parameters or locus specific mutation rates often need to be obtained from other sources. However, useful parameterizations can still be constructed using the method of Heidenreich et al. (1996, 1997) or the method of Sherman and Portier (1997), thus addressing this nonidentifiability problem. The method of Heidenreich et al. (1996, 1997) was used here.

Data Sources

Data from the National Toxicology Program (NTP) 2-year TCE carcinogenicity assay (NTP 1990) were used to fit model parameters. Treated animals received doses of 1000 mg/kg TCE by gavage, control animals were given corn oil (vehicle). Exposures started at age 8 weeks. Hepatocellular carcinomas were analyzed here.

For male mice, the control group consisted of 48 animals, with 8 animals developing carcinomas; and the dosed group consisted of 50 animals, with 31 animals developing carcinomas. In an initial analysis, it was assumed that the tumors were either all fatal (i.e. caused immediate death) or all incidental (i.e. did not cause death of the animals) for likelihood constructions. Results obtained for the incidental scenario were in better agreement with experimentally observed locus specific mutation rates (see below). The incidental tumor assumption is also supported by statistical tests provided in the NTP report (NTP 1990).

Model Parameterization

The following parameter combinations were used (see Heidenreich et al. 1996, 1997), due to the parameter nonidentifiability problem mentioned earlier:

$$\text{Parameter 1: } p_1 = \alpha - \beta - \mu$$

$$\text{Parameter 2: } p_2 = \nu X \mu$$

$$\text{Parameter 3: } p_3 = \alpha\sqrt{\mu / \nu X}$$

where:

α = cell division rate per day

β = cell death/differentiation rate per day

ν = first mutation rate (initiation) per day

X = number of susceptible cells

μ = second mutation rate (malignant conversion) per day.

Cell division rates can change over time. Therefore, in addition to these parameter combinations, the ratio of cell division rates α_2/α_1 across the change point at, say, time $t_1 = 56$ days is also necessary to calculate the hazard function. This can be determined, at least in principle, from the tumor data. However, preliminary analyses showed that the likelihood was very insensitive to p_3 and to the ratio α_2/α_1. Therefore, p_3 was fixed at a plausible value (see below) and the ratio of the αs fixed. This choice improved the convergence of the maximum likelihood estimation.

It is reasonable to assume that the second mutation rate μ is very small compared to $\alpha-\beta$; therefore p_1 approximately equals the net cell proliferation, and reflects the chronic promotion rate. Parameter p_2 is the product of the mutation rates, times the number of normal susceptible cells, and reflects the chronic initiation rate. The last parameter, p_3, has no particular biological meaning, but is proportional to the cell division rate α. This parameter is of particular interest for the risk assessment of TCE, which is believed to be mitogenic.

It is assumed that X, the number of normal susceptible cells, equals the total number of hepatocytes in the mouse liver, approximately 10^8 cells (Luebeck et al. 1997). It is further assumed that $\nu = \mu$, i.e. equality of the first and second mutation rates. Then,

$$p_3 = \alpha / \sqrt{X}$$

Thus, if α is known, then p_3 is known. A labeling index-derived cell division rate is available for hepatocellular foci in B6C3F1 mice (Klaunig 1993). The labeling index for control mice in this experiment (approximately 0.2) was converted into an estimate of α by means of the method of Moolgavkar and Luebeck (1992); thus p_3 was approximated as 3×10^{-6}. This value was used to obtain the maximum-likelihood estimates of model parameters shown in Table 1.

TABLE 1: Maximum-likelihood estimates of MVK model parameters, fit from NTP (1990) male B6C3F1 mouse data.

Male mice, fatal tumor assumption

Parameter*	Estimates	95% LCL	95% UCL
p_2	0.5259×10^{-9}	0.4188×10^{-10}	0.6600×10^{-8}
p_1 (c)	0.2840×10^{-1}	0.2150×10^{-1}	0.3751×10^{-1}
p_1 (d)	0.3466×10^{-1}	0.2754×10^{-1}	0.4362×10^{-1}

Male mice, incidental tumor assumption

Parameter*	Estimates	95% LCL	95% UCL
p_2	0.1950×10^{-5}	0.1177×10^{-6}	0.8313×10^{-5}
p_1 (c)	-0.3143×10^{-2}	-0.8543×10^{-2}	0.2228×10^{-1}
p_1 (d)	0.5926×10^{-2}	0.5121×10^{-3}	0.6852×10^{-1}

* $p_1(c) = p_1$ in controls, $p_1(d) = p_1$ in dosed animals.

These analyses demonstrate an increase in net cell proliferation. However, it is possible that TCE increases p_2, and not p_1.

As mentioned above, the incidental tumor scenario yields plausible estimates of background mutation rates. Because $\nu = \sqrt{p2 / X}$,

$$\nu_{fatal} = 2.3 \times 10^{-9} \text{ per day}$$

$$\nu_{incidental} = 1.4 \times 10^{-7} \text{ per day.}$$

SIMULATIONS

Three sets of simulations were performed. The first examines the sensitivity of the output of the MVK model to different sets of assumptions regarding the values of p_1 and p_2. The second examines the effect of different experimental design protocols on the ability of the model to distinguish the contribution to tumor hazard made by p_1 vs. p_2. The third examines the effects on excess risk estimates by different assumptions regarding dose-response in net cell proliferation.

Sensitivity of Model Results to Parameter Assumptions

The effect of changes in chronic initiation rate p_2 (2×, 4×, and 10× the background rate fit from the NTP data) on tumor hazard are shown in Figure 2. An increase of initiation rate of 10× over background results in approximately the same increase in tumor hazard. The effect of changes in chronic promotion rate p_1, relative to the fitted value for TCE (0.5× and 2× the TCE rate), are shown in Figure 3. A doubling of the promotion rate as fit from the TCE data results in a 1.5 order-of-magnitude increase in tumor hazard at the end of the experiment. Therefore, as can be seen from a comparison of these figures, relatively small changes in the promotion parameter result in larger increases in tumor hazard over the lifetime of animals as compared to increases in initiation rate. Therefore, in a "mixed" promoter/initiator mechanistic scenario, the MVK model indicates that the promotional mechanism may have a larger impact than the initiation mechanism on lifetime risk.

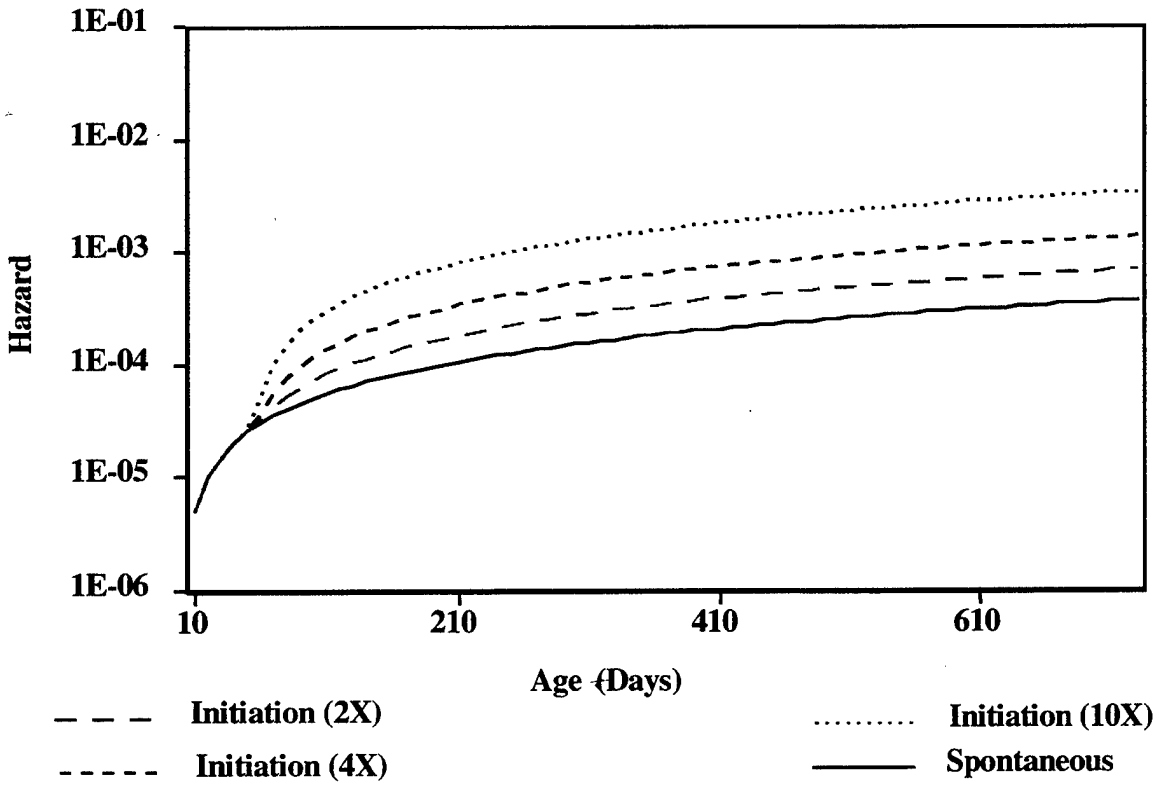

Figure 2: Sensitivity of tumor hazard to varying model parameters. Values of p_2 (initiation) were assumed to be 0.5×, 1.0×, and 2.0× the background initiation rate of $50[day]^{-1}$, with no changes in chronic promotion rate (p_1).

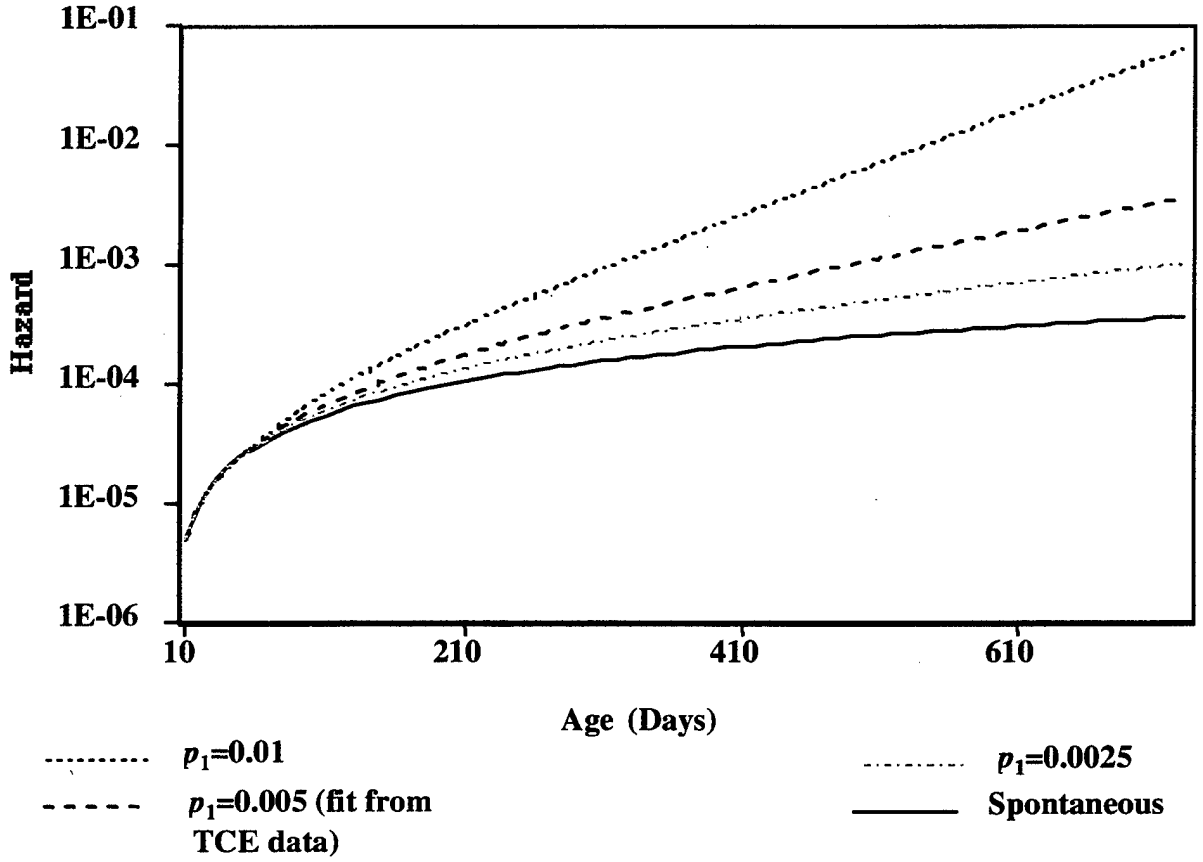

Figure 3: Sensitivity of tumor hazard to varying model parameters. Values of p_1 (promotion) were assumed to be 2×, 4×, and 10× the fitted values from the NTP data (0.005 $[day]^{-1}$), with no changes in chronic initiation rate (p_2).

A difficulty arises, however, when an attempt is made to evaluate the contribution to total hazard in a mixed-mechanism scenario from the individual components. Figure 4 illustrates this problem. This figure depicts the "individual" contribution to tumor hazard from the parameters fit from the NTP data, from an increase in background initiation rate of 4×, and from the combined mechanisms. It is not possible to differentiate the relative contribution to hazard from the components. Statistical tests were not employed here since hypothetical rather than data-based changes in parameters were made.

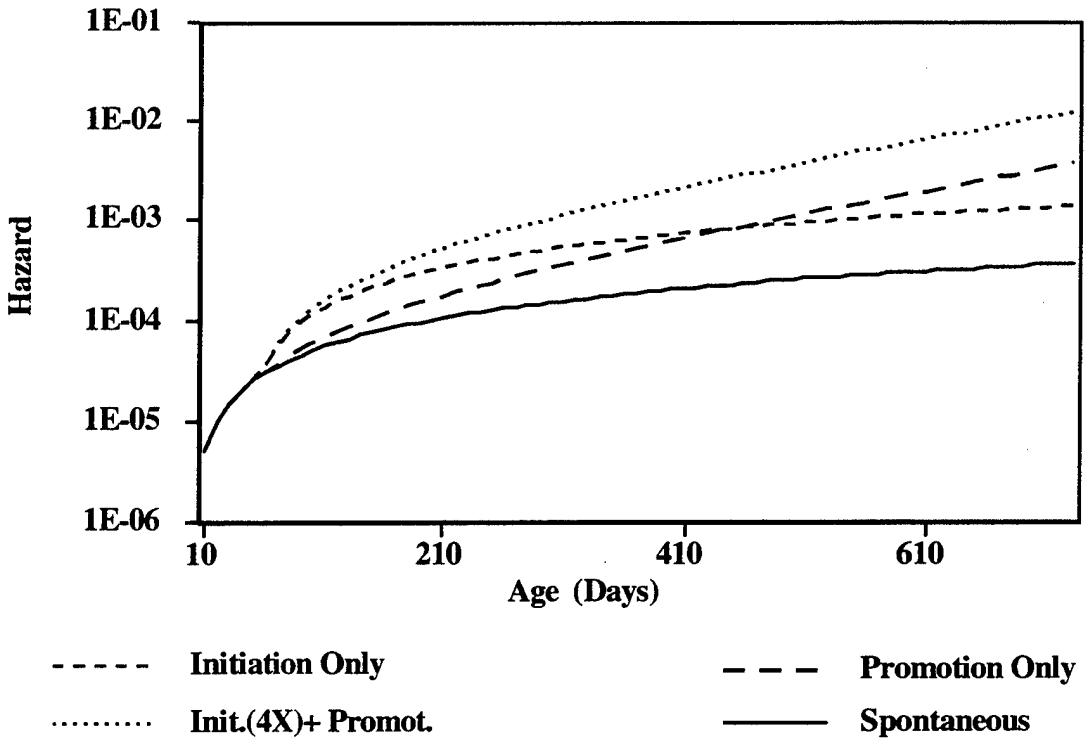

Figure 4: Sensitivity of tumor hazard to varying model parameters. Independent contribution to tumor hazard from fitted values of p_1 (promotion) and p_2 (initiation) fit from the NTP data are plotted. Additionally, tumor hazard that is associated with the joint effect of the fitted value of p_1 and 4X the fitted value of p_2 are plotted. Note that it is difficult to separate independent initiation and promotion effects.

Effect of Different Experimental Designs

In order to examine the effect of changes in experimental design on the ability of the model to discriminate mechanisms, Monte Carlo simulation was used to generate 1000 experiments within a particular design. The designs are summarized as:

1) n=50 each group (control and dosed), time of sacrifice=365 to 730 days (standard NTP design),

2) n=50 each group (control and dosed), time of sacrifice=56 to 500 days,

3) n=100 each group (control and dosed), time of sacrifice=56 to 500 days.

Sacrifice time points were generated by uniform random deviates in the respective time intervals. Parameters of the model were fit as described earlier; p_1 and p_2 were fit using maximum-likelihood estimation, and p_3 was calculated from published data .

Pearson correlation coefficients (r) were calculated between p_1 as an indication of promotional action, and p_2 as an indication of initiating action. As can be seen in Table 2a, a high degree of correlation is demonstrated between estimates of p_1 (promotion) and p_2 (initiation) based on 1000 Monte Carlo simulations of the experiment under the standard NTP design.

TABLE 2: Correlation coefficient matrices for parameters of the MVK model; calculated under different experimental design assumptions using the NTP (1990) male B6C3F1 mouse data (High correlation coefficients indicate the inability of the model to discriminate between parameters)

a) *n=50, time of sacrifice=365 to 730 days*

	p_1 (control)	p_1 (dosed)	p_2 (control)	p_2 (dosed)
p_1 (control)	1.00	0.88	-0.91	-0.93
p_1 (dosed)	0.88	1.00	-0.88	-0.89
p_2 (control)	-0.91	-0.88	1.00	0.93
p_2 (dosed)	-0.93	-0.89	0.93	1.00

b) *n=50, time of sacrifice=56 to 500 days*

	p_1 (control)	p_1 (dosed)	p_2 (control)	p_2 (dosed)
p_1 (control)	1.00	0.59	-0.69	-0.67
p_1 (dosed)	0.59	1.00	-0.68	-0.81
p_2 (control)	-0.69	-0.68	1.00	0.66
p_2 (dosed)	-0.67	-0.81	0.66	1.00

c) *n=100, time of sacrifice=56 to 500 days*

	p_1 (control)	p_1 (dosed)	p_2 (control)	p_2 (dosed)
p_1 (control)	1.00	0.77	-0.78	-0.85
p_1 (dosed)	0.77	1.00	-0.81	-0.87
p_2 (control)	-0.78	-0.81	1.00	0.79
p_2 (dosed)	-0.85	-0.87	0.79	1.00

Discrimination between the individual mechanisms is improved by increasing the time over which interim sacrifices are performed, as in Table 2b. The correlations are reduced appreciably due to information available at earlier time points when the differential behavior of the initiator

and the promotor is more pronounced. The correlations increase again if the number of experimental animals is increased, as in Table 2c.

Model Misspecifications using Dose-Response Assumptions

This analysis explored the effects of model misspecification on excess risk estimates. Since detailed dose-response information was not available for the oral route of exposure, tumor incidence data were simulated in hypothetical experiments that involved 4 dose groups of animals with 200 animals in each group, where doses are defined as divisions of the NTP dose (1000 mg/kg/d over lifetime) as a reference. All doses were assumed to start on day 56. Early deaths (other than tumor related) and sacrifices were assumed to be randomly distributed between 365 and 730 days. Tumors were assumed to be incidental. Assumed doses were:

1) controls (0 mg/kg/d)
2) low dose (10 mg/kg/d)
3) medium dose (100 mg/kg/d)
4) high dose (500 mg/kg/d)

The generalized model parameterization for the data generation was assumed to be as follows:

$p_1 = \delta_0 + \delta_1 D + \delta_2 D^2$

where:

$\delta_0 = 0$ [i.e. no net cell proliferation in controls]

$\delta_1, \delta_2 = 0.02$

D = dose

Additionally, the following were set according to the previous analysis:

$\alpha = 0.03$ [fixed]

$p_2 = 5.0 \times 10^{-6}$ [set at an equivalent level regardless of dose; i.e. no dose-response in mutation rates].

The value of 0.02 for δ_1 and δ_2 was obtained by assuming a quadratic function and adjusting the net cell proliferation rate (p_1 for treated male mice, incidental tumors) to reflect a maximum dose of 500 mg/kg/d, and to reflect the tumor incidence seen at 1000 mg/kg/d in the NTP experiment.

Note that the value of p_2 chosen reflects the results of a high-dose experiment; thus may overestimate the possible initiation rate at lower doses.

Under these assumptions 1000 experiments were generated using Monte Carlo simulation with observations at time points that represent sacrifices or times of death from other causes as described above. Hypothetical incidental tumors were sampled directly from the probability of tumor. The generated incidence time is the time to the appearance of the first malignant cell in the tissue (according to the MVK model). That time is then compared with the random time for death (sacrifice or death from other causes) and the status of the animal as tumor bearing or not tumor bearing is determined according to whether the incidence time is smaller or larger the time of death. Each experiment was analyzed using likelihood maximization, with 3 different model parameterizations:

Model A: quadratic dose-response in net cell proliferation ("correct" underlying model as defined)

$$p_{1A} = \delta_0 + \delta_1 \times 0 + \delta_2 D^2$$

Model B: linear dose-response in net cell proliferation

$$p_{1B} = \delta_0 + \delta_1 D + \delta_2 \times 0$$

Model C: linear-quadratic dose-response in net cell proliferation

$$p_{1C} = \delta_0 + \delta_1 D + \delta_2 D^2$$

Thus, Model A is correct, Model B is misspecified, and Model C is "overspecified" in terms of describing the simulated dose-response relationship in a parsimonious manner.

Table 3 presents the parameter estimation results under the simulated dose-response model assumptions. The parameter medians obtained from Model A closely coincide with the "true" values used for the underlying model, which provides a reliability check for the simulation. However, the considerable variance and skewness of the distributions obtained for δ_0 and δ_2 indicate that a wide variety of biologically implausible numerical combinations are possible. This is also observed for Model B. For Model C more symmetric distributions for δ_1 and δ_2 were obtained, although δ_0 is still highly asymmetric. Excess risks (probability of tumor in treated animals minus probability of tumor in control animals at 1.0 mg/kg/d) associated with the three

models are represented in Figure 5, where it can be seen that the median excess risk as estimated by the linear model (Model B) is overestimated by approximately 3 orders of magnitude relative to the median excess risk estimated by the quadratic model (Model A). Negative values evident in Figure 5 (Model C) are the result of the large variance of δs.

TABLE 3: Simulated MVK model parameter values under different net cell proliferation dose-response assumptions. Model A incorporates the defined hypothetical dose-response function. Models B and C are misspecified.

	δ_0	δ_1	δ_2	p_2
Model A (quadratic)				
Mean	-9.0×10^{-4}	NA	2.2×10^{-2}	$6.3\times10^{+1}$
Standard Error	2.5×10^{-4}	NA	6.2×10^{-4}	$3.0\times10^{+0}$
Median	-1.7×10^{-5}	NA	2.0×10^{-2}	$4.9\times10^{+1}$
Standard Deviation	5.3×10^{-3}	NA	1.3×10^{-2}	$6.4\times10^{+1}$
Sample Variance	2.8×10^{-5}	NA	1.7×10^{-4}	$4.0\times10^{+3}$
Skewness	$-5.0\times10^{+0}$	NA	$6.4\times10^{+0}$	$6.7\times10^{+0}$
Model B (linear)				
Mean	-9.8×10^{-4}	1.2×10^{-2}	NA	$6.0\times10^{+1}$
Standard Error	2.5×10^{-4}	3.2×10^{-4}	NA	$2.7\times10^{+0}$
Median	8.9×10^{-5}	1.0×10^{-2}	NA	$4.7\times10^{+1}$
Standard Deviation	5.3×10^{-3}	6.7×10^{-3}	NA	$5.7\times10^{+1}$
Sample Variance	2.8×10^{-5}	4.5×10^{-5}	NA	$3.2\times10^{+3}$
Skewness	$-4.4\times10^{+0}$	$5.8\times10^{+0}$	NA	$5.3\times10^{+0}$
Model C (linear-quadratic)				
Mean	-8.2×10^{-4}	-1.1×10^{-3}	2.5×10^{-2}	$6.1\times10^{+1}$
Standard Error	2.1×10^{-4}	1.4×10^{-3}	2.8×10^{-3}	$2.3\times10^{+0}$
Median	4.1×10^{-5}	6.9×10^{-4}	2.0×10^{-2}	$4.9\times10^{+1}$
Standard Deviation	4.0×10^{-3}	2.7×10^{-2}	5.3×10^{-2}	$4.4\times10^{+1}$
Sample Variance	1.6×10^{-5}	7.5×10^{-4}	2.8×10^{-3}	$1.9\times10^{+3}$
Skewness	$-1.5\times10^{+0}$	-5.8×10^{-1}	$1.1\times10^{+0}$	$2.3\times10^{+0}$

NA= not applicable

Figure 5a: Model A - Net Cell Proliferation Modeled as Quadratic (Correct Underlying Model)

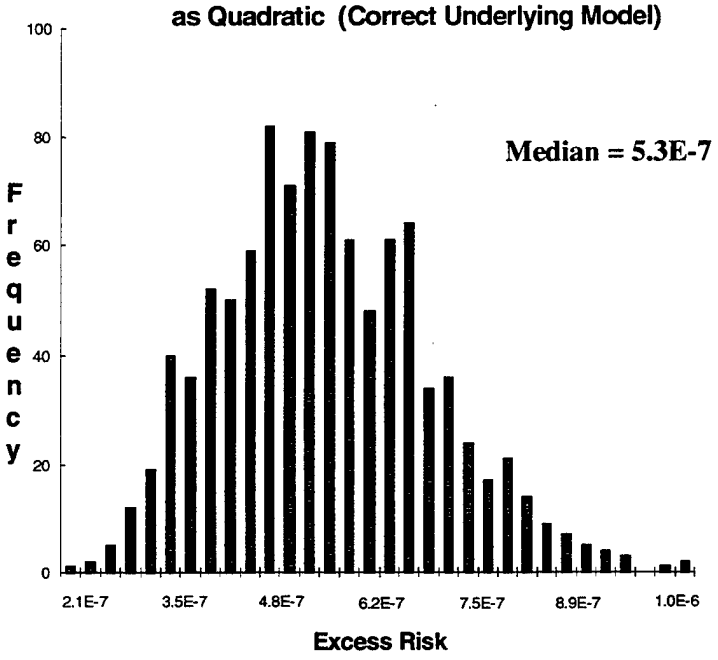

Figure 5b: Model B - Net Cell Proliferation Modeled as Linear

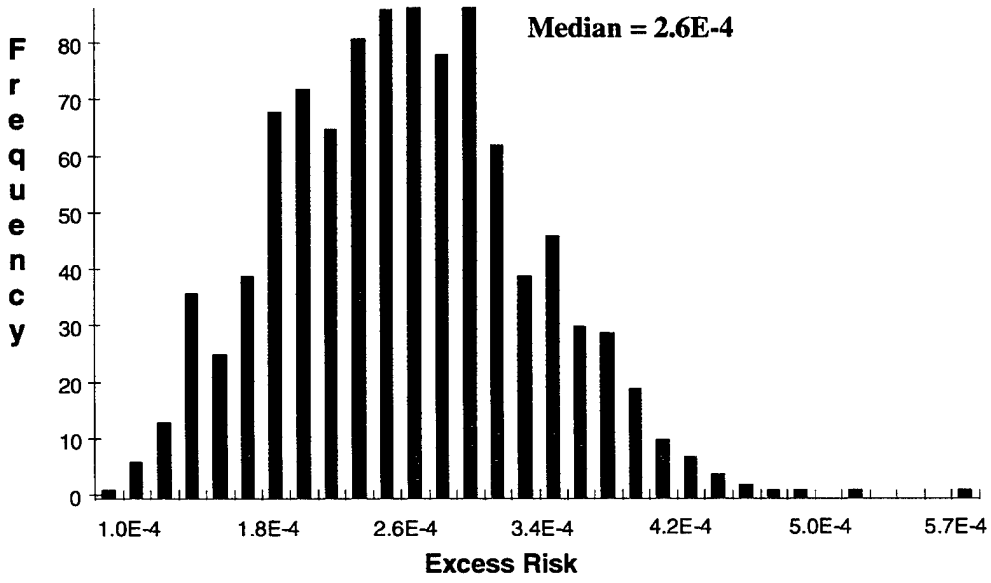

Figure 5c: Model C - Net Cell Proliferation Modeled as Linear-Quadratic

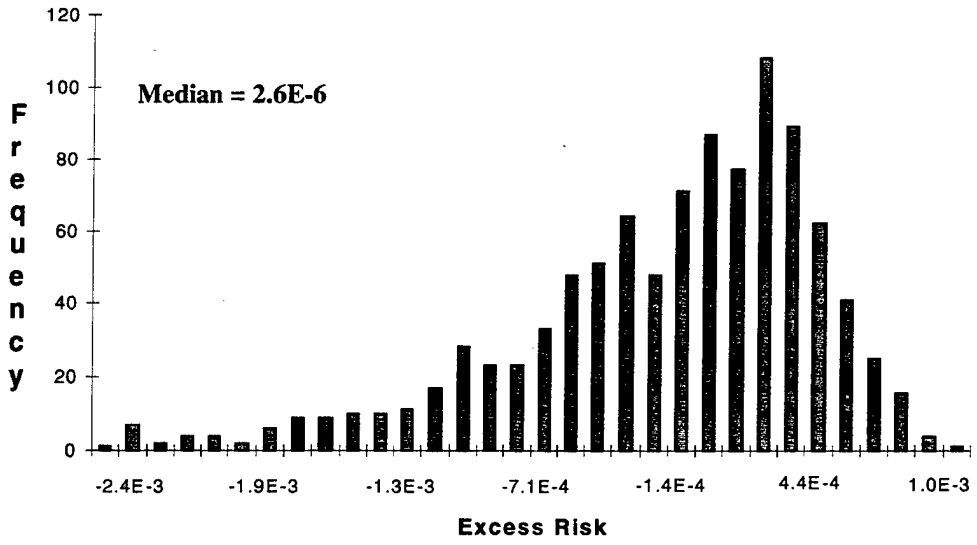

Figure 5: Simulated excess risk for TCE dose of 1 mg/kg/d (underlying model assumption = quadratic dose-response in net cell proliferation).

DISCUSSION

There are limitations on the interpretation of the results of this study that illustrate the difficulties involved with mechanistic modeling of carcinogens. The model was not fit to the results of an experiment designed for elucidation of mechanisms; rather, a combination of previously published data was used. As such, the results should be interpreted as an exploratory exercise, rather than a risk assessment.

Effects such as stimulation of cell proliferation and cell killing were not addressed here. The model results reflect tumor hazard in experimental mice, and numerous extrapolations with a great deal of associated uncertainty (including toxicokinetics) would be necessary to evaluate human risks. The model was not able to discriminate well between the respective contribution to tumor risk from initiating vs. promoting activity using standard published data applied to a chronic exposure scenario. It is clear that the majority of information available from typical

experimental designs is not adequate for mechanistic modeling. Experiments have been performed where preneoplastic liver foci have been quantified (Luebeck et al. 1995, Moolgavkar et al. 1996); it is expected that this type of information will be valuable for mechanistic modeling of TCE and its metabolites. Increasing the number of early sacrifices in experiments may also improve this information.

The results for the incidental analysis of the NTP data for TCE-induced tumors were compatible with the results in a similar study of tetrachloroethylene (Luebeck et al. 1997), which may have similar carcinogenic mechanisms as TCE. The MVK model did not appear to discriminate as to the relative importance of initiating or promoting activity when the TCE data were analyzed, although an analysis (not shown here) that assumed "pure" initiation did not yield plausible values for parameters (statistical tests were not employed here since the changes in parameter values were hypothetical). However, the data still provide useful mechanistic information if considered together with plausible biological information. For instance, the analysis that assumed incidental tumors yielded a mutation rate that was consistent with experimental values. Also, the incidental scenario is consistent with negative or zero net growth of intermediate lesions when not "promoted". The net cell proliferation rate can be assumed to be zero for the background, but seems to be elevated for exposed animals. It is not clear from the analysis whether this increase is due to an increase in α or a decrease in β, although the former is perhaps more biologically plausible. This information, considered along with other studies (Klaunig et al. 1991, Dees & Travis 1993), implicate a mitogenic mechanism in TCE carcinogenicity. Promotion as a result of TCE exposure may be a more important contributor to tumor hazard than genotoxicity over a lifetime of chronic exposure.

The results of the model misspecification simulation exercise suggest that it is difficult to reliably discern the underlying biological dose-response relationship (specified here as a quadratic threshold in the net cell proliferation) even at a relatively large sample size (800 animals). Note, however, that we assumed that the tumors were incidental; thus time-to-tumor information was not available, which reduces the potential available information.

A comparison of the excess risk values generated by the different models also reveals a strong dependence on the assumed mechanism. If a threshold or quadratic cell proliferation dose-response is appropriate for TCE, then only the explicit incorporation of this behavior into the dose-response function will reliably represent the excess risk at low doses. Our results also show that a linear-quadratic model fails to provide an unbiased estimate of the excess risk at low doses. The distribution is skewed toward the left, possibly allowing for negative excess risks (protective effects) for which there are minimal biological evidence. The linear model is grossly misspecified, and overpredicts the risk (as predicted by the correct underlying quadratic model) by approximately 3 orders of magnitude. Therefore, if TCE exhibits threshold behavior in cell proliferation at low doses, the use of a linear dose-response model will overpredict risks.

In summary:

- This simulation exercise demonstrates the range of uncertainties that result from different model assumptions applied to rodent toxicity data and demonstrates critical data needs.
- Promotion may be a more important contributor to tumor hazard than genotoxicity over a lifetime of chronic exposure in mice. In these simulations, a doubling of initiation rate resulted in a 5-fold increase in tumor hazard at day 730, whereas a doubling in promotion rate resulted in a 15-fold increase in tumor hazard at day 730.
- The MVK 2-mutation model fit to TCE data is highly sensitive to net cell proliferation rate ($\alpha-\beta$).
- The model was not able to discriminate well between the respective contribution to tumor risk from initiating vs. promoting action using standard published rodent data applied to a chronic exposure scenario.
- Improvements in discrimination resulted from simulating early sacrifices (56-500 days), as indicated by decreased correlation coefficients between initiation and promotion parameters.
- Use of a linear model results in implausible parameter fits and overestimates of risk (~3 orders of magnitude at the median) if the true dose-response relationship in cell proliferation is quadratic.

- Reductions in uncertainty would likely result from collection of intermediate foci data and other mechanistic information, as well as incorporating toxicokinetic and metabolite toxicity information into the TCE model.

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APPENDIX

MECHANISTIC STUDIES OF TCE AND METABOLITE LIVER CARCINOGENICITY

Appendix: Mechanistic studies of TCE and metabolite liver carcinogenicity

(Note: only original studies examining toxicology mechanisms are included, except for genotoxicity reviews)

Study[1]	Chemicals[2]	Species[3]	Exposure Route[4]	Doses[5]	*N*	Time	Endpoints[6]	Findings[7]
Anna 1994	TCE, DCA, PCE	B6C3F1 m mice, Athymic m nude mice	TCE, PCE- COG; DCA- DW	Controls, TCE- 800, PCE- 1700, DCA- 5000,	TCE- 110, PCE- 160, DCA- 110, Controls- 50	76 weeks	Liver tumors, H-ras and K-ras mutation	Increased liver tumors in treated animals, no sig. increase in H-ras activation with TCE, DCA, sig. decrease in H-ras and numerical increase in K-ras mutations with PCE
Bull 1990	TCA, DCA	B6C3F1 m & f mice	DW	Control, 1000, 2000, Phenobarb- 500	5 to 24 per treatment period	15 to 52 weeks	Liver tumors, histopathology	Males: Sig. dose-related increases in tumors, hockey stick DR w/DCA, linear DR w/TCA, increased liver weights, early hepatomegaly and cytomegaly w/DCA, increased lipofuscin w/TCA Females: No tumors, increased liver weights
Carter 1995	DCA	B6C3F1 m mice	DW	Control, 500, 5000	5 per sacrifice period per treatment group	0 to 15 days	Liver histopathology, SA, LI	Sig. increased liver weights after 10 days (due to hypertrophy), SA and LI sig. reduced in dosed groups initially, then transient increase in SA, continued reduction in LI, no sig. differences at 30 days; hepatocyte glycogen incorporation, cytotoxicity observed
Chang 1992	TCA, DCA, CH, (also other chlorinated compounds)	B6C3F1 m mice, Fischer 344 m rats, cultured mouse and rat hepatocytes, human CCRF-CEM lymphobasts	DW, direct application to cell cultures	Control DCA: 500, 5000; TCA: 50, 500	4 per per sacrifice period per treatment group	7 days to 30 weeks	DNA strand breaks, peroxisomal enzyme activity	None of tested compounds sig. increased DNA strand breaks in rodent liver or human lymphoblasts. DCA increased peroxisomal proliferation @ 5000 mg/L.
Channel 1993	TCA	WB344 hepatocyte cell line	Direct application	100 mg/ml	NA	100 hr	Cell cycle kinetics	TCA slightly prolonged S phase, reversible effect
Crebelli 1989	TCE	Exhaustive summary of mutagenicity assays up to 1989: bacterial, fungal, mammalian *in vitro* and *in vivo*	various	various	various	various	Mutagenicity	Author's conclusion is that TCE activated by microsomal fractions is "weakly mutagenic"
Daniel 1992	CH, DCA, (also chloroacetadehyde)	B6C3F1 m mice	DW	Control CH:1000 DCA:500	33, 40	30, 60, 104 weeks	Hyperplastic nodules, tumors	Sig. and similar increased tumor incidence for CH and DCA.
DeAngelo 1989	DCA, TCA, (also monochloroacetic acid)	B6C3F1 m mice, SD m rats	DW	Control, Corn oil, 1000 to 5000	6 per dose group	14 days	Increases in peroxisomal enzymes (3 indicators)	Corn oil only gavage increased PP in rat. DCA, TCA more effective in increasing PP in mouse than in rat. TCA more effective than DCA in mouse.

DeAngelo 1991	DCA	B6C3F1 m mice	DW	Control Acetic acid 50 500 3500 5000	50 per dose group	4, 15, 30, 45, 60 weeks	Liver pathology, carcinogenicity	Significant dose-response in liver neoplasia for 2 highest doses, threshold at 500 mg/L.
Dees 1993	TCE	B6C3F1 m & f mice	COG	Control, 100 250 500 1000	5 per dose group	10 days	Liver histopathology ^{3}H-thymidine labeling	Increased apoptosis in highest group, increases in eosinophilic staining in treated groups, increased DNA labeling in mature hepatocytes. No increases in lipid peroxidation.
Dees 1994	TCA	B6C3F1 m & f mice	COG	Control 100 250 500 1000	5 per dose group	11 days	Liver histopathology ^{3}H-thymidine labeling	Slight apoptosisand eosinophilic staining in highest group. Increased DNA labeling in mature hepatocytes. No increases in lipid peroxidation. Increases in mitotic figures. No differences between males and females. (Note differences from Bull et al. 1990)
Elcombe 1985	TCE	B6C3F1 and Adderly Park (Swiss) m mice, Osborne-Mendel and Adderly Park (Wistar) m rats	COG	Control 500 1000 1500	10 per dose group	10 days	Liver histopathology^{3} H-thymidine labeling, peroxisomal enzymes	Liver weight icreases seen in mice and rats; due to hypertrophy in rats and hypertrophy/hyperplasia in mice. No necrosis, sig. increased DNA synthesis and mitotic figures in mice. Increases in PP and peroxisome volume density seen in mice.
Elcombe 1985	TCE, TCA	Adderly Park (Swiss) m mice, Adderly Park (Wistar) m rats. Cultured rat, mouse, human hepatocytes	COG	Control TCE: 50 100 200 500 1000 2000 TCA: 10 20 50 100 200	4-5 per dose group	10 days	Kinetics, PP	TCE: Increases in PP seen in mice, none in rats, no increases in catalase. TCA: Increases in PP in both mice and rats, no increases in catalase. Intrinsic clearance for TCE in hepatocytes: mouse>rat>human. TCE increased PP in mouse and rat heaptocytes, none in human.
Fahrig 1995	TCE, TCA, DCA, CH, trichloroethanol	Exhaustive summary of mutagenicity assays up to 1995: bacterial, fungal, mammalian *in vitro* and *in vivo*	various	various	various	various	Mutagenicity	Author's conclusion there is evidence for mutagenicity of TCE, TCA, DCA, CH, but that studies are conflicting

Ferreira-Gonzales 1995	TCA, DCA	B6C3F1 m mice	DW	Control DCA:1000, 3500 TCA:4500	?	104 weeks	Liver tumors, K and H-*ras* mutations	Signif. Increase in tumors, H-61 ras mutations identified, equal incidence of mutations in spontaneous and DCA/TCA induced tumors. DCA caused shift in frequency of mutations.
Fuscoe 1996	DCA	B6C3F1 m mice	DW	Control 500 1000 2000 3500	10 per dose group	9 and 28 weeks (continuous), and 10, 26, and 31 weeks (stop-exposed)	Genotoxicity	Highest dose caused increased micronucleated polychromatic erythrocytes, micronucleated normochromatic erythrocytes, DNA crosslinking in leukocytes.
Goldsworthy 1987	TCE, TCA (also PCE and pentachloroethane, also TCE/PCE mix)	B6C3F1 m mice, F-344 m rats	COG	Control, Methyl cellulose control, positive control (WY14643), TCE:1000 TCA:500	5-6 per dose group	10 days	PP in liver and kidney	Vehicle had no effect on PP. Significant effects: TCA increased PP in rat liver, TCE increased PP in rat kidney. TCE and TCA incerased PP in mouse liver and kidney. No chemicals were as potent as WY.
Herren-Freund 1987	TCE, TCA, DCA, PB + ENU	B6C3F1 m mice	DW, ENU - IP	Controls ENU: 2.5, 10 mg/kg TCE: 3 40 TCA, DCA 2 5 PB: 500	22-33 per dose group	61 weeks	Tumors	TCE: no increase in tumors. TCA, DCA increased tumors with and without ENU. PB: no increase in tumors.
Klaunig 1989	TCE, TCA, CH, trichloroethanol, PB	B6C3F1 mouse and F344 rat hepatocytes	direct application	0.00001 to 0.003 M (determined as non-cytotoxic)	NA	4-24 hrs	Cytotoxicity (LDH release) Intercellular communication (dye coupling)	Rat hepatocytes more sensitive to cytoxicity. Transient inhibition in dye coupling seen w/ TCE and TCA in mouse cells
Klaunig 1991	TCE, PB, BB, unleaded gasoline	B6C3F1 m&f mice, F344 m&f rats	COG- TCE	TCE:500	3 per treatment period	3-14 days	Liver histopathology, ^{3}H-thymidine labeling	TCE: no increase in rat or female mouse hepatocyte labeling index, sig. Increases in male mouse hepatocytes, male rat renal cells, no increase in female rat or mouse renal cells.
Laib 1979	TCE, vinyl chloride	Wistar microsomes, newborn Wistar m&f rats	I	2000	2-3 per treatment period	4-10 weeks	Liver histopathology ATPase	No preneoplastic ATPase-deficient foci w/TCE

Larson 1992	TCA, DCA	B6C3F1 m mice, F344 m rats	DW	Metabolism: 5 20 100 Lipid peroxication: Control 100 300 1000 2000	2-5 per dose group	Single doses	Metabolism, lipid peroxidation	DCA metabolized more extensively than TCA in mice and rats, half-life shorter. Higher plasma DCA in rats. Both TCA and DCA increased lipid peroxidation; DCA more potent (threshold 300 mg/kg).
Nelson 1989	DCA, TCA	B6C3F1 m mice	Tween gavage	Control DCA: 10 500 TCA: 500	6-13 per dose group per time period	1-24 hours	PP, DNA breaks	Single-strand DNA breaks induced by single doses of DCA and TCA. PP induced only by repeated doses. Induction of breaks not related to PP.
Nelson 1990	DCA, TCA	B6C3F1 m mice	DW	Control 1000 2000	4-12 per dose	37-52 weeks	c-myc and c-Hras expression	c-H-ras expression elevated in carcinomas from both treatments relative to hyperplastic nodules and other tissue. C-myc levels higher in TCA induced carcinomas compared to DCA induced carcinomas.
Okino 1991	TCE, also ethanol and phenobarbital	Wistar m rats	TCE: I PB: IP EtOH: DW	TCE: 500 2000 8000 PB:80 mg/kg/d EtOH: 2g/d	5 per dose group	3 weeks	Histopathology enzyme action	PB and EtOH pretreatments enhanced TCE hepatoxicity (all doses). PB pretreated: TCE decreased metabolism, benzene aromatic hydroxylase activity, and P 450 content. EtOH pretreated: increased metabolism, benzene aromatic hydroxylase activity, no change P 450 content.
Pereira 1996	DCA, TCA	B6C3F1 f mice	DW	Control 2.0 mmol/L 6.67 20	40-134 per dose	360-576 days	Liver histopathology tumors, BRDU labeling index, GST-π	Dose response second-order for DCA, linear for TCA. DCA foci eosinophilic, GST-π positive. TCA foci basophilic, GST-π negative.
Perreira 1996	DCA, TCA, +MNU	B6C3F1 f mice	DW, IP - MNU	Controls MNU: 25 mg/kg TCA, DCA: 2.0 mmol/L 6.67 20	10-40 per dose	31-52 weeks	Liver histopathology tumors, GST-π	Dose response second-order for DCA, linear for TCA. DCA foci eosinophilic, GST-π positive. TCA foci basophilic, GST-π negative. DCA tumors regressed on cessation of exposure, TCA tumors did not.
Richmond 1991	DCA	B6C3F1 m mice	DW	Control 5000	5 mice per dose per treatment period	4-60 weeks	Tumor markers	Hyperplastic nodules expressed markers less in hyperplastic nodules as compared to tumors, except for c-jun. Nodules contained nests of marker-positive cells; none were detected in adjacent normal tissue.

Sanchez 1990	DCA, TCA	B6C3F1 m mice, Swiss-Webster m&f mice	DW	Control 300 100 2000	4-15 per dose per treatment period	2-14 days	Liver histopathology[3] H-thymidine labeling	DCA caused focal necrosis, TCA did not. Highest dose of both TCA and DCa caused increased labeling.
Snyder 1995	DCA	B6C3F1 m mice	DW	Control 500 5000	5 per sacrifice period per treatment group	5-30 days	Liver histopathology apoptosis (*in situ* nick end-labeling)	Down regulation of apoptosis in high treatment group.
Stott 1982	TCE	B6C3F1 m mice, Osborne-Mendel m rats	I, COG	I: 10 600 COG: Control mice: 250 500 1200 2400 rats: 1100	10-16 per dose group	6 hours I, 3 days- 3-weeks COG	Metabolism, liver and kidney toxicity, DNA alkylation	Mice metabolized more TCE to toxic intermediates than rats. Liver toxicity in mice (doses>250), no sig. toxicity in rats. No kidney toxicity in either. No sig. DNA alkylation observed.
Templin 1995	TCE	Fischer 344 m rats, beagle m dogs, human m blood	Tween gavage	0.15- 0.76 mmol/kg	4 per dose group	0.25-240 hours	Metabolism, bile deposition, TCA plasma binding	Peak conc. of TCA in dog blood higher than rat, but formation rate lower. DCA not detected in either.
Tsai 1996	DCA, PB	B6C3F1 m mice	DW	Control DCA: 3500 PB: 1%	3 per treatment period	0-90 days	Hepatocyte response to growth factors (hepatocyte, epidermal, fibroblast, TGF-β1), ^{3}H-thymidine labeling	DCA & PB depressed DNA synthesis, DCA-treated hepatocytes reponsive to growth factors (in contrast to PB).

Notes:

1. First author and date: see **References**.
2. TCE= trichloroethylene, TCA= trichloroacetic acid, DCA= dichloroacetic acid, DCVC=dichlorovinylcysteine and conjugates, CH= chloral hydrate, PCE=perchloroethylene (tetrachloroethylene), ENU=ethylnitrosurea, PB=phenobarbital, BB=sodium barbital.
3. m= male, f= female
4. I= inhalation, COG= corn oil gavage, WG= water gavage, DW= drinking water, IP=intraperitoneal.

5. Inhalation study units= parts per million (ppm), gavage study units= milligrams per kilogram per day (mg/kg×d), drinking water units= milligrams/liter (mg/L).
6. SA= specific activity of ^{3}H-thymidine incorporated into hepatocyte DNA, LI= hepatocyte labeling index of ^{3}H-thymidine treated liver slices, PP= peroxisomal proliferation
7. Sig.= statistically significant at α=0.05.