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Using Mode of Action to Assess Health Risks from Mixtures of Chemical/Physical Agents (CU-1073)

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13. ABSTRACT ***(Maximum 200 Words)***

Mixtures of carcinogenic chemicals are a major problem in ground water and soils on DoD and DOE facilities. While there is frequently data available for interactions between chemicals to judge risks from short term exposures, data that describe how interactions influence the development of cancer are very rare. This is largely because of the high cost associated with conducting complex interaction studies over the lifetime of experimental animals. Therefore, it is important that the limited resources that are available for studying interactions be directed towards the development of general principles that can be applied to wide variety of circumstances. The co-occurrence of chlorinated solvents at DoD and DOE facilities such as trichloroethylene (TCE), tetrachloroethylene (PERC) and carbon tetrachloride (CT) is a case in point.

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Project Title: Using Mode of Action to Assess Health Risk for Mixtures of Chemical/Physical Agents.

Performing Organizations: Pacific Northwest National Laboratory (PNNL) Richland, WA and Wright Patterson Air Force Base (WPAFB) Dayton, OH.

Project Background: Mixtures of carcinogenic chemicals are a major problem in ground water and soils on DoD and DOE facilities. While there is frequently data available for interactions between chemicals to judge risks from short term exposures, data that describe how interactions influence the development of cancer are very rare. This is largely because of the high cost associated with conducting complex interaction studies over the lifetime of experimental animals. Therefore, it is important that the limited resources that are available for studying interactions be directed towards the development of general principles that can be applied to a wide variety of circumstances. The co-occurrence of chlorinated solvents at DoD and DOE facilities such as trichloroethylene (TCE), tetrachloroethylene (PERC) and carbon tetrachloride (CT) is a case in point. Two metabolites of these solvents, dichloroacetate (DCA) and trichloroacetate (TCA) act as tumor promoters in the liver, but by different mechanisms. CT acts by a third mechanism of killing normal cells and encouraging the growth of resistant tumor cells by the reparative process that ensues. Synergism would be expected when chemicals with differing mechanisms of action are involved in treatments based on theoretical considerations. However, evidence to date suggests that DCA and TCA act on distinct populations of tumor cells. This suggests that their effects would be no more than additive. It is hypothesized that CT acts indiscriminately in stimulating the growth of different tumor cell types. Thus, it is predicted that DCA and TCA would not add significantly to the numbers of tumors produced by CT. Chlorinated solvents appear to be an ideal set in which to explore the limits on interaction between non-genotoxic carcinogens.

The hypothesis this project addresses is whether classifying the modes of action represented in a mixture will provide a simpler and more accurate means of predicting the hazards that the mixture poses over a range of exposure situations. If this is the case, knowledge about the dose-response characteristics of a particular mode of action at low doses should be applicable for estimating the risks associated with the combination. The advantage of this approach is that while number of chemicals present in the mixture may be large, the number of modes of action responsible for the biological effects is small. Each mode of action may have many mechanisms that might contribute to changes in cell birth/death processes, but establishing mechanisms for every chemical would be very expensive. The modes of action represented by the three

chemicals included in this study are general to chemical carcinogenesis. Thus, the approach that would result from proving our hypothesis should be broadly applicable to any mixture of chemicals and/or physical causes of cancer. The seven top chlorinated hydrocarbon solvents found on DOE facilities produce liver cancer by non-genotoxic mechanisms. Two others are clearly genotoxic. Therefore, all modes of action are represented among these contaminants.

Objective: The overall technical objectives of this project are to: (a) provide a scientific basis for estimating the risk for liver cancer induction by mixtures of chlorinated hydrocarbon solvents in hazardous waste sites and contaminated ground water, (b) test the hypothesis that interaction between non-genotoxic modes of action can be meaningfully predicted from knowledge of the mode of action and the dose-response relationships found with the individual components of the mixture, and (c) develop an experimental design to validate the approach, based on the results of these studies, using solvents that independently generate the metabolites responsible for the cancer induction.

Waste sites under the control of DoD and DOE contain high concentrations of chlorinated solvents, usually in the form of complex mixtures. Chlorinated ethylene (e.g. trichloroethylene or tetrachloroethylene) and chlorinated ethanes (e.g. 1,1,1-trichloroethane) are most common. Dichloroacetate (DCA) and trichloroacetate (TCA) are the metabolites of these solvents that produce cancer in the liver. Both metabolites are tumor promoters. Chloroform and carbon tetrachloride represent cytotoxic chlorinated solvents. If the premise of this proposal is correct, it should be possible to estimate the cancer risks of these mixtures based on knowledge of the modes of action of these key metabolites and pharmacokinetics of their production and elimination from the body.

Technical Approach: The specific objectives during the period of this report were to establish a chemically initiated animal that develops liver cancer and to conduct experiments with single and/or multiple chemical combinations (DCA, TCA, or CT) in the drinking water in the model. The primary descriptive parameters are the relative effects of the treatments on the average number of tumors/animal (defined as all visible lesions at necropsy) and mean tumor size. At a first approximation these two parameters are treated as independent variables, with tumor numbers thought of as being a function of tumor initiation and tumor size being one of tumor promotion. The sequence of experiments first focused on identifying interactions in binary mixtures and using that data to determine whether we can predict the results of a limited number of tertiary mixtures. The binary interaction of most interest is the interaction between DCA and TCA. In a sense, these compounds have very similar modes of action, although their

mechanisms are clearly different since different tumor phenotypes are promoted. The experiments in which the doses of DCA and TCA are varied are necessary to interpret carcinogenesis data for the single solvents that produce both metabolites as well as for understanding how mixtures of the solvents interact to induce liver cancer. The interactions of these two are to be evaluated against a single dose of CT to limit costs of the project. Studies of the interactions between mechanisms and/or modes of action (toxicodynamics) will be coupled with studies of how the mixture modifies the delivery of effective doses of the active metabolites of each chemical to their target (pharmacokinetics). The complexity of this task is considerably reduced because methods used in the development of physiologically-based pharmacokinetic (PBPK) models for DCA and TCA as metabolites of TRI at both WPAFB and PNNL are directly transferable to this effort.

Project Accomplishments: The major research activity during FY 98 was the initiation/ promotion study started in FY97 using B6C3F1 mice with DCA, TCA, and CT as promoters of vinyl carbamate-initiated liver cancer. The study was divided into four segments for logistical purposes (Table 1). The experiments were started using mice initiated with 3 mg/kg vinyl carbamate (VC) at two weeks of age. The mice in each experiment were started on their respective drinking water treatments one week later (at weaning). For three of the experiments, selected mice were scheduled for sacrificed at 18, 24, 30 and 36 weeks of treatment. Animals in Experiment IV were scheduled for sacrifice at weeks 24 and 36. At sacrifice the mice were weighed and the livers removed and weighed. Livers were examined carefully, all lesions were identified and measured, and the lesions with liver tissue were sliced and prepared in tissue cassettes. These samples were preserved in 10% neutral buffered formaldehyde (NBF) for 24 hours before being transferred to 70% ethanol. Some liver tumor tissue was frozen at -78%C for molecular biology assays if needed.

A modification of the study design was made necessary by the initial results with CT. The doses of CT used in the initial experiments were selected to be just below those used in the NCI cancer bioassay; 20, 100 and 500 mg/kg. Doses of 100 and 500 mg/kg were found to produce such large numbers of tumors that counting and sizing lesions is next to impossible. A repeat of these experiments is being conducted utilizing 5, 20 and 50 mg/kg per day. These new doses were selected on the basis of separately conducted studies that cell replication rates within the liver of B6C3F1 mice were significantly increased at 20 mg/kg, but 5 mg/kg did not. A single dose of 100 mg/kg CT produced a 3-day labeling index >0.9 so the maximum dose chosen was 50 mg/kg. The design of this new segment is shown in Figure 2. The restart of these experiments may delay the publication of our results by a few months.

Terminal body weight data and tumor incidence and tumor size data have been summarized for all experiments that have been completed. Statistical analysis of the data is in progress, but some general trends are apparent in the data and will be briefly discussed in this report. Slides of liver tumors are in the process of being prepared for histological evaluation of tumor type and immunohistochemical evaluations of c-Jun expression.

All three compounds produced dose-response relationships similar to that observed in non-initiated mice, but in a shorter time frame and with greater multiplicity of tumors/mouse. The time courses of tumor development, both in terms of numbers of tumors/mouse and in tumor size, are depicted for all three chemicals in Figures 1-3.

DCA has its major effect on tumor numbers at both 0.5 and 2 g/L, with no significant effect at 0.1 g/L. This time dose-response relationship faithfully mirrors the differences in latency observed at these same two doses in non-initiated mice with DCA. Therefore, the VC-initiated model does not distort the basic nature of what is an effective dose; it simply increases the number and size of lesions that can be observed at shorter latencies.

TCA substantially increases both the numbers and size of tumors that result from treatment. These responses are much more regular, reflecting the linear dose-response curve associated with this chemical in non-initiated mice. It is noteworthy that similar relationships of dose-response with DCA and TCA have been observed in N-methyl-N-nitrosourea initiated mice (Pereira et al., 1997). Therefore, this concordance is not tumor initiator specific.

Responses to CT were much more dramatic than with either DCA or TCA. The numbers of tumors induced by 500 mg/kg became too large to accurately count by 30 months. Even the dose of 100 mg/kg produced 40 tumors/mouse in this time frame. Of greater concern is the fact that tumor size plateaus at these two doses at 24 weeks. Although there was no excess mortality at this time, these high doses have clearly produced a maximal response and will make interaction studies very difficult to interpret. Therefore, this experiment has been reinitiated at lower doses.

Analysis of interactions between DCA, TCA and CT is complex at this early stage in the project. Data can be expressed in a variety of ways, but we have chosen to focus on interactions through time to provide a better perspective of data consistency. It also serves to illustrate that the interactions being studied are complex. Although interactions with CT and the other two compounds have been completed, they are not presented here, because we do not believe them to be valid because of the apparently saturated response at the two highest doses. Therefore, the

only data we feel is valid is the two time points at the lowest dose of CT used (20 mg/kg). At this dose there was no significant interactions between CT and DCA or TCA.

There does appear to be significant interactions between DCA and TCA. In Figure 4 the effects of a varied dose of DCA superimposed on a fixed dose of TCA is shown. A very substantial increase in the number of liver tumors was observed at 24 weeks of treatment at the two lowest doses of DCA. At 36 weeks, there were no differences. As strange as this result might seem, it may be rationalized by the fact that DCA stimulates the growth of a different cell population than TCA (Stauber and Bull, 1997; Stauber et al., 1998). As indicated above, DCA's effects on numbers were substantially greater than on tumor size. This interaction might be explained by an effect of the combined exposure on DCA's metabolism. Prior work has shown that the metabolism of DCA is significantly affected by autoinhibition. This usually occurs at higher doses of DCA. It is particularly important to note that this apparent interaction does not occur at 36 weeks, suggesting that there are finite numbers of tumor cell types whose growth is stimulated by the two compounds. The convergence of the tumor numbers at 36 weeks is probably at least partially attributable to the significant inhibition of the growth rate of the tumors produced by TCA promotion by high doses of DCA. This inhibition was clearly dose related.

The effect of varying dose rates of TCA on the growth of tumors promoted by DCA is more interesting. Here small doses of TCA clearly suppressed the number of DCA-induced tumors. High doses of TCA did not affect tumor numbers. None of the TCA treatments affected tumor size. Therefore, suppression of tumor numbers by TCA is independent of an effect on growth rate of lesions. It is important to remember that the effect of high doses of DCA, given alone, disproportionately increases the rate and number of tumors with a much-shortened latency. Tumorigenesis at these doses of DCA clearly involves mechanisms that are not occurring at 0.1 and 0.5 g/L. In large part the large differences in latency with such a small difference in dose is attributable to the very large increases in blood levels of DCA that are produced as a result of autoinhibition of metabolism in this region (Gonzalez-Leon et al., 1997). In mice the blood levels of DCA increase from 2-3 μM at 0.5 g/L to 300-500 μM at 2 g/L (Kato-Weinstein et al., 1998). These latter concentrations are in the range in which the pyruvate dehydrogenase kinase is inhibited by DCA (Pratt and Roche, 1979)

These results provide strong support for the hypothesis that the use of mice pre-initiated for cancer can simplify study of the toxicodynamics of tumor promotion. It is very important to recognize that the descriptive data derived from these studies are not appropriately used in

conventional risk assessment. However, these data can be important in developing formal biologically based models for low doses of chemicals that act as tumor promoters. The best evidence to support this use in modeling is the fact that the general character of relative latency and tumor multiplicity seen in studies of DCA and TCA in uninitiated mice was faithfully reproduced in these experiments. In addition, even in the initiated mice, it was clear that the population of responsive tumor cells was finite, reaching a maximum with both DCA and TCA. Therefore, an early conclusion is that the probability of these compounds acting as carcinogens are a function of the rate at which these cells are produced in an organ. In contrast, there appeared to be no upper limit to the number of tumors that could be produced by carbon tetrachloride. This is very consistent with the indiscriminate stimulus cell killing and reparative hyperplasia provides for tumor growth in the liver. Several authors have shown carbon tetrachloride and other cytotoxic agents are by far a more effective means of stimulating tumor growth than mitogenic agents (Ledda-Columbano et al., 1992). The initiation permits those processes to be studied and modeled and should provide very useful insight into the low dose behavior of this general class of chemical carcinogens. However, the incidence data (or more appropriately tumor multiplicity data) must be adjusted to conform to spontaneous initiation rates.

These preliminary data suggest that the induction of liver cancer from mixtures of solvents may have predictable outcomes. The principal concern from data collected is the apparent interactions between low doses of DCA superimposed on a high dose of TCA to increase tumor numbers. As stated in our original research plan, we intend to fully investigate the pharmacokinetics of DCA under these conditions as that the most likely explanation. However, it may be an important finding for explaining why certain characteristics of trichloroethylene tumors are more similar to DCA than TCA, despite that very low levels of DCA and high levels of TCA are produced from this single solvent. Other interactions appeared to be primarily inhibitory. In particular, the inhibition promotion of tumors by high doses of DCA inhibited by low doses of TCA. This was not an expected result, but it appears to be highly significant. Therefore, an assumption of additivity of low dose responses to tumor promoters does not seem to hold generally. Unfortunately, our experimental design did not address interactions between low doses of tumor promoting agents, as we needed to limit costs. However, continuation of this work to look at low dose interactions between DCA and TCA might shed significant light on probable cancer risks from low dose exposure to chlorinated ethane and ethylene solvents.

Publications/Presentations

No technical report or journal articles were published during this report period, but abstracts were prepared and the following presentations were made:

Oral Presentations

Bull, R. J. Risk Assessment for Mixtures. In: Third Annual SERDP Symposium: Building Bridges to the 21st Century, Dec. 3-4, 1997. Washington, DC.

Bull, R. J. 1998. Using Mode of Action to Assess Health Risks from Mixtures of Chemical/Physical Agents. Strategic Environmental Research And Development Program (SERDP) and Environmental Security Technology Certification Program (ESTCP) Joint Annual. In-progress Review : Cleanup Thrust Area, May 12-15, 1998. Arlington, VA.

Poster Presentations

Bull, R. J., L. B. Sasser, J. H. Miller and G. A. Orner. 1997. Risk Assessment for Mixtures. In: Third Annual SERDP Symposium: Building Bridges to the 21st Century, Dec. 3-4, Washington, DC.

Bull, R. J., J. Fisher, J. C. Parker, L. B. Sasser, and J. H. Miller. 1998. Using Mode of Action to Assess Health Risks From Mixtures of Chemical/Physical Agents. In: Fourth Annual SERDP Technical Symposium and Workshop, Dec. 1-3, Arlington, VA.

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Kato-Weinstein, J., Lingohr, M.K., Thrall, B.D., and Bull, R.J. (1998) Effects of dichloroacetate-treatment on carbohydrate metabolism in B6C3F1 mice. Toxicology 130:141-154.

Ledda-Columbano, G.M, Coni, P., Curto, M., Giacomini, L., Faa, G., Sarma, D.S.R. and Columbano, A. (1992) Mitogen-induced liver hyperplasia does not substitute for compensatory regeneration during promotion of chemical carcinogenesis. Carcinogenesis 13:379-383.

Pereira, M.A. and Phelps, J.B. (1996) Promotion by dichloroacetic acid and trichloroacetic acid of N-methyl-N-nitrosourea-initiated cancer in the liver of female B6C3F1 mice. *Cancer Lett.* 102:133-141.

Pratt, M.L.and Roche, T.E. (1979). Mechanism of pyruvate inhibition of kidney pyruvate dehydrogenasea kinase and synergistic inhibition by pyruvate and ADP. *J Biol Chem* 254:7191-7196.

Stauber, A.J. and Bull, R.J. (1997) Differences in phenotype and cell replicative behavior of hepatic tumors inducted by dichloroacetate (DCA) and trichloroacetate (TCA). *Toxicol. Appl. Pharmacol.* 144:235-246.

Stauber, A.J., Bull, R.J. and Thrall, B.D. (1998) Dichloroacetate and trichloroacetate promote clonal expansion of anchorage-independent hepatocytes *in vivo* and *in vitro*. *Toxicol. Appl. Pharmacol.* 150:287-294.

Table 1. Initial Design and Phasing of Mode of Action Studies.

Group No.	VC mg/kg	DCA g/L	TCA g/L	CT mg/kg	N/Sac	Sac Times	Total N
Experiment 1							
1	0	0	0	0	5	18 24 30 36	20
2	3	0	0	0	10	18 24 30 36	40
3	3	0.1	0	0	10	24 36	20
4	3	0.5	0	0	10	24 36	20
5	3	2.0	0	0	10	18 24 30 36	40
Experiment 2							
1	0	0	0	0	5	18 24 30 36	20
2	3	0	0	0	10	18 24 30 36	40
6	3	0	0.1	0	10	24 36	20
7	3	0	0.5	0	10	24 36	20
8	3	0	2.0	0	10	18 24 30 36	40
Experiment 3							
9	3	0	0	0(G)	5	24 36	10
10	3	0	0	20	5	24 36	10
11	3	0	0	100	5	24 36	10
12	3	0	0	500	5	18 24 30 36	20
14	3	0.1	0	500	5	24 36	10
15	3	0.5	0	500	5	24 36	10
16	3	2.0	0	500	5	18 24 30 36	20
18	3	0	0.1	500	5	24 36	10
19	3	0	0.5	500	5	24 36	10
20	3	0	2.0	500	5	18 24 30 36	20
Experiment 4							
9	3	0	0	0(G)	5	24 36	10
10	3	0	0	20	5	24 36	10
11	3	0	0	100	5	24 36	10
12	3	0	0	500	5	18 24 30 36	20
14	3	0.1	0	500	5	24 36	10
15	3	0.5	0	500	5	24 36	10
16	3	2.0	0	500	5	18 24 30 36	20
18	3	0	0.1	500	5	24 36	10
19	3	0	0.5	500	5	24 36	10
20	3	0	2.0	500	5	18 24 30 36	20
Experiment 4							
29	3	0.0	2.0	0	10	24 36	20
30	3	0.1	2.0	0	10	24 36	20
31	3	0.5	2.0	0	10	24 36	20
32	3	2.0	2.0	0	10	24 36	20
33	3	2.0	0.0	0	10	24 36	20
34	3	2.0	0.1	0	10	24 36	20
35	3	2.0	0.5	0	10	24 36	20

Table 2. Modification of study to accomodate lower doses of carbon tetrachloride

Group No.	VC mg/kg	DCA g/L	TCA g/L	CT mg/kg	N/Sac	Sac Times	Total N
Experiment 5							
1	0	0	0	0	5	18 24 30 36	20
9	3	0	0	0(G)	10	24 36	20
36	3	0	0	5	10	24 36	20
10	3	0	0	20	10	24 36	20
37	3	0	0	50	10	18 24 30 36	40
38	3	0.1	0	50	10	24 36	20
39	3	0.5	0	50	10	24 36	20
40	3	2.0	0	50	10	18 24 30 36	40
41	3	0	0.1	50	10	24 36	20
42	3	0	0.5	50	10	24 36	20
43	3	0	2.0	50	10	18 24 30 36	40

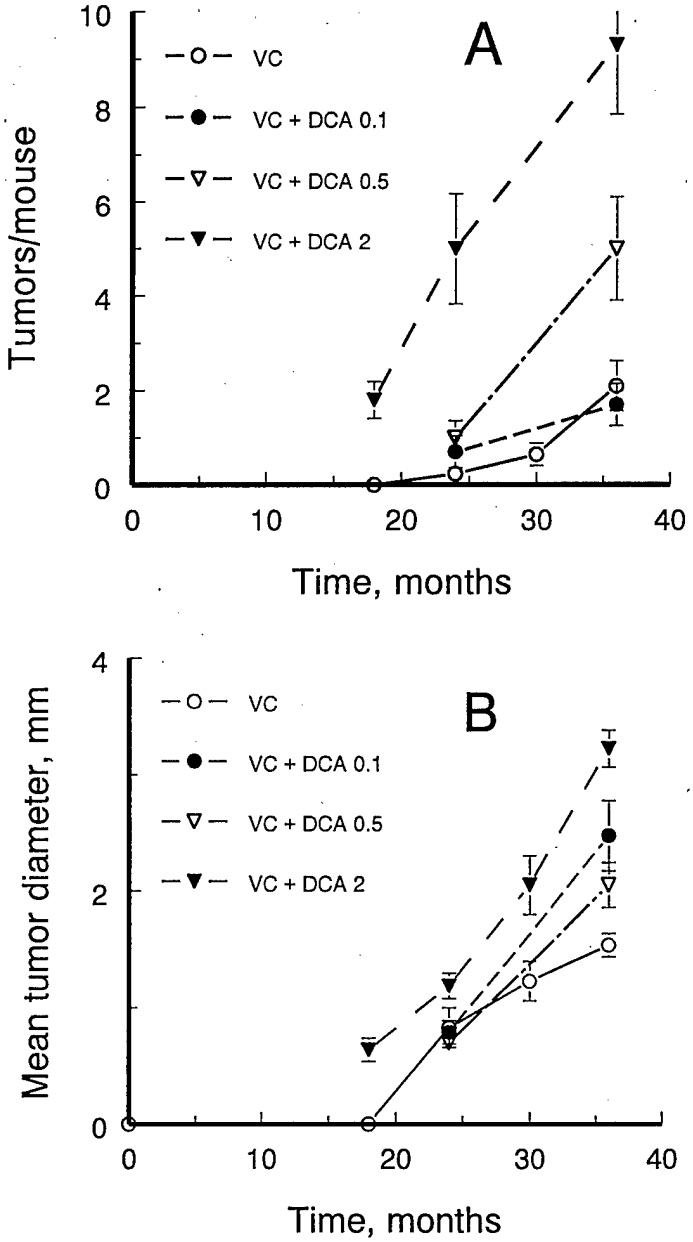

Figure 1. Time course of tumor development in vinyl carbamate-initiated male B6C3F1 mice treated with varying concentrations of dichloroacetate (DCA). Panel A depicts changes in tumor numbers, panel B changes in tumor size.

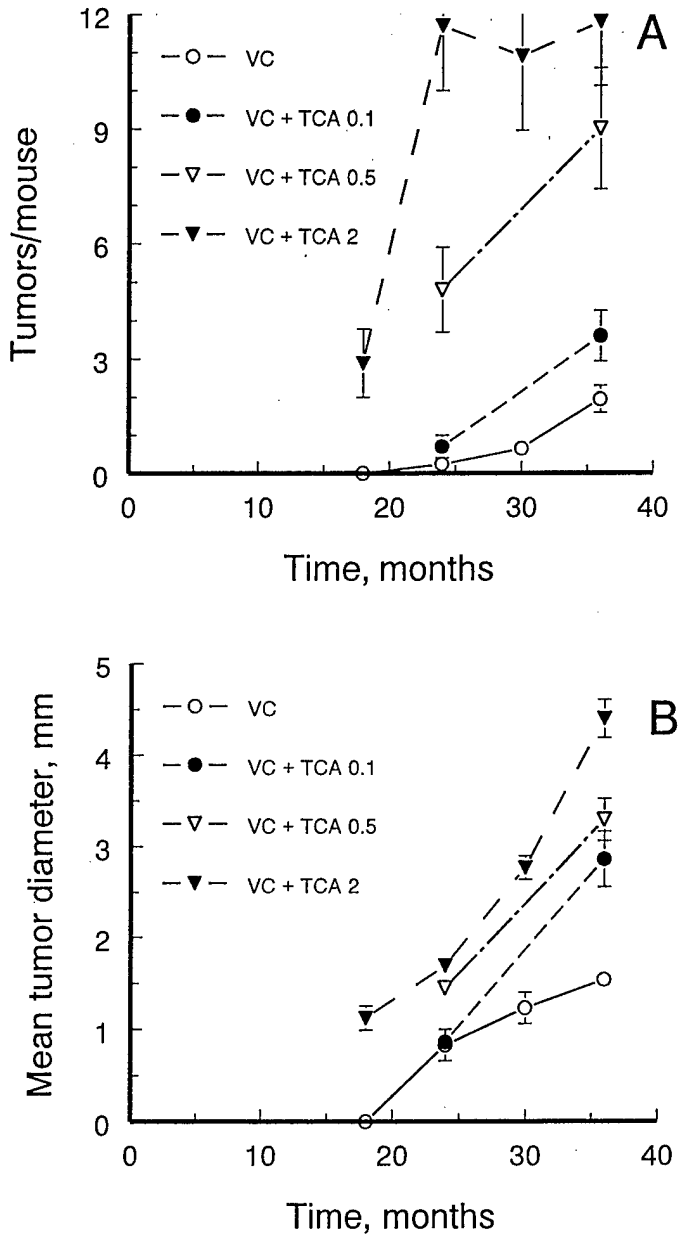

Figure 2. Time course of tumor development in vinyl carbamate-initiated male B6C3F1 mice treated with varying doses of trichloroacetate (TCA). Panel A depicts changes in tumor numbers, panel B changes in tumor size.

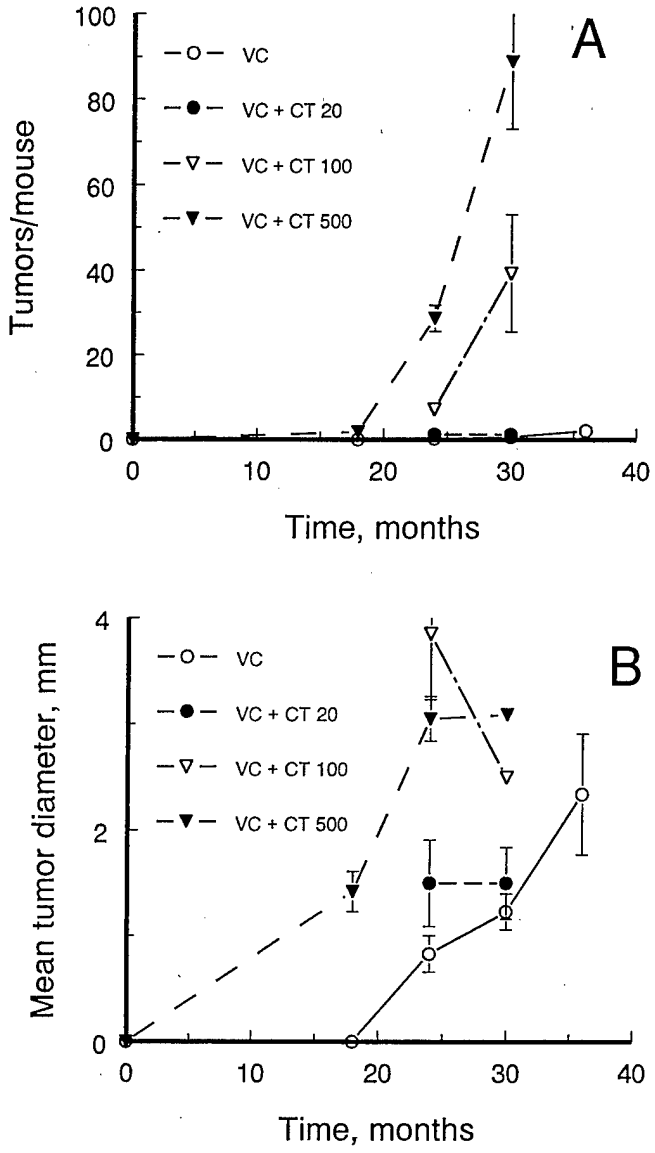

Figure 3. Time course of tumor development in vinyl carbamate-initiated male B6C3F1 mice treated with varying doses of carbon tetrachloride (CT). Panel A depicts changes in tumor numbers, panel B in tumor size.

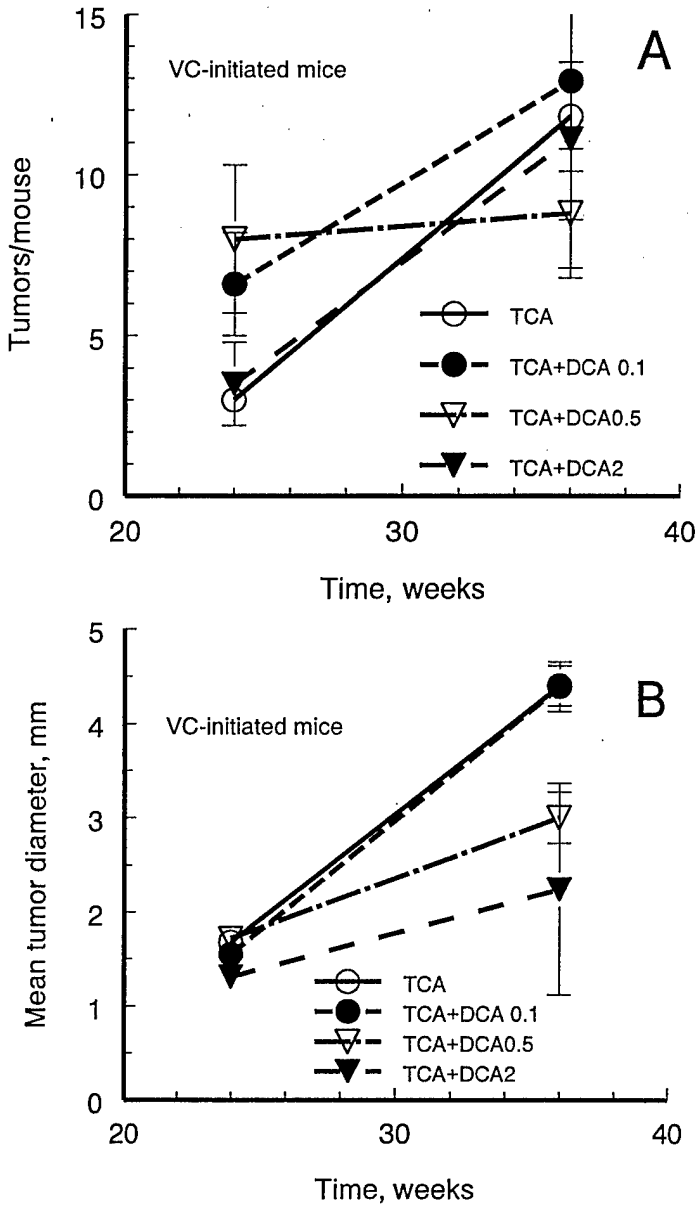

Figure 4. Illustration of the interactions of varying doses of DCA in drinking water on the tumor promoting activity of a fixed dose of TCA (2 g/L). Panel A depicts effects on tumor number, panel B effects on tumor size.

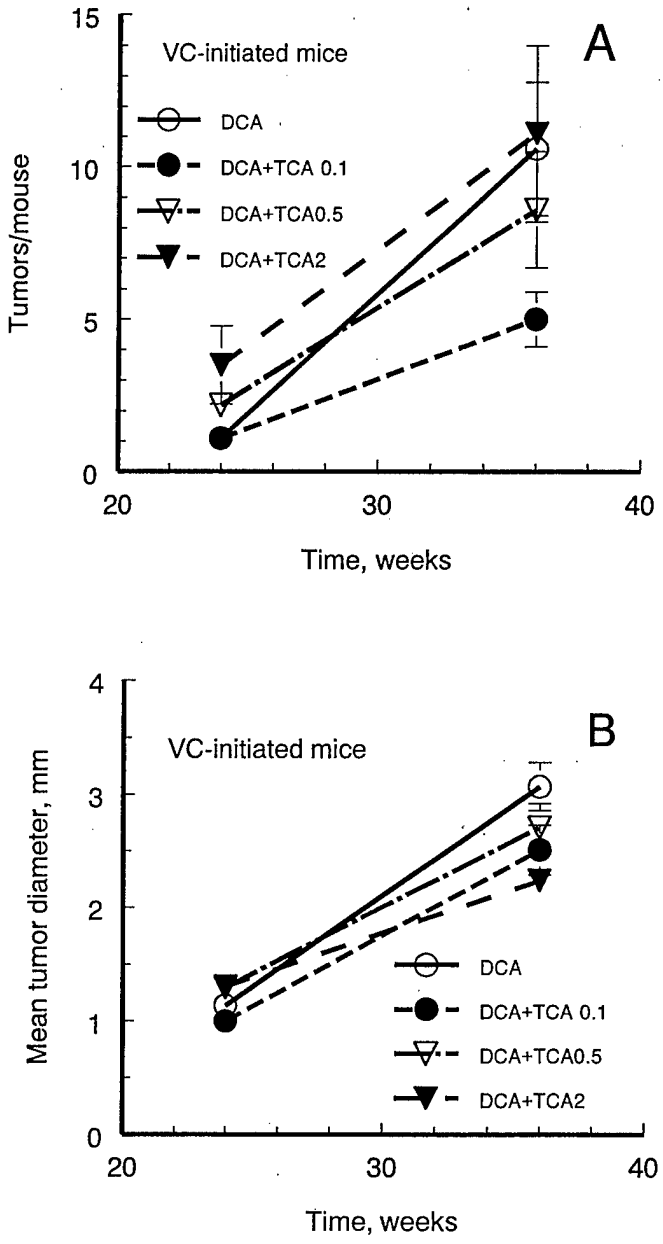

Figure 5. Illustration of the interactions of varying doses of TCA in drinking water on the tumor promoting activity of a fixed dose of DCA (2 g/L). Panel A depicts effects on tumor number, panel B effects on tumor size.