FINAL REPORT

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MECHANISMS INVOLVED IN TRICHLOROETHYLENE INDUCED LIVER CANCER: IMPORTANCE TO ENVIRONMENTAL CLEANUP

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Executive Summary

Trichloroethylene (TCE) is a common contaminant of groundwater as a result of poor disposal practices of the past. As a consequence, this solvent is the focus of many clean-up operations of hazardous waste sites. The finding that TCE induces liver cancer in mice has been a primary driver for current environmental regulations of this contaminant. Under the proposed cancer risk guidelines of the Environmental Protection Agency, identifying the dose-response behavior of key events involved in carcinogenic responses can be used for developing alternative risk assessments, which ultimately impact environmental standards and remediation costs.

A critical issue in addressing the mechanism by which TCE induces liver cancer is to identify the metabolites produced by TCE that contribute to the tumor response. It has been proposed that dichloroacetate (DCA) and trichloroacetate (TCA) are potential metabolites that have been produced from TCE, and both metabolites are carcinogenic in mice. Classically, TCA was considered the active metabolite in inducing liver cancer from TCE exposure. TCA falls into a broad category of chemicals known as "peroxisome proliferators," which utilize a mechanism of tumor induction that is thought to be specific to rodents. In contrast, DCA induces tumors in multiple species through mechanisms that are distinct from TCA. Therefore, understanding the relative contributions of TCA and DCA in TCE-induced liver cancer is an important variable when considering the potential risk to humans.

This task was accomplished primarily through three approaches. First, metabolism and kinetic studies were performed to evaluate the potential formation rates of DCA and TCA in mice exposed to TCE. Second, a comparative study was performed to evaluate whether the pattern of mutations in the H-ras gene in tumors induced by TCE, DCA, and TCA would reveal the relative roles of these metabolites in tumor induction. Third, studies were performed using isolated mouse liver cells in an effort to understand which signaling pathways are modulated by

these chemicals and how these pathways regulate cell division. Results from these experiments demonstrated that because DCA is very rapidly metabolized within the liver, blood levels of DCA after administration of carcinogenic doses of either DCA or TCE are much lower than previously appreciated. However, studies using isolated liver cells as a model system demonstrated that even low micromolar concentrations of DCA are sufficient to promote the growth of cells with precancerous characteristics.

Comparisons of the mutation patterns in the H-ras gene in tumors induced by TCA, DCA, and TCE suggest that the tumors induced by TCE cannot be accounted for solely by formation of TCA. However, these studies also revealed that H-ras mutations are a late event in the formation of mouse liver tumors and therefore are not directly predictive of the role of either metabolite in tumor formation. Using the c-Jun protein as a marker that distinguishes the phenotype of tumors induced by TCA or DCA, it was found that tumors induced by TCE had a mixed phenotype, which is also inconsistent with TCA acting as the sole active metabolite in TCE-induced liver cancer. The mechanism for TCA-induced liver cell growth can be attributed to activation of a nuclear receptor (PPAR), which is expressed at much higher levels in mice than in humans. There is general consensus that this mechanism is specific to rodents and poses little risk to humans.

Studies of the mechanism of DCA-induced effects on liver cells are less definitive, although modulation of metabolic pathways that lead to accumulation of liver glycogen levels appear to be involved. These pathways are likely to have a threshold above the typical environmental exposures humans are likely to encounter. However, the elimination of DCA from the blood of exposed animals is dependent on an enzyme (GSTz) whose expression varies dramatically throughout the human population. Although the contribution of DCA in TCE-induced cancer appears to be much less than that of TCA, it cannot be ruled out. Therefore, the risk to humans should not be unequivocally dismissed.

1.0 Research Objectives

Trichloroethylene (TCE) is a common contaminant of groundwater as a result of poor disposal practices of the past. As a consequence, this solvent is the focus of many clean-up operations of uncontrolled hazardous waste sites. TCE is carcinogenic in both mice and rats, but at different sites, the liver and kidney, respectively (NCI 1976; NTP 1988; NTP 1990). Liver-tumor induction in mice has been the tumor most critical from the standpoint of environmental regulation (Bull 2000). Under the proposed cancer risk guidelines of the Environmental Protection Agency (EPA 1996), identifying the dose-response behavior of key events involved in carcinogenic responses can be used for developing alternative risk assessments.

A major difficulty in developing alternative approaches for TCE is the fact that three of its metabolites are capable of inducing liver cancer in mice (Bull et al. 1990; Daniel et al. 1992; DeAngelo et al. 1999; Pereria 1996). Two of these metabolites have distinct modes of action, dichloroacetate (DCA) and trichloroacetate (TCA). The third metabolite, chloral hydrate, is probably active as a result of its conversion to one or both of these two metabolites. Ordinarily, the first approach to assigning causality to a metabolite in tumorigenesis would be an attempt to measure its concentration in the body and associate that with tumorigenic concentrations observed when the compound is itself administered. This can be done with relative ease with TCA. However, it has been more difficult with DCA since blood levels of this metabolite after exposure to carcinogenic doses of DCA fall rapidly below detection limits (Kato-Weinstein et al. 1998; Merdink et al. 1998).

Mutations in the ras protooncogene have been used to determine if distinct patterns of DNA-sequence alterations can provide indications of the type of DNA damage that might be produced by carcinogens. The presence of ras mutations in chemically-induced tumors was suggested as a means of determining whether a chemical was genotoxic (Wiseman et al. 1986). However, the

discovery that spontaneous tumors also contain this oncogene indicated that this assumption may not be correct (Fox and Watanabe 1985). Several non-genotoxic carcinogens have been shown to produce tumors with a H-ras mutation frequency considerably below those that result spontaneously (Maronpot et al. 1995). Among these chemicals are a class called peroxisome proliferators, of which TCA and TCE are members. DCA and TCE were found to induce tumors with similar H-ras mutation spectra (Anna et al. 1994), whereas only limited data have been available on TCA (Fereira-Gonzalez et al. 1995). Thus, a major focus of this research was to evaluate whether the pattern and frequency of H-ras mutations in TCE-induced tumors could be explained by the same parameters in tumors induced by the metabolites TCA or DCA.

The present project was organized around three interrelated objectives:

The *first objective* addressed the pharmacokinetic questions regarding the formation and elimination of DCA and TCA in mice administered TCE and whether levels of these metabolites may account for the tumors induced by TCE. The *second objective* was to investigate potential molecular mechanisms by which TCA and DCA may, in the absence of directly causing mutations, promote the clonal growth and expansion of precancerous cell populations within mouse liver. The *third objective* was to investigate whether the genotype of tumors induced by TCA and DCA can be used to establish the relative roles of these metabolites in TCE-induced cancer. In particular, the focus of the latter studies was to compare the incidence and spectra of mutations in the H-ras gene (codon 61) to determine if the reported similarities in the genotype of DCA- and TCE-induced tumors have a causal relationship.

2.0 Methods and Results

In the present project, we have evaluated the relative importance of TCA and DCA in mouse-liver tumor induction by TCE. To address this question, three primary approaches have been utilized:

1. Studied the metabolism and kinetics of DCA and TCA formation to determine the potential formation and bioavailability of these metabolites in relationship to tumor formation induced by TCE
2. Investigated mutations within the H-ras gene as a potential biomarker of TCA and DCA-mediated tumor formation in comparison with tumors induced by TCE
3. Utilized protein expression patterns as phenotypic markers of TCA and DCA-mediated tumor formation for comparison with tumors induced by TCE.

2.1 Summary of Pharmacokinetic Findings

The pharmacokinetic experiments conducted in this project were primarily short-term studies of DCA and TCA metabolism intended to evaluate the potential formation rates of these metabolites and the effects that chronic exposure have on their formation and elimination. The primary conclusion that can be drawn from these experiments is that the blood concentrations of DCA that correspond to carcinogenic doses (when administered directly in drinking water) are much lower than previously thought. DCA is metabolized in both rodents and most humans very rapidly with a half-life at low doses in the 10–40 min range. We have shown that prior treatment with DCA substantially inhibits its metabolism, increasing its half-life to more than 10 h in the rat (Gonzalez-Leon et al. 1999).

There are dramatic differences in DCA in the blood stream of mice given a minimally carcinogenic dose (0.5 g/L) and those of a high dose that produces tumors with a short latency (2 g/L). The peak blood concentrations arising from this small four-fold increase in concentration in drinking water vary by up to 100-fold. During the daytime, blood concentrations of DCA are

seen to fall rapidly to undetectable levels in the group that was provided 0.5 g/L in their water. The important conclusion from these data is that, apparently, much lower blood concentrations of DCA are associated with tumor formation than had been previously appreciated. The concentrations of DCA seen in the blood of mice included in these experiments (2–3 µM) are associated with a lifetime tumor incidence of 79% (Daniel et al. 1992). Therefore, it can be surmised that DCA is active as a carcinogen when blood concentrations are in the submicromolar range (for example, 0.1 g/L might be expected to give a 10–20% tumor incidence, and blood levels corresponding to this dose peak in a range of 0.5 µM). These concentrations are significantly lower than the detection limits of standard analytical methods, so the contribution of DCA in TCE-induced tumors cannot be discounted based solely on classical dosimetry. For this reason, molecular approaches to evaluate biomarkers of DCA metabolism *per se* are needed to further evaluate this issue.

2.2 Summary of *In vitro* Findings

Short-term mechanistic studies were also conducted using isolated hepatocytes to investigate the potential signaling pathways modulated by TCA and DCA that may account for the effects of these metabolites in control of hepatocellular growth and survival. Using isolated hepatocytes as a model system, this project was the first to find that the mitogen-activated protein kinase (MAPK) pathway is rapidly activated by TCA and other peroxisome proliferators, and that this pathway is necessary for the mitogenic effects of these agents on hepatocytes (Mouhno and Thrall 1999). In contrast, this pathway is not directly activated by DCA in the isolated hepatocyte model (Lingohr et al., submitted for publication). The primary findings from the *in vitro* studies with DCA are that low concentrations of DCA ($\leq$ 10 µM), when given chronically, have the potential to promote the survival and expansion of a subpopulation of hepatocytes with anchorage-independent growth characteristics both *in vitro* and *in vivo* (Stauber et al. 1998). While a mechanism for the expansion of initiated cells is not yet clear, alterations in glycogen metabolism and the subsequent feedback alterations in insulin-receptor pathways may be

involved (Lingohr et al. 2001). A potential candidate kinase involved is phosphatidylinositol 3-kinase because studies using inhibitors of this kinase indicate it is involved in the glycogen accumulation and insulin receptor downregulation responses induced by chronic DCA exposure (Lingohr et al., submitted for publication). However, further studies will be necessary to more closely evaluate these potential molecular mechanisms. Because detailed descriptions of the methods used and the results obtained from these experiments have been published in peer-reviewed literature (refer to Section 6.0), these results are not reiterated here.

2.3 Description of Chronic Tumor Studies

A summary of the methods and results from the tumor studies is provided in the following sections. These experiments were conducted to provide a molecular comparison at the genotype and phenotype level of the tumors induced by administration of DCA, TCA, or TCE. A detailed description of these experiments has been submitted for publication (Bull et al., submitted).

2.3.1 Experimental Animals

Male B6C3F1 mice were purchased from Charles River Laboratories (Wilmington, MA) at 4–6 weeks of age for Experiment #1, and at 11 days (on arrival) for Experiment #2. Protocols and animal care were approved by the Institutional Animal Care and Use Committee (IACUC) at Pacific Northwest National Laboratory. Animals were housed 4–6 per cage in shoebox cages and had free access to NIH-07 rodent chow and drinking water at all times. Control, TCA, and DCA drinking water solutions were prepared from deionized water and adjusted with sodium hydroxide to pH 7.0 ± 0.2. Animal rooms were maintained on a 12-h light-dark cycle with temperatures controlled to 22–24°C and relative humidity between 35% and 65%.

Experiment 1. After a 1-week acclimation period, animals were randomly assigned to treatment groups and given 0, 0.5, or 2 g/L DCA or 2 g/L TCA in their drinking water for the duration of the study. A subset of mice was sampled after 52 weeks, and the remainder were treated for an

additional 35 weeks. Tumors were identified at necropsy, their diameters measured in two dimensions (longest and shortest) to obtain a mean diameter. Histopathological examination was limited to 15 randomly selected tumors to ensure that non-neoplastic lesions were not being misclassified.

*Experiment 2***.** DCA and TCA were administered as a mixture to male B6C3F1 mice in drinking water. Twenty animals were assigned to each of 10 groups that received the following concentrations of DCA or TCA in their drinking water for 52 weeks: 0; 0.5 g/L TCA; 2 g/L TCA; 0.1 g/L DCA; 0.5 g/L DCA; 2 g/L DCA; 0.1 g/L DCA + 0.5 g/L TCA; 0.5 g/L DCA + 0.5 g/L TCA; 0.1 g/L DCA + 2 g/L TCA; or 0.5 g/L DCA + 2 g/L TCA. The animals were sacrificed at 52 weeks, the livers were examined for gross lesions as indicated above, and the histological sections were made for immunostaining and examination by a pathologist.

*Experiment 3***.** TCE was administered by gavage in a 5% Alkamuls® in a distilled water vehicle to a group of 50 mice for a period of 79 weeks. A control group of 15 mice received an equivalent volume of the vehicle by the same method of administration. At the time of sacrifice, the livers were removed, tumors identified, the size of the lesions measured, and the tissues sectioned for examination by a pathologist and for immunostaining.

2.3.2 Tumor Sampling, PCR Amplification, and Sequencing

At the conclusion of each experiment, animals were euthanized, livers were removed, and macroscopically visible lesions (tumors) were identified, measured, and separated from surrounding tissue. A portion of tissue was excised from 25 tumors per treatment group (where available) frozen in liquid nitrogen and stored at -80°C until used for *ras* mutation analysis. Remaining portions of the tumor were either snap-frozen in liquid nitrogen for use in Western blotting, or were fixed in 10% neutral buffered formalin for 24 h, then transferred into 70% ethanol until paraffin-embedded and examined histologically. Tumor tissues (and nontumor in

experiment 1) for H-*ras* analysis were digested overnight at 50° in DNA lysis buffer (50 mM Tris-Cl, pH 8.0, 20 mM NaCl, 1 mM EDTA, 1% SDS, 1 mg/mL proteinase K), proteinase heat inactivated by boiling, diluted 1:10 in water, and 1–4 µL used as template for PCR amplification. DNA was amplified using the primer pairs GCCGCTGTAGAAGCTATGA and CTTGGTGTTGTTGATGGCAAATACA to generate a 469 b.p. section of H-*ras* containing the first and second exon. PCR reaction mixtures contained 4 mM magnesium chloride, 10 mM tris, 50 mM potassium chloride, 0.2 mM deoxynucleotides, 0.2 µM of each primer, and 0.01 U/µL *Thermus aquaticus* DNA polymerase (Perkin-Elmer, Norwalk, Connecticut). A second amplification of an internal sequence containing codon 61 and adding an M13 sequence to both ends was then conducted (primers: TGTAAAACGACGGC-CAGTACAGCCCAGGTCTTGTA and CAGGAAACAGCTATGACCGTTGATGGCAAATAC). After amplification, PCR products were purified using Microcon 100-filter units (Amicon, Beverly, MA) and sequenced on an automated cycle sequencer (ABI 377 DNA sequencer). PCR products were sequenced in the forward direction using Perkin Elmer-Applied Biosystems dye primers and confirmed by sequencing in the reverse direction using either dye primer (Experiment 1) or dRhodamine terminator (Experiment 2) cycle sequencing. DNA from several mutant tumors was reamplified and cloned using a TOPO-TA Cloning Kit (Invitrogen, Carlsbad, California), providing an additional degree of confidence in our mutation detection.

2.3.3 Analysis

Sequencing chromatograms were compared using Sequencher software (Ver. 3.0, Gene Codes Corporation, Ann Arbor, Michigan). Western blots were scanned on a flatbed scanner and quantified on a Power Macintosh 7100 using the public-domain software NIH Image, Version 1.57. Statistical analyses were conducted using Sigma Stat Version 2.0 (Jandel Scientific, San Rafael, California) except as otherwise noted. Animal weights were compared by one-way ANOVA followed by the Tukey Test for pairwise comparisons. Tumor incidence was compared using Fisher's Exact test. Tumor size, multiplicity, and liver somatic indices were

compared using the Kruskal-Wallis nonparametric one-way analysis of variance on ranks. Mutation frequency comparisons were made by Chi-square analysis, and mutation spectra were compared using a mutation-analysis program described by Cariello et al. (1994). A p-value of less than or equal to 0.05 was considered significant.

2.3.4 Immunohistochemistry

The serial liver sections were stained with the c-Jun antibody, SC-45 (Santa Cruz Biotechnology, Santa Cruz, California) in a 1 to 25 dilution by methods previously described (Stauber and Bull 1997). This antibody was raised against the sequence TPTPTQFLCPKNVTD which includes Thr 91 and 93 of mouse c-Jun. These residues are phosphorylated by JNK, and this activity is thought to lead to dephosphorylation of c-Jun in the DNA-binding domain (Nakano et al. 1994), which leads to activation of c-Jun as a transcription factor. Therefore, this is potentially a deactivated form of c-Jun (Kato-Weinstein et al. 2000) that seems to accumulate in mouse-liver tumors.

2.3.5 Summary of Results of Chronic Tumor Studies

All treatments gave rise to tumorigenic responses in the liver, consistent with our past experience. The magnitude of the response to 2 g/L DCA and TCA were about 50% of that seen in past experiments (Bull et al. 1990; Stauber and Bull 1997; DeAngelo et al. 1999). The tumor yield with TCE was slightly higher than was expected from prior NCI (1976) and NTP (1990) bioassays. All gross lesions in this experiment were examined by a pathologist and subjected to sequencing of the H-ras codon 61 and a random sample stained for c-Jun.

Tumor incidence and multiplicity (number of nodules, adenomas, and carcinomas per animal) were significantly higher in animals treated with 2 g/L DCA or TCA for 52 weeks than in control mice. In addition, tumors were induced in animals treated with 0.5 or 2 g/L DCA for 87 weeks. Mixtures of DCA and TCA increased tumor induction in all combinations. In similar fashion, TCE at a daily dose of 1 g/kg per day for 79 weeks increased the gross tumor response

significantly over the concurrent vehicle control. The only treatment that did not increase tumor incidence or multiplicity was 0.1 g/L of DCA in drinking water for 52 weeks.

The dose-response relationships between tumor responses produced by DCA or TCA alone or in combination are displayed in Figure 1. In this figure, the results of both experiments using DCA given alone are combined as being more representative than found in the DCA alone treatment group in the mixture study. The experiments using mixtures of DCA and TCA in drinking water produced responses that were very close to additive when a low dose of DCA (0.1 g/L) was combined with higher dose levels of TCA (0.5 and 2 g/L).

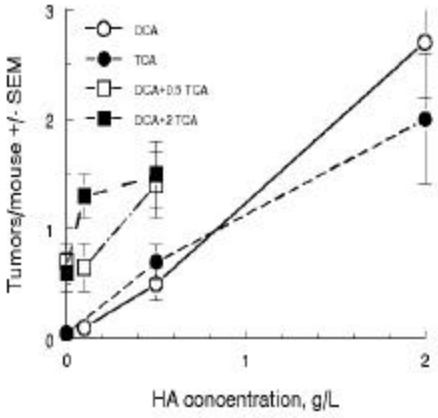

Figure 1. Dose Response Relationship for Tumor Induction By DCA, TCA and Combined DCA+TCA Treatments.

Table 1 indicates the relative frequency at which tumors produced by TCE, DCA and TCA expressed c-Jun that was distributed primarily in the cytosolic compartment of cells. Earlier work by Stauber and Bull (1997) indicated that the occurrence of a c-Jun negative lesion was diagnostic of TCA, whereas almost all lesions induced by DCA were c-Jun positive. This result was generally borne out in the present study, but more c-Jun tumors were observed when DCA was administered alone. Nevertheless, approximately 50% of DCA-induced tumors were c-Jun+. When either mixtures of DCA or TCA were administered, a large fraction of the tumors displayed a mixed c-Jun phenotype. While half the lesions seen with DCA alone were c-Jun+, the mixtures gave rise to tumors that were c-Jun+ in one part of the tumor and c-Jun- in other portions. When TCE was administered, 42% of the lesions displayed a c-Jun+ phenotype, only 34% exhibited a c-Jun- phenotype, and 24% were of the mixed phenotype.

Table 1. Nodules and Tumors Induced by TCA, DCA and TCE that Express a c-Jun+ Phenotype

Treatment	c-Jun+	c-Jun-	Mixed	Total Tumors
TCA 0.5 g/L	0 (0)	14 (1.0)[a]	0 (0)	14
2.0 g/L	0 (0)	12 (1.0)	0 (0)	12
DCA 0.1 g/L	1 (0.5)	1 (0.5)	0 (0)	2
0.5 g/L	3 (0.43)	4 (0.57)	0 (0)	7
2 g/L	14 (0.45)	17 (0.55)	0 (0)	31
TCA 0.5 +				
DCA 0.1 g/L	0 (0)	12 (0.86)	2 (0.14)	14
DCA 0.5 g/L	0 (0)	13 (0.81)	3 (0.19)	16
TCA 2 g/L +				
DCA 0.1 g/L	1 (0.04)	25 (0.92)	1 (0.04)	27
DCA 0.5 g/L	1 (0.04)	12 (0.44)	14 (0.52)	27
TCE 1.0 g/kg bw	16 (0.42)	13 (0.34)	9 (0.24)	38

[a] Frequency ( )

Mutation frequencies and spectra from tumors from animals treated with TCE, DCA, and TCA are shown in Figure 2. No mutations were detected in DNA from normal tissue of B6C3F1 mice. Few tumors were observed in control mice sampled at the times examined in this study (9 control tumors were sequenced; of these, only 2 contained a codon 61 mutation). Therefore, for statistical purposes, mutation data from this study were compared to historical control data (Maronpot et al. 1995). However, we have chosen to depict mutation spectra in a way that includes the wild-type sequence (CAA) as it provides a simultaneous view of the mutation frequency.

The H-*ras* codon 61 mutation frequency in tumors of mice differed significantly ($P<0.05$) between TCE and TCA (Fig. 2). Only 23% of the tumors from TCE-treated animals had a mutation in codon 61, whereas 53% of the tumors from TCA-treated animals had mutations. This difference in mutation frequency was statistically different ($p<0.05$) by Fisher's exact test.

Tumors from DCA-treated mice were intermediate in their frequency of H-ras mutations, but this response was not significantly different from control. In all cases, these frequencies are below the frequency of codon 61 mutations in spontaneous tumors in this strain of mice (56%) (Maronpot et al. 1995). The mutation frequency reported by Ferreira-Gonzalez et al. (1995) was 55% in 11 hepatocellular carcinomas of male B6C3F1 mice treated with 4.5 g/L TCA for 104 weeks. Our study used lower doses and was of a shorter duration than the Fereira-Gonzalez study. The mutation spectrum of the TCA-treated animals from the current study is not significantly different from that of the historical controls with respect to the AAA sequence at codon 61, the most common mutation.

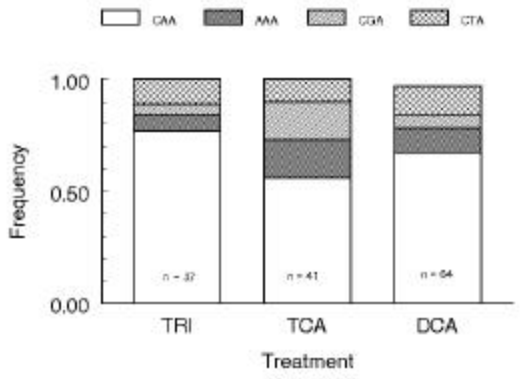

Figure 2. H-ras Codon 61 Mutation Frequency In Tumors Induced by Trichloroethylene (TRI), Trichloroacetate (TCA) or Dichloroacetate (DCA).

When the H-*ras* mutation frequencies for all studies of DCA-induced tumors was analyzed according to the length of treatment, an interesting pattern emerged (Figure 3). With the exception of the very highest dose of DCA (5 g/L), the mutation frequencies in DCA and spontaneous tumors increase gradually over time. Additional evidence that H-*ras* mutations may be a late event in TCE and/or DCA-induced hepatocarcinogenesis is provided by a limited subset of tumors that were both sequenced and classified histologically. Only 8 of 34 (24%) of the adenomas contained codon 61 mutations while 9 of 15 (60%) carcinomas contained mutant H-*ras* at this codon. The percentage of mutant sequence within each tumor (as judged by the ratio of mutant to wild-type peak heights) was lower than would be expected if the tumors were the result of clonal expansion of cells bearing mutant *ras*. Most tumors contained less than 50% mutant sequence, and only one was completely without wild-type sequence. The average (± SE) ranged from 38 ± 5% in the 0.5 g/L DCA treatment group to 49 ± 13% in the 2 g/L DCA for 87 weeks group. There were no

significant differences in this measure classified by treatment duration or dose.

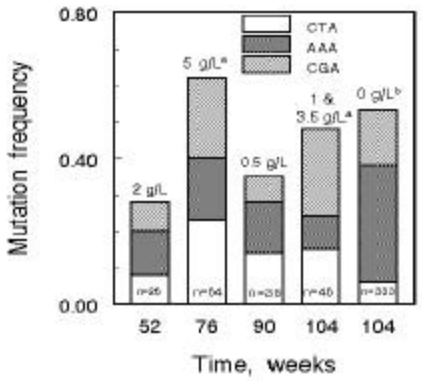

Figure 3. Relationship between DCA Treatment Duration and Dose with H-ras Mutation Frequency.

These results strongly suggest that TCA is not solely responsible for liver tumors when mice are treated with TCE. The liver-tumor response has been exclusively attributed to TCA in the past (Elcombe 1985). However, the c-Jun positive phenotype is not produced when TCA is administered alone to B6C3F1 mice (Stauber and Bull 1997), nor by other peroxisome proliferators. Furthermore, the metabolites display the same proclivity for producing these same phenotypes in stimulating the growth of colonies from mouse hepatocyte suspensions on soft agar (Stauber et al. 1998). In the TCE-treated mice, 2/3 of the tumors recovered expressed the c-Jun positive phenotype exclusively or in part. A caution must be expressed that this does not implicate c-Jun in the development of the tumor because the antibody used in this study recognizes a form of the transcription factor that is largely located in the cytosol that surrounds the nucleus rather than in the nucleus (Kato-Weinstein et al. 2000). Other antibodies give a different pattern to the staining because they interact differently to phosphorylated and non-phosphorylated forms of the antibody at different epitopes.

The H-*ras* codon 61 mutation frequencies and spectra was the second test of the hypothesis that TCA was a primary, if not exclusive, contributor to TCE-induced liver tumors in mice. This

was based on the simple hypothesis that there should be congruence between the mutation frequency and spectra if TCA were solely responsible for the tumors. Clearly, this was not the case as the H-*ras* codon mutation frequency was significantly lower in TCE-induced tumors than TCA-induced tumors. The mutation frequency seen in DCA-induced tumors was not significantly different from that of TCE, although examination of the data also makes it difficult to attribute the tumors exclusively to DCA. Therefore, these data support the hypothesis that neither metabolite is the exclusive cause of liver tumors in TCE-treated mice.

In summary, the occurrence of two distinct c-Jun phenotypes of liver tumors in mice treated with TCE is inconsistent with TCA being solely responsible for these tumors. It is probable that DCA contributes to the development of liver tumors in mice treated with TCE, but mixtures of DCA and TCA tend to produce tumors with a mixed phenotype, whereas TCE produced hepatic tumors that appeared uniformly Jun+. Therefore, the possibility of a third mechanism cannot be ruled out. H-*ras* codon 61 mutation frequencies and spectra support these conclusions. However, *ras*-dependent signaling pathways are activated in all hepatic tumors examined, irrespective of H-*ras* mutations.

3.0 Relevance, Impact, and Technology Transfer

How does this new scientific knowledge focus on critical DOE environmental management problems? Cleanup costs for chlorinated solvents found on DOE sites are most frequently driven by TCE because it is the most widespread contaminant and is generally present at the highest concentrations. A presentation by James Cogliano of EPA at the 1999 Society of Toxicology meeting indicates that EPA has accepted the concept of nonlinear extrapolation for liver-tumor

induction by TCE. Results from this project contributed to the body of scientific data that led to these decisions. Thus, this project ended with its major technical objectives satisfied.

To what extent does the new scientific knowledge bridge the gap between broad fundamental research that has wide-ranging applications and the timeliness to meet needs-driven applied technology development? While this project had little focus on development of new technologies, a novel use of magnetic resonance imaging was developed that allowed for imaging liver tumor growth in mice (Miller et al. 2000). This technique was shown to be useful for analyzing tumor growth as well as tumor regression in individual tumors without the need for large-scale serial sacrifice studies. The technique used in this project should have additional applications in a variety of cancer research areas, including applications for monitoring the efficiency of chemotherapy in mouse models. Results obtained from this project also provided new knowledge at the fundamental-science level, which may be applicable to a better understanding of the risks associated with other chlorinated solvents. Since DCA and TCA are common metabolites of a number of chlorinated solvents, the fundamental knowledge gained by this research has a wider range of application than just for TCE.

What is the project's impact on individuals, laboratories, departments and institutions? What new capacity, equipment or expertise has been developed? How has this research advanced our understanding in this area? In addition to contributing to postdoctoral and graduate student education, the fundamental science gained from this project has contributed to new scientific directions for the investigators involved. In particular, the cell-signaling pathways identified in this project have led to new concepts in liver cell signaling that are now being pursued at the

molecular level on other projects. One manuscript generated from this project recently received national recognition as the best paper published in the journal *Toxicology and Applied Pharmacology* (awarded by the Society of Toxicology, March, 2001). It is anticipated that these new capabilities and research directions will ultimately lead to a better basic understanding of liver-cell function, which is more broadly applicable to a number of disease states.

4.0 Project Productivity

While the specific methods and approaches used were modified as results warranted during progression of this project, the scientific intent of the original aims of this project were met within the proposed budget and schedule.

5.0 Personnel Supported

The following investigators contributed to this research through partial support by this project:

Investigator		**Role on Project**
Richard J. Bull	PNNL	Principal Investigator
Brian D. Thrall	PNNL	Co-Investigator
Lyle B. Sasser	PNNL	Collaborator
Irvin R. Schultz	PNNL	Collaborator
John H. Miller	PNNL	Collaborator
Gayle Orner	PNNL	Postdoctoral Fellow
Barbara J. Mounho	PNNL	Postdoctoral Fellow

Graduate Student Research Contributions:

Partial funding by this project contributed to the education and training of graduate students:

Stauber, AJ. Mechanisms of hepatic tumor induction by dichloroacetate and trichloroacetate differ. Effects on cell division, cell survival, and clonal expansion in the liver of male B6C3F1 mice. Ph.D. Dissertation, Washington State University (Ph.D. granted 1998).

Kato-Weinstein, J. Factors involved in the hepatic effects of brominated and chlorinated haloacetates in B6C3F1 mice. Ph.D. Dissertation, Washington State University (Ph.D. granted 1999).

Merdink, J. Formation of Dichloroacetate from Trichloroacetate. Implications to the Risk Assessment of Trichloroethylene. Ph.D. Dissertation, Washington State University (Ph.D. granted 2000).

Gonzalez, A. Modification of Haloacetate Metabolism in Rodents by Pretreatment in Drinking Water. Ph.D. Dissertation, Washington State University (Ph.D. granted 1999).

Lingohr, MK. Dichloroacetate modulates glycogen metabolism and insulin signaling proteins *in vivo* and *in vitro*. Ph.D. Dissertation, Washington State University (Ph.D. granted 5/00).

6.0 Publications

Peer-Reviewed Publications Resulting From the 3-year Research Period:

Manuscripts Published or In Press*:*

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.

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

Merdink, J.L., Gonzalez-Leon, A., Bull, R.J. and Schultz, I.R. 1998. "The extent of dichloroacetate formation from trichloroethylene, chloral hydrate, trichloroacetate, and trichloroethanol in B6C3F1 mice." *Toxicological Sciences* **45**:33–41.

Mounho, B.J. and Thrall, B.D. 1999. "Peroxisome proliferator-induced activation of extracellular signal-regulated kinase (Erk) pathway contributes to hepatocellular clonal expansion." *Toxicol. Appl. Pharmacol.* **159**:125–133.[(a)]

Barton, H.A., Bull, R., Schultz, I., Andersen, M.E. 1999. "Dichloroacetate (DCA) dosimetry: interpreting DCA-induced liver cancer dose response and the potential for DCA to contribute to trichloroethylene-induced liver cancer." *Toxicology Lett.* **106**:9–21.

Gonzalez-Leon, A., Merdink, J.L., Schultz, I.R., Bull, R.J. 1999. "Effect of pretreatment with dichloroacetate or trichloroacetate in drinking water on the pharmacokinetics of a subsequent challenge dose in B6C3F1 mice." *Chem.-Biol. Interactions* **123**:239–253.

Bull, R.J. 2000. "Mode of action of liver tumor induction by trichloroethylene and its metabolites, trichloroacetate and dichloroacetate." *Environ. Health Perspect.* **108** (suppl. 2).

Kato-Weinstein, J., Stauber, A.J., Orner, G.A., Thrall, B.D. and Bull, R.J. 2000. "Differential effects of dihalogenated and trihalogenated acetates in the liver of B6C3F1 mice." *J. Appl. Toxicol*. **20**, (in press).

Merdink, J.L., Bull, R.J. and Schultz, I.R. 2000. "Trapping and identification of the dichloroacetate radical from the reductive dehalogenation of trichloroacetate by mouse and rat liver microsomes." *Free Radical Biol. Med*. **29**:125–130.

Merdink, J.L., Bull, R.J. and Schultz, I.R. 2001. "Toxicokinetics of bromodichloroacetate in B6C3F1 mice." *J. Appl. Toxicol*. **21**:53–57.

Miller, J. H., Minard, K. M., Wind, R. A., Orner, G. A., & Bull, R. J. 2000. "*In vivo* MRI

(a) This publication received the Society of Toxicology Board of Publications Award for "Best Paper" in *Toxicology and Applied Pharmacology*.

measurements of tumor growth induced by dichloroacetate: Implications for mode of action." *Toxicology*, ***145***, 115–125.

Manuscripts Submitted for Publication:

Lingohr, MA, Bull, RJ and Thrall, BD. "Dichloroacetate treatment stimulates glycogen accumulation in isolated mouse hepatocytes independent of insulin through a pathway involving phosphatidylinositol 3-kinase." (Submitted to *Toxicological Sciences*).

Bull, RJ, Orner, GA, Malone, JA, Cheng, RS, Stauber, AJ, Sasser, LB, Smith, MK and Thrall, BD. "The contribution of dichloroacetate and trichloroacetate to liver tumor induction in mice by trichloroethylene." (Submitted to Toxicological Sciences).

7.0 Interactions

Participation/Presentations at Meetings, Workshops, Conferences, and Seminars.

In addition to the peer-reviewed manuscripts published or submitted, the following abstracts were presented at regional and national scientific meetings:

1. Smith, MK, RJ Bull and BD Thrall. 1996. "Effects of dichloroacetate (DCA) on intracellular proteins involved in the transmission of signals to the nucleus." *PANWAT Proc*. 13:8.
2. Orner, GA, MK Smith, RJ Bull and BD Thrall. 1996. "Effects of trichloroacetate and clofibric acid on DNA binding activity towards the SP1 concensus sequence." *PANWAT Proc*. 13:22.
3. Kato, J, BD Thrall and RJ Bull. 1996. "Expression of hepatic GST-II and c-Jun immunoreactive protein in DCA-induced tumor and nontumor tissues." *PANWAT Proc*. 13:25.
4. Orner, GA, MK Smith, RJ Bull and BD Thrall. 1997. "Effects of trichloroacetic acid (TCA) and clofibric acid (CFA) on DNA binding activity towards the Sp1 consensus sequence." T*oxicologist* 354:1797.
5. Kato-Weinstein, J, BD Thrall and RJ Bull. 1997. "Detection of c-Jun immunoreactive protein in mouse liver tumor." *Toxicologist* 222:1128.
6. Smith, MK, BD Thrall and RJ Bull. 1997. "Dichloroacetate (DCA) modulates insulin signaling." *Toxicologist* 223:1133.
7. Stauber, AJ, RJ Bull and BD Thrall. 1997. "Dichloroacetate and trichloroacetate promote clonal expansion of initiated phenotypes in mouse hepatocytes in vitro." Conference on *Mechanisms of Susceptibility to Mouse Liver Carcinogenesis*, September 8–10, Chapel Hill, North Carolina.
8. Kato-Weinstein, J, BD Thrall and RJ Bull. 1997. "Alterations in carbohydrate metabolism with haloacetate treatment." *PANWAT*, 14:15.
9. Orner, GA, LC Stillwell, RS Cheng, LB Sasser, RJ Bull and BD Thrall. 1997. "Comparison of H-ras mutation spectra in tumors of trichloroacetate and dichloroacetate-treated B6C3F1 mice." *PANWAT*, 14:29.
10. Lingohr, MK, BD Thrall and RJ Bull. 1997. "Dichloroacetate (DCA) modulates the insulin signaling pathway in mouse liver cells." *PANWAT* 14:34.

11. Stauber, AJ, RJ Bull and BD Thrall. 1998. "Dichloroacetate and trichloroacetate promote clonal expansion of anchorage-independent hepatocytes." *Toxicologist* 42:62.
12. Stauber, AJ, LB Sasser, RJ Bull, GA Orner and BD Thrall. 1998. *Anchorage-independent colony formation in vitro can detect both in vivo tumor initiation and promotion.* American Association for Cancer Research, New Orleans, Louisiana.
13. Mounho, BJ and BD Thrall. 1998. "Tumor promotion by peroxisome proliferators may involve the activation of mitogen-activated protein kinases (ERK1/ERK2)." *Toxicologist* 42:51.
14. Orner, GA, Stillwell, LC, Cheng, RS, Sasser, LB, Bull, RJ and BD Thrall. 1998. "Effects of trichloroacetate (TCA) and dichloroacetate (DCA) on H-ras in male B6C3F1 mice." *Toxicologist* 42:60.
15. Lingohr, MK, Thrall, BD and RJ Bull. 1998. "Dichloroacetate affects proteins involved in insulin signaling in mouse liver cells." *Toxicologist* 42:61.
16. Kato-Weinstein, J, Thrall, BD and RJ Bull. 1998. "The effect of haloacetates on carbohydrate metabolism in male B6C3F1 mice." *Toxicologist* 42:908.
17. Mounho, BJ and Thrall, BD. 1999. "Activation of the ERK pathway by peroxisome proliferators." *Toxicologist*, 48:645.
18. Thrall, BD, Mounho, BJ, Bull, RJ, and Lingohr, MK. 2000. "Evidence for divergent signaling pathways in regulation of receptor mediated effects of peroxisome proliferators." *Toxicologist* 54: 1512.

8.0 Transitions (not applicable)

9.0 Patents (none)

10.0 Future Work

Based on the results of this study, future experiments were designed and proposed to address the role that a specific metabolic enzyme (glutathione S-transferase zeta) has in metabolism and elimination of DCA in human samples. Because this enzyme is polymorphic in human populations and appears to be reversibly inactivated by DCA, its activity in humans and its role in the adverse effects of DCA is important to evaluate. Since funding for this project was not renewed, follow-on studies are not anticipated.

11.0 Literature Cited

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