INHIBITION AND RECOVERY OF RAT HEPATIC GLUTATHIONE S-TRANSFERASE ZETA AND ALTERATION OF TYROSINE METABOLISM FOLLOWING DICHLOROACETATE EXPOSURE AND WITHDRAWAL

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Support
Running title: Inhibition and recovery of hepatic GSTz by DCA.

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Number of text pages, 22
Number of tables, 2
Number of figures, 4
Number of references, 40
Number of words in abstract, 249
Number of words in Introduction, 713
Number of words in Discussion, 655

Abbreviations:

GSTz1-1, glutathione S-transferase zeta; DCA, dichloroacetate; MA, maleylacetone.
Abstract

Dichloroacetate (DCA) is an investigational drug for certain metabolic disorders, a by-product of water chlorination and a metabolite of certain industrial solvents and drugs. DCA is biotransformed to glyoxylate by glutathione S-transferase zeta (GSTz1-1), which is identical to maleylacetaceta te isomerase (MAAI), an enzyme of tyrosine catabolism. Clinically relevant doses of DCA (mg/kg/d) decrease the activity and expression of GSTz1-1, which alters tyrosine metabolism and may cause hepatic and neurological toxicity. The effect of environmental DCA doses (µg/kg/d) on tyrosine metabolism and GSTz1-1 is unknown, as is the time course of recovery from perturbation following sub-chronic DCA administration. Male Sprague-Dawley rats (200 g) were exposed to 0 µg, 2.5 µg, 250 µg or 50 mg DCA/kg/d in drinking water for up to 12 weeks. Recovery was followed after the 8 week exposure. GSTz specific activity and protein expression (Western immunoblotting) were decreased in a dose-dependent manner by 12 weeks exposure. Enzyme activity and expression decreased 95% after one week administration of high dose DCA. Eight weeks after cessation of high dose DCA, GSTz activity had returned to control levels. At the 2.5 or 250 µg/kg/d doses, enzyme activity also decreased after 8 weeks exposure and returned to control levels one week after DCA was withdrawn. Urinary excretion of the tyrosine catabolite maleylacetone increased from undetectable amounts in control rats to 60-75 µg/kg/24h in animals exposed to 50 mg/kg/d DCA. The liver:body weight ratio increased in the high dose group after 8 weeks of DCA. These studies demonstrate that short term administration of DCA inhibits rat liver GSTz across the wide concentration range to which humans are exposed.
Introduction

Dichloroacetate (DCA) is an unusual xenobiotic that has impacted both clinical therapeutics and environmental toxicology. It has been used as an investigational drug for treating several acquired and congenital metabolic and cardiovascular diseases at acute or chronic doses that typically range between 25 mg/kg/d and 100 mg/kg/d. These concentrations are thousands of times greater than those to which most humans are usually exposed (Stacpoole et al., 1998).

Many haloacetates, including DCA and its mono- and tri-chloro analogs, are distributed ubiquitously in the biosphere, including lakes, groundwater, drinking water, glacial ice, fog, rain, precipitation, air and soil (reviewed in Stacpoole et al, 1998 and Ammini and Stacpoole, 2003). DCA is one of the most prevalent haloacetates found in samples from these sources and has been implicated as a toxin to plants and trees at atmospheric exposure levels (Hoekstra et al., 1999; Rompp et al., 2001). DCA is also a by-product of drinking water disinfection (Krasner et al., 1989; Mughal, 1992) and a metabolite of two widely used chlorinated industrial solvents, trichloroethylene and tetrachloroethylene (Coleman et al., 1976; Elcombe 1985; Odum et al., 1988; Westrick et al., 1984) and of certain drugs (Stacpoole et al., 1998). Humans may be exposed to DCA by chlorination of municipal drinking water or by groundwater contamination at certain Superfund sites. The World Health Organization has set a target safety level for DCA in the drinking water of 50 µg/L, while the United States Environmental Protection Agency limit is 60 µg/L for the total of five halogenated acetic acids in drinking water (WHO 1996). Consequently, drinking water is considered the major route of exposure to this compound by humans and may approximate 2-4 µg/kg/d (Stacpoole et al., 1998).
Toxicological studies of DCA in animals have usually employed chronic exposure levels in the mg/kg/d dose range that greatly exceed levels typical of human environmental exposure. These studies have identified liver, kidney, nervous system, testes and eye as susceptible target tissues in rodents and dogs (reviewed in Stacpoole et al., 1998 and Ammini et al., 2003). DCA is carcinogenic in mice (Anna et al., 1994; Bull et al. 1990; DeAngelo et al., 1991; Herren-Freund, 1987; Pereira 1996) and rats (DeAngelo, 1996; Richmond et al, 1995). Clinical case reports have also indicated that DCA is a reversible peripheral neurotoxin and hepatotoxin (DeAngelo et al., 1991 and 1996; Mather et al., 1990; Moore et al., 1979; Spruijt et al., 2001; Stacpoole et al., 1998).

Glutathione S-transferase zeta (GSTz1-1) catalyzes the glutathione-dependent conversion of DCA to glyoxylate (James et al., 1997; Tong et al., 1998; Anderson et al., 1999; Tzeng et al., 2000) (Fig. 1). GSTz1-1 is identical to maleylacetoacetate isomerase (MAAI) (Blackburn et al., 1998), which catalyzes the penultimate reaction in the catabolism of tyrosine and phenylalanine (Fig. 1). Liver expresses the highest amount of total GSTz1-1 protein per milligram cytosolic protein, followed by brain and lung. DCA is a mechanism-based inhibitor of GSTz1-1 (Anderson et al., 1999 and 2002; Tzeng et al., 2000) at clinically relevant doses and increases the urinary excretion of maleylacetone (MA), an endogenous substrate for the isomerase (Cornett et al., 1999; Ammini et al., 2003).

Human GSTz1-1 is reported to undergo covalent modification of the active site cysteine-16 in vitro by DCA (Anderson et al., 1999; 2002). Inactivation of GSTz1-1 is considered to be irreversible, since human GSTz1a-1a inactivated in vitro by DCA could not be restored by dialysis of the inactivated protein against 0.1 M potassium phosphate.
buffer (Tzeng et al., 2000). In vivo studies also suggest that inactivation of GSTz1-1 by DCA is irreversible, since immunoreactive GSTz expression decreased in livers of dosed Fischer 344 rats and Sprague-Dawley rats (Anderson et al., 1999; Ammini et al., 2003). Recovery of protein expression apparently requires protein synthesis (Schultz et al., 2002) and thus should lead to a restoration of enzyme expression and activity. Anderson et al. (1999) reported recovery of GSTz expression 8-12 days after a single administration of 45 mg/kg/d DCA. Recovery after withdrawal of sub-chronic exposure to DCA at any exposure level has not been investigated.

Here we report results obtained in rats administered DCA at exposure levels that span the environmental to therapeutic concentration range. Recovery was examined following 8 weeks exposure to the compound. We determined the effect of DCA on drinking water consumption, body weight, liver weight and the time course of inactivation and recovery of hepatic GSTz activity and expression. We also measured the urinary excretion of DCA and MA.
Materials and Methods

Chemicals and antibodies. Dichloroacetic acid-1-\(^{14}\)C (specific activity 52 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO), and converted to its sodium salt by addition of NaHCO\(_3\). Unlabeled clinical grade sodium DCA was purchased from TCI America (Portland, OR). Polyclonal chicken anti-mouse GST\(_z\)-1-1 antibodies were kindly supplied by Dr. Markus Grompe (Oregon Health Sciences University). ECL Western Blotting Detection Reagents was purchased from Amersham Biosciences Corporation (Piscataway, NJ). In this study, mineral water (Zephyrhills brand) purchased from a local grocer was used as drinking water to avoid contamination by DCA present in municipal water. The maleylacetone was synthesized as described previously (Cornett et al., 1999). BF\(_3\)–MeOH was from Aldrich Chemical Company Inc. (reagent grade; Milwaukee, WI) and methylene chloride was from Fisher Scientific (pesticide grade; Pittsburgh, PA). All other chemicals used in this study were of high purity and were bought from commercial suppliers.

Animals and administration. Male Sprague-Dawley rats (200 g, 2 months old) were purchased from Harlan (Indianapolis, IN). Animals were housed individually under constant conditions of temperature and humidity, and maintained on a 12 h light/dark cycles. They were placed in separate cages with free access to unchlorinated spring water (to minimize uncontrolled exposure to DCA) and food. Animals were randomly assigned to 22 treatment groups (group A to V) of 6-12 rats each (Table 1). During the week before exposure to DCA, the water consumption and body weight of each rat were monitored. DCA was provided in drinking water ad libitum.
The DCA concentrations were adjusted three times per week for each animal, based on its water intake and body weight, to reach a dose of 2.5 µg, 250 µg or 50 mg DCA per kg body weight per day. The lowest and highest DCA concentrations were chosen to reflect those expected from typical environmental and clinical exposure levels, respectively (Stacpoole, et al 1998). The intermediate dose of 250 µg was chosen both to reflect potential DCA environmental exposure to heavily contaminated (Superfund) groundwater sites and to aide in elucidating concentration-dependent trends in DCA’s effects on hepatic GSTz.

Rats were divided into 2 series: the rats of Series 1 were administered DCA for up to 8 weeks, and DCA were then withdrawn from drinking water for up to 8 weeks (group A to R, Table 1); the rats of Series 2 received DCA for 12 weeks (group S-V, Table 1). Animals were observed daily and fresh drinking water was prepared at least weekly. Animals were anesthetized with carbon dioxide and were sacrificed by decapitation at the times indicated in Table 1.

**Preparation of liver cytosol.** The liver was removed immediately after sacrifice and cytosolic fractions were prepared by differential centrifugation, as previously reported (James et al., 1997). The cytosol supernatant was stored in 3 ml aliquots at –80°C and its protein concentration was determined (Lowry et al., 1951).

**Enzyme specific activity.** Hepatic GSTz enzyme activity was measured by our published method (James et al., 1997). Liver cytosolic fractions were incubated in a reaction mixture containing an enzyme-saturating concentration of 14C-labeled DCA (0.2 mM) and glutathione (1 mM). The percent conversion of 14C-DCA to 14C-glyoxylate was
determined by HPLC. The specific enzyme activity was defined as nmoles glyoxylate formed/min/mg protein.

**Expression and purification of human GSTz1-1.** Human GSTz1-1 was used as standard for Western immunoblotting in this study. The cDNA of GSTz1-1 was inserted into plasmid pQE30 (QIAGEN) using the BamH1 and Hind III sites. The constructed plasmid containing GSTz1-1 was transferred into *E. coli* host strain M15 (QIAGEN). The M15 cells were incubated in LB medium at 37°C and the protein GSTz1-1 was induced in the present of 1 mM isopropylthiogalactoside (IPTG) for 4 h. The cells were then harvested, lysed in buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0) under native conditions, and centrifuged. The supernatants were pooled and applied onto Ni-NTA resins in a column procedure (QIAGEN) and the column was washed (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0). The protein was eluted (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 100 mM imidazole, pH 8.0) and concentrated using a Centrifugal Filter Devices with nominal molecular weight limits of 10,000 (Millipore). The buffer was exchanged with 10 mM sodium phosphate containing 1 mM DTT and 10% glycerol, pH 8.0 and the protein concentration was determined (Bio-Rad Protein Assay).

**Immunoblotting.** Cytosol samples or human GSTz1-1 were heated in SDS PAGE sample buffer at 95°C for 5 min and 40 µg of total protein from each of the cytosol samples and 0.5 µg protein from the pure human GSTz1-1 were resolved by SDS-PAGE on a polyacrylamide gel (12%, Bio-Rad Laboratories, Hercules, CA). The proteins were then transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). After blocking for 1 h in 5% fat free milk in TBS-T buffer (Bio-Rad Laboratories,
Hercules, CA), the membrane was incubated with chicken anti-mouse GSTz1-1 polyclonal antibody (primary antibody) for 1 h, followed by donkey anti-chicken IgG secondary antibody (Research Diagnostics Inc.) for 2 h. Detection was performed using ECL Western Blotting Detection Reagents (Amersham Biosciences Corp., Piscataway, NJ) and bands were quantitated by image analysis software (ScanAnalysis).

**Determination of urinary DCA and MA.** Urine samples and the internal standard 2-oxohexanoic acid (OHA) were derivatized to their methyl esters by reacting with 12%BF₃–MeOH complex. DCA and MA were extracted with methylene chloride. The concentrations of DCA and MA were measured by GC/MS (Yan et al., 1997; Shroads et al., 2004), using a Hewlett-Packard (Palo Alto, CA, USA) 5890 series II plus Gas Chromatograph and a 5972A series Mass Selective detector.

**Statistical analysis.** Means, standard deviations and statistical significance for the various experiments were carried out using Excel software (Microsoft, Seattle, WA). For comparing mean values of two treatment groups, one-tailed Student’s *t*-test was used to determine significance of data. A *p* value of ≤0.05 was considered to be statistically significant.
Results

Age-dependent changes in hepatic GSTz. In untreated rats, the activity and expression of GSTz changed with age and body weight of the rats (Table 2). Twenty-four week old rats weighed 20% more than 12 week old rats from the same source, purchased at the same time. However, hepatic GSTz activity was higher in the 12-week old rats than any of the older age groups studied (p<0.005). Hepatic GSTz protein expression, analyzed by immunoblotting, followed the same trend, but because of higher experimental variability between replicates in the western blots the difference was not statistically significant. The experimental error in GSTz activity measurement was less than 2%, while the experimental error in measuring GSTz protein expression by immunoblotting was up to 15%, which meant that differences in protein expression less than this could not be determined reliably.

Inactivation and restoration of hepatic GSTz activity and protein expression. Enzyme activity with DCA as substrate was reduced 95% by 1 week of dosing with 50 mg/kg DCA (p<0.0001), and remained low over the 8 week exposure period (Fig. 2). GSTz protein expression, shown in Fig 3, was barely detectable after 1 week’s treatment, falling over 95% (p<0.0001), and remained suppressed over the 8 week exposure. After withdrawal of DCA, GSTz activity returned to 59% of control by 1 week and returned completely to control levels by 8 weeks. After DCA withdrawal, GSTz protein expression increased gradually, although mean expression remained below control animals by 8 weeks.

The lower doses of DCA, 2.5 µg/kg/d or 250 µg/kg/d, had no significant effect on enzyme activity after 4 weeks treatment, but activity had decreased significantly by 8
week’s exposure to DCA (p<0.05). Similar trends were found for GSTz protein expression, but did not attain statistical significance. One week after withdrawal of these doses, GSTz activity had returned to the control levels.

With the particular aim of confirming the reduction in GSTz activity following low dose exposure to DCA, four additional groups (S-V, Table 1) were treated with DCA for 12 weeks. The GSTz activity and protein expression in these groups showed a clear dose-dependent reduction (Fig. 4).

**Drinking water consumption, body weight, and liver weight.** Consumption of drinking water was measured in each rat group that was studied over 1 (A and B) or 16 (O, P, W and R) weeks (Table 1). The highest water consumption (133.5 ml/kg/d) occurred in the 50 mg DCA/kg/d group by 1 week exposure, and was 20% greater (p<0.05) than the amount consumed by control animals (111.1 ml/kg/d). Otherwise, water consumption among the groups was similar.

The body weight of each rat was monitored three times a week. There was no effect of any dose level of DCA on body weight during the 16 week study. DCA did not alter liver weight in animals exposed to the 2.5 µg/kg/d or 250 µg/kg/d dose. The 50 mg/kg/d DCA dose increased the liver-body weight ratio 21% (p<0.005) above control by 8 weeks of exposure, but this ratio returned to control levels 8 weeks after discontinuing DCA.

**Urinary DCA and MA.** Rats excreted DCA in urine at a rate of 4.5 mg/kg/24 h after one week of 50 mg/kg/d dose, and the amount increased to 5.5 mg/kg/24 h after 8 weeks of exposure. Only trace amounts of DCA (less than 10 µg/kg/24 h) were excreted one week after discontinuing DCA administration. No DCA was measured in urine samples
(limit of detection, 1 µg/ml) from animals receiving either the 2.5 µg/kg/d or 250 µg/kg/d doses.

Rats that received 50 mg/kg/d DCA excreted MA at a rate of 60-75 µg/kg/24 h during the 8 week dosing period. MA excretion decreased below detectable limits (0.2 µg/ml) one week after DCA withdrawal. No MA was detected in the urine of rats receiving either the 2.5 µg/kg/d or 250 µg/kg/d doses.

**Discussion**

At the 50 mg/kg/d DCA dose, both GSTz enzyme activity and protein expression decreased significantly (p<0.0001) and remained low during the entire 8 weeks exposure (Figs. 2 and 3). After withdrawal of DCA, GSTz enzyme activity and expression gradually increased. High doses of DCA are known to alter rat hepatic GSTz activity and protein expression (James *et al*., 1997; Anderson *et al*., 1999; Cornett *et al*., 1999; Ammini *et al*., 2003), but the time required for restoration of enzyme activity and expression after sub-chronic treatment has not been determined previously. The results of this study suggest that recovery of protein expression lagged behind enzyme activity in the high dose animals, for reasons that are unclear. Possibly, liver cytosol of control animals contains enzymatically inactive GSTz protein that is still recognized by the antibody, and this is not the case in rats recovering from high dose DCA. It is noteworthy that, in the present study, even the lowest dose of DCA (2.5 µg/kg/d) reduced the hepatic GSTz activity after 8 to 12 weeks exposure. This suggests that DCA levels found in some municipal water sources may be sufficient to alter the hepatic GSTz activity and, hence, amino acid metabolism in humans.
The hepatic specific activity of GSTz in control animals decreased with age, falling 20% between 12 and 24 weeks of age during the period of study (p<0.05; Table 2). This is consistent with our previous observation that the plasma elimination half-life of 50 mg/kg/d DCA was 5.4 hr in young adult rats (3-4 months; 180-265 g) vs. 9.7 hr in old rats (16 months; 580-690 g) (James et al., 1998). Similarly, Schultz et al (2002) showed that 60 week old mice had lower GSTz expression and reduced capacity to metabolize DCA than 8 week old animals. Together, these data indicate that hepatic GSTz levels decline with age in rodents, and reinforced the need for age and weight-matched controls in studies of the biotransformation and pharmacological effects of DCA.

Few studies have examined the effect of DCA on water consumption in animals. However, DeAngelo et al. (1989) and Mather et al. (1990) demonstrated that DCA caused a dose-dependent decrease in water consumption in exposed rats. We found that high dose DCA actually resulted in an initially higher water consumption than that measured in control animals.

Mather et al. (1990) reported that high dose (500 and 5000 ppm) DCA for 90 days in drinking water blunted weight gain in rats, and similar results were obtained by Pereira et al. (1997) in mice. The mechanism was considered to be due to the consequences of liver carcinogenesis. In contrast, we found no effect of DCA on body weight at any of the exposure levels used in this study. DCA may cause hepatic hypertrophy or hyperplasia in rats (Anderson et al., 1975; Mariash and Schwartz, 1986; Mather et al., 1990; Smith et al., 1992). Although high dose DCA (50 mg/kg/d) also increased liver weight and the liver to body weight ratio in this study, these indices returned to baseline levels after DCA was withdrawn.
Events leading to the loss of GSTz1 from the liver following administration of DCA are incompletely understood. There is evidence from in vitro studies that in the presence of GSH, DCA itself can bind covalently to GSTz1, and it has been postulated that this leads to destruction of the enzyme (Anderson et al. 2002). Other candidates for forming adducts to GSTz1, which may lead to its destruction, are MA and maleylacetoacetate. These are the physiologically important substrates for GSTz1 and both are electrophilic molecules. MA inhibits hepatic GSTz in vitro in a dose-dependent manner (Cornett et al., 1999) that is only partly reversible following removal of MA by dialysis (Lantum et al., 2003; Ammini et al., 2003). The present study was not designed to resolve the mechanism by which GSTz1 is lost from the liver following DCA administration, but rather to explore the lowest exposure levels associated with this loss. However, MA is a reactive molecule that can form adducts with many cellular nucleophiles, and may therefore cause generalized tissue damage. Consequently it is noteworthy that urinary MA levels remained elevated above control values in rats that received 50 mg/kg/d DCA for the duration of administration. This suggests internal exposure occurs to high concentrations of this electrophile, and probably also its precursor, maleylacetoacetate. MA levels dropped to below detection limits by one week after cessation of DCA exposure. This suggested that, although GSTz1 protein levels remained depressed at one week after DCA was withdrawn, there was sufficient enzyme to metabolize the levels of maleylacetoacetate and MA normally produced from tyrosine catabolism. It should be noted, however, that both MA and maleylacetoacetate are unstable molecules and the limit of chemical detection by our present analytical method may not correspond to the limit of toxicity from these substances.
In conclusion, the results of this investigation may have important implications about the effects of chronic DCA exposure on human health. Daily, oral administration of DCA at a concentration typical of that present in municipal drinking water supplies (reviewed in Stacpoole, et al., 1998) significantly decreased the activity and expression of hepatic GSTz. Thus, exposure of humans and other species to so called “environmental” levels of this compound may perturb certain fundamental pathways of intermediary metabolism in ways that may lead to cumulative toxicity.
Acknowledgements

Preliminary accounts were presented at meetings of the International Society for the Study of Xenobiotics (Dijon, France, 2003; Providence, RI, 2003). We thank Dr. M. Grompe (Oregon Health Sciences University) for providing us chicken anti-mouse GSTz1-1 antibodies.
References


Footnotes

This work was supported in part by NIH Grants ES07375 and ES07355.
Legends for Figures

Fig. 1. Phenylalanine and tyrosine catabolism produces the alkylating agents maleylacetoacetate and fumarylacetoacetate, and their decarboxylation products, maleylacetone and fumarylacetone. Maleylacetoacetate, maleylacetone and DCA are substrates for maleylacetoacetate isomerase (GSTz1-1).

Fig. 2. Inactivation of hepatic GSTz enzyme specific activity by DCA and its recovery after withdrawal of DCA. Rats were given 0 µg, 2.5 µg ( ), 250 µg ( ) or 50 mg ( ) DCA/kg/day in drinking water for up to 8 weeks, followed by no DCA for up to 8 weeks. At each time point, one group of rats served as controls (no DCA exposure). Data are expressed as mean percent of control ± SD (n = 6 for 1, 4 or 16 week administration and n = 12 for 8 or 9 week administration) from the matched control group. An asterisk indicates a significant change relative to the matched controls (p<0.05).

Fig. 3. Inhibition of hepatic GSTz protein expression by DCA and its recovery after withdrawal of DCA. Rats were given 0 µg, 2.5 µg ( ), 250 µg ( ) or 50 mg ( ) DCA/kg/day in drinking water for up to 8 weeks, followed by no DCA for up to 8 weeks. At each time point, one group of rats served as controls (no DCA exposure). Data are shown as mean percent of control ± SD (n = 6 for 1, 4 or 16 week administration and n = 12 for 8 or 9 week administration) from matched control group. An asterisk indicates a significant change relative to the matched controls (p<0.05). Also shown is a representative Western immunoblot for hepatic GSTz. Lanes 1, 8 and 12 contain 0.5 µg recombinant hGSTz (mol wt. 26,000). Lanes 2-7 contain 40 µg cytosolic protein from 8
week controls (group G) and lanes 9-11 contain 40 µg cytosolic protein from rats treated for 8 weeks with 2.5 µg/kg (group H).

Fig. 4. Hepatic GSTz enzyme specific activity (□) and protein expression (■) are inhibited by DCA in a dose-dependent manner. Rats were administered 0 µg, 2.5 µg, 250 µg or 50 mg DCA/kg/d for 12 weeks. Data are shown as mean percent of control ± SD (n = 6) from matched control group. An asterisk indicates a significant change relative to the matched controls (p<0.05). Also shown is a representative Western immunoblot to GSTz of liver cytosol from rats treated for 12 weeks of DCA at the indicated doses. Each lane contains 40 µg cytosolic protein.
TABLE 1. Experiment design.

<table>
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<tr>
<th>Age (week)</th>
<th>9</th>
<th>12</th>
<th>16</th>
<th>17</th>
<th>24</th>
<th>20</th>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Administration</strong></td>
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<td>Without DCA</td>
<td>With DCA</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Week number</strong></td>
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<td>4</td>
<td>8</td>
<td>9</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
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<td>A</td>
<td>C</td>
<td>G</td>
<td>K</td>
<td>O</td>
<td>S</td>
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<td>2.5 µg/kg/d DCA</td>
<td>D</td>
<td>H</td>
<td>L</td>
<td>P</td>
<td>T</td>
<td></td>
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<tr>
<td>250 µg/kg/d DCA</td>
<td>E</td>
<td>I</td>
<td>M</td>
<td>Q</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>50 mg/kg/d DCA</td>
<td>B</td>
<td>F</td>
<td>J</td>
<td>N</td>
<td>R</td>
<td>V</td>
</tr>
</tbody>
</table>

Male Sprague-Dawley rats (200 g, 7-week old) were randomly assigned to one of the 22 administration groups (A to V, n = 6/group). After receiving bottled water for one week, they (8-week old) were given low (2.5 µg and 250 µg/kg/day) dose DCA or high (50 mg/kg/day) dose DCA in bottled water (free from chlorination by-products including DCA). Rats of Series 1 were administered DCA for up to 8 weeks (1 week for group A and B, 4 weeks for group C-F; 8 weeks for group G-R) and after that they were given drinking water with no DCA for up to 8 weeks (1 week for group K-N and 8 weeks for group O-R) for recovery studies. Rats of Series 2 received DCA for 12 weeks (group S-V). Animals were sacrificed on the last day of the indicated week time point.
TABLE 2. GSTz enzyme activity and protein expression in control rats.

<table>
<thead>
<tr>
<th>Age (week)</th>
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<th>16</th>
<th>17</th>
<th>24</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>363±20</td>
<td>407±19</td>
<td>414±26</td>
<td>434±29</td>
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<tr>
<td>Enzyme specific activity (nmol/min/mg)</td>
<td>2.11±0.27</td>
<td>1.68±0.10a</td>
<td>1.63±0.07a</td>
<td>1.62±0.23a</td>
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<tr>
<td>Protein expression (relative amount)</td>
<td>100±25</td>
<td>99±19</td>
<td>91±20</td>
<td>81±17</td>
</tr>
</tbody>
</table>

Seven week old rats (~200 g) were given bottled water for up to 17 weeks and were sacrificed on the last day of the indicated week time (age) point. Data are expressed as mean ± SD (n = 6/group).

a  p<0.005 relative to 12 week old rat groups.
Figure 1

4-Hydroxy-phenylpyruvate \rightarrow Tyrosine

\[ \text{4-hydroxyphenylpyruvate dioxygenase} \]

Homogentisate \rightarrow 1,2-homogentisate dioxygenase

\[ \text{1,2-homogentisate dioxygenase} \]

Maleylacetone \rightarrow dehydrogenase

\[ \text{decarboxylase} \]

Fumarylacetone \rightarrow maleylacetocetate isomerase (MAAI)

\[ \text{MAAI also known as GSTz} \]

4-Maleylacetocetate (cis) \rightarrow 4-phenylalanine monooxygenase

\[ \text{4-phenylalanine monooxygenase} \]

Dichloroacetate \rightarrow

4-Phenylalanine

4-4-hydroxyphenylpyruvate

4-hydroxyphenylpyruvate

4-hydroxyphenylpyruvate

4-hydroxyphenylpyruvate

4-hydroxyphenylpyruvate
Figure 2

The figure shows a bar graph comparing GSTz specific activity (% of matched controls) across different weeks with and without DCA administration. The x-axis represents weeks (Week 1, Week 4, Week 8, Week 9, Week 16) and the y-axis represents GSTz specific activity. The graph includes bars for DCA administration and No DCA conditions, with asterisks indicating statistical significance. Week 16 shows a significant decrease in GSTz specific activity when DCA is administered compared to the no DCA condition.
Figure 3

The graph shows the relative amount of protein expression (% of matched controls) over different weeks. There are two conditions: DCA administration and No DCA. The graph includes error bars to indicate variability.

- Week 1: The expression is significantly lower than the matched controls (indicated by asterisks).
- Week 4: There is a moderate increase in expression compared to the matched controls.
- Week 8: The expression is close to the matched controls.
- Week 9: There is a slight decrease in expression compared to the matched controls.
- Week 16: The expression is significantly lower than the matched controls (indicated by asterisks).

The asterisks (*) indicate statistically significant differences from the matched controls.
Figure 4

![Graph showing GSTz specific activity and expression (% of matched controls) for different DCA doses (2.5 µg/kg/d, 250 µg/kg/d, 50 mg/kg/d). The graph indicates significant differences (*) between the control (0) and treated groups.](image)

![Western blot image showing protein expression levels for different DCA doses.](image)