

## **Supplemental Material.**

The Dichloroacetate Dilemma: Environmental Hazard vs. Therapeutic Goldmine – Both or Neither?

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**Table of Contents.**

Supplemental Text A.....page 3

Supplemental Text B.....page 5

Supplemental Text C.....page 6

Supplemental Text D.....page 7

Supplemental Text E.....page 9

Supplemental Text F.....page 11

Supplemental Text G.....page 12

Supplemental Text H.....page 14

Supplemental References.....page 15

Supplemental Figure 1.....page 27

Supplemental Figure 2.....page 28

Supplemental Figure 3.....page 29

Supplemental Figure 4.....page 30

Supplemental Material, Text A.

DCA is considered *possibly carcinogenic to humans (Group 2B)* (WHO IARC 2004) based largely upon several independent studies that have confirmed the ability of DCA to increase the incidence of hepatocellular adenomas and/or carcinomas in male B6C3F mice (Bull et al. 1990; DeAngelo et al. 1991; Daniel et al. 1992; Ferreira-Gonzalez et al. 1995; Herren-Freund et al. 1997; DeAngelo et al. 1999) and F344 rats (Pereira 1996; Pereira and Phelps 1996) and in female B6C3F mice (US EPA 1991; Richmond et al. 1995; DeAngelo et al. 1996). Daily exposure levels associated with increased tumor incidence range from 0.5 g/L (~80 mg/kg) to 5 g/L (~800 mg/kg), although a daily dose of only 0.05 g/L (~8 mg/kg) was found in one study to increase the multiplicity of tumors in male mice (DeAngelo et al. 1999).

Hypomethylation and mutagenicity of DNA, glycogen accumulation, peroxisomeproliferation and chronic injury and repair, among other mechanisms, have been invoked to explain DCA's hepatic carcinogenicity in rodents (WHO 2004), without consensus. Accumulated evidence indicates that DCA is, at best, a weak mutagen and does not appear to be genotoxic at concentrations relevant to environmental exposure (WHO 2000; Moore and Harrington-Brock 2000). DCA is also a weak stimulator of peroxisome proliferation in rodent livers (Mather et al. 1990; Daniel et al. 1992; DeAngelo et al. 1999) and activates mouse and human peroxisome proliferator-activated receptors (PPARs) (Maloney and Waxman 1999; Walgren et al. 2000). However, inconsistencies between DCA doses required to induce hepatic tumorigenesis and peroxisome proliferation (DeAngelo et al. 1999) and between the effects of DCA and PPARs on the expression of the proto-oncogene c-Jun (Sakai et al. 1995; Stauber and Bull 1997; Stauber et al. 1998) make it unlikely that peroxisome proliferation contributes importantly to the DCA carcinogenesis in rodents. Furthermore, current evidence suggests that humans are less susceptible to the peroxisome proliferative effects of several chlorinated xenobiotics than are rats or mice (US EPA 1998; US EPA 2003).

Hepatic glycogen accumulation increases in rodents exposed to DCA (Kato-Weinstein et al. 1998; Linghor et al. 2001) and it has been postulated that this effect may be causally associated with hepatic tumor formulation (Kato-Weinstein et al. 1998). The significance of this association is made more intriguing by the fact that patients with certain inborn errors of glycogen metabolism are also more prone to develop benign and malignant liver tumors (Bannasch et al. 1980), although the mechanisms accounting for this association are obscure. However, DCA exerts multiple effects on glycogen metabolism that depend upon exposure level, tissue type and nutritional status of the host (Stacpoole 1989; Kato-Weinstein et al. 1998; Linghor et al. 2001), rendering any association between DCA's effects on glycogen metabolism and tumor formation uncertain.

DCA has long been recognized to exert numerous other effects on intermediary metabolism through multiple sites and mechanisms of action (reviewed in Stacpoole 1989). Some of these have been shown to be associated with desired therapeutic effects on carbohydrate (Stacpoole et al. 1978; Stacpoole et al. 1983a) and lipid (Stacpoole et al. 1978, Moore et al. 1979; Stacpoole et al. 1983b) metabolism; while others are considered to underlie its toxicity towards hepatic (Cornett et al. 1999; Linghor et al. 2001; Schultz et al. 2002) and various other tissues and organ systems (Williams et al. 2006; Calcutt et al. 2009).

Supplemental Material, Text B.

Recipients have included healthy subjects undergoing pharmacokinetic investigations or short-term exercise studies, and children and adults with acquired or congenital metabolic or cardiovascular diseases (reviewed in Bersin and Stacpoole 1997; Planche et al. 2005; Stacpoole et al. 2008a). Randomized controlled trials in children (Agbenyega et al. 2003; Stacpoole et al. 2006) or adults (Stacpoole et al. 1992; Kaufmann et al. 2006) with acquired or congenital lactic acidosis in which DCA was administered either intravenously as repeat 50 mg/kg doses over 1-2 days or orally at daily doses of 25 mg/kg for up to 6 months showed no significant worsening of liver function compared to placebo administration. Long-term follow-up (exceeding 10 years in several cases) of daily DCA administration of several children with various genetic mitochondrial diseases has also failed to find significant worsening of hemodynamic, hepatic, hematological, renal or electrolyte function (Stacpoole et al. 2008b). Open label studies of ~25 mg/kg/day DCA in young children with mitochondrial diseases have demonstrated generally good acute and chronic tolerability, sustained reductions in blood and cerebrospinal fluid lactate concentrations and possible stabilization of clinical status in certain disease categories (Berendzen et al. 2006; Stacpoole et al. 2008b).

Supplemental Material, Text C.

In 2006, Kaufman, et al. (Kaufmann et al. 2006) prematurely terminated a randomized controlled trial of 25 mg/kg/day DCA in patients with a mitochondrial DNA point mutation that causes mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) because of new onset or significant worsening of peripheral neuropathy within a few weeks or months of beginning treatment. Partial or complete resolution of the neuropathy occurred within 3-9 months in most cases. In a rat model of DCA neuropathy, oral DCA doses of 50-500 mg/kg/d for up to 16 weeks induced tactile allodynia and thermal hypoalgesia (Calcutt et al. 2009). Nerve conduction slowing was associated with reduction in the diameter of myelinated nerve axons, altered membrane lipid composition in spinal cord and sciatic nerve and increased concentrations of the oxidative stress markers malondialdehyde and 4-hydroxynonenal (Landgraf et al. 2007; Calcutt et al. 2009). Although earlier in vitro studies suggested that DCA induced a reversible dysmyelination of rat neurons (Felitsyn et al. 2007), no alteration of myelin formation was observed in vivo (Calcutt et al. 2009).

Supplemental Material, Text D.

Physiologically-based pharmacokinetic (PBPK) models of DCA for rodents (Keys et al, 2004) and humans (Li et al, 2008) have been developed in an attempt to clarify the toxicological consequences of the mechanism-based (suicide) inhibition of cytoplasmic GSTz1/MAAI by the compound. These models are based in part on the assumptions that 1) the enzyme inhibitory effect of DCA is reversible and 2) alternate pathways of DCA metabolism may exist and assume greater importance when cytosolic GSTz1/MAAI is irreversibly inhibited. Inhibition and recovery of cytosolic GSTz1/MAAI occurs across the DCA environmental to clinical exposure range (Schultz et al. 2002; Guo et al. 2006), thus incorporating dose levels that increase cancer incidence in rodents. This begs the question whether the proximate cause of tumorigenicity is DCA per se, rather than a metabolite. In addition, alternate routes of DCA metabolism may exist and help explain the relative “plateauing” of plasma levels and half-life observed repeatedly in humans chronically treated with the compound (Stacpoole et al 1998 and unpub. obs.). One such pathway is the reductive dehalogenation of DCA to monochloroacetate (MCA) which has been demonstrated in humans (Stacpoole et al. 1998) and rats (Shroads et al. 2008) to account for <1% of an administered clinically relevant dose, but which may assume greater importance with age of the host (Schultz et al. 2002; Shroads et al. 2008). At least one site for the biotransformation of DCA to MCA is the circulation, although the mechanism for this conversion is unknown (Shroads et al. 2008). The recent discovery of a mitochondrial GSTz1/MAAI capable of biotransforming DCA to glyoxalate (Li et al. 2010) raises that possibility of another alternative route of DCA metabolism, although both the cytoplasmic and mitochondrial enzymes from rat liver appear to be equally susceptible to inhibition by high doses (500 mg/kg/d) of DCA. It is unknown whether mitochondrial GSTz1 possesses MAAI activity and, thus, might participate in tyrosine catabolism. The PBPK model for DCA kinetics in humans (Li et al 2008) indicates that inhibition of cytosolic GSTz1/MAAI assumes greater importance for the biotransformation of

high (mg/kg) rather than low ( $\mu\text{g}/\text{kg}$ ), doses of the compound. This assumption is not entirely consistent with a recent comparative pharmacokinetic investigation of 5 days oral administration of 2.5  $\mu\text{g}/\text{kg}$  vs. 25 mg/kg DCA to healthy adults (Shroads et al. 2010). In this study, repeat dosing led to inhibition of plasma DCA clearance at both exposure levels, although the magnitude of the delay in clearance was directly associated with both dose and GSTz1/MAAI genotype.

Recently, Li and colleagues (Li et al. 2010) demonstrated the presence of GSTz1/MAAI in the mitochondrial matrix of rat and human liver. Enzyme specific activity in mitochondria is approximately one-third of that measured in hepatic cytoplasm and appears equally susceptible to inhibition by DCA, compared to the cytosolic protein

Supplemental Material, Text E.

In contrast, delta-aminolevulinate levels in DCA-treated patients do not reach those found in tyrosinemic individuals. Neither maleylacetone nor delta-aminolevulinate has been detected in the urine of humans exposed sub-acutely or chronically to environmental levels of DCA. This is not surprising, given the weak or absent evidence from population-based studies for an association between chlorinated water (WHO IARC Monograph 1991; NTP Report 1992) or TCE (Triebig et al. 1982; Triebig et al. 1983; Lee et al. 2003) exposure and either liver cancer or peripheral neuropathy. Thus, based on present data, the liver and nervous system appear to be the only *clinically* significant targets of DCA's adverse effects, and then only upon chronic administration of doses ~10,000 times higher than those achieved from environmental exposure.

To illustrate the magnitude separating the impact of chronic exposure to environmental versus clinical levels of DCA, consider the hypothetical case of a young child with a genetic mitochondrial disease who represents the population most often treated with DCA and for the longest duration (Berendzen et al. 2006; Stacpoole et al. 2008b). These patients typically are small-for-age; thus, their annual gain in height and body mass is significantly less than for healthy peers. A 10-year-old child with a mitochondrial disease might weigh 30 kg at the time DCA treatment begins and usually receives a daily dose of 25 mg/kg/day of the sodium salt, or 20 mg/kg/day of the DCA anion. Assuming a typical annual weight gain of 2 kg, this child would have received over 2.8 kg of the DCA molecule after 10 years of continuous treatment. In contrast, had that individual been exposed to DCA solely from environmental sources, or ~2-4 µg/kg/day, the cumulative dose would be only 284-568 mg, or 0.01-0.02% of the amount of DCA given as a medicinal agent.

Indeed, the long history of human exposure to clinical doses of DCA begs the question about their relevance to the development of an appropriate risk assessment for environmental

levels of this molecule. In this author's opinion, the fact that mg/kg doses of DCA administered to sick children and adults, sometimes for over a decade, has identified reversible changes in liver and peripheral nerve function as the only clinically significant adverse effects argues strongly against the likelihood that chronic exposure to vastly smaller environmental doses of DCA harbors significant human health risk. The important caveat to this conclusion is that very few patients have received chronic DCA sufficiently long to appropriately evaluate its carcinogenic potential at any exposure level.

Supplemental Material, Text F.

These data indicate that age may be a critical risk factor for neurological and perhaps other toxicities associated with DCA. Consistent with this notion are the results from the long-term follow up of pediatric patients originally enrolled in the trial of DCA for congenital lactic acidosis (Stacpoole et al. 2008b), many of whom were 6-10 years older than when they enrolled originally. Repeat nerve conduction velocity testing subsequently disclosed statistically significant worsening of certain indices of electrical function, despite no clinical symptomatology, in all but a few subjects. However, these findings require careful interpretation. Peripheral neuropathy is very common in children and adults with genetic mitochondrial diseases (Hays et al. 2006; Kaufmann et al. 2006; Stickler et al. 2006) and most of the patients who participated in the MELAS and congenital lactic acidosis trials had evidence of electrical conduction abnormalities prior to treatment with DCA. Liver dysfunction is also frequently present in persons with genetic mitochondrial diseases (Bindoff 2006). Thus, older individuals in general and particularly adults with congenital mitochondrial diseases appear to represent populations that may be at increased risk for both toxicity *and* benefit from clinically relevant exposure levels of DCA.

Short-term oral or intravenous administration of DCA may increase urinary oxalate excretion, although concentrations typically remain within the normal range. In a randomized controlled trial of intravenous DCA vs. placebo in adult patients with acquired causes of lactic acidosis, autopsy examination of a limited number of tissue specimens disclosed a statistically significant increase in oxalate crystal formation in kidneys in DCA-treated patients, but no between-group differences in crystal formation in heart, thyroid or liver were found (Stacpoole et al. 1992).

Supplemental Material, Text G.

The notion that a simple, inexpensive chemical might have widespread utility as a safe and selective anti-cancer treatment stimulated a burst of excitement in the lay community (Anonymous 2007; Pearson 2007). Several centers in North America are initiating clinical trials of DCA in various human cancers. (Garon 2009; Dunbar and Stacpoole 2010; Michelakis et al. 2010). In a phase 1, open label trial of 5 middle-aged patients with glioblastoma multiforme (GBM), a highly malignant brain cancer that shows marked over-expression of PDK2, oral administration of DCA at doses between 6.25 mg/kg to 25 mg/kg twice daily for up to 15 months caused peripheral neuropathy as the only adverse drug effect and only at exposure levels exceeding 12.5 mg/kg/d (Michelakis et al. 2010). Biochemical investigation of DCA's effects in brain tumor tissue excised before and after DCA treatment revealed that DCA depolarized mitochondria, increased PDH activity and production of mitochondrial reactive oxygen species (probably from Complex I of the respiratory chain) and induced apoptosis in GBM cells. DCA also inhibited hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), promoted p53 activation and suppressed angiogenesis in vitro and in vivo, presumably secondary to HIF-1 $\alpha$  inhibition. Four patients who received twice daily doses of 6.25 mg/kg DCA for at least 3 months had mean  $\pm$  SD plasma DCA trough levels of  $0.44 \pm 0.16$  mM, similar to the  $K_i$  of DCA for PDK2 (Bowker-Kinley et al. 1998).

Caution should be applied in predicting DCA's pharmacodynamic and toxicological effects solely from plasma DCA concentrations. At first glance, it would appear that circulating DCA levels necessary to inhibit PDKs far exceed those sufficient to inhibit GSTz1/MAAI (Cornett et al. 1999; Schultz et al. 2002; Guo et al. 2006) or to cause hepatocellular toxicity in animals (DeAngelo et al. 1999). However, the concentrations of DCA required for half-maximal activation of PDH in intact rat heart and rat heart mitochondria are approximately 100  $\mu$ M and 4  $\mu$ M, respectively (Whitehouse and Randle 1973). Moreover, DCA readily enters mitochondria

via the pyruvate carrier system and is reported to be concentrated in the matrix water of mitochondria (Kerby et al. 1976; Evans 1982). Consequently, plasma DCA levels may underestimate intra-mitochondrial concentrations in many tissues.

Supplemental Material, Text H.

Thus, several life-threatening congenital or acquired diseases may be amenable to therapeutic intervention with DCA, providing appropriate consideration is given to subject age and genetic factors that influence its metabolism and, therefore, its dosing, in these populations. A common pathological thread linking these disorders is an acquired or congenital abnormality of mitochondrial oxidative metabolism and energetics. Furthermore, the therapeutic target shared by these conditions is the PDH complex that, in turn, is central to DCA's pharmacodynamic action and, indirectly, is associated with its biotransformation through glycine removal (Suppl. Fig. 1)

An expected consequence of DCA's ability to stimulate oxidative phosphorylation by up-regulating PDH and increasing flux through the Krebs cycle and respiratory chain is the generation of increased reactive oxygen species (ROS). In cancer and pulmonary arterial hypertension, this increased production of ROS by tumor and pulmonary artery smooth muscle cells appears to underlie DCA's therapeutic effects. In contrast, ROS accumulation may also explain some of DCA's adverse effects on fetal development (Williams et al. 2006) and on liver (Hassoun and Ray 2003, Hassoun and Dey 2008, Hassoun et al. 2010, Larson and Bull 1992) and peripheral nerve function (Calcutt et al. 2009). In addition, the apoptotic effect of DCA exhibited in human cancers also contrasts with the inhibitory action of the compound on spontaneous apoptosis in normal hepatocytes from B6C3F1 male mice exposed to DCA in drinking water (Stauber and Bull 1997, Snyder et al. 1995). DCA stimulates PDH activity in rodent liver (Evans 1982), so it might also be expected to increase ROS production and therefore promote, rather than inhibit, apoptosis. Although speculative, significant differences in intracellular anti-oxidant defense mechanisms among tissues and species may contribute to the differential effects of DCA on ROS-mediated cellular toxicity.

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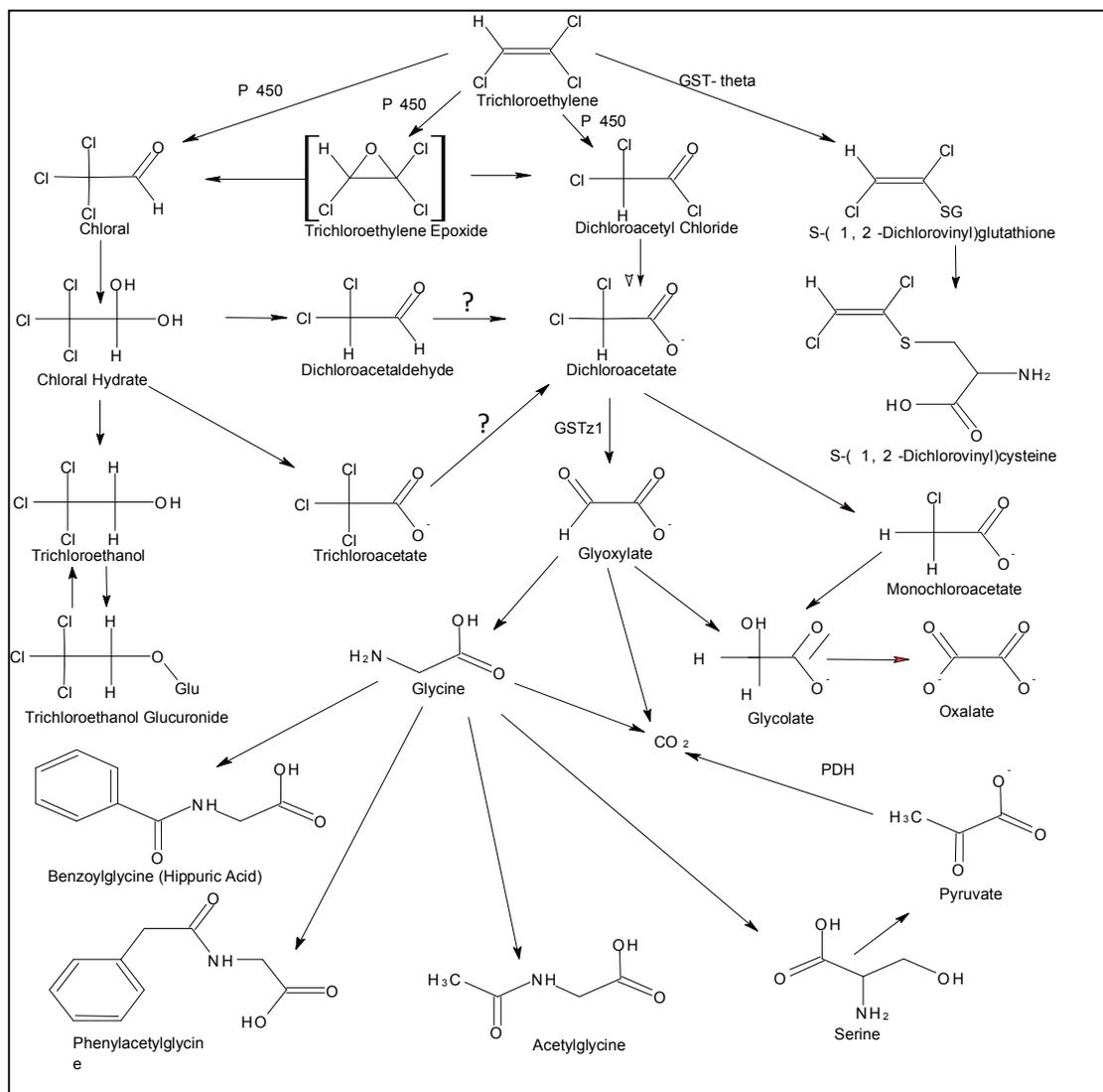
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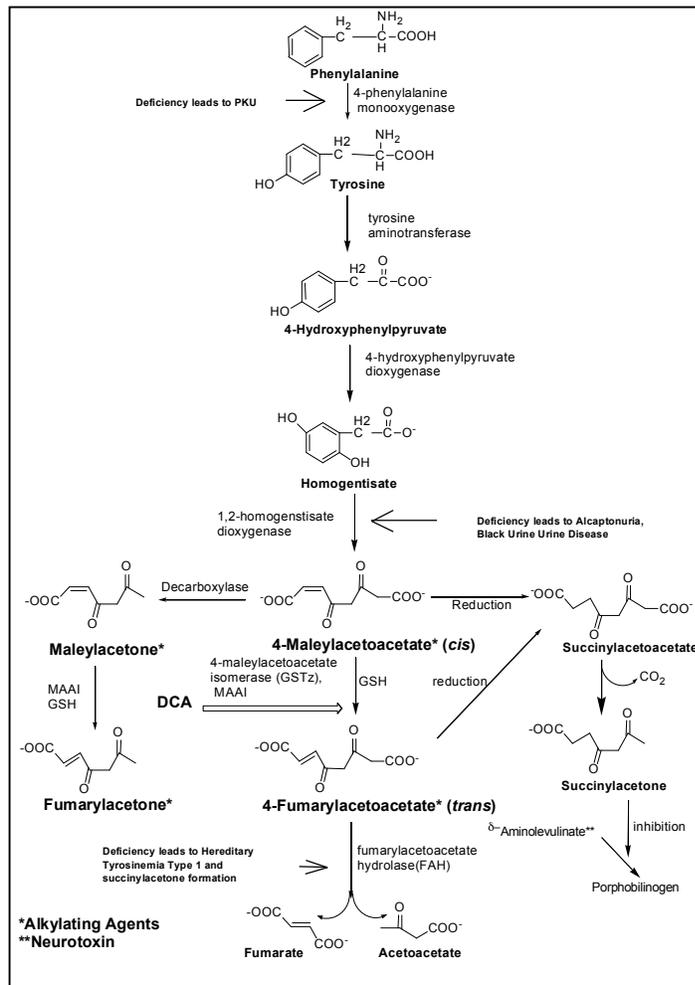
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Supplemental Material, Fig. 1.



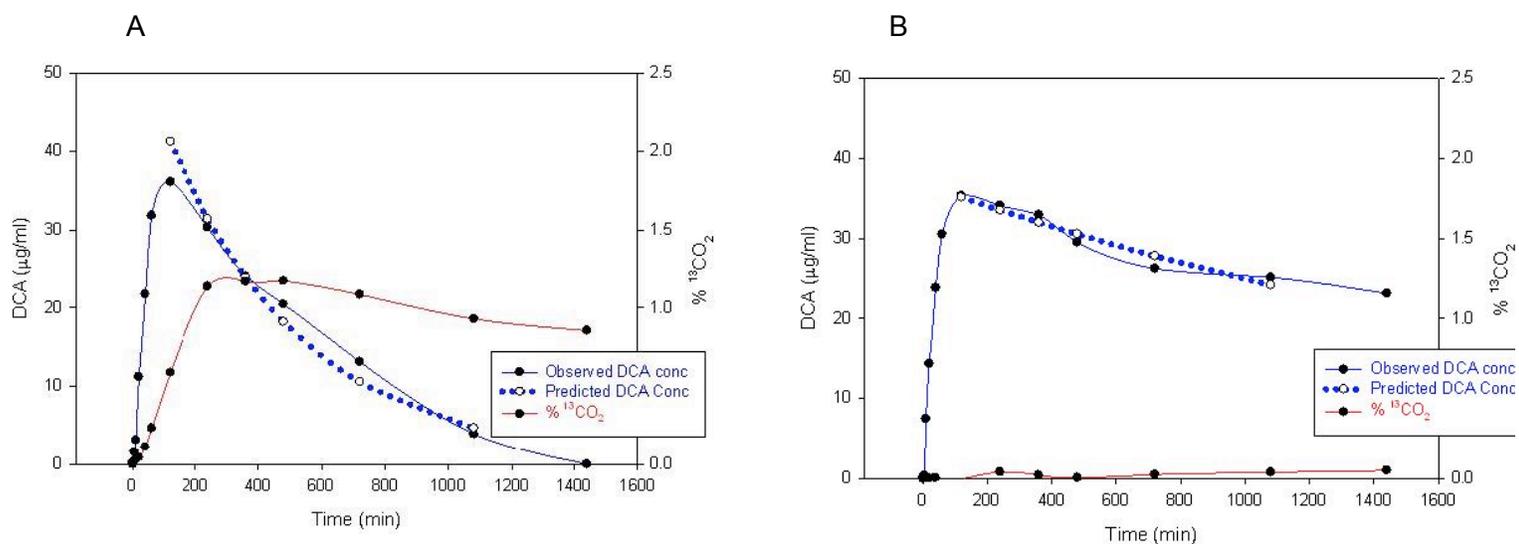
Supplemental Material, Fig. 1. Biotransformation of TCE and DCA. Abbreviations: GST, glutathione transferase; PDH, pyruvate dehydrogenase.

Supplemental Material, Fig. 2.



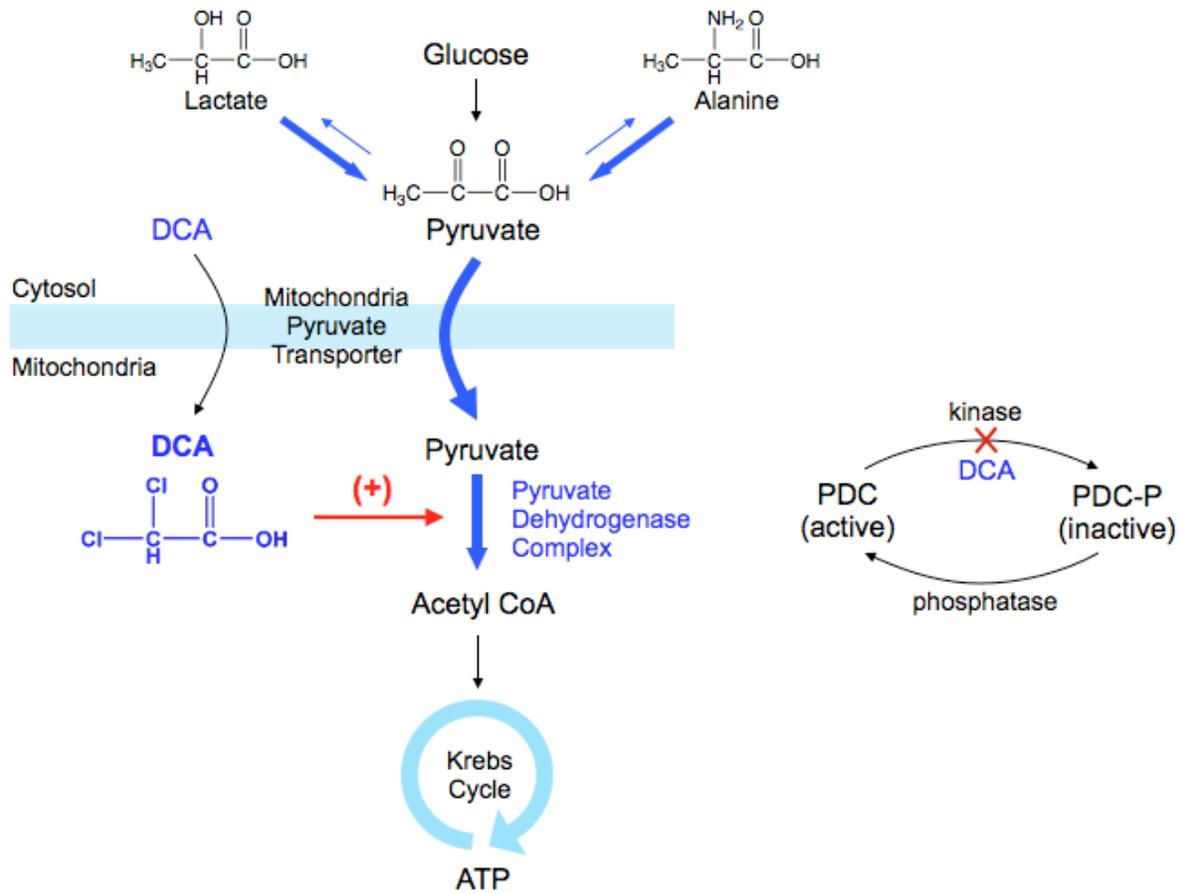
Supplemental Material, Fig. 2. Phenylalanine/tyrosine catabolic pathway. Shown are known inborn errors of metabolism associated with this pathway and the site of inhibition by DCA. Abbreviations: PKU, phenylketonuria; GSTz, glutathione transferase zeta. (Adapted from Ammini and Stacpoole 2003).

Supplemental Material, Fig. 3.



Supplemental Material, Fig. 3. Influence of GSTz1/MAAI haplotype on the kinetics and biotransformation of DCA. Two healthy adults received oral DCA at a dose of 25 mg/kg/day. 1, 2- <sup>13</sup>C-DCA was administered as that fifth dose and the kinetics and metabolism of <sup>13</sup>C-DCA were determined. Panel A shows the 24 hour DCA plasma concentration profile and CO<sub>2</sub> content in exhaled air of a subject who is homozygous for the wildtype (EGT/EGT) allele for GSTz1/MAAI. Panel B shows the same findings in a subject who is heterozygous for the KGM allele (KGM/KGT), in which a Val99Met substitution confers a change in structure of the enzyme protein that causes marked slowing of DCA kinetics and metabolism. Urinary accumulation of maleylacetone (MA) and delta-aminolevulinic acid (δ-ALA) was higher in the “slow” DCA metabolizer compared to the “fast” metabolizer, consistent with decreased GSTz1/MAAI activity. (Adapted from Shroads et al. 2010). Kinetic analysis and modeling of the data were performed using WinNonlin 4.1 (Pharsight, Inc., Mountain View, CA).

Supplemental Material, Fig. 4.



Supplemental Material, Fig. 4. Site and mechanism of action of DCA. DCA is transported into mitochondria via the pyruvate transporter system and inhibits pyruvate dehydrogenase kinase, thereby maintaining the pyruvate dehydrogenase complex (PDC) in its unphosphorylated, active form. Stimulation of PDC directly increases the oxidative removal of pyruvate and of lactate and alanine, which are in equilibrium with pyruvate. The acetyl CoA derived from the oxidation of these molecules helps maintain carbon flux through the Krebs (tricarboxylic acid) cycle. The PDC-catalyzed step and dehydrogenases of the Krebs cycle generate reducing equivalents in the forms of reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH<sub>2</sub>). These, in turn, provide electrons and protons for the respiratory chain, leading to the reduction of molecular oxygen to water and the synthesis of ATP. (Adapted from Li et al. 2010)