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Role of dichloroacetate in the treatment of genetic mitochondrial diseases ☆

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ABSTRACT

Dichloroacetate (DCA) is an investigational drug for the treatment of genetic mitochondrial diseases. Its primary site of action is the pyruvate dehydrogenase (PDH) complex, which it stimulates by altering its phosphorylation state and stability. DCA is metabolized by and inhibits the bifunctional zeta-1 family isoform of glutathione transferase/maleylacetoacetate isomerase. Polymorphic variants of this enzyme differ in their kinetic properties toward DCA, thereby influencing its biotransformation and toxicity, both of which are also influenced by subject age. Results from open label studies and controlled clinical trials suggest chronic oral DCA is generally well-tolerated by young children and may be particularly effective in patients with PDH deficiency. Recent in vitro data indicate that a combined DCA and gene therapy approach may also hold promise for the treatment of this devastating condition.

Contents

1. Introduction

Clinical investigations of dichloroacetate (DCA), administered as an ionic complex with sodium or other cations, have spanned 50 years [1]. Interest has grown over the last two decades about the potential utility of DCA in the treatment of genetic mitochondrial diseases that has culminated in the recent publication of the primary outcome results of two controlled clinical trials of the drug in affected children [2] and adults [3]. These studies and related translational research on DCA have raised important questions about its place in the treatment of these devastating conditions.

This review summarizes new information on the pharmacology of DCA most relevant to its potential in congenital mitochondrial disorders in which age, disease category and pharmacogenetics emerge as criteria that may define the selection of future patients for chronic drug exposure. These findings, together with mechanistic insight into the downstream biochemical consequences of pyruvate dehydrogenase (PDH) deficiency, provide support for the concept that

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children with mutations in PDH and perhaps in other components of the PDH complex may be particularly responsive to and tolerant of long-term DCA when administered alone or in combination with other treatment modalities.

2. Site and mechanism of action

Pyruvate plays a central role in carbohydrate and energy metabolism [Fig. 1]. Its reversible decarboxylation to acetyl coenzyme A (CoA) by the PDH complex and its decarboxylation to oxaloacetate by pyruvate carboxylase provides entry of glucose-derived carbon atoms to the tricarboxylic acid (TCA) cycle. Reducing equivalents (reduced nicotinamide adenine dinucleotide, NADH; reduced flavin adenine dinucleotide, $FADH_2$) generated by the PDH-catalyzed step and by various reactions of the TCA cycle provide electrons for the mitochondrial electron transport system and for the ultimate synthesis of ATP from ADP and inorganic phosphate by Complex V (ATP synthase) of the respiratory chain.

Under aerobic conditions, the activity of the PDH complex is rate-limiting for the mitochondrial oxidation of glucose and pyruvate and for lactate and alanine, which are in equilibrium with pyruvate. It thus serves a critical role as a determinant of the efficient conversion of carbohydrate fuel into energy. Rapid regulation of the PDH multi-enzyme complex is controlled primarily by reversible phosphorylation of serine residues located in the α subunit of the heterotetrameric ($\alpha_2\beta_2$) first (E1) component of the complex. When the E1α subunit is unphosphorylated, PDH functions as an α-ketoacid decarboxylase in the presence of the obligate cofactor thiamine pyrophosphate to oxidize pyruvate. It also catalyzes the subsequent reductive acetylation of the lipoyl moiety of dihydrolipoamide transacetylase [E2; Fig. 2]. In humans, four isoforms of pyruvate dehydrogenase kinase (PDK 1–4) phosphorylate E1α and render the entire complex inactive. PDK activity is inhibited by pyruvate and by an increase in the ADP+Pi/ATP ratio and is stimulated by an increase in the ratio of NADH/NAD+ or of acetyl CoA/CoA. Dephosphorylation and activation of E1α is mediated in humans by two isoforms of PDH phosphase (PDP 1 and 2), which are themselves dependent upon calcium and magnesium ions for activity.

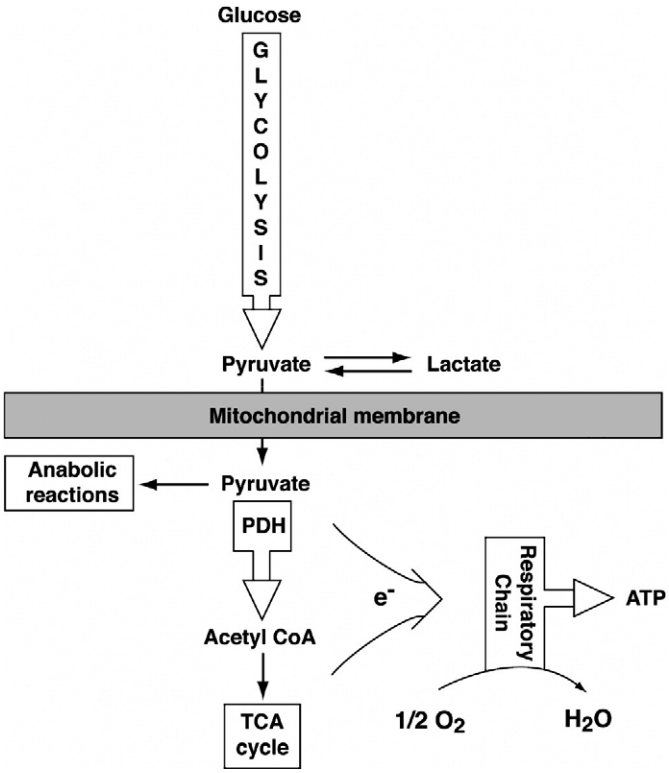

Fig. 1. Pathways of pyruvate metabolism and oxidative phosphorylation. Pyruvate may be reduced to lactate in the cytoplasm or may be transported into the mitochondria for anabolic reactions, such as gluconeogenesis and lipogenesis, or for oxidation to acetyl CoA by the pyruvate dehydrogenase (PDH) complex (PDC). Reducing equivalents (NADH, FADH) are generated by reactions catalyzed by the PDC and the tricarboxylic acid cycle and donate electrons (e^-) that enter the respiratory chain at NADH ubiquinone oxidoreductase (complex I) or at succinate ubiquinone oxidoreductase (complex II). Cytochrome *c* oxidase (complex IV) catalyses the reduction of molecular oxygen to water and ATP synthase (complex V) generates ATP from ADP.

DCA is an analog of pyruvate. It enters the circulation rapidly after an oral dose and has an oral bioavailability approaching unity [4]. The drug is transported across cell membranes (including the blood-brain barrier) by the monocarboxylate transport system for which lactate, pyruvate and ketone bodies are natural substrates [5]. DCA also competes with pyruvate for entry into mitochondria via the mitochondrial pyruvate transporter. Stimulation of PDH activity generally occurs within minutes following oral or parenteral administration and is reflected by a reduction in blood lactate concentration, the magnitude of which is dependent upon the pretreatment lactate level and the dose of DCA [6]. Careful examination of the crystal structure of PDK2 (which has the widest tissue expression among the kinases) and its interaction with natural and synthetic ligands has demonstrated that DCA and pyruvate share a common binding site in the center of the N-terminal regulatory domain [7]. DCA, in the presence of ADP, induces changes in the active site that lead to uncompetitive inhibition of PDK2 and subsequent activation of the PDH complex. The relative sensitivity of PDKs to DCA inhibition is reported to be: PDK2 ≈ PDK4 > PDK1 ≫ PDK3 [8].

For many years, the ability of DCA to rapidly activate the PDH complex by inhibiting PDK activity was considered the primary mechanism that rationalized its use in acquired or congenital conditions in which the efficient conversion of carbohydrate-derived fuel into energy was perturbed. However, as we shall review later in this article, a second mode of action of DCA on the PDH complex may underlie its sustained pharmacodynamic effects during chronic exposure.

3. Biotransformation and kinetics

The primary site of DCA metabolism is the liver, where the zeta-1 family isoform of glutathione transferase (GSTz1) dechlorinates DCA to glyoxylate in a cytoplasmic reaction that requires glutathione but does not consume it [Fig. 3; Ref. [5]]. A very minor route of DCA metabolism that accounts for <1% of an administered dose is reductive dehalogenation to monochloracetate, which may occur within the plasma compartment [9]. Monochloracetate is potentially highly neuro-toxic [10], although the site and mechanism responsible for its toxicity is unknown. The primacy of the liver in metabolizing DCA was strikingly demonstrated in patients undergoing liver transplantation, in whom clearance (Cl) of DCA from the plasma was virtually eliminated during the anhepatic phase of surgery [11].

GSTz1 is a bifunctional enzyme. As maleylacetoacetate isomerase (MAAI), it also catalyzes the penultimate step in tyrosine catabolism by converting maleylacetoacetate and maleylacetone to fumarylacetoacetate and fumarylacetone, respectively [Fig. 4; Ref. [5]]. Several important inborn errors of metabolism are associated with the tyrosine catabolic pathway. Loss-of-function mutations in fumarylacetoacetate hydrolase, the terminal enzyme in the pathway, leads to hereditary tyrosinemia type I. As a result of hydrolase inhibition, fumarylacetoacetate and maleylacetoacetate accumulate as do their ketone analogs, fumarylacetone and maleylacetone. These intermediaries possess reactive groups that make them potent alkylating agents and putative renal and hepatic toxins. Hydrolase inhibition also diverts carbon precursors to form succinylacetoacetate and succinylacetone, which inhibits a proximate step in heme synthesis and causes accumulation of the heme precursor δ-aminolevulinate (δ-

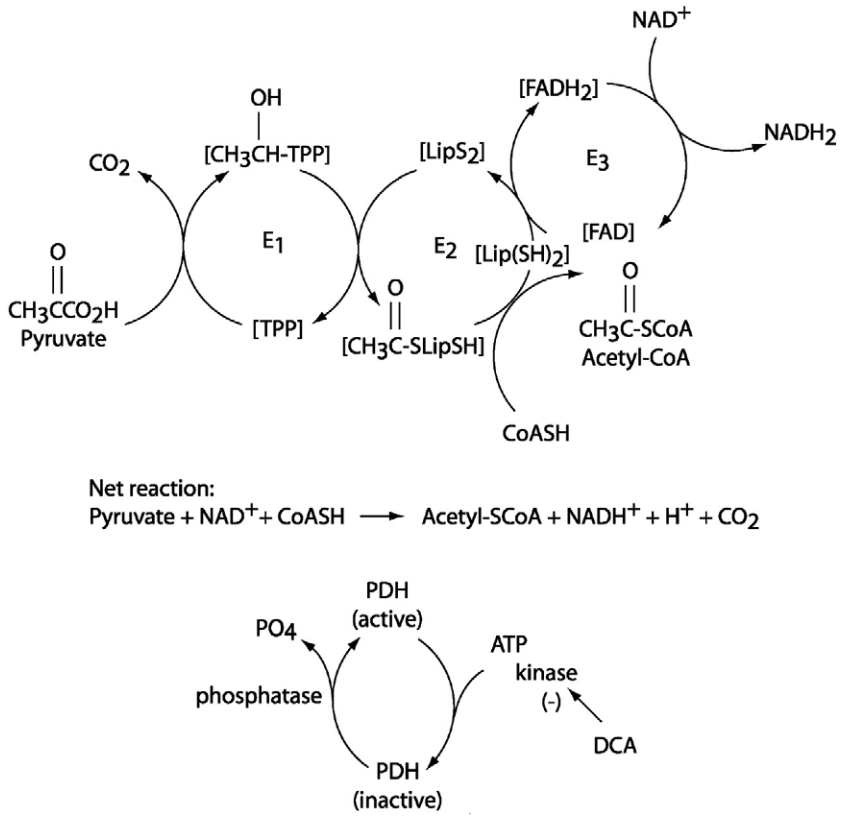

Fig. 2. The pyruvate dehydrogenase (PDH) multi-enzyme complex (PDC). Pyruvate is decarboxylated by the PDH subunit (E_1) in the presence of thiamin pyrophosphate (TPP). The resulting hydroxyethyl–TPP complex reacts with oxidized lipoamide ($LipS_2$), the prosthetic group of dihydrolipoamide transacetylase (E_2), to form acetyl lipoamide. In turn, this intermediate reacts with reduced coenzyme A (CoASH) to yield acetyl CoA and reduced lipoamide ($Lip(SH)_2$). The cycle of reaction is completed when reduced lipoamide is reoxidized by the flavoprotein, dihydrolipoamide dehydrogenase (E_3). Finally, the reduced flavoprotein is oxidized by NAD and transfers reducing equivalents to the respiratory chain via NADH. PDC is regulated, in part, by reversible phosphorylation, in which the phosphorylated enzyme is inactive. Increases in the intramitochondrial ratios of NADH/NAD and acetyl CoA/CoA also stimulate kinase mediated phosphorylation of PDC. The drug dichloroacetate (DCA) inhibits the kinase responsible for phosphorylating PDH, thus 'locking' the enzyme in its unphosphorylated, catalytically active state.

ALA). Accumulation of δ-ALA is thought to contribute to the neuropathic manifestations of tyrosinemia type I.

Repeated exposure of rodents to DCA causes a post-transcriptional and reversible pharmacological "knockdown" of hepatic GSTz1/MAAI protein expression and catalytic activity [12,13]. This coincides with modest changes in the peak plasma concentration (Cmax) of DCA but in marked increases in plasma half-life (t1/2) and the area under the plasma concentration curve (AUC) and a fall in Cl [9]. A similar inhibitory effect of repeated DCA exposure on its plasma kinetics has been demonstrated repeatedly in humans [9]. In both rodents and humans, DCA-induced inhibition of GSTz1/MAAI also leads to accumulation in plasma and/or urine of maleylacetone (and presumably maleylacetoacetate) and δ-ALA [Table 1].

4. Pharmacogenetics

The human gene for GSTz1/MAAI has three non-synonymous single nucleotide polymorphisms (SNPs): G94>A (rs 3177427) Glu→Lyr at position 32; G124>A (rs 7972) Gly→Arg at position 42; and C245>T (rs1046428) Thr→Met at position 82 that show different activity toward various xenobiotic haloacids [14–16]. Differences in the frequency of polymorphic variants of the GSTz1/MAAI gene may occur among racially or ethnically diverse groups. They may also differ in their kinetic properties toward DCA, thereby influencing its biotransformation and toxicity. To investigate this possibility we determined the allele and haplotype frequencies for the three non-synonymous SNPs of GSTz1/MAAI in 438 genomic DNA samples from Caucasians, African-Americans, Hispanics and Asians that were obtained from the Center for Pharmacogenomics or the General Clinical Research Center DNA repository at the University of Florida and from Coriell Cell Repositories (Coriell Institute for Medical Research, New Jersey). Allele and haplotype frequencies for the four racial/ethnic groups are shown in Tables 2 and 3. Due to significant linkage disequilibrium among the three SNPs, there were four common haplotypes among eight possible variants. In the populations studied, the EGT haplotype was the most common (0.50–0.56) with EGM and KGT being intermediate and KRT being the least common (0 to 0.05), consistent with previous findings [12]. The allele frequencies for these SNPs accord with those reported in the NCBI db SNP. The most common haplotype among populations was EGT (53% overall), whereas the KRT haplotype occurred with the lowest frequency (≤5%).

To determine possible kinetic differences in the biotransformation of DCA to glyoxylate, we expressed and purified four variants of the GSTz1/MAAI protein. The proteins corresponded to the following haplotypes: 1) KGT (Z1B), 2) EGM (Z1D), 3) EGT (Z1C) and 4) KRT (Z1A) shown in Table 4. Purified proteins corresponding to these haplotypes were then analyzed kinetically to determine the K_m and V_{max} for both DCA and glutathione, using a coupled assay system and spectrofluorometric detection [14,16]. The K_m and V_{max} values for DCA were approximately 10-fold higher for the rare KRT haplotype compared to those for the other variant proteins. Because the KRT haplotype is distinguished by the Gly42Arg SNP, the data suggest this is the variant leading to the functional differences observed. Accordingly, the small percentage of humans who possesses the KRT haplotype may exhibit considerably different rates of DCA biotransformation and, hence, an altered toxicological response to the compound.

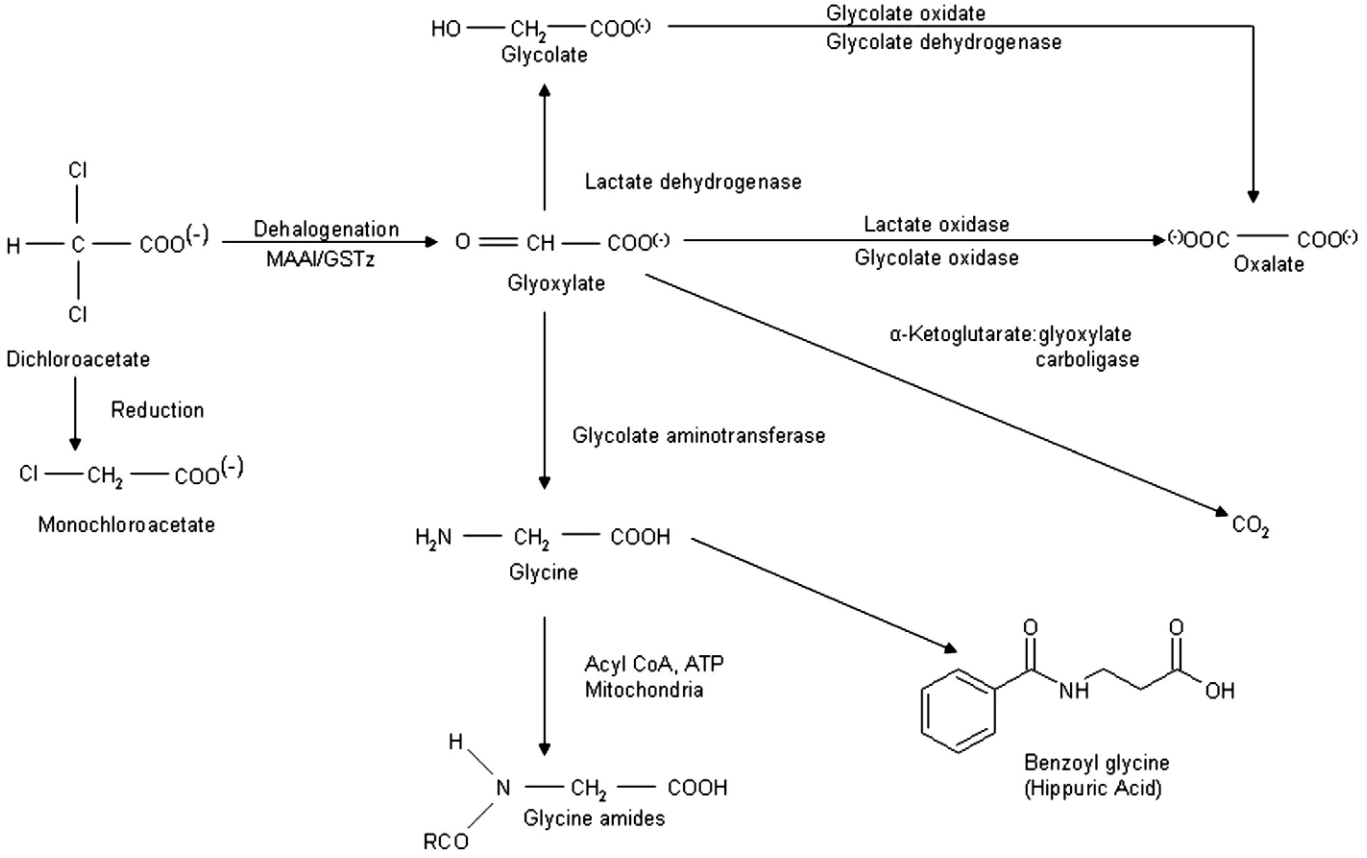

Fig. 3. Biotransformation of DCA. The major route of metabolism is dechlorination to glyoxylate, whereby DCA enters the general carbon pool of the host. The conversion is catalyzed in cytosol by the bifunctional enzyme zeta-1 isoform of glutathione transferase (GSTz1)/maleylacetoacetate isomerase (MAAI). Reductive dehalogenation of DCA to monochloroacetate normally represents a very minor pathway of biotransformation. End products of DCA metabolism include carbon dioxide, oxalate and various glycine and hippuriate derivatives excreted in the urine.

It is noteworthy that the K_m and V_{max} value for DCA for the KRT polymorphism are increased proportionately. Thus, it could be argued that the biological consequence of decreased substrate affinity for the enzyme is neutralized by the increased maximal velocity of the reaction. We speculate that, under these circumstances, the change in K_m may override that in V_{max}, because substrate-protein affinity is required before enzyme-mediated catalysis can occur. If this is the case, it can be postulated that the KRT haplotype, relative to the other known GSTz1/MAAI haplotypes, may lead to 1) a decreased rate of DCA biotransformation and glyoxylate formation; 2) a decreased plasma CL and increased t1/2 of DCA; 3) an increased plasma and tissue accumulation of monochloracetate, maleylacetoacetate and maleylacetone; and 4) consequently, an increased risk of toxicity to chronic DCA exposure.

5. Toxicology

Although several tissues and organ systems in rodents have been identified as susceptible to DCA toxicity [10], those that so far appear to be relevant to human safety are the liver and nervous system. DCA can cause hepatocellular inflammation, hyperplasia and carcinoma [9] in doses similar to those used clinically. In contrast, data from both open label studies [9,17,18] and randomized, placebo-controlled trials [2,3,18–21] indicate that acute intravenous or chronic oral administration of the drug to children or adults causes symptomatic, mild (usually ≤two-fold the upper limit of normal) and readily reversible elevation of hepatic transaminases in a small proportion of patients as the only manifestation of hepatoxicity. In both rats [13] and humans [2] chronic oral DCA increases the urinary accumulation of maleylacetone several-fold above baseline levels, but it is unknown whether this putative hepatotoxin is causally related to the induction of toxicity in either species.

Central nervous system (CNS) effects of DCA in humans have been limited to transient drowsiness or to a sensation of calmness in a few adult subjects. DCA appears to have less sedative effect than what occurs with conventional CNS depressant drugs, based on results of tests employed in the evaluation of minor tranquilizers, benzodiazepines and barbiturates [4], but may have properties similar to those of axiolytics. However, the most problematic adverse effect of DCA on the nervous system is reversible peripheral neuropathy, which has been documented in dogs, rats and humans [10] and which is currently considered the most limiting factor when considering chronic administration of the drug. A series of recently published laboratory and clinical studies have helped elucidate the mechanisms that may contribute to this toxicity.

5.1. Dysmyelination

DCA induces a dose- and exposure-dependent reversible inhibition in the expression of myelin-related proteins following its administration to myelinating co-cultures of neonatal rat Schwann cells and dorsal root ganglia neurons [22]. DCA exposure decreases the expression of myelin basic protein (MBP), protein zero (P0), myelin-associated glycoprotein (MAG) and peripheral myelin protein 22 (PMP22) [Fig. 5]. DCA does not alter steady-state levels of intermediate filament proteins, but promotes the formation of anti-neurofilament antibody reactive whirls that are present in certain hereditary neuropathies [23] and that could potentially disrupt axonal transport. The drug has modest effects on neuronal and glial cell vitality, as determined by the release of lactate dehydrogenase.

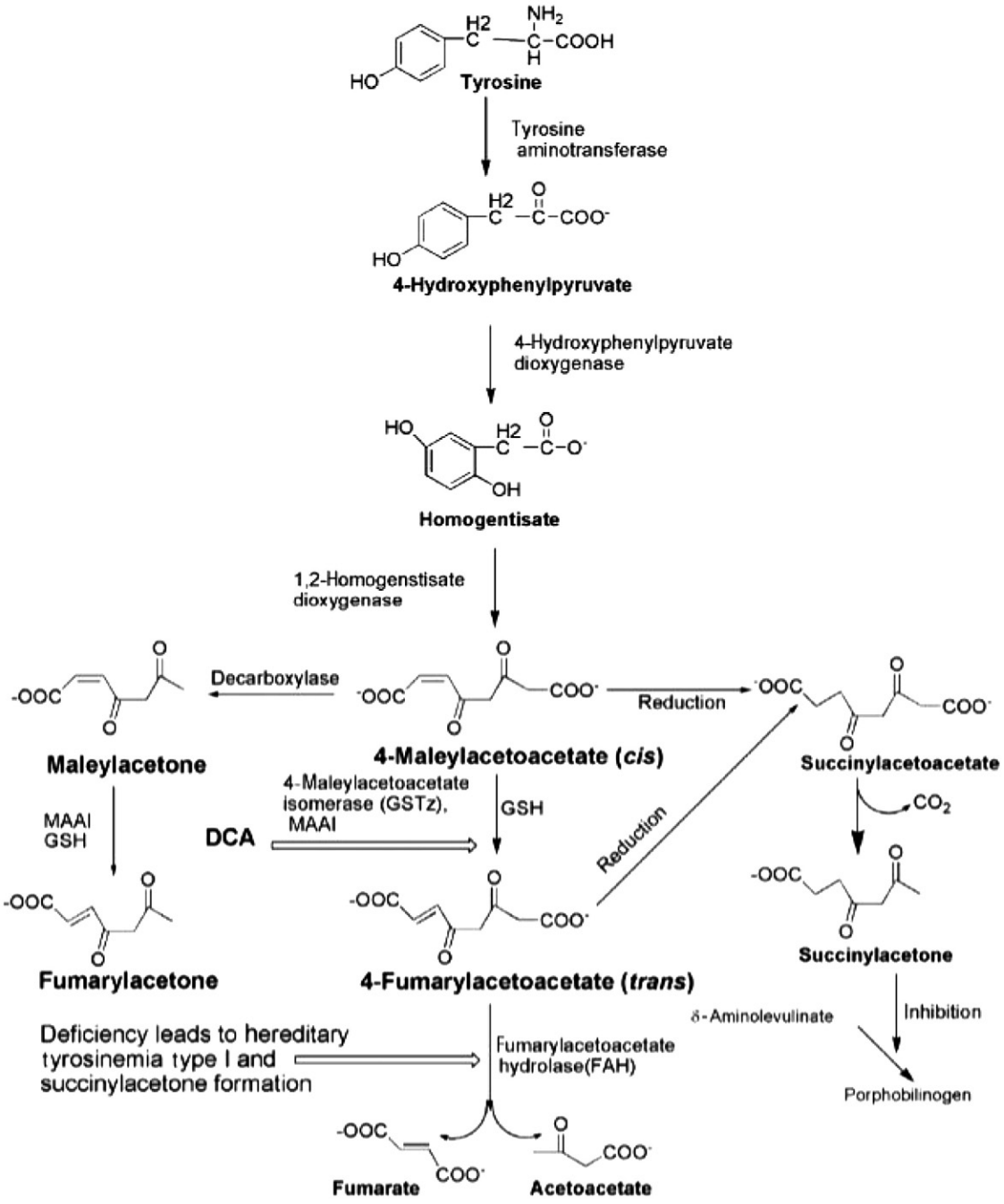

Fig. 4. Tyrosine catabolic pathway and site of action of DCA. DCA depletes maleylacetoacetate isomerase (MAAI), causing accumulation of maleylacetoacetate, maleylacetone, fumarylacetoacetate and fumarylacetone. MAAI is identical to the z-1 isoform of glutathione transferase (GSTz1), which biotransforms DCA to glyoxylate. Succinylacetone also accumulates as a result of DCA and is a known inhibitor of δ-aminolevulinate dehydratase, causing buildup of δ-aminolevulinate and inhibition of heme biosynthesis. Perturbation of heme metabolism is thought to cause the neuropathic complications in patients with tyrosinemia type I.

5.2. Age

Two randomized, double-blind, placebo-controlled trials have evaluated chronic, oral DCA in patients with genetic mitochondrial diseases who were treated for up to six months with 12.5 mg/kg every 12 h by mouth or feeding tube [2,3]. Serial quantitative assessment of peripheral nerve conduction velocity and amplitude of the upper and lower extremities were conducted in the participants of both trials. In one study conducted at the University of Florida, patients with congenital lactic acidosis, whose mean age at entry was 5.6±5 years, showed no difference in peripheral nerve function by clinical or quantitative assessment between the 21 children who received placebo and the 22 subjects who received DCA for six months [2]. This trial recruited a clinically and genetically heterogeneous group of patients who had defects in the PDH complex or in one or more complexes of the respiratory chain or who had a pathological mitochondrial DNA point mutation. This latter group included six patients with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS). In contrast, a controlled clinical trial conducted at Columbia University in 30 older adolescents and adults (mean age at entry 30±14 years) who harbored the common A4332G point mutation for MELAS was terminated prematurely because of a high incidence of worsening or new-onset peripheral neuropathy that developed within weeks or a few months of DCA administration [3].

Table 1
Pharmacokinetic and pharmacodynamic consequences of GSTz1/MAAI inhibition by DCA

Substrate	Variable	Consequence
	Cmax	↑
	t1/2	↑↑↑
	AUC	↑↑↑
	Cl	↓↓↓
	Oxalate	↑
	Monochloroacetate	↑
Tyrosine	Maleylacetone	↑↑↑
	δ-aminolevulinate	↑

Changes in oxalate, monochloroacetate and maleylacetone have been reported in both plasma and urine, depending on the experimental conditions, while changes in δ-aminolevulinate have been reported only in urine.
Abbreviations: Cmax, peak plasma concentration; t1/2, plasma half-life; AUC, area under the plasma concentration curve; Cl, plasma clearance.

Peripheral neuropathy is prevalent among individuals with genetic mitochondrial diseases and most of the subjects who participated in the trials at Florida [24] and Columbia [25] had electrical evidence of peripheral neuropathy prior to receiving DCA. Therefore, given the inherent vulnerability of this population, it is impossible to differentiate the relative impacts of drug and disease progression on peripheral nerve function. However, the striking difference between the pediatric and adult populations in their apparent susceptibility to DCA toxicity is unlikely due to disease genotype, since the children who had congenital lactic acidosis due to MELAS tolerated DCA as well as the other patients studied at Florida.

Therefore we hypothesized that subject age might be an important determinant in DCA neurotoxicity. To test this postulate, we conducted preliminary experiments in different age groups of rats exposed to DCA in drinking water [26]. The drug induced a sensory-motor peripheral neuropathy involving the hind limbs that was directly dependent upon age and drug dose. Associated morphometric studies suggest that the neuropathy is associated with axonal atrophy [26] and with changes in the lipid composition of the spinal cord and sciatic nerve of dosed animals [27].

The apparent age-dependent variability in inter-species tolerance to chronic DCA administration may reflect differences in its kinetics and/or biotransformation. Therefore, we determined the plasma kinetics of DCA in five children and four adults who participated in the trials conducted at Florida and Columbia and who each received 12.5 mg/kg DCA twice daily for six months [9]. As summarized in Table 5, there were no significant differences in any pharmacokinetic parameter between the age groups after receiving the initial dose. However marked differences in the kinetics of plasma DCA between the age groups did occur after six months of continuous exposure to 25 mg/kg/day DCA. Figs. 6 and 7 represent the mean values ± standard deviations of the individual time versus concentration curves for the subjects studied at Florida and Columbia, respectively. The patient groups exhibited closely similar kinetics behavior, which is consistent with the data presented in Table 5. In contrast, the CL of the drug was markedly delayed in the adult patients, compared to the children, after six months of treatment. Indeed, comparison of the values at time 0 clearly demonstrate that residual DCA is carried over from the previous dose in the adult patients and is not cleared within the 12-hour dosing interval. When the data from both patient groups were combined there were no correlations between age and any kinetics parameter measured after the first DCA dose, but there were moderate to strong correlations between these indices following six months of drug exposure [Table 6].

We also found similar age-dependency in the kinetics of DCA across a broad (5 week–15 month) age spectrum in rats [9]. In these animals the age-related decrease in plasma drug clearance was associated with increased concentrations of urinary DCA, oxalate (an end product of DCA metabolism) and maleylacetone and increased concentrations of plasma maleylacetone and both urinary and plasma levels of monochloroacetate.

Together these data indicate that age is closely correlated with 1) a delay in the plasma clearance of DCA; 2) accumulation of potentially toxic tyrosine (maleylacetone) and DCA (monochloroacetate) catabolites; and 3) an increased susceptibility to the drug's peripheral neuropathic effects. It remains unclear whether the neuropathy is due to tissue accumulation of DCA or other molecules. The results obtained from the cell culture experiments suggest that DCA itself may be capable of producing abnormal myelination of CNS cells, since rat Schwann cells and dorsal root ganglian neurons do not express detectable GSTz1/MAAI protein levels in tissue culture (unpublished experiments).

It could be argued that six months exposure is too brief to assess the consequences of long-term administration of DCA, particularly when there remains underlying concern about its carcinogenic potential, as demonstrated in certain rodent strains [5,10]. Accordingly, we have continued to evaluate the tolerability of the drug in children who participated in the original randomized clinical trial at Florida and have been followed subsequently on open label DCA [19]. In this study, patients who initially received placebo for six months were crossed over to DCA and all subjects were continued on the double-blind trial for an additional 12 months. At the end of this phase, 36 of the original 43 subjects chose to continue receiving open label DCA and to return every six months for evaluation. Exposure of this group to DCA totaled 110.42 patient-years through May 2005 and their mean three year survival was 79%. There were no clinically significant changes in biochemical metabolic indices (including

Table 2
Minor allele frequencies of three non-synonymous SNPs of GSTz1/MAAI in human populations

Polymorphisms	Racial/ethnic group			
	Caucasians	African-Americans	Hispanics	Asians
	(*N*=106)	(*N*=105)	(*N*=121)	(*N*=106)
SNP G94>A (rs3177427)	A (0.30)	A (0.32)	A (0.30)	A (0.39)
Published MAF[a]	A (0.27–0.31)	A (0.19–0.35)	A (0.23)	A (0.48–0.50)
SNP G124>A (rs7972)	A (0.05)	A (0.03)	A (0.05)	A (0.01)
Published MAF	A (0.08–0.12)	A (0.01–0.03)	A (0.04)	A (0.00)
SNP C245>T (rs1046428)	T (0.21)	T (0.11)	T (0.28)	T (0.05)
Published MAF	T (0.17–0.19)	T (0.10–0.14)	T (0.24)	T (0.01–0.08)

[a] MAF (minor allele frequency) reported in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/index.html).

Table 3
Haplotype frequencies of three non-synonymous SNPs of GSTz1/MAAI in human populations

Haplotypes	Haplotype frequency				
	All	African-American	Asians	Caucasians	Hispanics
	(*n*=438)	(*n*=105)	(*n*=106)	(*n*=106)	*N*=121
KRT (Z1A)	0.03	0.03	0.00	0.05	0.04
KGT (Z1B)	0.27	0.30	0.38	0.23	0.17
EGM (Z1D)	0.17	0.11	0.04	0.20	0.28
EGT (Z1C)	0.53	0.55	0.56	0.50	0.50

Table 4
Kinetic parameters of four GSTz1/MAAI haplotypes

Haplotype	DCA		Glutathione	
	K_m	V_{max}	K_m	V_{max}
	(μM)	(ρmol/min/mg)	(μM)	(ρmol/min/mg)
KGT (Z1B)	28±2	46±1	14±1	49±2
EGM (Z1D)	27±3	52±1	12±1	47±1
EGT (Z1C)	37±6	53±3	12±1	56±2
KRT (Z1A)	370±15	500±17	184±13	538±20

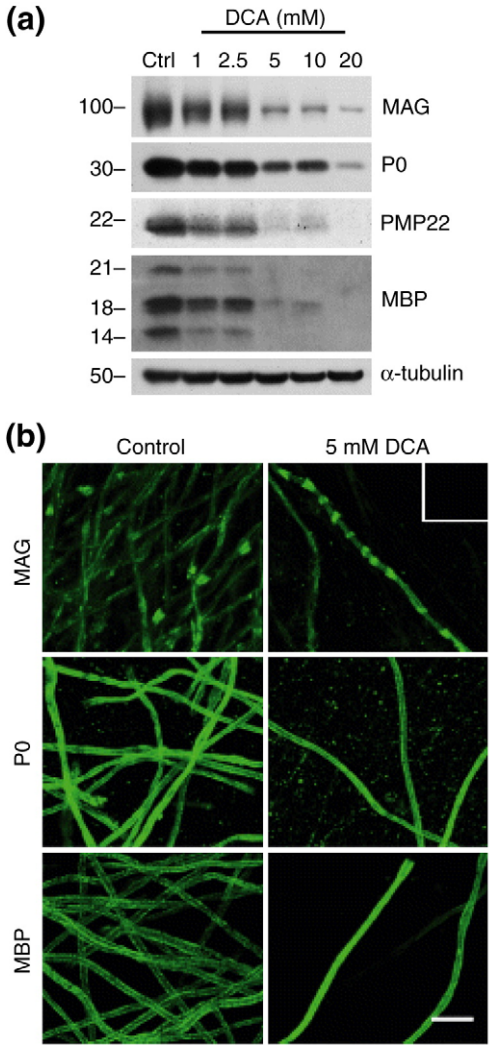

Fig. 5. Reduction of myelin proteins in myelinating Schwann cell-dorsal root ganglion (SC-DRG) neuron co-cultures after DCA exposure. (a) Myelinating SC-DRG neuron cultures were treated with DCA for 12 days at the indicated concentrations and whole protein lysates (10 μg/lane for MAG and P0; 20 μg/lane for PMP22 and MBP) were analyzed by Western blotting with the indicated antibodies. Tubulin is shown as a loading control. Molecular mass in kDa. (b) Representative cultures were immunolabeled with antibodies against MAG, P0 and MBP. Sample incubated with non-specific rabbit IgG is shown in the upper right corner. Scale bar, 10 μm.

hepatic transaminases) and only serum total protein (2% increase; $p=0.04$) and 24-hour urinary oxalate (22% increase; $p=0.004$) concentrations achieved statistical significance. Both the basal ($p<0.001$) and carbohydrate meal-induced ($p=0.004$) rises in blood

Table 5
Plasma DCA kinetics in humans

Parameters (units)	First dose		Six months	
	Children	Adults	Children	Adults
No. subjects	5	4	5	4
Age (years)	5.2±1.8	24±10	5.7±1.8	24.5±10
t1/2 (h)	2.5±0.4	2.1±1.5	6.4±3.4	21±5.8
Cmax (μg/ml)	23±9.1	25±6.6	35±10	53±18
$AUC_{0-\infty}$ (μg/ml · h)	83±33	70±18	340±130	1500±700
Clearance (ml/h)	150±60	180±46	37±14	8.3±4.6

Subjects received 12.5 mg/kg of DCA. Data are mean±standard deviation of results obtained after the first DCA dose and after six months of daily exposure to 12.5 mg/kg every 12 h. Kinetic parameters were determined from a non-compartmental pharmacokinetic model using WinNonLin software (version 5.01). Abbreviations are t1/2, terminal half-life; Cmax, maximum plasma concentration; $AUC_{0-\infty}$, area under the curve from time 0 extrapolated to infinity; and CL, total body clearance.

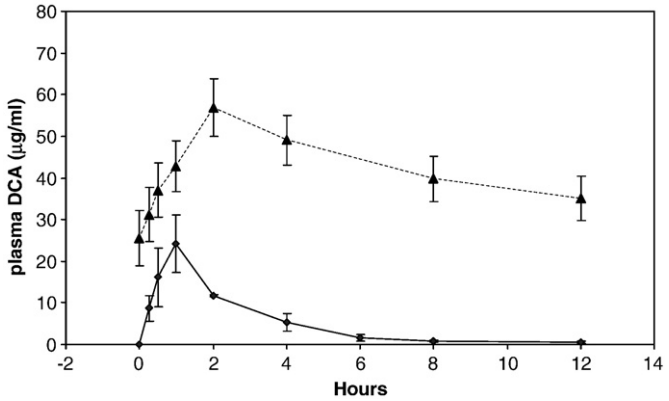

Fig. 6. Concentration–time profile of ^{13}C-DCA in plasma of samples obtained in 5 previously drug-naïve young children (ages 2.2–7.1 years) after receiving an initial dose of 12.5 mg/kg (♦) and after 6 months of 12.5 mg/kg administered twice daily (▲).

lactate were blunted by DCA and the median cerebrospinal fluid (CSF) lactate also decreased over time ($p=0.04$), indicating that the lactate-lowering effect of DCA was sustained.

However, in contrast to the apparent lack of an effect of DCA on nerve conduction during the first six months of treatment [2], we found that long-term exposure to the drug was associated with a decrease in the mean conduction velocity and an increase in the mean distal latency in peroneal nerves (both $p<0.001$). It is reasonable to assume that the peripheral neuropathy observed in these subjects reflects both the chronicity of their primary mitochondrial disease and their exposure to DCA, although the relative impact of each on peripheral nerve conduction cannot be quantified.

6. Future therapeutic role of DCA

Of the three controlled clinical trials of DCA conducted for genetic mitochondrial diseases, two demonstrated biochemical improvement in terms of blood or CSF lactate concentrations or both and showed no significant adverse effects of the drug, compared to placebo [2,21]. These trials recruited populations of patients (mostly children) who were extremely genetically and clinically heterogeneous, thus precluding evaluation of subgroups. The third trial, conducted in older subjects with a common pathological point mutation in mtDNA, ended prematurely because of drug-associated neurotoxicity and before any valid assessment could be made of other clinical or biochemical endpoints [3]. Thus, despite the controlled nature of these trials, they have several shortcomings that have confounded

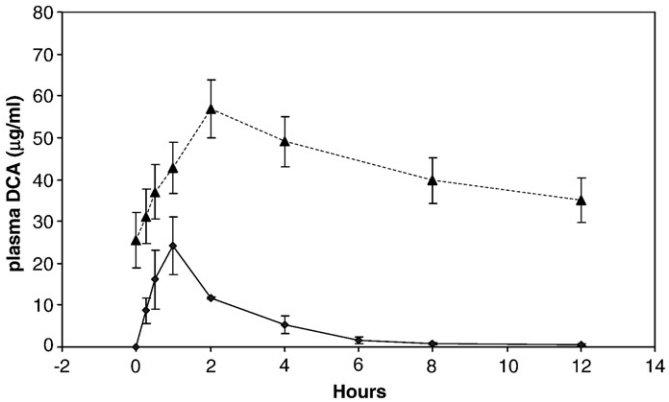

Fig. 7. Concentration–time profile of ^{12}C-DCA in plasma of samples obtained in 5 previously drug-naïve older subjects (ages 14.0–33.9 years) after receiving an initial dose of 12.5 mg/kg (♦) and after 6 months of 12.5 mg/kg administered twice daily (▲).

Table 6
Correlations between age and pharmacokinetic parameters

Parameters	Correlation with age (R^2) [2-sided p-value]	
	First dose (N=9)	Six months (N=9)
Cmax	0.08 [0.47]	0.40 [0.066]
t1/2	0.19 [0.23]	0.63 [0.012]
$AUC_{0-\infty}$	0.14 [0.32]	0.75 [0.003]
CL	0.11 [0.38]	0.67 [0.007]

Correlations between age and kinetic parameters were made from data obtained after the first DCA dose and after six months of daily exposure to 12.5 mg/kg every 12 h. Abbreviations are t1/2, terminal half-life; Cmax, maximum plasma concentration; $AUC_{0-\infty}$, area under the curve from time 0 extrapolated to infinity; and CL, total body clearance.

evaluation of the therapeutic potential of DCA in any well-defined population of patients with mitochondrial disease.

We have proposed that DCA should be most effective in patients with congenital defects in the PDH complex, in particular, with mutations in the E1α subunit [28]. This hypothesis is founded upon both the primary site and mechanism of action of DCA on fuel metabolism and on the sum of the evidence regarding drug safety and efficacy from both open label and controlled trials [Table 7]. Published information is available for at least 46 patients with PDH complex deficiency world-wide who were treated with DCA [28]. These subjects ranged in age from newborn to middle age, but the median age was 17 months. Developmental delay and muscle weakness were the most common reported clinical signs and both pretreatment lactate concentrations in blood (mean ± SD; 5.2 ± 3.8) and CSF (6.0 ± 4.1) were elevated. Mean residual PDH was 30 ± 19% of normal for the reference laboratory. Twenty-two patients had a primary defect in the E1α subunit of the enzyme.

DCA was reported to be generally well-tolerated in this predominately pediatric population and its administration was associated with significant decreases in blood and CSF lactate concentrations. The six deaths that occurred in patients who received DCA were not thought to be drug-related and it could not be determined with certainty whether the deterioration of hepatic, neurological or neuromuscular function observed in a few patients was due to DCA, progression of underlying disease or both.

We continue to follow four children (three females) with PDH deficiency at the University of Florida. Their clinical presentations and courses through May 2005 have been summarized [28]. Three have continued to receive oral DCA (25/mg/kg/day) through January 2008 and have maintained normal venous blood lactate levels and stable clinical conditions, with the exception of one 16-year-old girl who has experienced some behavioral instability associated with menarche and another 12-year-old girl who was withdrawn from DCA after over 8 years of treatment because of worsening peripheral nerve conduction.

Together, these data suggest that the next appropriate step in the evaluation of DCA is a controlled clinical trial in patients with E1α deficiency and perhaps with other defects in the PDH complex. Eligibility should be restricted to young children for two reasons: first, to intervene as early as possible in the course of their degenerative disease and second, to mitigate the age-dependent decline in the kinetics and biotransformation of the drug and thus decrease the likelihood of toxicity. A potential exclusion criterion is the presence of the "high K_m" haplotype for GSTz1/MAAI, although there currently are insufficient data to determine the impact of this variant on the in vivo kinetics and disposition of DCA in humans. Other aspects of the design and outcome measures of such a clinical trial have been summarized [27].

An additional potential role for DCA in the treatment of genetic mitochondrial diseases is as adjunctive therapy, in combination with other nutritional or pharmacological interventions or with gene therapy. In the case of PDH deficiency, the use of ketogenic diets [reviewed in Ref. [29]] has long been inculcated into the therapeutic regimen of affected subjects, based on the rationale that a high fat intake provides an alternative fuel source when carbohydrate metabolism is severely impaired. Despite the logic of this approach and encouraging anecdotal reports, the chronic safety and efficacy of ketogenic diets have not been evaluated in a controlled manner.

Coenzyme Q10 (CoQ10) is another attractive candidate for use in conjunction with DCA. CoQ10 is a common component of nutriceutical "cocktails" frequently administered to patients with presumed or proven mitochondrial diseases and appears to be generally well-tolerated. The rationale for considering CoQ10 treatment of patients with genetic mitochondrial disorders is predicated on the following facts: 1) primary CoQ10 deficiency is a very rare cause of mitochondrial disease and anecdotal reports indicate that oral replacement therapy is effective; 2) CoQ10 is required for efficient transfer of electrons from complexes I and II to complex III of the respiratory chain; and 3) CoQ10 may act as an antioxidant to scavenge free radicals generated by a respiratory chain damaged by mutations in one or more complexes.

To our knowledge, the combination of DCA and CoQ10 as treatment for genetic mitochondrial diseases has not been studied. There is no a priori basis for assuming that patients with PDH deficiency suffer from a secondary deficiency in the availability or action of CoQ10. Indeed, preliminary evidence obtained in cultured fibroblasts from patients with E1α deficiency suggest that excess accumulation of reactive oxygen species may not be a feature of the disease [30]. This might be expected, given the inhibition of PDH and resultant depression of TCA cycle (and, by inference, respiratory chain) activity expressed by these cells [31]. New information about the therapeutic role of CoQ10 should be gained from the first phase 3 trial of CoQ10 in children with genetic mitochondrial diseases that is currently underway [32].

The PDH complex is an attractive target for gene therapy because of its central role in cellular energetics and the fact that all its components are encoded by nuclear DNA [33]. Thus, evolutionary mechanisms exist for importing each component into mitochondria. Adeno-associated virus (AAV) is a nonpathogenic parvovirus to which most humans have been exposed. Recombinant AAV vectors have been successfully used to transduce a variety of genes in several different cell types in vitro and the ability of these vectors to medicate persistent transgene expression has been validated in vivo in a

Table 7
Summary data on patients with PDH complex deficiency treated with DCA

Indices	Result	Number of patients[a]
Baseline		
Gender (M/F)	15/25	40
Median age (months)	17	38
E1α deficiency	22	46
Residual PDH activity (% normal)	30 ± 19	28
Blood lactate (mmol/l)	5 ± 4	33
CSF lactate (mmol/l)	6 ± 4	23
DCA		
Dose (mg/kg)	42 ± 4	46
Dose (range)	10d–9y9m	26
Biochemical effects		
Blood lactate (% decrease)	36 ± 26	22
CSF lactate (% decrease)	33 ± 25	11
Adverse events (# patients)		46
Deaths	6	
Abnormal liver function	3	
Worsened peripheral neuropathy	4	
Changes in motor function	4	
Tremors	2	

Data from Ref. [28].
[a] Number of patients in whom a result was reported expressed as a mean ± standard deviation.

Table 8
Effect of scAAV serotype vectors 2 and 6, with and without DCA, on PDH activity in fibroblasts

Cell line	% of parallel control				
	w/AAV2-PDH	w/AAV6-PDH	w/DCA	w/DCA+AAV2-PDH	w/DCA+AAV6-PDH
Control 1	9	−3	36	46	38
Control 2	−4	8	32	40	44
Control 3	12	22	42	50	45
Patient 1	78	73	44	91	86
Patient 2	48	63	59	81	80
Patient 3	8	7	28	58	47

Skin fibroblasts from three controls and three patients with PDH E1α deficiency were cultured with or without scAAV2-PDH/or scAAV6-PDH transduction for 10 days at 1000 vector particles per cell and with or without 5 mM DCA added at day nine for 24 h. Cells were harvested and total PDH activity was determined ( ). Negative percentages refer to values of enzyme activity in transduced cells that were less than respective non-transduced controls. Abbreviations are: AAV, adeno-associated virus (serotype 2 or 6); DCA, dichloroacetate; PDH, pyruvate dehydrogenase.

number of mammalian species, including humans. AAV-mediated human gene transfer has been used in early phase clinical trials for several diseases, including cystic fibrosis [34], α 1 anti-trypsin deficiency [35] and lipofucinosis [36]. We recently demonstrated that self-complementary adeno-associated virus (scAAV) can deliver and express the PDH E1α subunit gene in primary cultures of skin fibroblasts from both healthy control subjects and patients who have E1α deficiency due to defined mutations in the subunit [37]. Maximum transduction efficiency was achieved using scAAV2 and scAAV6 serotypes. Both these vectors increased E1α expression 40–60% in control and patient cells and increased PDH activity in two of these patient cell lines. Subsequent exposure of the transduced fibroblasts to 5 mm DCA for 24 h increased PDH activity up to 91% of the activity measured in untreated control cells [Table 8]. In addition, DCA increased the expression of the E1α protein and, to variable extents, that of the other components of the PDH complex in both transduced and non-transduced cells [Fig. 8]. This latter finding was not entirely unexpected, because earlier studies had suggested that short-term exposure of rats [38] or cultured human fibroblasts [39] somehow stabilizes the E1α protein and reduces its turnover. The combined effects of AAV-mediated transduction followed by DCA resulted in additive or supra-additive effects on PDH activity in the congenitally enzyme deficient cells.

These results beg the question whether combined gene and pharmacological therapy holds promise for treating patients with E1α deficiency and perhaps other mutations in the PDH complex. They also emphasize the importance of extending such preliminary findings to an animal model of PDH deficiency. In this regard, a transgenic murive model of E1α deficiency has been described [40] in which the defect is lethal to male mouse embryos but gives rise to viable female offspring that exhibit developmental delay and other neurological defects[40,41]. This and future animal models may provide exceptional opportunities to determine the feasibility of

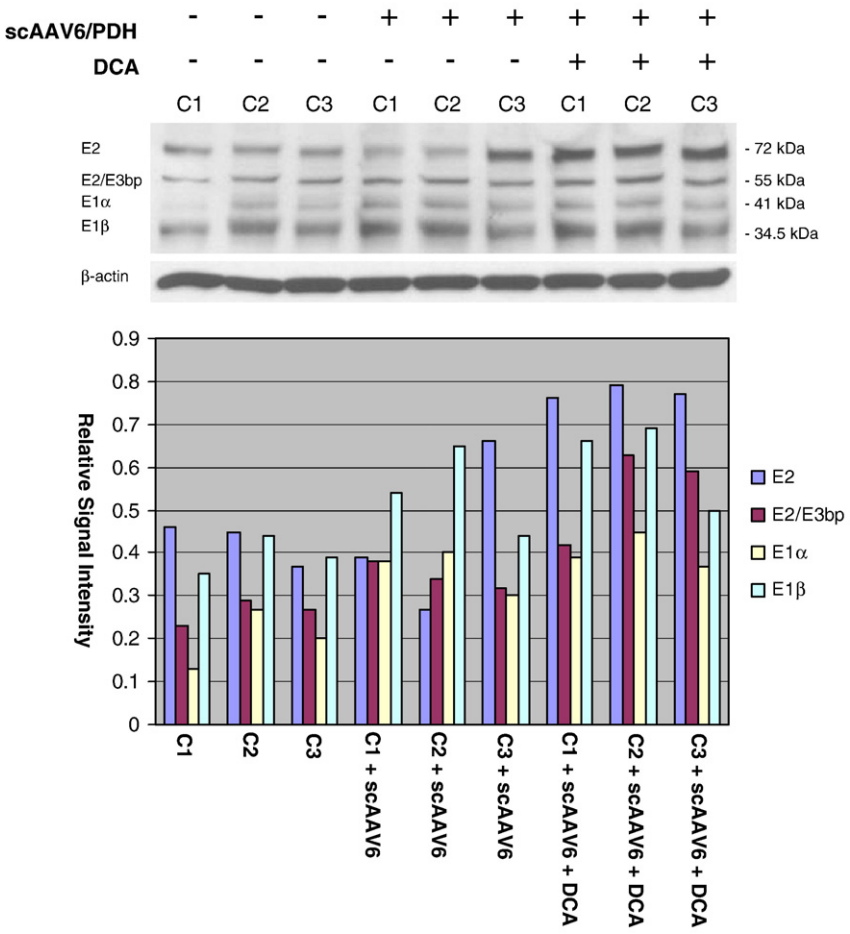

Fig. 8. Effect of scAAV serotype 6, with or without DCA, on expression of PDH complex proteins. Top panel: Immunoblot shows relative changes in steady-state levels of PDH E1α, E1β, E2 and E2/E3bp after AAV vectors delivery and/or combination of DCA administration. Cell lysates (15 μg) from three healthy subjects (C1, C2 and C3) were separated on 10% SDS-PAGE gels and immunoblotted with monoclonal Abs against E1α, E1β, E2 and E2/E3bp. Bottom panel: Quantitative analysis of immunoblot data. Using ImageJ the pixel densities in each band (E2, E2/E3bp, E1α and E1β) from the gel were quantitated and the amount of protein was standardized to the amount of β-actin (E2, E2/E3bp, E1α and E1β/β-actin) in each lane, respectively. Data are means of 2 independent experiments.

novel therapeutic approaches for PDH deficiency prior to their ultimate evaluation in affected humans.

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