available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Evaluation of dichloroacetate treatment in a murine model of hereditary tyrosinemia type 1

Chantale Langlois [a], *Rossana Jorquera* [a,✠], *Milton Finegold* [b], *Albert L. Shroads* [c], *Peter W. Stacpoole* [d], *Robert M. Tanguay* [a,*]

[a] *Laboratory of Cellular and Developmental Genetics, CREFSIP, Department of Medicine, University Laval, Que., Canada G1K 7P4*
[b] *Department of Pathology, Texas's Children Hospital, Houston, TX 77030, USA*
[c] *College of Pharmacy, Division of Endocrinology and Metabolism, Department of Medicine, College of Medicine, University of Florida, Gainesville, FL 32610, USA*
[d] *College of Pharmacy, Department of Medicine, Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, FL 32610, USA*

ARTICLE INFO

Article history:
Received 1 December 2005
Accepted 22 February 2006

Keywords:
Hereditary tyrosinemia type 1
Fumarylacetoacetate hydrolase
Dichloroacetate
Maleylacetoacetate isomerase
NTBC

Abbreviations:
HTI, hereditary tyrosinemia type 1
DCA, dichloroacetate
MAAI, maleylacetoacetate isomerase
FAH, fumarylacetoacetate hydrolase
NTBC, 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione
MAA, maleylacetoacetate
FAA, fumarylacetoacetate
MA, maleylacetone
FA, fumarylacetone

ABSTRACT

Hereditary tyrosinemia type 1 (HT1) is an autosomal recessive disease severely affecting liver and kidney and is caused by a deficiency in fumarylacetoacetate hydrolase (FAH). Administration of 2-(2-nitro-4-trifluoro-methylbenzyol)-1,3 cyclohexanedione (NTBC) improves the HT1 phenotype but some patients do not respond to NTBC therapy. The objective of the present study was to evaluate whether administration of dichloroacetate, an inhibitor of maleyl acetoacetate isomerase (MAAI) to FAH-knockout mice could prevent acute pathological injury caused by NTBC withdrawal. DCA (0.5 and 5 g/L) was given in combination with a standard diet or with a tyrosine-restricted diet. With the low-tyrosine diet body weight loss and most of hepatic and renal injuries were prevented regardless the DCA dose. The administration of DCA with a standard diet did not prevent damage nor the oxidative stress response nor the AFP induction seen in FAH-knockout mice. DCA was shown to inhibit hepatic MAAI activity to 86% (0.5 g/L) and 94% (5 g/L) of untreated wild-type mice. Interestingly, $FAH^{-/-}$ mice deprived of NTBC (NTBC-OFF) and NTBC-treated FAH-knockout mice had similar low hepatic MAAI activity levels, corresponding to 10–20% of control. Thus the failure of DCA treatment in $FAH^{-/-}$ mice seems to be attributed to the residual MAAI activity, high enough to lead to FAA accumulation and HT1 phenotype.

* *Corresponding author*. Tel.: +1 418 656 3339; fax: +1 418 656 5036.
E-mail address: robert.tanguay@rsvs.ulaval.ca (R.M. Tanguay).

✠ This paper is dedicated to the memory of Dr. Rossana Jorquera who died on 18th January 2006.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.02.017

SAA, succinylacetoacetate
SA, succinylacetone
δ-ALAD, δ-aminolevulinic acid dehydratase
δ-ALA, δ-aminolevulinic acid
GSH, reduced glutathione
GSSG, oxidized glutathione
HPPD, 4-hydroxyphenylpyruvate dioxygenase
AP, alkaline phosphatase
GGT, γ-glutamyl-transferase
GC–MS, gas chromatography–mass spectrometry
γ-GCS, gamma-glutamyl cysteine synthetase
MAT, methionine adenosyl transferase
NMO, NAD(P)H:quinone oxidoreductase 1
HO-1, heme oxygenase 1
DTNB, 5,5′-dithiobis (2-nitrobenzoic acid)
NADPH, nicotinamide adenine dinucleotide phosphate (reduced form)
HGA, homogentisate

1. Introduction

The most severe disease of the tyrosine catabolic pathway (Fig. 1) is hereditary tyrosinemia type I (HT1, OMIM 276700), an autosomal recessive disease characterized by progressive liver and kidney damage and neurological crisis [1–3]. In the acute form, symptoms appear in early childhood and are characterized by a rapid deterioration of hepatic and renal functions, leading to death during the first year because of liver failure. The chronic form includes symptoms such as progressive hepatic dysfunction during infancy and death from liver cirrhosis or hepatocarcinoma [4]. Additional features of the disease include renal dysfunction and severe neurological crisis [5].

HT1 is caused by the deficiency of fumarylacetoacetate hydrolase (FAH, EC 3.7.1.2) the last enzyme of the tyrosine catabolic pathway [6–8]. FAH catalyses the hydrolysis of fumarylacetoacetate (FAA) into fumarate and acetoacetate. A deficiency in FAH causes accumulation of FAA, maleylacetoacetate (MAA), succinylacetone (SA) and tyrosine (Fig. 1). The metabolite FAA has been proposed to be the main effector of the disease. Mutagenic, cytostatic and apoptogenic activities of FAA, but not MAA nor SA, have been demonstrated using V79 Chinese hamster cells [9–11]. The mutagenicity of FAA in vivo has also been inferred from studies in a mouse model of HT1 [12] and FAA may also be involved in development of hepatocellular carcinoma in HT1. Other metabolites of tyrosine breakdown may also play a pathogenic role in HT1. SAA and SA are the main FAA-derived metabolites in HT1 (Fig. 1), and an accumulation of SA after reduction of SAA can cause porphyria-like neurological crisis by inhibition of δ-aminolevulinic acid dehydratase (δ-ALAD, EC 4.2.1.24) [5]. Prevention of FAA accumulation should have beneficial therapeutic effects in HT1. A dietary restriction in tyrosine and phenylalanine is given to HT1 patients to limit the accumulation of the toxic metabolite FAA, but liver transplantation [13] remains the ultimate treatment for the prevention of the acute phenotype and hepatocarcinoma. However, this alternative is problematic because of poor organ availability and risks of complications [14,15]. Since 1992, drug therapy with 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC), an inhibitor of hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.7) [16,17] (Fig. 1), is used for treatment in HT1 patients [16,18]. NTBC rapidly improves liver and kidney functions but does not completely prevent the formation of hepatocarcinoma in long-term therapy [19,20]. NTBC has also been administered to FAH-knockout mice to prevent neonatal death but hepatocarcinoma was observed in 50% of NTBC-treated mice at 10 months of age [21]. Despite the beneficial clinical effects of NTBC, the occurrence of hepatocarcinoma in HT1 emphasizes the importance of the study of HT1 for improving treatment of this disease. As an alternative approach, we evaluated whether chemical inhibition of maleylacetoacetate isomerase (MAAI, EC 5.2.1.2, Fig. 1), another enzyme of the tyrosine degradation pathway acting upstream of FAH, could replace or supplement NTBC treatment. We hypothesized that inhibition of MAAI with dichloroacetate (DCA) would prevent the accumulation of FAA and might ameliorate the acute phenotype of HT1.

DCA is a major by-product of water chlorination and is also used as an investigational drug for several acquired and

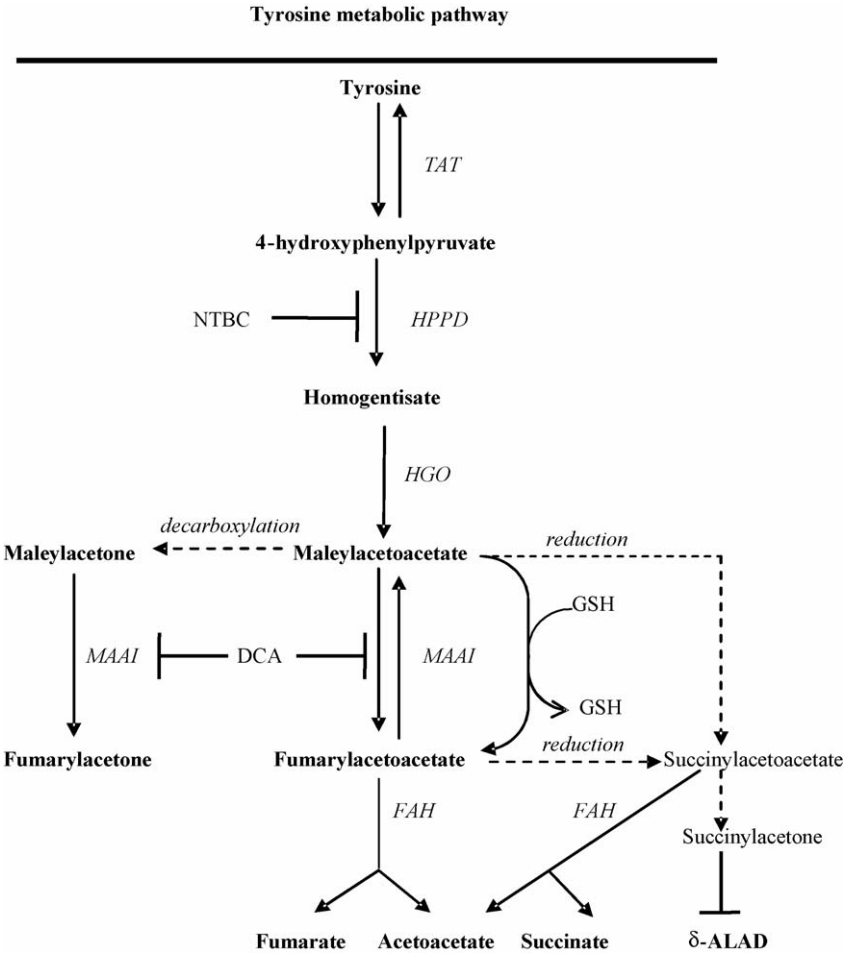

Fig. 1 – The five enzymatic reactions of the tyrosine catabolic pathway. The GSH-dependent non-enzymatic bypass for MAA transformation in FAA [48] is also indicated. δ-ALAD is inhibited by succinylacetone causing δ-ALA accumulation and inhibition of heme biosynthesis. Doted line represents a pathway with no known enzyme. TAT: tyrosine aminotransferase; HPPD: 4-hydroxyphenylpyruvate dioxygenase; HGO: homogentisate dioxygenase; MAAI: maleylacetoacetate isomerase; FAH: fumarylacetoacetate hydrolase; NTBC: 2-(2-nitro-4-trifluoromethylbenzoyl)1,3-cyclohexanedione; DCA: dichloroacetate; δ-ALAD: δ-aminolevulinic acid dehydratase; δ-ALA: δ-aminolevulinic acid.

congenital metabolic diseases [22,23]. DCA has also been proposed as a neuroprotective agent in experimental models of cerebral ischemia [24]. DCA is mainly biotransformed to glyoxylate by gluthatione S-transferase (GSTZ) [25,26] in a GSH-dependent manner. GSTZ is identical to MAAI [27], which catalyses isomerisation of MAA into FAA (Fig. 1). It has also been demonstrated in vitro that DCA inhibits the enzyme GSTZ in a GSH-dependent and irreversible manner [26,28,29]. DCA inhibits its own metabolism and the tyrosine degradation pathway (Fig. 1). MAAI activity is reduced in a time- and dose-dependent manner by DCA in mice [30] and rats [28,29,31]. Enzyme activity was virtually eliminated with a single dose of 1 g/kg/day DCA in rats [31]. A maximal inhibition of 95% of MAAI activity has been observed in rats after a 5-day administration of 0.3 mmol DCA/kg/day [29]. Protein levels of MAAI are also reduced by DCA in rats with a dose >4 mg/kg/day [32] and with 0.3 mmol/kg [29]. A study by Tzeng et al. [28] reported a reduction of MAAI activity in human liver cytosol with 0.5 mM DCA, but another study [31] showed no significant inhibition of the enzyme. Hepatocarcinogenicity of DCA (80 mg/kg/day) was reported in rats [33,34] and in mice [34–40]. However, hepatocarcinogenicity of DCA in humans has not been demonstrated [41,42]. Based on the ability of DCA to inhibit MAAI, we evaluated whether it could be used as an alternative treatment for HTI using the FAH-knockout mouse model.

2. Materials and methods

2.1. Chemicals

DCA (sodium salt) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and dissolved in distilled water at 0.5 g/L (~0.08 g/kg/day) and 5 g/L (~0.8 g/kg/day) (pH 5). NTBC kindly provided by S. Lindstedt (Gothenburg University, Sweden) was solubilized in water (7.5 mg/L, pH 7). Protein assay reagents and SDS-Page molecular weight standards (low range) were purchased from Bio-Rad (Hercules, CA). For the glutathione determination assay, reduced glutathione

(GSH), oxidized glutathione (GSSG), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH), 5-sulfosalicylic acid, triethanolamine and glutathione reductase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2-Vinylpyridine was purchased from Aldrich Chemical Co. For MAAI activity determination, ascorbic acid and homogentisate (HGA) were purchased from Sigma Chemical Co. and ferrous sulfate, from BDH Co. (Toronto, Canada).

2.2. Animals and treatment

Three-month old male FAH-knockout ($FAH^{\Delta exon5}$, referred to here as $Fah^{-/-}$ mice) was used and was genotyped by PCR as described in [43] using 50 ng of DNA isolated from mice hair bulbs [44]. Pregnant females and newborn $Fah^{-/-}$ mice were treated with NTBC in drinking water until the beginning of the experiments. A first group of $Fah^{-/-}$ mice received ad libitum a standard rodent chow diet (Charles River Rodent, Agribrands St. Hubert, QC, Canada) containing 0.51% tyrosine and 0.82% phenylalanine. Mice were withdrawn from NTBC and instead given 0.5 g/L DCA (0.08 g/kg/day; DCA 0.5 group; $n = 6$) or 5 g/L DCA (0.8 g/kg/day; DCA 5 group; $n = 8$). As control groups, five $Fah^{-/-}$ mice received NTBC (NTBC group), nine $Fah^{-/-}$ mice were taken off NTBC (NTBC-OFF group), and four $Fah^{+/+}$ mice (untreated group) given water. DCA (0.5 g/L) and (5 g/L) was administered to three and four $Fah^{+/+}$ mice, respectively. A second similar experiment was done but this time with a low-tyrosine diet (Harlan TD 96105, Wilmington, DE, USA) containing no tyrosine and 0.3% phenylalanine. All mice were weighed three times per week, examined for hair appearance, corneal opacification and general motility to evaluate their health status, and were sacrificed after 5 weeks of treatment. Mice were anaesthetized by intraperitoneal injection of ketamine–xylazine (0.2 mL/10 g body weight) and sacrificed by cardiac puncture. Blood, urine, liver and kidney were collected.

2.3. Histology

At sacrifice, 1 mm slices of liver and kidney were cut for histology. The remaining tissues were frozen at −80 °C. Liver and kidney slices were fixed in 10% PBS-buffered formaldehyde, pH 7.4, for a period of 24 h and kept in ethanol 80% at 4 °C after dehydration. Tissues were embedded in paraffin wax at 58 °C and 4 μm sections were rehydrated and stained with hematoxylin-eosin and with a polyclonal rabbit antibody to rat FAH [7,45]. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and methanol. Avidin and biotin pretreatment was used to prevent endogenous staining. The secondary antibody was biotinylated goat anti-rabbit IgG (BA-1000; VectorLabs, Burlingame CA, USA). Color development was performed with the AEC detection kit from Ventana Medical Systems (Tucson, AR, USA).

2.4. Biochemical analysis

Blood was collected at the time of sacrifice and put in serum separator tubes (Microtainer Brand; Becton Dickinson, New Jersey, USA), which contain a polymer barrier material allowing separation of serum and cells during centrifugation (5000 rpm for 10 min). Serum levels of alkaline phosphatase (AP), γ-glutamyl-transferase (GGT), urea, glucose and creatinine were quantified by using an automated analyzer. Serum levels of succinylacetone were measured by the δ-aminolevulinate dehydratase (δ-ALAD) inhibition assay [46].

Urine was collected weekly from each mouse in a group, pooled and kept at −80 °C until analysis. DCA and its metabolites as well as tyrosine metabolites (maleylacetone, MA; fumarylacetone, FA; SA, fumarate, acetoacetate) and lactate were measured by gas chromatography–mass spectrometry (GC–MS) as described [47].

2.5. Immunoblotting

Liver and kidney were homogenized (10% w/v) in phosphate buffer, pH 7.4 and were centrifuged 30 min at 10,000 rpm. Supernatants were collected and protein concentrations determined using the Bio-Rad protein assay (Biorad, Hercules, CA). The rest of the homogenates were frozen at −80 °C for further analysis. Proteins (20 μg) were separated on a 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane (Bio-Trace, Pall Gelman Laboratory; Pensacola, FL) and membranes blocked in a tris-buffered saline solution containing 0.1% Tween 20 and 5% non-fat dry milk for 1 h. Coomassie blue staining of gels was used as a protein loading control. Primary rabbit polyclonal antibodies raised against enzymes implicated in tyrosine metabolism were anti-MAAI, anti-HPPD and anti-homogentisate dioxygenase (HGO) (made in our lab from fused cloned protein in the pET30 vector (Novagene, Madison, WI). Other primary rabbit polyclonal antibodies were anti-α-fetoprotein (AFP, a gift from Dr. L. Bélanger, CRHQC, QC, Canada), anti-gamma-glutamyl cysteine synthetase (γ-GCS, catalytic subunit; Neo Markers, Fremont, CA), anti-methionine adenosyl transferase I/III (MAT; a gift from Dr. Jose Mato, University de Navarra, Spain), and anti-heme oxygenase 1 (HO-1, Stressgen, Victora, CA). Primary mouse monoclonal antibodies against NAD(P)H:quinone oxidoreductase 1 (NMO, a gift from Dr. D. Siegel, University of Colorado, Denver, CA) was also used. As secondary antibodies, goat anti-rabbit peroxidase (Jackson ImmunoResearch Lab, West Grove, PA) or anti-mouse (Molecular Probes, Eugene, OR) coupled to horseradish was used. Incubations with different antibodies were followed by several washes in tris-buffered saline solution containing 0.1% Tween 20. The detection of proteins was done by using a chemiluminescence kit (Perkin-Elmer; Boston, MA) on X-ray films (Fuji Photo Film Co., Tokyo, Japan). Films were scanned and signal intensities estimated using NIH Image software.

2.6. MAAI activity

MAAI activity in mouse liver was determined by a spectrophotometric assay [48]. Transformation of HGA to MAA was started with the addition of 50 μg of extract of *E. coli*-expressed HGO to the reaction cuvette containing 2 mM ascorbate, 50 μM ferrous sulfate and 50 μM of GSH in a phosphate 10 mM buffer (pH 7.4). The reaction was followed at 330 nm. When a plateau was achieved, 25 μg of purified FAH (recombinant His-tag fused protein expressed in *E. coli* synthesized in our lab) and

400 μg of cytosolic proteins, obtained after centrifugation of a 10% homogenate at 10,000 rpm, were added to the reaction cuvette (1 mL final volume). Transformation of MAA in FAA, catalyzed by MAAI, was followed at 330 nm. MAA concentration was measured by its absorbance coefficient (13,500/M/cm). Adding 50 μM of GSH to the cuvette should not affect the MAAI activity since the GSH concentration of liver cells is 5 mM.

2.7. Glutathione determination

For total glutathione determination (GSH and GSSG), livers were homogenized (10% w/v) in a 3% 5-sulfosalicylic acid buffer for deproteinization [49]. One microliter of liver homogenate was incubated with 0.21 mM NADPH, 0.6 mM DTNB and 100 mM triethanolamine in a final volume of 1 mL of stock buffer containing 125 mM sodium phosphate pH 7.5 and 6.3 mM Na_4-EDTA. Ten units of glutathione reductase were added to the cuvette and 5-thio-2-nitrobenzoic acid formation was followed by absorbance measurements at 412 nm. GSSG was determined specifically by a different assay [49]. Liver homogenate (10 μL) was added to 400 mM triethanolamine and 5 μL of 2-vinylpyridine was added to the tube to prevent GSH oxidation. Tubes were mixed and kept at room temperature for 1 h. Following the incubation, 0.2 mM of NADPH and 0.6 mM of DTNB were added to the mixture. Zero was set and 10 units of glutathione reductase were added to initiate the reaction. The reaction was followed at 412 nm. Total glutathione, GSH and GSSG contents were determined by comparison to standard curves generated with known amounts of GSH or GSSG.

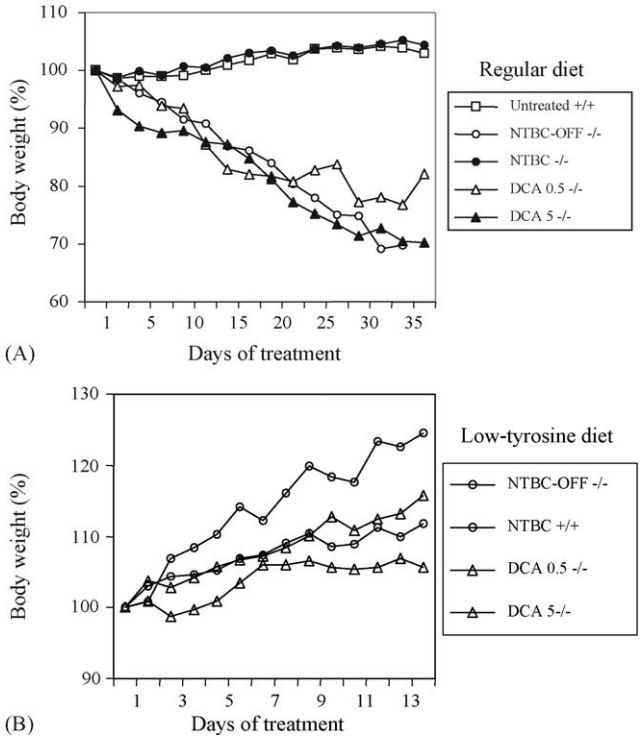

Fig. 2 – Body weight curves (%) for DCA-treated FAH-knockout mice fed with a regular (A) or tyrosine-low diet (B) over 35 days. Mice were weighed three times a week. The untreated group (□) are $Fah^{+/+}$ mice and other groups are $Fah^{-/-}$ mice (NTBC-OFF (○), NTBC (●), DCA 0.5 (△), DCA 5 (▲)).

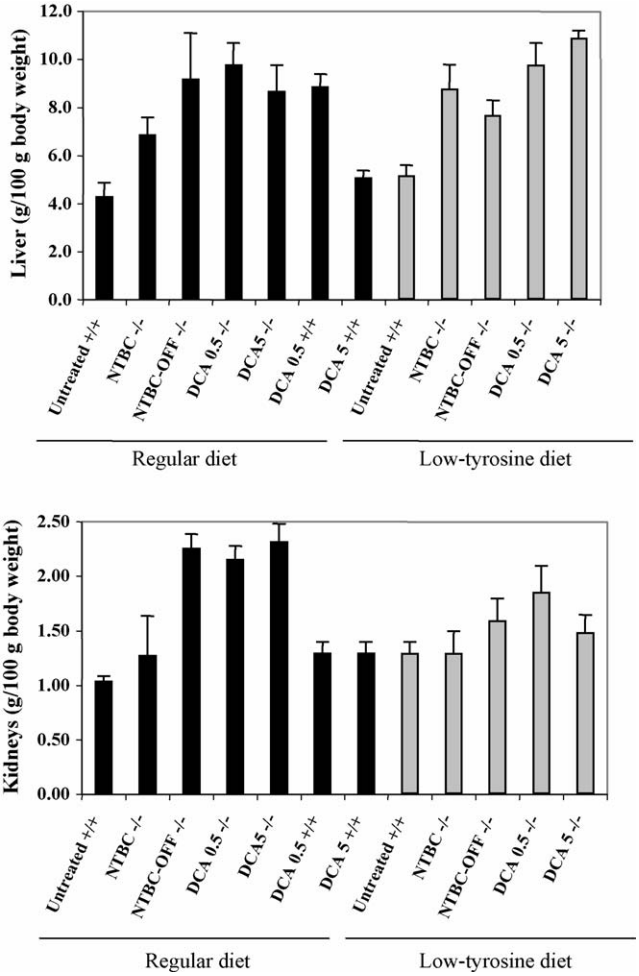

Fig. 3 – Relative liver (left) and kidneys (right) weight (g/100 g body weight) for male FAH wild type and knockout mice fed on a regular- or a low-tyrosine diet during 35 days.

3. Results

3.1. DCA does not prevent body weight loss in FAH-knockout mice after NTBC withdrawal

When NTBC treatment is discontinued, $Fah^{-/-}$ mice begin to show a progressive decrease in body weight [21]. At the end of the experiment (day 35), $Fah^{-/-}$ mice deprived of NTBC (NTBC-OFF) on a regular diet had lost 30% of body weight (Fig. 2A). The DCA 5 group displayed similar body weight loss. The DCA 0.5 group displayed a final loss of 20% body weight but all mice from this group gradually regained body weight mostly during the last week of the treatment perhaps as a result of injured liver. At higher doses of DCA (10 g/L), $Fah^{-/-}$ mice became ill and died before the end of the experiment (data not shown). In

comparison, all mice on a low-tyrosine diet (NTBC, NTBC-OFF, DCA 0.5, DCA 5) gained 5–25% of body weight during the same treatment period (Fig. 2B).

3.2. DCA does not prevent hepatic and renal damage after NTBC discontinuation

FAH is mainly expressed in liver and kidney both of which show tissue damage [7,16,50,51]. Macroscopic (Fig. 3) and microscopic evaluations (summarized in Table 1 and illustrated in Fig. 4) were done on these tissues. NTBC-OFF group showed high relative liver weight (g liver/g body weight) regardless of the diet. DCA treatment did not prevent this high relative liver weight. $Fah^{+/+}$ mice treated with DCA showed a similar increase in relative liver weight.

Microscopically, the NTBC-OFF group showed abnormal hepatic features with diffuse necroinflammation and focal bile duct proliferation (Fig. 4 C) compared to the untreated mouse control group ($Fah^{+/+}$ mice). NTBC-treated $Fah^{-/-}$ mice showed a few small foci of necroinflammation, minimal focal micro- and macrosteatosis and minimal nuclear pleomorphism (Fig. 4B). The DCA 0.5 treated group displayed milder damage than the other groups. The intensity of damage in the DCA 5 g/L was similar to that observed in the NTBC-OFF group (Fig. 4D). Administration of DCA (5 g/L) also caused hepatic abnormalities in $Fah^{+/+}$ mice (Fig. 4A). In contrast, all groups (including those treated with DCA) fed with a low-tyrosine diet showed a lower degree of liver injury than those treated and fed the regular diet.

Kidneys of $Fah^{-/-}$ mice deprived of NTBC and fed with a regular diet were generally swollen, pale and showed a high relative weight compared to untreated $Fah^{+/+}$ mice or NTBC-treated $Fah^{-/-}$ mice (Fig. 3). Similar macroscopic alterations were observed in kidney of DCA-treated $Fah^{-/-}$ mice (0.5 and 5 g/L), but not in DCA-treated $Fah^{+/+}$ mice. All mice on the low-tyrosine diet, including those treated with DCA (0.5 and 5 g/L), showed normal or reduced kidney size. Results from the histological analysis of the kidneys are summarized in Table 1 and illustrated in Fig. 5. NTBC treatment had a beneficial effect on size and visual appearance of the kidneys as kidneys of the NTBC-treated $Fah^{-/-}$ mice were normal. NTBC discontinuation induced rare foci of tubular injury (NTBC-OFF; Fig. 5A) and administration of DCA (0.5 or 5 g/L) did not prevent renal damage (Fig. 5B). A low-tyrosine diet significantly improved kidneys histology regardless of the treatment.

3.3. DCA-treated $Fah^{-/-}$ mice do not show improvement of hepatic and renal functions

To determine whether DCA could prevent the development of the biochemical HT1 phenotype, several markers were analyzed in the serum and urine of $Fah^{-/-}$ mice. These results are summarized in Table 2. Liver function was assessed by serum GGT, AP, glucose and urea levels. Renal function was

Table 1 – Liver and kidneys histological findings

Diet	FAH genotype	Nb of mice	Treatment	Liver histology	Kidney histology
Reg	+/+	4	Untreated	Normal with small foci of inflammation	Normal
Reg	−/−	6	NTBC	Small foci of necroinflammation, moderate micro and macrosteatosis and nuclear pleomorphism	Normal
Reg	−/−	9	NTBC-OFF	Diffuse necroinflammation and focal bile duct proliferation	Rare foci of tubular injury, nuclear size and chromatin variation with cytoplasmic vacuoles
Reg	−/−	3	DCA 0.5	Diffuse hepatocellular injury (milder than NTBC-OFF on regular diet)	Proximal tubular vacuolization, focal anisocytosis and protein casts
Reg	−/−	6	DCA 5	Diffuse hepatocellular swelling, massive necrosis and abundant glycogen	Focal tubular dilatation, proximal tubular vacuoles, single cell necrosis and nuclear variation
Reg	+/+	3	DCA 0.5	Normal	Normal
Reg	+/+	4	DCA 5	Hepatocellular swelling and small foci of inflammation	Normal
Low	+/+	3	Untreated	Normal	Normal
Low	−/−	4	NTBC	Normal with mild focal of inflammation	Proximal tubular vacuolization
Low	−/−	4	NTBC-OFF	Abundant glycogen, focal steatosis, anisocytosis and diffuse mild hepatocellular swelling (less injury than NTBC-OFF on regular diet)	3 mice on 4 were normal; one mouse has tubular vacuolization
Low	−/−	3	DCA 0.5	Extensive bile duct proliferation and diffuse mild hepatocellular swelling	Tubular vacuolization
Low	−/−	3	DCA 5	Hepatocellular swelling, rare focal necrosis and inflammation	Tubular vacuolization

Note: regular diet is indicated by Reg and low-tyrosine diet, by Low.

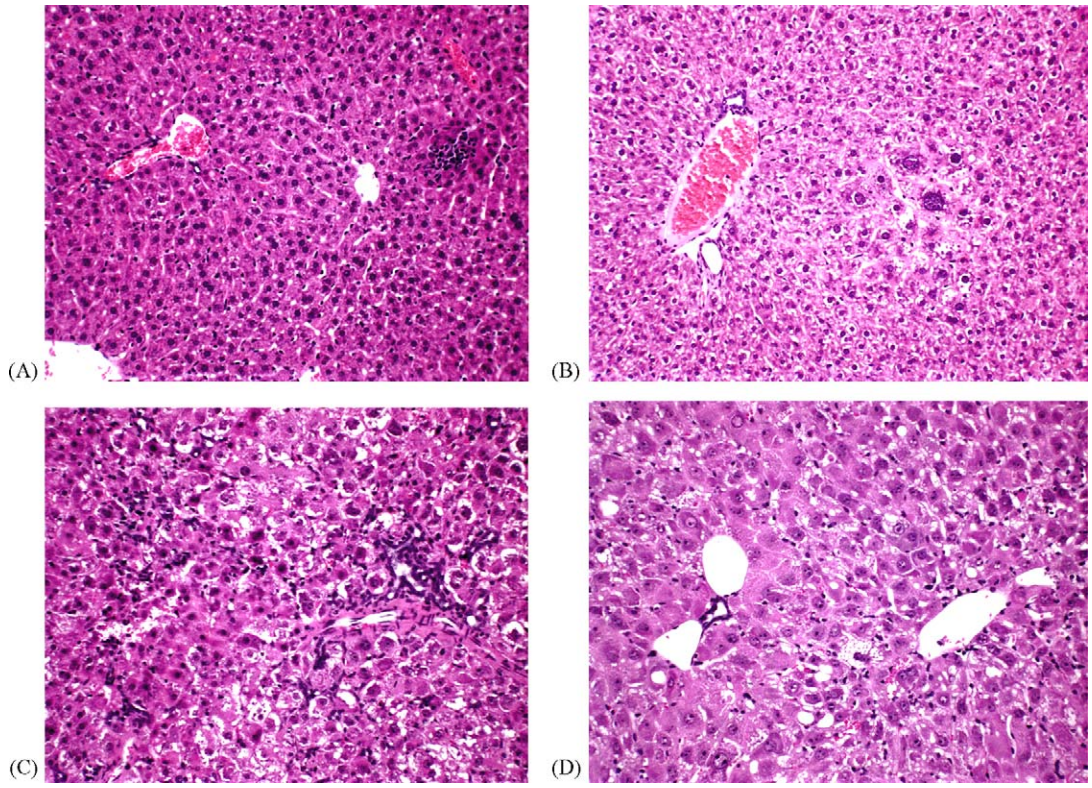

Fig. 4 – Liver histology for $Fah^{-/-}$ mice compared to $FAH^{+/+}$ mice under regular diet (200×). Panels are liver sections of untreated $Fah^{+/+}$ (A), NTBC-treated $Fah^{-/-}$ mice (B), NTBC-OFF-treated mice $Fah^{-/-}$ (C) and DCA (5 g/L)-treated $Fah^{-/-}$ mice (D). Microscopic analysis of untreated $Fah^{+/+}$ liver is shown as control and is normal. Liver section of NTBC-OFF mice displays diffuse necroinflammation and focal bile duct proliferation. Liver section of DCA (5 g/L)-treated $Fah^{-/-/-}$ mouse shows diffuse hepatocellular swelling with massive necrosis.

assessed by serum creatinine. Tyrosine and SA, a hallmark metabolite of HT1, were measured to assess the function of the tyrosine degradation pathway.

NTBC-OFF and DCA (0.5 and 5 g/L) $Fah^{-/-}$ mice had higher GGT levels than untreated $Fah^{+/+}$ and $Fah^{-/-}$ mice on NTBC. However, GGT levels were four-fold (DCA 0.5) and 2.5-fold higher (DCA 5) than in NTBC-OFF mice, indicating a higher degree of liver damage (Table 2). Levels of AP in DCA 0.5 and DCA 5 groups were also higher than in NTBC-OFF mice (1079 and 1215 U/L, respectively, versus 540 U/L). Low levels of glucose are usually found in HT1 [3] and hypoglycemia was also noted in NTBC-OFF and DCA-treated (0.5 and 5 g/L) mice. Levels of urea were 2.5-fold lower for the NTBC-OFF group compared to controls on regular diet. That result suggests a hepatic cause of the abnormal cycle of urea. DCA administration increases levels of urea to 3.9 mmol/L (DCA 0.5 g/L) and 4.2 mmol/L (DCA 5 g/L). Creatinine levels were normal for the majority of $Fah^{-/-}$ and $Fah^{+/+}$ mice. Only the DCA 5 group presents a slight increase in creatinine, indicative of renal damage most likely of glomerular origin. Levels of SA were 0.20 μmol/L for NTBC-OFF mice compared to 0.30 μmol/L for DCA 5 mice. Tyrosine levels in the NTBC-OFF group were nine-fold higher than controls ($Fah^{+/+}$ mice). Tyrosine levels were higher in the DCA groups than in the NTBC-OFF $Fah^{-/-}$ mice. The low-tyrosine diet prevented elevations of tyrosine. This diet improved the levels of most biochemical markers evaluated here (phosphate, GGT, urea, tyrosine and glucose) in comparison with the groups fed a regular diet (Table 2).

3.4. *DCA does not prevent the oxidative stress response and AFP induction in the liver*

As shown in the western blot of Fig. 6A, the liver of NTBC-OFF $Fah^{-/-}$ mice fed with the regular diet showed a striking oxidative stress response, as assessed by an increase in the protein levels of NMO-1, HO-1 and γGCS. NMO-1 is a phase II-detoxification enzyme and marker of oxidative burst, which has already been reported to be induced in murine HTI models [2,52,53]. The anti-NMO antibody used here recognized two NMO isoenzymes, the slower migrating band corresponding to the inducible NMO-1 isoform, which is only detected in the liver of NTBC-OFF and DCA (0.5 or 5 g/L)-treated $Fah^{-/-}$ mice fed with the regular diet (Fig. 6A). HO-1 catalyzes the conversion of heme to bilirubin and is inducible by oxidative stress as a protective mechanism (for a review [54]). γGCS catalyzes the first step of GSH synthesis, and is inducible due to excessive GSH consumption or oxidation. This oxidative stress response was induced to the same extent in $Fah^{-/-}$ mice treated with DCA (0.5 or 5 g/L) and fed with the regular diet (Fig. 6A).

In contrast, neither NMO-1 nor HO-1 was induced in the liver of NTBC-OFF or DCA-treated $Fah^{-/-}$ mice fed with the low-tyrosine diet, although slightly elevated γGCS protein

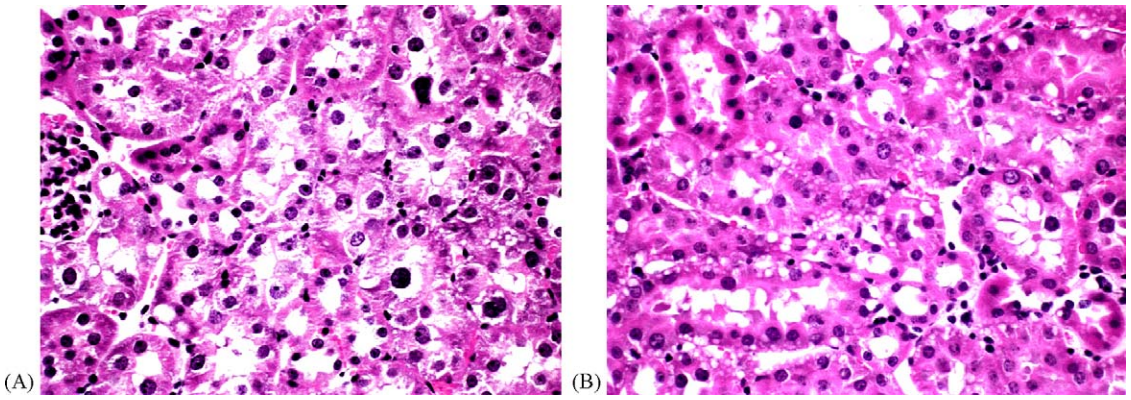

Fig. 5 – Histology of kidneys of NTBC-OFF $Fah^{-/-}$ mice (A) compared to DCA (5 g/L)-treated $Fah^{-/-}$ mice (B) (400×). The kidney section of the NTBC-OFF mice displays nuclear size and chromatin variation with tubular vacuolization. The kidney section of the DCA (5 g/L)-treated $Fah^{-/-}$ mice shows focal single cell necrosis with nuclear pleomorphism.

levels were observed in these mice (Fig. 6A). None of the markers of the oxidative stress response was induced in NTBC-treated $Fah^{-/-}$ mice, untreated or DCA-treated $Fah^{+/+}$, regardless of the diet. The western blot analysis also showed that the expression of AFP, a marker of liver cell immaturity and/or genetic dysregulation leading to malignancy, was exclusively expressed in NTBC-OFF and DCA (0.5 or 5 g/L)-treated $Fah^{-/-}$ mice fed with a regular diet.

3.5. *DCA reduces MAAI activity in the liver*

In view of the lack of phenotypic effects of the DCA treament, we tested if indeed DCA inhibited hepatic MAAI in vivo. The expression of three enzymes sequentially involved in the tyrosine catabolic pathway, HPPD, HGO and MAAI, was analyzed by western blot in mice treated or not with DCA. As shown in Fig. 7A, discontinuation of NTBC treatment caused a general decrease in protein levels of HPPD, HGO and MAAI in the liver of $Fah^{-/-}$ mice fed the regular diet. A similar decrease in the levels of these proteins was noticed in the liver of DCA (0.5 or 5 g/L)-treated $Fah^{-/-}$ mice. A densitometric analysis of immunoreactive MAAI in NTBC-OFF and DCA-treated $Fah^{-/-}$ mice showed that its expression was reduced to ~50% of levels found in NTBC-treated $Fah^{-/-}$ mice or untreated wild-type mice fed with the regular diet. Treatment of $Fah^{-/-}$ mice on the regular diet with DCA (0.5 or 5 g/L) did not cause a further decrease in MAAI protein levels as reported in normal rodents [32,29,55]. However, the expected DCA-induced degradation of the MAAI protein was evident after DCA treatment of $Fah^{+/+}$ mice, as the densitometric analysis shows a reduction to ~80% and 30% of control MAAI values after treatment of $Fah^{+/+}$ mice with the low (0.5 g/L) and high (5 g/L) DCA dose, respectively.

When $Fah^{-/-}$ mice were fed with the low-tyrosine diet and NTBC was discontinued, protein levels of HPPD, HGO and MAAI were unchanged or slightly decreased compared to control mice (Fig. 7A). MAAI expression in the liver of NTBC-OFF $Fah^{-/-}$ mice decreased lightly to ~90% of control values (Fig. 7A). Treatment of $Fah^{-/-}$ mice fed on the low-tyrosine diet with DCA caused the expected decrease of MAAI protein levels, which corresponded to ~35% and 10% of control levels after DCA treatment with 0.5 and 5 g/L, respectively.

Table 2 – Biochemical analysis in serum

Diet	FAH	No of mice	Treatment	AP (U/L)	GGT (U/L)	Glucose (mmol/L)	Creatinine (μmol/L)	Urea (mmol/L)	Tyrosine (μmol/L)	SA (μmol/L)
Reg	+/+	4	Untreated	61 ± 7	4 ± 0	12.9 ± 1.5	15 ± 3	5.2 ± 0.2	74 ± 8	0 ± 0
Reg	−/−	6	NTBC	43 ± 9	4 ± 0	12.0 ± 1.9	17 ± 4	6.4 ± 1.0	457 ± 53	0.1 ± 0.1
Reg	−/−	9	NTBC-OFF	540 ± 333	46 ± 10	4.8 ± 0.2	19 ± 9	2.0 ± 0.3	696 ± 232	0.2 ± 0.1
Reg	−/−	3	DCA 0.5	1079 ± 40	191 ± 17	5.2 ± 0.4	18 ± 6	3.9 ± 0.1	840 ± 54	NA
Reg	−/−	6	DCA 5	1215 ± 337	115 ± 21	4.7 ± 1.6	23 ± 4	4.2 ± 0.9	840 ± 54	0.3 ± 0.1
Reg	+/+	3	DCA 0.5	70 ± 1	4 ± 0	10.3 ± 0.4	15 ± 0	7.4 ± 0.3	NA	NA
Reg	+/+	4	DCA 5	86 ± 14	4 ± 0	10.4 ± 1.6	20 ± 2	7.7 ± 0.4	87 ± 22	0.1 ± 0.1
Low	+/+	3	Untreated	17 ± 13	4 ± 0	16.7 ± 5.3	17 ± 2	4.4 ± 1.0	100 ± 18	0 ± 0
Low	−/−	4	NTBC	54 ± 9	6 ± 3	16.8 ± 0.9	17 ± 2	5.3 ± 0.5	381 ± 91	0 ± 0
Low	−/−	4	NTBC-OFF	50 ± 5	4 ± 0	12.8 ± 3.7	26 ± 6	5.4 ± 0.8	91 ± 8	0.1 ± 0.10
Low	−/−	3	DCA 0.5	54 ± 9	4 ± 0	9.5 ± 1.5	19 ± 0	5.1 ± 0.1	NA	NA
Low	−/−	3	DCA 5	49 ± 1	4 ± 0	8.8 ± 1.2	17 ± 3	4.3 ± 0.4	NA	NA

Note: Mean and standard deviation are indicated. Regular diet is indicated by Reg and low-tyrosine diet, by Low. Data represent male treated mice (AP – as in text AP: alkaline phosphatase; GGT: γ-glutamyl transferase; NA; not available).

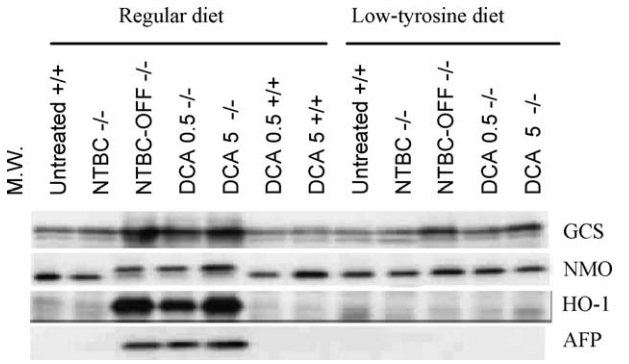

Fig. 6 – Western blot analysis of oxidative stress proteins in liver of *Fah*$^{-/-}$ and *Fah*$^{+/+}$ mice, with or without DCA treatment, after 5 weeks of treatment, fed a regular or low-tyrosine diet. FAH genotype (−/− or +/+) is indicated following name group.

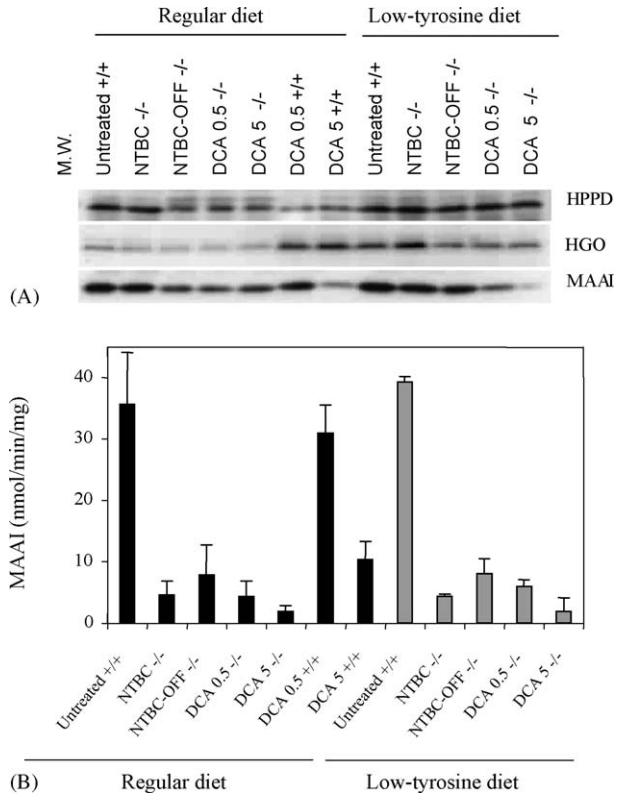

Fig. 7 – Immunoblot analysis of enzymes of the tyrosine degradation pathway (A) and enzymatic activity of MAAI (B) in liver of *Fah*$^{-/-}$ and *Fah*$^{+/+}$ mice, with or without DCA treatment, after 5 weeks of treatment, fed a regular or low-tyrosine diet FAH genotype (−/− or +/+) are indicated following name group. Means and standard deviations of MAAI activity (nmol/min/mg) are shown in panel B.

Since, in normal rodents, DCA has been reported to inhibit MAAI by direct inhibition and by degradation of the protein, the activity of hepatic MAAI was also measured. As shown in Fig. 7B, a striking decrease in MAAI activity occurred in the liver of all *Fah*$^{-/-}$ mice, regardless of the treatment or diet. Relative to the activity found in the liver of untreated wild-type mice, the residual MAAI activity in *Fah*$^{-/-}$ mice was ~15–20% in the NTBC and NTBC-OFF groups, ~10% in the low (0.5 g/L) DCA group and ~5% in the high (5 g/L) DCA group. In *Fah*$^{+/+}$ mice treated with DCA, a marked decrease in MAAI activity was only observed after treatment with the high DCA dose, the residual activity corresponding to ~30% of control values.

3.6. *Tyrosine catabolism and DCA degradation products*

The excretion of tyrosine metabolites and DCA degradation products in the urine of *Fah*$^{-/-}$ mice treated or not with DCA was analyzed by GS–MS. Tyrosine metabolites MA, FA and SA were not detected nor in the urine of untreated wild-type mice nor in NTBC-treated FAH$^{-/-}$ mice (Table 3), which indicates an efficient HPPD inhibition by NTBC. In contrast, MA, FA and SA were found in the urine of NTBC-OFF mice and DCA-treated mice. SA excretion was at its highest during the first week of NTBC discontinuation. A general tendency to decline was observed in excreted SA, MA and FA levels

Table 3 – Tyrosine metabolites found in urine of FAH-knockout mice treated or not with DCA and fed with a regular diet

Genotype	Treatment group	Week of treatment	Concentration (μg/mL)		
			MA	FA	SA
FAH$^{+/+}$	Untreated	–	0	0	0
FAH$^{-/-}$	NTBC	1	0	0	0
		3	0	0	0
		5	0	0	0
FAH$^{-/-}$	NTBC-OFF	1	3.7	2.3	8.4
		3	2.3	3.1	3.9
		5	1.8	1.3	2.5
FAH$^{-/-}$	DCA (0.5 g/L)	1	2.1	3.0	5.7
		3	1.4	1.9	3.4
		5	2.0	1.8	1.4
FAH$^{-/-}$	DCA (5 g/L)	2	4.9	4.5	12.3

Table 4 – DCA, DCA metabolites in urine of FAH-knockout mice treated or not with DCA and fed with a regular diet

Genotype	Treatment group	Week of treatment	Concentration (μg/mL)			
			DCA	Glyoxylate	Oxalate	Hippurate
$FAH^{+/+}$	Untreated	–	0	2.5	87.0	619.8
$FAH^{-/-}$	NTBC	1	0	1.7	113.1	532.8
		3	0	2.1	107.0	460.1
		5	0	2.5	133.2	396.0
$FAH^{-/-}$	NTBC-OFF	1	0	4.9	273.1	500.0
		3	0	4.7	138.2	534.4
		5	0	1.8	45.2	186.2
$FAH^{-/-}$	DCA (0.5 g/L)	1	0	5.1	179.2	724.8
		3	0	2.8	141.9	485.0
		5	15.8	2.9	65.7	313.3
$FAH^{-/-}$	DCA (5 g/L)	2	4045.1	4.0	230.3	445.2

from weeks 1–5. In DCA (0.5 g/L)-treated mice, urinary SA levels were slightly lower than in NTBC-OFF mice, but showed a similar decline with time (Table 3). $Fah^{-/-}$ mice treated with the high DCA dose (5 g/L) presented a poor urinary miction capacity and enough urine could only be collected at the second week of treatment. At this time point, urinary SA, MA, and FA levels were higher than the corresponding metabolite levels observed in $Fah^{-/-}$ mice treated with the low DCA dose (0.5 g/L).

Unmetabolized DCA was only found in the urine of $Fah^{-/-}$ mice after 5 weeks of treatment with the low dose (0.5 g/L) and at 2 weeks at the high dose (5 g/L). Metabolites derived from DCA biotransformation, but also generated endogenously, such as glyoxylate, oxalate and hippuric acid, were found in the urine of all mice. Hippurate was the main excreted metabolite, followed by oxalate and glyoxylate. Urinary glyoxylate, the main product of the oxidative dechlorination of DCA catalyzed by MAAI [26], was excreted at a relatively high concentration (≈5 μg/mL) in DCA (0.5 g/L)-treated mice during the first week of treatment, but similar urinary glyoxylate levels were observed in NTBC-OFF mice up to week 3 of treatment. From weeks 3–5 of DCA treatment, glyoxylate excretion decreased and stabilized to around 3 μg/mL, a value slightly higher than that observed in urine of NTBC-OFF mice after 5 weeks of NTBC discontinuation (1.8 μg/mL).

Other DCA metabolites, such as oxalate and hippurate, formed from the dehydrogenation or transamination of glyoxylate [23], respectively, were generally excreted at maximal levels during the first week of DCA 0.5 treatment and then declined to minimal levels at week 5 (Table 4). However, this was also the case for the endogenously formed oxalate and hippurate found in the urine of NTBC-OFF mice. Treatment of $Fah^{-/-}$ mice with the high DCA dose (5 g/L) resulted on similar excretion levels of oxalate and hippurate than those found in mice treated with the low DCA dose (Table 4).

3.7. DCA alters the GSH/GSSG ratio only when administered at high dose

Since DCA treatment has been shown to increase GSH content in the liver of male rats [56] GSH and GSSG levels were measured in $FAH^{-/-}$ mice treated or not with DCA. As shown in Fig. 8, slightly higher GSH levels were found in the liver

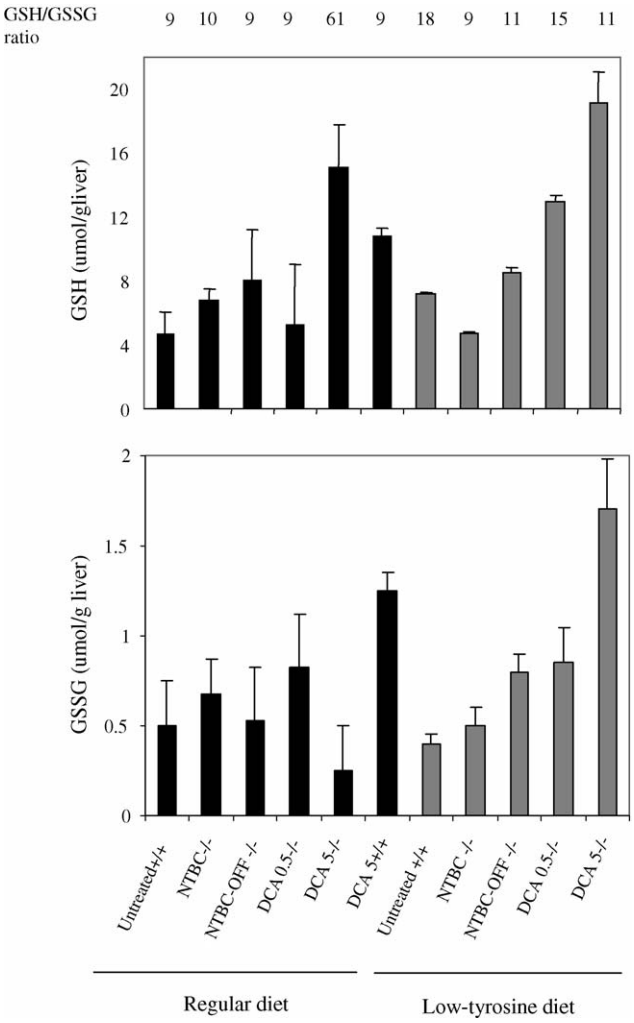

Fig. 8 – Hepatic GSH (top) and GSSG (bottom) contents in $Fah^{-/-}$ mice treated with DCA at the end of a 35 days treatment. GSH:GSSG ratios are indicated on top of the columns of the GSH top graph. FAH genotype (−/− or +/+) is indicated following name group.

of NTBC-treated and NTBC-OFF $FAH^{-/-}$ mice compared to untreated wild-type mice fed with a regular diet. GSH contents near control levels were observed in DCA (0.5 g/L)-treated mice, but these increased almost four-fold with the high DCA dose (5 g/L). In $FAH^{+/+}$ mice, hepatic GSH levels were 2.3-fold higher after treatment with the high DCA dose. When mice were fed with the low-tyrosine diet, slightly higher levels of hepatic GSH were observed in NTBC-treated and NTBC-OFF $FAH^{-/-}$ mice compared to similar groups fed with the regular diet, but a marked increase in GSH content (four-times the control levels) was observed after treatment of $FAH^{-/-}$ mice with DCA (0.5 g/L).

The GSH/GSSG ratio, an indicator of the cellular redox state, is around 10 under normal physiological conditions [57]. As shown on top of Fig. 8, only $FAH^{-/-}$ mice treated with the high DCA dose (5 g/L) showed a marked alteration in the GSH/GSSH ratio, which increased almost six times over control values due to an increase in GSH contents and a parallel decrease in GSSG levels.

4. Discussion

Mice on a regular diet but treated with NTBC did not develop the HT1 phenotype. DCA-treated groups (0.5 and 5 g/L) fed on a regular diet for a 5-week period presented a phenotype similar to the one observed after removal of NTBC. First, similar progressive body weight losses were seen in NTBC-OFF and DCA 5 g/L groups (~30% at day 35). Mice receiving the 0.5 g/L DCA dose lost ~20% of body weight. Second, renal damage observed in $Fah^{-/-}$ mice fed with a regular diet were not prevented with five weeks administration of DCA (0.5 and 5 g/L). NTBC-OFF and DCA-treated (0.5 and 5 g/L) mice showed a higher relative kidney weight with a moderate increase of creatinine. Higher creatinine levels were observed in DCA 5 g/L mice although no nephrotoxicity was observed at that dose in $Fah^{+/+}$ mice. Third, the livers of DCA-treated (0.5 and 5 g/L) mice displayed severe abnormalities with a high relative weight and microscopic alterations such as diffuse hepatocellular injury. Interestingly, microscopic alterations were less severe in 0.5 g/L DCA-treated mice than in the NTBC-OFF group. This finding suggests a possible beneficial clinical effect of DCA 0.5 g/L in HT1. However, liver functions of DCA 0.5 mice were not improved in comparison to NTBC-OFF mice.

Our observations indicate some toxic effects to $Fah^{-/-}$ mice by DCA. DCA 5 g/L induced more severe microscopic liver damage. DCA-treated mice showed several abnormalities of liver functions: serum AP and GGT levels were higher in DCA 0.5 and DCA 5 mice, respectively, compared to untreated $Fah^{+/+}$ and NTBC $Fah^{-/-}$ mice.

Western blot analysis of livers from $Fah^{-/-}$ mice off NTBC or treated with DCA (0.5 and 5) showed an oxidative stress response as evidenced by the induction of NMO 1, HO-1 and γGCS. The DCA 0.5 group showed a lighter HO-1 induction than NTBC-OFF mice. DCA treatment (0.5 and 5 g/L) did not prevent the expression of alpha foeto protein (AFP) a feature also observed in NTBC-OFF mice. The oxidative stress response found in the liver of NTBC-OFF mice is likely caused by the toxic metabolites (FAA, MAA, FA and MA). The absence of FAH activity and the reduced activity of MAAI found in NTBC-OFF mice explain the accumulation of FAA, MAA, FA, MA and SA reported in several studies. FAA, MAA, FA and MA are alkylating compounds. FAA accumulation in HT1 causes oxidative stress and induces apoptosis [10,11,58–60] and perturbation of gene expression (NMO, TAT, HPPD, G-6P) [61]. These could include misregulation of MAAI. MAA is an electrophilic compound and could be toxic to cells; MA (>150 μM) was proposed to be associated with DCA-toxicities in part because it is cytotoxic to hepatocytes in vitro [62]. FA has a similar structure to FAA and MAA but their roles in DCA toxicity remain unknown. Moreover, the mutagenicity of FAA is enhanced by GSH depletion [10]. Decreased levels of GSH in the plasma of a HTI patient were also reported [63]. Decreased GSH contents are also associated to an oxidative stress environment. Replenishment of GSH is insured by de novo synthesis of GSH or reduction of GSSG. A moderate up-regulation of hepatic GSH contents was seen in NTBC-OFF $FAH^{-/-}$ mice on a regular and a restrictive diet. Higher hepatic GSH levels (14 μmol/g liver) were found in the DCA 5 groups. This group fed on a regular diet also showed a high GSH/GSSG ratio (value of 61) indicating an abnormal redox status and an impairment to stabilize cellular homeostasis. High hepatic GSH levels in the DCA (5 g/L) treated mice could be explained by a reduction of GSSG (Fig. 8) and up-regulation of γGCS proteins found in these mice. γGCS catalyses the first step of GSH synthesis and its up-regulation causes GSH synthesis, which is a major component of daily defense against toxicity of xenobiotic compounds and oxidants. As DCA is a known xenobiotic compound, high doses of DCA (5 g/L) could account for elevated hepatic GSH contents. Increase of GSH contents may protect cells from an oxidative stress milieu. However, the high hepatic GSH levels seem to be inefficient to prevent the oxidative stress response (NMO and HO-1) and protect cells from liver damages. Moreover, higher level of GSH could induce a higher MAAI activity since MAAI uses GSH to transform MAA in FAA. Different levels of GSH might influence MAAI activity. However, no relation between GSH levels and MAAI activity was observed in all groups including DCA groups. So, it seems that DCA metabolism does not induce GSH depletion.

Inhibition of MAAI by DCA should induce an accumulation of its substrate, MAA. DCA inhibited MAAI causing an accumulation of tyrosine metabolites in a dose-dependent manner (except for SA). The excretion of DCA in urine may explain the reduction of DCA biotransformation products at day 24. The higher induction of HO-1 in DCA 5 mice on the regular diet may result from a more severe oxidative stress caused by hepatotoxicity of DCA and/or a higher accumulation of the toxic metabolites. Such accumulation might be caused by an incomplete inhibition of MAAI in DCA (0.5 and 5 g/L)-treated mice. However, spectrophotometric assays of hepatic MAAI activity showed a 94% (DCA 5) and 86% (DCA 0.5) inhibition of the enzyme. Curiously, reduced MAAI activity was seen in NTBC as well as NTBC-OFF $Fah^{-/-}$ groups regardless of the diets. Edward and Knox [64] showed that adducts can be formed between FAA and GSH and are potential inhibitors of MAAI. Similar findings have been reported [65] for the FAA homologue fumarylpyruvate, and

GSH. These findings could explain the lower levels of MAAI activity observed in the NTBC-OFF $Fah^{-/-}$ group fed a regular diet. Other studies proposed that the tyrosine catabolic pathway in NTBC-treated mice was still partially open and this could explain the incomplete protection by the drug causing residual FAA accumulation and formation of adducts with GSH. This phenomenon remains unexplained at this time.

DCA metabolism was found to be severely impaired in the DCA 5 group compared to NTBC-OFF and DCA 0.5. Lower MAAI activity is consistent with that result. Urine excretion of DCA in DCA (0.5 g/L)-treated mice appeared at day 24 of treatment and glyoxylate levels were reduced by 1.4-fold at that time. Considering that MAAI transforms DCA in glyoxylate, 24 days of treatment were required to induce enough MAAI inhibition to cause a reduction in DCA transformation in glyoxylate. DCA-treated groups (0.5 and 5 g/L) accumulated biotransformation products in a dose-dependent manner and the major DCA by-products found were glycine conjugates (hippuric acid and phenylacetylglycine) and oxalate. As seen in MAAI-knockout mice, DCA was not actively metabolized to either glyoxylate or monochloroacetate [32]. All of these compounds were excreted in a DCA dose-dependent manner (except for phenylacetylglycine).

The low-tyrosine diet strikingly improved the HTI phenotype in NTBC-OFF mice. All mice (including NTBC-OFF and DCA groups) showed a gain of body weight (up to 25%) at the end of the experiment. Biochemical markers of liver function (GGT and AP) were markedly improved. In addition, these mice showed no induction of oxidative stress proteins (HO-1 and NMO). Although the low-tyrosine diet reduced nephromegaly and counteracted most of the renal tubular lesions efficiently (except for tubular vacuolization) in all $FAH^{-/-}$ mice, dietary treatment did not ameliorate renal functions of the NTBC-OFF group. A combination of NTBC and a tyrosine-restrictive diet gave the best results to prevent the HTI phenotype. This finding supports the importance of a better prevention in accumulation of toxic metabolites for the treatment of HTI.

In summary, the inefficiency of DCA treatment to improve the appearance of the HTI phenotype may be attributed to the residual MAAI activity leading to FAA accumulation. Recently, a MAAI-knockout mouse model [48] was created. These mice breed normally and appeared healthy but accumulated high levels of FAA and SA. This study with MAAI-knockout mice showed a GSH-dependent non-enzymatic bypass of MAA. Although the non-enzymatic bypass would not be as efficient as the enzyme-mediated reaction, it seems to be important enough to allow FAA accumulation in mice double mutant for MAAI and FAH [48] fed a regular diet. These mice showed an altered phenotype with predominant renal injuries and died rapidly. This finding suggests that even a complete block of MAAI in HT1 patients with DCA might not block the GSH non-enzymatic formation of FAA and HTI phenotype. Another explanation for the inefficiency of DCA as a treatment for HTI could be the hepatotoxicity of DCA as observed in rodents [22]. This could explain the milder HTI phenotype of mice treated with a low dose of DCA (0.5 g/L) compared to the high dose (5 g/L). Thus, our data indicate that DCA is not a favorable alternative for HTI treatment.

Acknowledgments

We wish to thank Dr. S. Lindstedt (Gottenburg, Sweden) for the gift of NTBC and Dr. A. Bergeron and D. Orejuela for revision of the manuscript. This work was supported by a grant from CIHR (MOP-11081 to RMT), a partial studentship from the Foundation Marc Bourgie to CL, and by ES07355 from the National Institute of Health (PWS).

REFERENCES

[1] Kvittingen E. Hereditary tyrosinemia type I-an overview. Scand J Clin Lab Invest 1986;46:27–34.
[2] Russo PA, Mitchell GA, Tanguay RM. Tyrosinemia: a review. Pediatr Dev Pathol 2001;4:212–21.
[3] Mitchell GA, Grompe M, Lambert M, Tanguay RM. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. Hypertyrosinemia in the metabolic and molecular bases of inherited disease, New York: McGraw-Hill; 2001. 1777–1805.
[4] Russo P, O'Regan S. Visceral pathology of hereditary tyrosinemia type I. Am J Hum Genet 1990;47:317–24.
[5] Mitchell G, Larochelle J, Lambert M, Michaud J, Grenier A, Ogier H, et al. Neurologic crisis in hereditary tyrosinemia. New Engl J Med 1990;322:432–7.
[6] Lindblad B, Lindstedt S, Steen G. On the enzymic defects in hereditary tyrosinemia. Proc Natl Acad Sci USA 1977;74:4641–5.
[7] Tanguay RM, Valet JP, Lescault A, Duband JL, Laberge C, Lettre F, et al. Different molecular basis for fumarylacetoacetate hydrolase deficiency in the two clinical forms of hereditary tyrosinemia (type I). Am J Hum Genet 1990;47:308–16.
[8] Phaneuf D, Lambert M, Laframboise R, Mitchell G, Lettre F, Tanguay RM. Type I hereditary tyrosinemia. Evidence for molecular heterogeneity and identification of a causal mutation in a French Canadian patient. J Clin Invest 1992;90:1185–92.
[9] Tanguay RM, Jorquera R, Poudrier J, St-Louis M. Tyrosine and its catabolites: from disease to cancer. Acta Biochim Pol 1996;43:209–16.
[10] Jorquera R, Tanguay RM. The mutagenicity of the tyrosine metabolite, fumarylacetoacetate, is enhanced by glutathione depletion. Bioch Biophys Res Commun 1997;232:42–8.
[11] Jorquera R, Tanguay RM. Cyclin B-dependent kinase and caspase-1 activation precedes mitochondrial dysfunction in fumarylacetoacetate-induced apoptosis. FASEB J 1999;13:2284–98.
[12] Manning K, Al-Dhalimy M, Finegold M, Grompe M. In vivo suppressor mutations correct a murine model of hereditary tyrosinemia type I. Proc Natl Acad Sci USA 1999;96:11928–33.
[13] Paradis K, Weber A, Seidman EG, Larochelle J, Garel L, Lenaerts C, et al. Liver transplantation for hereditary tyrosinemia: the Quebec experience. Am J Hum Genet 1990;47:338–42.
[14] Kuang AA, Rosenthal P, Roberts JP, Renz JF, Stock P, Ascher NL, et al. Decreased mortality from technical failure improves results in pediatric liver transplantation. Arch Surg 1996;131:887–92.
[15] Cacciarelli TV, Esquivel CO, Moore DH, Cox KL, Berquist WE, Concepcion W, et al. So SK Factors affecting survival after orthotopic liver transplantation in infants. Transplantation 1997;64:242–8.

[16] Lindstedt S, Holme E, Lock EA, Hjalmarson O, Strandvik B. Treatment of hereditary tyrosinaemia type I by inhibition of 4-hydroxyphenylpyruvate dioxygenase. Lancet 1992;340:813–7.
[17] Ellis MK, Whitfield AC, Gowans LA, Auton TR, Provan WM, Lock EA, et al. Inhibition of 4-hydroxyphenylpyruvate dioxygenase by 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione and 2-(2-chloro-4-methanesulfonylbenzyol)-cyclohexane-1,3-dione. Toxicol Appl Pharmacol 1995;133:12–9.
[18] Holme E, Lindstedt S. Tyrosinaemia type I and NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexanedione). J Inherit Metab Dis 1998;21:507–17.
[19] Holme E, Lindstedt S. Nontransplant treatment of tyrosinemia. Clin Liver Dis 2000;4:805–14.
[20] van Spronsen FJ, Bijleveld CMA, van Maldegem BT, Wijburg FA. Hepatocellular carcinoma in hereditary tyrosinemia type I despite 2-2nitro-4-3 trifluoro-methylbenzoyl-1,3-cyclohexanedione treatment. J Pediatr Gastroenterol Nutr 2005;40:90–3.
[21] Grompe M, Lindstedt S, Al-Dhalimy M, Kennaway NG, Papaconstantinou J, Torres-Ramos CA, et al. Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditary tyrosinamia type I. Nature Genet 1995;10:453–60.
[22] Stacpoole PW. The pharmacology of dichloroacetate. Metabolism 1989;38:1124–44.
[23] Stacpoole PW, Henderson GN, Yan Z, Cornett R, James MO. Pharmacolkinetics, metabolism and toxicology of dichloroacetate. Drug Metab Rev 1998;30:499–539.
[24] Pelling J, Sutherland G, Brown RA, Curry S. Protective effect of dichloroacetae in a rat model of forebrain ischemia. Neurosci Lett 1996;208:21–4.
[25] James MO, Cornett R, Yan Z, Henderson GN, Stacpoole PW. Glutathione-dependent conversion to glyoxylate, a major pathway of dichloroacetate biotransformation in hepatic cytosol from humans and rats, is reduced in dichloroacetate-treated rats. Drug Metab Dispos 1997;25:1223–7.
[26] Tong Z, Board PG, Anders MW. Gluthathione transferase zeta catalyses the oxygenation of the carcinogen dichloroacetic acid to glyoxylic acid. Biochem J 1998;331:371–4.
[27] Fernandez-Canon JM, Penalva MA. Characterization of a fungal maleylacetoacetate isomerase gene and identification of its human homologue. J Biol Chem 1998;273:329–37.
[28] Tzeng HF, Blackburn AC, Board PG, Anders MW. Polymorphism- and species-dependent inactivation of glutathione transferase zeta by dichloroacetate. Chem Res Toxicol 2000;3:231–6.
[29] Anderson WB, Board PG, Gargano B, Anders MW. Inactivation of glutathione transferase zeta by dichloroacetic acid and other fluorine-lacking alpha-haloalkanoic acids. Chem Res Toxicol 1999;12:1144–9.
[30] Schultz IR, Merdink JL, Gonzalez-Leon A, Bull RJ. Dichloroacetate toxicokinetics and disruption of tyrosine catabolism in B6C3F1 mice: dose-response relationships and age as a modifying factor. Toxicology 2002;173:229–47.
[31] Cornett R, James MO, Henderson GN, Cheung J, Shroads AL, Stacpoole PW. Inhibition of glutathion S-transferase zeta and tyrosine metabolism by dichloroacetate: a potential unifying mechanism for its altered biotransformation and toxicity. Biochem Biophys Res Commun 1999;262:752–6.
[32] Ammini CV, Fernandez-Canon J, Shroads AL, Cornett R, Cheung J, James MO, et al. Pharmacologic or genetic ablation of maleyacetoacetate isomerase increases levels of toxic tyrosine catabolites in rodents. Biochem Pharmacol 2003;66:2029–38.
[33] DeAngelo AB, Daniel FB, Most BM, Olson GR. The carcinogenicity of dichloroacetic acid in the male Fisher 344 rat. Toxicology 1996;114:207–21.
[34] Komulainen H. Experimental cancer studies of chlorinated by-products. Toxicology 2004;198:239–48.
[35] Herren-Freund SL, Pereira MA, Khoury MD, Olson G. The carcinogenicity of trichloroethylene and its metabolites trichloroacetic acid and dichloroacetic acid, in mouse liver. Toxicol Appl Pharmacol 1987;90:183–9.
[36] Bull RJ, Sanchez IM, Nelson MA, Larson JL, Lansing AJ. Liver tumor induction in B6C3F1 mice by dichloroacetate and trichloroacetate. Toxicology 1990;63:342–59.
[37] DeAngelo AB, George MH, House DE. Hepatocarcinogenicity in the male B6C3F1 mouse following a lifetime exposure to dichloroacetic acid in the drinking water: dose-response determination and modes of action. J Toxicol Environ Health A 1999;58:485–507.
[38] DeAngelo AB, Daniel FB, Stober JA, Olson GR. The carcinogenicity of dichloroacetic acid in the male B6C3F1 mouse. Fundam Appl Toxicol 1991;16:337–47.
[39] Daniel FB, DeAngelo AB, Stober JA, Olson GR, Page NP. Hepatocarcinogenicity of chloral hydrate, 2-chloroacetaldehyde, and dichloroacetic acid in the male B6C3F1 mouse. Fundam Appl Toxicol 1992;19:159–68.
[40] Pereira MA. Carcinogenic activity of dichloroacetic acid and trichloroacetic acid in the liver of female B6C3F1 mice. Fundam Appl Toxicol 1996;31:192–9.
[41] Stacpoole PW, Greene YJ. Dichloroacetate. Diabetes Care 1992;15:785–91.
[42] Walgren JE, Kurtz DT, McMillan JM. The effect of the trichloroethylene metabolites trichloroacetate and dichloroacetate on peroxisome proliferation and DNA synthesis in cultured human hepatocytes. Cell Biol Toxicol 2000;16:257–73.
[43] Grompe M, Al-Dhalimy M, Finegold M, Ou CN, Burlingame T, Kennaway NG, et al. Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. Genes Dev 1993;7:2298–307.
[44] Schmitteckert EM, Prokop CM, Hedrich HJ. DNA detection in hair of transgenic mice-a simple technique minimizing the distress on the animals. Lab Anim 1999;33:385–9.
[45] Labelle Y, Puymirat J, Tanguay RM. Localization of cells in the rat brain expressing fumarylacetoacetate hydrolase, the deficient enzyme in hereditary tyrosinemia type 1. Biochim Biophys Acta 1993;1180(3):250–6.
[46] Grenier A, Lescault A, Laberge C, Gagne R, Mamer O. Detection of succinylacetone and the use of its measurement in mass screening for hereditary tyrosinemia. Clin Chim Acta 1982;123:93–9.
[47] Shroads AL, Henderson GN, Cheung J, Greywoode J, James MO, Stacpoole PW. Unified gas chromatographic–mass spectrophotometric method for quantitating tyrosine metabolites in urine and plasma. J Chromatogr B Anal Technol Biomed Life Sci 2004;808:153–61.
[48] Fernandez-Canon JM, Baetscher MW, Finegold M, Burlingame T, Gibson KM, Grompe M. Maleylacetoacetate isomerase (MAAI/GSTZ)-deficient mice reveal a glutathione-dependent nonenzymatic bypass in tyrosine catabolism. Mol Cell Biol 2002;22:4943–51.
[49] Anderson ME. Determination of glutathione and glutathione disulfide in biological sample. Methods Enzymol 1985;113:548–55.
[50] Ruppert S, Kelsey G, Schedl A, Schmid E, Thies E, Schutz G. Deficiency of an enzyme of tyrosine metabolism underlies altered gene expression in newborn liver of lethal albino mice. Genes Dev 1992;6:1430–43.
[51] Prive L. Pathological findings in patients with tyrosinemia. Can Med Assoc J 1967;97:1073.

[52] Kelsey G, Ruppert S, Beermann F, Grund C, Tanguay RM, Schutz G. Rescue of mice homozygous for lethal albino deletions: implications for an animal model for the human liver disease tyrosinemia type I. Genes Dev 1993;7:2285–97.
[53] Liang HC, Shertzer HG, Nebert DW. Oxidative stress response in liver of an untreated newborn mouse having a 1,2-centimorgan deletion on chromosome 7. Biochem Biophys Res Commun 1992;182(3):1160–5.
[54] Takahashi T, Morita K, Akagi R, Sassa S. Heme oxygenase-1: a novel therapeutic target in oxidative stress tissue injuries. Curr Med Chem 2004;11:1545–61.
[55] Schultz IR, Sylvester SR. Stereospecific toxicokinetics of bromochloro- and chlorofluoroacetate: effect of GST-zeta depletion. Toxicol Appl Pharmacol 2001;175(2): 104–13.
[56] Yang HM, Davis ME. Dichloroacetic acid pretreatment of male and female rats increases chloroform-induced hepatotoxicity. Toxicology 1997;124(1):63–72.
[57] Griffith OW. Biologic and pharmacologic regulation of mammalian glutathione synthesis. Free Radic Biol Med 1999;27(9/10):922–35.
[58] Jorquera R, Tanguay RM. Fumarylacetoacetate, the metabolite accumulating in hereditary tyrosinemia, activates the ERK pathway and induces mitotic abnormalities and genome instability. Hum Mol Genet 2001;10:1741–52.
[59] Kubo S, Sun M, Miyahara M, Umeyama K, Urakami K, Yamamoto T, et al. Hepatocyte injury in tyrosinemia type I is induced by fumarylacetoacetate and is inhibited by caspase inhibitors. Proc Natl Acad Sci USA 1998;95(16):9552–7.
[60] Bergeron A, Jorquera R, Tanguay RM. Hereditary tyrosinemia: an endoplasmic reticulum stress disorder? Med Sci (Paris) 2003;19:976–80.
[61] Luijerink MC, Jacobs SM, van Beurden EA, Koornneff LP, Klomp LW, Berger R, et al. Extensive changes in liver gene expression induced by hereditary tyrosinemia type I are not normalized by treatment with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC). J Hepatol 2003;39:901–9.
[62] Lantum HB, Cornejo J, Pierce RH, Anders MW. Perturbation of maleyacetoacetic acid metabolism in rats with dichloroacetic acid-induced glutathione transferase zeta deficiency. Toxicol Sci 2003;74:192–202.
[63] Stoner E, Starkman H, Wellner D, Wellner VP, Sassa S, Rifkind AB, et al. Biochemical studies of a patient with hereditary hepatorenal tyrosinemia: evidence of glutathione deficiency. Pediatr Es 1984;18(12):1332–6.
[64] Edwards SW, Knox WE. Homogentisate metabolism: the isomerization of maleyacetoacetate by an enzyme which requires glutathione. J Biol Chem 1956;220:79–91.
[65] Lack L. Enzymic *cis–trans* isomerization of maleypyruvic acid. J Biol Chem 1961;236:2835–40.