



## Bezafibrate can be a new treatment option for mitochondrial fatty acid oxidation disorders: Evaluation by in vitro probe acylcarnitine assay

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### ABSTRACT

**Background:** The number of patients with mitochondrial fatty acid oxidation (FAO) disorders is recently becoming larger with the spread of newborn mass screening. Despite the advances in metabolic and molecular characterization of FAO disorders, the therapeutic studies are still limited. It was reported recently that bezafibrate (BEZ), an agonist of peroxisome proliferating activator receptor (PPAR), can restore FAO activity in cells from carnitine palmitoyltransferase-2 (CPT2) and very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiencies as well as clinical symptoms in the adult patients.

**Methods:** In this study, the therapeutic effect of BEZ was determined by in vitro probe acylcarnitine (IVP) assay using cultured fibroblasts and tandem mass spectrometry on various FAO disorders. The clinical trial of BEZ treatment for a boy with the intermediate form of glutaric acidemia type 2 (GA2) was also performed.

**Results:** The effect of BEZ was proven in cells from various FAO disorders including GA2, deficiencies of VLCAD, medium-chain acyl-CoA dehydrogenase, CPT2, carnitine acylcarnitine translocase and trifunctional protein, by the IVP assay. The aberrantly elevated long- or medium-chain acylcarnitines that are characteristic for each FAO disorder were clearly corrected by the presence of BEZ (0.4 mmol/L) in culture medium. Moreover, daily administration of BEZ in a 2-year-old boy with GA2 dramatically improved his motor and cognitive skills, accompanied by sustained reduction of C4, C8, C10 and C12 acylcarnitines in blood, and normalized the urinary organic acid profile. No major adverse effects have been observed.

**Conclusion:** These results indicate that BEZ could be a new treatment option for FAO disorders.

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### 1. Introduction

Mitochondrial  $\beta$ -oxidation (FAO) is an essential energy producing pathway, particularly during the reduced energy supply from carbohydrate due to prolonged starvation or low caloric intake during infection, diarrhea or febrile illness. A number of FAO disorders have been recognized with the spread of tandem mass spectrometry (MS/MS) in the field of study of inborn metabolic disease as well as neonatal mass screening [1,2]. Many of them show episodic attacks like lethargy, acute encephalopathy or even sudden death due to energy production insufficiency.

It is considered that the FAO system consists of the following four groups: 1) carnitine cycle, which activates long-chain fatty acids for undergoing  $\beta$ -oxidation, including carnitine transporter (OCTN2),

carnitine palmitoyltransferase-1 or -2 (CPT1 or CPT2, respectively, EC 2.3.1.21), or carnitine acylcarnitine translocase (CACT, EC 2.3.1.21); 2) long-chain FAO, whose enzymes are connected to the mitochondrial inner membrane, including very-long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.99.13) deficiency, and trifunctional protein (TFP, EC 1.1.1.211 and EC 2.3.1.16); 3) medium-chain FAO, whose enzymes are located in the mitochondrial matrix, including medium- and short-chain acyl-CoA dehydrogenases (MCAD, EC 1.3.99.3 and SCAD, EC 1.3.8.1) respectively), enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, or medium- and short-chain 3-ketothiolase (MCKAT and SCKAT, respectively); and 4) electron transfer system, from the dehydrogenases to respiratory chain, including electron transferring flavoprotein (ETF, EC 1.5.8.2) and ETF dehydrogenase (ETFDH, EC 1.5.5.1) [3–5].

Clinical features of FAO disorders can be roughly divided into the following three types: 1) severe form (neonatal form): patients present life-threatening illness with profound hypoglycemia, liver failure or hyperammonemia, and are often fatal in early infancy; 2) intermediate

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form (juvenile form): patients have intermittent episodic attacks like lethargy, encephalopathy, or even sudden death often onset in infancy or young childhood; 3) mild form (myopathic form): the patients may often show late onset after school ages or adulthood with episodes of hypotonia, myalgia, lethargy, myopathy-like symptoms, or liver dysfunction [6].

In vitro probe acylcarnitine profiling (IVP) assay was developed to evaluate FAO disorders recently [7,8]. Acylcarnitine (AC) profiles in the special culture medium as below after incubating with fatty acids as substrates are determined by MS/MS. Bezafibrate (BEZ) is a hypolipidemic drug, which is an agonist of peroxisome proliferating activator receptor (PPAR), and is claimed to act for induction of several FAO enzymes [9–11].

In this study, the effect of BEZ on various FAO disorders was evaluated using the IVP assay. Furthermore, we report an in vivo trial of BEZ on a boy with the intermediate form of GA2, presenting dramatic improvement with BEZ.

## 2. Materials and methods

### 2.1. Subjects and skin fibroblasts

Fibroblasts from 10 Japanese children with FAO disorders, one each of severe and intermediate forms of GA2, 2 each of severe and myopathic (mild) forms of VLCAD deficiency, one each of deficiencies of MCAD, CPT2, CACT, and TFP as well as 6 controls (healthy volunteers, passages 3 to 16) were used. The clinical types and genotypes are shown in Table 1. The child with MCAD deficiency was detected in a newborn mass screening and non-symptomatic, while one with the intermediate form of CPT2 deficiency had liver dysfunction in infancy. The child with the intermediate form of CACT deficiency had

two life-threatening episodes in infancy, and after that no episodes were noted with normal development [12]. The child with TFP deficiency had an episode of liver failure in infancy, and then intermittent episodes of myalgia or hypotonia particularly following infection.

The clinical types and genotypes are shown in Table 1. In all cases, at least one allele has missense mutation, although the other alleles had missense or truncated mutations. In CACT deficiency (case 9), a missense mutation in an initiation codon (c.3G>A) in SLC25A29 was detected, but this could harbor a residual activity (Fukao et al., unpublished data).

### 2.2. In vitro probe assay with BEZ

Fibroblasts were cultured in 75 cm<sup>2</sup> flasks (Iwaki, Tokyo, Japan) containing modified Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 2 mmol/L of L-glutamine (Nacalai Tesque, Kyoto, Japan), 10% FBS (Sigma, St Louis, MO, USA) and 1% penicillin/streptomycin (Sigma) at 37 °C in a humidified 5% CO<sub>2</sub>/95% air incubator [13].

Fibroblasts harvested by trypsinization were seeded onto 6-well microplates (35 mm i.d., Iwaki, Japan) with the fresh above medium (2 mL/per well) until they reached confluence. Thereafter, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA) and cultured for 96 h in 1 mL of experimental substrate (experimental medium). The experimental medium is MEM containing bovine serum albumin (0.4% essential fatty acid-free BSA; Sigma), L-carnitine (0.4 mmol/L; Sigma), unlabeled palmitic acid (0.2 mmol/L; Nacalai Tesque) and 1% penicillin/streptomycin without L-glutamine, in the presence or absence of BEZ (0.4 mmol/L; Sigma). AC profiles in the culture medium were analyzed after 96 h. The experiments for each case were performed in triplicate.

### 2.3. Quantitative acylcarnitine analysis

ACs in culture medium supernatants were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA) as described previously [13]. Briefly, methanol (200 µL) including an isotopically-labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 µL of the supernatant from culture medium. The portions were placed on ice for 30 min, and centrifuged at 1000×g for 10 min. Then, 150 µL of the supernatant was dried under a nitrogen stream, and butyl-derivatized with 50 µL of 3N n-butanol-HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 µL of 80% acetonitrile:water (4:1 v/v). The ACs in 10 µL of the resultant aliquots were analyzed using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

Protein concentrations were measured by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction. The AC concentrations are expressed as nmol/mg protein.

### 2.4. Organic acid analysis using GC/MS

Urinary organic acids were analyzed according to the previous method [14]. Briefly, 40 µg of tropate (IS-2) and 20 µg each of heptadecanoate (IS-1) and tetracosane (C24) as internal standards were added to a urine specimen containing 0.2 mg creatinine. The samples were oxime-derivatized, and solvent extracted with ethylacetate, and trimethylsilylated (TMS-derivatization). The resultant aliquots were subjected to GC/MS (Shimadzu GC/MS QP2010 Plus, Kyoto, Japan), with a DB-5 column of 0.25 mm I.D×30 m, 1 µm film thickness (J&W, Folsom, CA). The temperature program was from 100 °C to 290 °C at a rate of 4 °C/min).

**Table 1**  
Clinical types and genotypes of patients with mitochondrial fatty acid oxidation disorders investigated.

Disease & case No.	Phenotype	Gene	Genotype, nucleotides (amino acids)	
			Allele 1	Allele 2
<b>GA2</b>				
1 (B)	Severe	ETFA	c.799G>A (G267R)	c.7C>T (R3X)
2 (C)	Intermediate	ETFDH	c.1217G>A (S406N)	c.1675C>T (R559X)
<b>VLCAD deficiency</b>				
3 (D)	Severe	ACADV	c.553G>A (G185S)	IVS9 + 1g>c
4 (E)	Severe	ACADV	c.454G>A (G152S)	c.997insT (A333fsX358)
5 (F)	Myopathic	ACADV	c.790A>G (K264E)	c.997insT (A333fsX358)
6 (G)	Myopathic	ACADV	c.1144A>C (K382Q)	c.1339G>A (G447R)
<b>MCAD deficiency</b>				
7 (H)	Non-symptomatic	ACADM	c.134A>G (Q45R)	c.449delCTGA (T150fsX153)
<b>CPT2 deficiency</b>				
8 (I)	Intermediate	CPT2	c.151A>G (R51G)	c.520G>A (E174K)
<b>CACT deficiency</b>				
9 (J)	Intermediate	SLC25A29	c.3G>A (M11)	IVS4 + 1g>t
<b>TFP deficiency</b>				
10 (K)	Intermediate	HADHB	c.739C>T (R247C)	c.817delG (D273fsX292)

Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; GA2, glutaric acidemia type 2; VLCAD, very-long-chain acyl-CoA dehydrogenase; CPT2, carnitine palmitoyltransferase-2; TFP, mitochondrial trifunctional protein; CACT, carnitine acylcarnitine translocase. Case 2 (C) is a boy with GA2 who underwent the clinical trial of BEZ. Non-symptomatic case 7 (H) was detected in the newborn mass screening. Severe, intermediate, and myopathic forms are mentioned in the text. (B) to (K) correspond to those of Fig. 1.

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