Valproic acid induces antioxidant effects in X-linked adrenoleukodystrophy

Stéphane Fourcade1,2, Montserrat Ruiz1,2, Cristina Guilera1,2, Eric Hahnen3,4, Lars Brichta3†, Alba Naudi5, Manuel Portero-Otín5, Georges Dauremont6, Nathalie Cartier7, Ronald Wanders8,9, Stephan Kemp8,9, Jean Louis Mandel10,11,12, Bruniilde Wirth3, Reinald Pampolina5, Patrick Aubourg7 and Aurora Pujol1,2,13,*

1Neurometabolic Disease Lab, Institut de Neuropathology, Institut d'Investigacio Biomèdica de Bellvitge (IDIBELL), Hospital de Llobregat, Barcelona, Spain, 2Center for Biomedical Research on Rare Diseases (CIBERER), Barcelona, Spain, 3Institute of Human Genetics, Institute of Genetics and Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany, 4Institute of Neuropathology, University Hospital Erlangen, Schwabachanlage 6, 91054 Erlangen, Germany, 5Department de Medicina Experimental, Universitat de Lleida-IRB-LLEIDA, Lleida, Spain, 6University of Ghent, Ghent, Belgium, 7INSERM U745 and University René Descartes Paris 5, Hôpital Saint-Vincent de Paul, Paris, France, 8Laboratory Genetic Metabolic Diseases, Department of Clinical Chemistry and 9Department of Pediatrics, Emma Children's Hospital, Academic Medical Center, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands, 10Chaire de Génétique Humaine, Collège de France, Illkirch F-67400, France, 11Institut de Génétique et de Biologie Moléculaire et Cellulaire, Inserm U596, CNRS, UMR7104, Université Louis Pasteur, Strasbourg, Illkirch F-67400, France, 12Institut Clinique de la Souris, 67404 Illkirch, France and 13Catalan Institution of Research and Advanced Studies (ICREA), Barcelona, Spain

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X-linked adrenoleukodystrophy (X-ALD) is a fatal, axonal demyelinating, neurometabolic disease. It results from the functional loss of a member of the peroxisomal ATP-binding cassette transporter subfamily D (ABCD1), which is involved in the metabolism of very long-chain fatty acids (VLCFA). Oxidative damage of proteins caused by excess of the hexacosanoic acid, the most prevalent VLCFA accumulating in X-ALD, is an early event in the neurodegenerative cascade. We demonstrate here that valproic acid (VPA), a widely used anti-epileptic drug with histone deacetylase inhibitor properties, induced the expression of the functionally overlapping ABCD2 peroxisomal transporter. VPA corrected the oxidative damage and decreased the levels of monounsaturated VLCFA (C26:1 n-9), but not saturated VLCFA. Overexpression of ABCD2 alone prevented oxidative lesions to proteins in a mouse model of X-ALD. A 6-month pilot trial of VPA in X-ALD patients resulted in reversion of the oxidative damage of proteins in peripheral blood mononuclear cells. Thus, we propose VPA as a promising novel therapeutic approach that warrants further clinical investigation in X-ALD.
In our study, we decided to investigate the effect of valproic acid (VPA), an unspecific HDAC inhibitor, in patients with X-ALD. VPA is able to cross the blood–brain barrier and has been in clinical use for more than 3 decades to treat epilepsy, mood disorders and migraine (24,25). VPA has demonstrated to increase neurite outgrowth and to exert neuroprotective effects on glutamate-induced excitotoxicity in vitro (26,27). Significant clinical improvements have been recently reported on a subset of spinal muscular atrophy patients upon VPA treatment (28–30).

Oxidative stress might be a major factor in the pathogenesis of adrenoleukodystrophy. In a mouse model of X-ALD, in which we observe an ‘AMN-like’ phenotype (20,21), we have demonstrated oxidative damage to proteins in the spinal cord (31). This occurs as early as 3.5 months of age, >1 year before neurological symptoms or neuropathological signs appear. We have also shown that an excess of saturated VLCFA triggers free radical production in vitro and ex vivo in an organotypic spinal cord slice culture system (31).

In the present study, we performed in vivo and in vitro experiments to show that VPA induces ABCD2 gene expression in the fibroblasts of X-ALD patients and reduces oxidative damage to proteins. Antioxidant effects on peripheral mononuclear patient cells indicate that VPA might constitute a promising novel therapeutic approach for X-ALD, particularly for patients suffering from AMN.

RESULTS

VPA induces ABCD2 gene expression in human X-ALD fibroblasts via an HDAC inhibitory mechanism

Human X-ALD fibroblasts were cultured in the presence of VPA (2 mM) for 2 and 12 days, as described previously (12), and the effect of VPA on the expression of members of the ABCD transporter subfamily was measured. A 2.8-fold increase in ABCD2 mRNA levels in X-ALD fibroblasts was observed after 2 days of treatment with VPA (Fig. 1A). Similar results were obtained when fibroblasts were treated with VPA for longer periods of time (12 days of culture, data not shown). Much like ABCD2, the ABCD3 peroxisomal transporter has been shown to be able to correct the defect in peroxisomal β-oxidation in ABCD1-deficient cells from X-ALD patients, when it is overexpressed by transient transfection (32). Less information is available on ABCD4, except for the finding that ABCD4 expression levels in the brain of X-ALD patients are inversely correlated with phenotype severity (33). In X-ALD fibroblasts cultures, we were able to measure a 1.4-fold increase in ABCD3 mRNA levels after treatment with VPA. In contrast, ABCD4 mRNA expression was not affected by VPA (Fig. 1A). As to investigate whether induction of ABCD2 expression by VPA was mediated through HDAC inhibitory mechanisms, we used a control drug (34). The molecular structure of valpromide (VPD or 2-propylpentanamide), the amide derivative of VPA (2-propylpentanoic acid), is almost identical to that of VPA. However, VPD does not exhibit HDAC inhibitory properties (35,36). ABCD2 gene expression was not induced by VPD (Fig. 1B), suggesting that the effect of VPA on
ABCD2 gene transcription was most likely mediated through inhibition of HDAC.

**ABCD2 overexpression in Abcd1<sup>−/−</sup>/Abcd2<sup>−/−</sup> mice prevents protein oxidative damage in the spinal cord**

There is mounting evidence that oxidative burden plays a major role in the initial steps of the X-ALD pathogenesis cascade (31,37–39). We recently demonstrated oxidative damage of proteins in vivo in the spinal cord of X-ALD mice at 3.5 months of age, well before the onset of neuropathological signs or neurological symptoms, which can be detected at 16 and 20 months, respectively (31). Specific and well-characterized markers of direct oxidation of carbonyl residues such as glutamic semialdehyde (GSA) (derived from the metal-catalyzed oxidation of proline and arginine) and aminoadipic semialdehyde (AASA) (which results from lysine oxidation) (40) were used. Both carbohydrates and polyunsaturated fatty acids, when reacting with free radicals, generate highly reactive dicarbonyl compounds. These in turn produce specific, non-enzymatic adducts when reacting with proteins, such as N<sup>2</sup>-carboxymethyl-lysine (CML), N<sup>2</sup>-carboxyethyl-lysine (CEL) and N<sup>2</sup>-malondialdehyde-lysine (MDAL) (41). The lipid peroxidation product MDAL has been shown to accumulate in the Abcd1 null mouse spinal cord as early as 3.5 months of age. At 12 months of age, additional markers of protein oxidative lesions, such as AASA, GSA (direct carboxylation), CEL and CML (glycoxidation/lipoxidation), are also increased (31). To determine whether ABCD2 overexpression is able to ameliorate oxidative lesions in vivo, we used a transgenic mouse line overexpressing ABCD2 under the control of the ubiquitous chicken β-actin promoter (20). These mice were crossed with the double-knockout Abcd1<sup>−/−</sup>/Abcd2<sup>−/−</sup> mice to generate triple mutant mice (Abcd1<sup>−/−</sup>/Abcd2<sup>−/−</sup>/TgAbcd2<sup>+</sup>). We had formerly reported that expression levels of the ABCD2 protein in spinal cord increased 5–10-fold over the baseline level and prevented accumulation of saturated VLCFA in organs and the onset of axonal degeneration (20). Here, we quantified oxidative lesion markers in double and triple mutants, and detected even higher amounts of MDAL in the Abcd1<sup>−/−</sup>/Abcd2<sup>−/−</sup> mice compared with Abcd1<sup>−/−</sup> mice (Fig. 1C). This suggests that loss of function of ABCD2 contributes to the lipoxidative lesions that are observed in double-knockout Abcd1<sup>−/−</sup>/Abcd2<sup>−/−</sup> mice. Most importantly, overexpression of ABCD2 normalized the levels of all markers of oxidative damage in spinal cord from the Abcd1<sup>−/−</sup>/Abcd2<sup>−/−</sup>/TgAbcd2<sup>+</sup> mice (Fig. 1C).

**VPA corrects levels of oxidative lesion markers in X-ALD fibroblasts**

In X-ALD patients, the same types of protein oxidative lesions as described in spinal cord occur in fibroblasts and are exacerbated by C26:0 exposure (31). To investigate whether the observed VPA induction of ABCD2 expression correlated with amelioration of the oxidative damage to proteins, we quantified the markers of each pathway of protein oxidative damage (31). After 7 days of treatment of X-ALD fibroblasts with VPA, the carboxylation and glycoxidation/lipoxidation markers were reduced compared with untreated X-ALD fibroblasts.
markers of oxidative damage were significantly decreased or normalized (Fig. 1D).

Effects of VPA on fatty acid metabolism

As VPA is able to stimulate expression of ABCD2 mRNA, the expected consequence would be normalization of the saturated VLCFA levels, as it has been shown in vitro (17,18,42) and in vivo after ABCD2 overexpression (20).

Increased levels of saturated hexacosanoic acid (C26:0), and tetracosanoic acid (C24:0), together with an increased C26:0/C22:0 ratio, are traditionally considered the most reliable diagnostic biochemical markers for X-ALD. VPA did not change the cellular levels of saturated VLCFA, such as C26:0 (Fig. 2A) or C24:0 (data not shown) in fibroblasts. In contrast, VPA reduced the abnormal accumulation of C26:1ω9, a monounsaturated VLCFA by 40% (Fig. 2A). C26:1ω9 also accumulates in the plasma and brain of X-ALD patients, although to a lesser extent (~2-fold over the baseline) than saturated C26:0, whose levels can be 5–7-fold higher in patients than in control individuals (2,43). This raises the possibility that the antioxidant effects observed after VPA treatment might be at least partially mediated by a decrease in unsaturated VLCFA.

**Ex vivo effects of VPA on ABCD2 gene expression and VLCFA levels**

As the half-life of VPA in rodents is very short (2.5 h in rodents compared with 8–10 h in humans) (44), we used an ex vivo rather than an in vivo system to assess the effects of VPA on ABCD2 gene. Rat organotypic hippocampal slice cultures (OHSCs) are often used as a drug screening and drug validation tool (45,46). Treating rat and mouse OHSCs with 2 mM of VPA for 2 days resulted in a 2.1-fold increase in ABCD2 gene expression (Fig. 2B). A similar effect (induction just below 2-fold) was found in OHSCs from Abcd1 null mice. To investigate whether VPA could also induce ABCD2 expression in the adult human hippocampal brain, slices obtained after epilepsy surgery were cultured on the same media used for the rat OHSC experiments and were treated with 2 mM VPA for 48 h. Treatment induced on average a 4-fold increase in ABCD2 gene expression (Fig. 2B). Doses of VPA of 0.8 mM were sufficient to double the expression levels of ABCD2 (Supplementary Material, Fig. S1). Interspecies differences might account for the higher increase in ABCD2 in human slices. Next, we investigated whether C26:1ω9 and C26:0 were modulated by VPA. OHSCs from 4-week-old wild-type, Abcd1-knockout and double-knockout Abcd1/Abcd2 mice were generated. As both C26:0 and C26:1ω9 are increased in Abcd1–/– and Abcd1–/Abcd2–/– derived cells, the brain specimens from double-knockout mice might offer information on the effects of VPA, independent of ABCD2 expression. After 12 days incubation with VPA, expression of the Abcd2 gene increased about 2-fold in Wt and Abcd1–/– OHSCs (Fig. 2B), but was not measurable in OHSCs from Abcd1–/Abcd2–/– mice, most likely due to mRNA decay (data not shown) (20). The concentration of C26:1ω9, but not of C26:0, decreased by 25% in Abcd1–/– OHSCs (Fig. 2C). This decrease in C26:1ω9 concent-

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**Figure 2.** Effect of VPA on VLCFA levels in human X-ALD fibroblasts (A). C26:0 and C26:1ω9 levels in control (n = 4) and X-ALD (n = 5) fibroblasts treated with VPA for 12 days. Fatty acids are expressed as percentage of total fatty acids analyzed compared with levels in control. C26:0 and C26:1ω9 levels in control correspond to 0.24 and 0.50 nmol/mg of protein. ABCD2 is induced by VPA in OHSCs. (B) ABCD2 expression level in rat (n = 3), Wt and Abcd1–/– mice (n = 4 [genotype]) OHSCs and human organotypic hippocampal brain slices (n = 2) treated with VPA (2 mM) for 2 days. ABCD2 expression levels were compared with control tissue by quantitative RT-PCR and normalized to 36B4. Data are represented as mean ± SD. Effects of VPA on C26:1ω9 in Abcd1–/– mice OHSCs are mediated via ABCD2. (C) C26:0 and C26:1ω9 levels in OHSCs from Wt (n = 3), Abcd1–/– (n = 4) and Abcd1–/Abcd2–/– (n = 3) mice treated with VPA for 12 days. Fatty acids are expressed as percentage of total fatty acids analyzed and compared with levels in control. C26:0 and C26:1ω9 levels in Wt correspond to 0.48 and 0.17 nmol/mg of protein. C26:1ω9 generates ROS in human fibroblasts (D). Control (n = 4) and X-ALD human fibroblasts (n = 5) were treated with C18:1ω9 (50 μM), C24:1ω9 (50 μM), C26:0 (25 or 50 μM) or C26:1ω9 (25 or 50 μM) for 24 h, then intracellular ROS using the probe DCFH were measured. Significant differences have been determined by ANOVA followed by Tukey’s HSD post hoc (*P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001).
VPA treatment induces elongase gene expression

As an HDAC inhibitor, VPA is thought to act at the transcriptional level on some 10% of coding genes (47). As the fatty acid content of the cell is a product of fatty acid synthesis and degradation, we hypothesized that VPA could induce the expression of genes involved in the synthesis of saturated VLCFA and eventually ‘mask’ the expected benefit from the increased catabolism of C26:0 mediated via ABCD2 up-regulation.

In mammalian tissues, VLCFA are synthesized from LCFA by elongases. Seven human elongases have been identified (ELOVL1–7), strongly conserved genes between mouse and human genomes (48,49). The effect of VPA on the level of expression of different elongase genes was evaluated by real-time quantitative PCR in human X-ALD fibroblasts. The expression of ELOVL3, ELOVL4 and ELOVL5 genes was increased between 1.5- and 2.5-fold after VPA treatment, both in X-ALD and control fibroblasts (Fig. 3). ELOVL4 has been reported to catalyze the elongation of C26:0 to C28:0 and C28:0 to C30:0 (50,51), whereas ELOVL3 mediates the elongation of C22:0 to C24:0 and C24:0 to C26:0 in vivo (48). Interestingly, ELOVL3 transcriptional regulation has been shown to depend on the expression of ABCD2 in the mouse liver (52). In contrast, the expression of ELOVL1 gene was not sensitive to VPA administration. ELOVL2, ELOVL6 and ELOVL7 expression was not detected in human fibroblasts.

We next tested the influence of VPA on the enzyme stearoyl-coenzyme A desaturase (SCD), which is also involved in the synthesis of monounsaturated fatty acids from saturated fatty acids. In human fibroblasts, VPA did not modify the expression of the SCD gene (Fig. 3). These results suggest that VPA could stimulate the elongation of saturated fatty acids via increased transcription of ELOVL3.

DISCUSSION

Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54). In the X-ALD mouse model that mimics AMN, Oxidative damage is a common feature of neurodegenerative disease, with a proven role in the pathology of diseases, such as amyotrophic lateral sclerosis and Parkinson’s disease (53,54).
free radicals due to the accumulation of hexacosanoic acid and to an impaired response of the antioxidant defense system(s) to oxidative insults. The latter defect is illustrated by the following experiments: (i) X-ALD fibroblasts die more quickly following glutathione depletion than their control cell lines; and (ii) excess C26:0 produces ROS in X-ALD and control fibroblasts, but oxidative lesions of proteins are generated in X-ALD cells only (31). Although initial experiments have demonstrated the role of saturated hexacosanoic acid C26:0 in triggering oxidative stress and damage in fibroblasts, data from the current study reveal that the monounsaturated C26:1 fatty acid, which also accumulates in X-ALD, is a more powerful inducer of ROS in vitro than C26:0. Moreover, we show that VPA ameliorates oxidative lesions to proteins without affecting levels of hexacosanoic acid, but decreasing the levels of C26:1ω9 instead.

These results indicate that the excess saturated VLCFA might not be the sole cause of oxidative damage in X-ALD and underline the role played by C26:1ω9 and possibly, by other fatty acids transported across the peroxisomal membranes by the ABCD1 transporter. This is in line with recent observations indicating that ABCD1 is capable of importing a range of LCFA and VLCFA when overexpressed in yeast (6). These findings are of particular relevance to Lorenzo’s oil, the dietary therapy in current use for X-ALD. The oil is a 4:1 mixture of oleic acid (C18:1) and erucic acid (C22:1), both on a glycerol backbone. Treatment with Lorenzo’s oil normalizes saturated VLCFA within 4–6 weeks, without halting neurological progression (7) or ameliorating oxidative damage as measured by thiobarbituric acid reactive substances in plasma (55). Administration of Lorenzo’s oil results in a marked increase in C26:1ω9 to C32:1ω9 fatty acids in the plasma of treated patients (56). This effect is attributed to an increase in long-chain monounsaturated substrates and subsequent increase in ω9-VLCFA elongation from erucic and oleic fatty acids. In view of the presented data, Lorenzo’s oil failure to reduce oxidative damage in spite of the decrease in C26:0 could be due to the counteracting effects of lowering C26:0 in one hand, while simultaneously increasing C26:1 levels on the other.

The concept of overlapping functions between ABCD1 and ABCD2 transporters gains strength in this work. We had previously shown that overexpression of ABCD2 prevented accumulation of saturated VLCFA in target organs and disease onset in the mouse, and we currently present compelling evidences indicating that overexpression of ABCD2 prevents oxidative damage to proteins. In this context, amelioration of C26:1ω9 levels instead of C26:0 was somewhat unexpected, as we had shown formerly that a transgenic mouse exhibiting constitutive ABCD2 overexpression could normalize the C26:0 levels in tissues (20). This discrepancy could owe to different mechanisms or the combination of them: (i) VPA raising the synthesis of saturated VLCFA, via increase in transcription of saturated VLCFA elongases (ELOVL3 and ELOVL4); and (ii) levels of induction of ABCD2 achieved by VPA are insufficient to perform with efficacy the import of C26:0 and degradation in peroxisomes. In fibroblasts, the levels of ABCD2 induction were ~3-fold in contrast to in vivo observations, in which ABCD2 protein expression increased 5–10-fold (20). This suggests a higher

Figure 4. Small pilot trial on X-ALD patients. Effects of VPA on X-ALD patients after 6 months of treatment. (A) Induction of ABCD2 mRNA expression in human mononuclear cells from five X-ALD patients treated with VPA for 6 months. (B) Increased oxidative lesion markers in peripheral mononuclear cells of X-ALD. Quantification by GC/MS of AASA, GSA, CML, CEL and MDAL in human mononuclear cells from five X-ALD patients and four controls sex- and age-matched. (C) VPA ameliorates oxidative markers in human mononuclear cells from X-ALD patients. AASA, GSA, CML, CEL and MDAL were quantified by GC/MS before (t = 0) and at the end of the trial with VPA (t = 6). Significant differences have been determined by ANOVA followed by Tukey’s HSD post hoc (∗P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001).

amyloid precursor protein and synaptophysin in axonal swellings appear. Additional evidence of the contribution of oxidative damage to the neurodegenerative cascade of X-ALD has been reported in post-mortem brain samples from patients with cerebral ALD, which show increased immunoreactivity to markers of lipoxidative damage (37), and in the plasma of X-ALD patients using the thiobarbituric acid test (39). Thus, oxidative damage could be linked to both the initiation and the progression of X-ALD neurodegeneration. The origin of this oxidative damage is being intensively studied. Current evidence points to the excessive production of
affinity of the transporter for the C26:1 VLCFA, a matter that deserves further investigation. Nevertheless, this increase in ABCD2 expression was sufficient to decrease C26:1 level, correlating with an important decrease in levels of the markers of protein oxidative lesions. In a similar manner, VPA treatment in the clinical setting corrects oxidative damage in peripheral mononuclear cells, independently of VLCFA levels (both saturated and monounsaturated fatty acids), in spite of a mild ABCD2 induction. This opens several possibilities: (i) the shown levels of ABCD2 induction are sufficient to correct oxidative stress by, for instance, transporting an unidentified substrate of ABCD1; (ii) peripheral mononuclear cells are simply not the best cells to analyze the effect of VPA on the VLCFA levels, despite an effect of oxidative lesions; or (iii) VPA might exert antioxidant effects independently of ABCD2. Indeed, VPA has repeatedly been associated with neuroprotective and antioxidant effects in vitro and in vivo, including descriptions of beneficial effects through enhancing mitochondria respiration rate and function in long-term in vivo treatments (57–59). On the other hand, it has been sporadically reported to be associated with hepatotoxicity due to oxidative burden (60). These are adverse effects that can easily be controlled and reverted by using l-carnitine (61). VPA has therefore always been considered a very safe drug. Our results support the engagement of AMN patients in longer, larger trials to allow clinical evaluation of potential therapeutic benefits.

MATERIALS AND METHODS

Chemicals

Chemicals and glassware analytical-grade chloroform, methanol, n-hexane, acetonitrile, ammonium, hydrochloric acid (fuming, 37%) and acetic acid (glacial, 100%) were obtained from Merck (Darmstadt, Germany). Deionized water was passed through a MilliQ Labo system (Millipore, Bedford, MA, USA). Tetracosanoic acid (C24:1ω9), hexacosanoic acid (C26:0) and VPA were purchased from Sigma (St Louis, MO, USA). Deuterium-labeled free fatty acid internal standards 3,3,5,5′-H4-C22:0 (isotopic purity: >98%), 3,3,5,5′-H4-C24:0 (isotopic purity: >98%) and 3,3,5,5′-H4-C26:0 (isotopic purity: >98%) were obtained from Dr H.J. ten Brink, Free University Hospital (Amsterdam, The Netherlands) (62). All glassware was acid washed and prior to use rinsed with chloroform containing 1% acetic acid. 6-Carboxy-2′, 7′-dichlorodihydrofluorescein diacetate, diacetoxymethyl-ester (H2-DCFDA) and VPD were, respectively, purchased from Molecular Probes and Lancaster synthesis.

Mouse breeding

The genotyping of Abcd1−/− and Abcd2−/− and transgenic overexpressing ABCD2 mice has been described previously (20,21). To obtain double heterozygous mutants, we crossed Abcd1 null females (mutation is on the X chromosome), with Abcd2−/− males. Double heterozygous in the F1 generation were intercrossed to obtain double-knockout mice and wild-type littermate controls; the offspring obeyed Mendelian ratios. Mice used for OHSC and fatty acid analysis, and quantification of oxidative lesion markers were on a pure C57BL/6J background (backcrossed at least 12 generations into C57BL/6J). Only male littermates were used for all the experiments. All animals were housed under the same controlled conditions, between 22 and 25°C, on a 12 h light/dark cycle, with free access to food and water. Animals were sacrificed and tissues including brain, spinal cord, sciatic nerve, adrenal gland and liver were snap-frozen in liquid nitrogen and conserved at −80°C. All methods employed in this work are in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996) and with the Ethics Committee of IDIBELL and the Generalitat de Catalunya.

Cell culture

Control and X-ALD human fibroblasts were grown in DMEM containing 10% of fetal bovine serum, 100 U/ml penicillin and 100 mg streptomycin, at 37°C in humidified 95% air/5% CO2. Unless otherwise stated, experiments were carried out with cells at 95% of confluence. Skin biopsies to prepare the fibroblastic lines were collected according to the institutional guidelines for sampling including informed consent of the persons involved or their representatives.

Organotypic hippocampal slice culture

The hippocampus from 4-week-old mice was immediately removed and placed in ice-cold dissecting media (pH 7.15). Next, the hippocampus was cut into 200 μm thick slices using a McIlwain tissue chopper to generate the organotypic slice cultures and placed into a sterile Petri dish with Grey’s balanced salt solution. Hippocampal brain slices were transferred onto Millicell-CM-cultured plate inserts (Millipore). The inserts were placed into wells of a six-well plate containing 1.0 ml of medium containing 50% MEM with Earl’s salts and glucose (6 mg/ml) (Gibco-BRL) (63).

OHSCs derived from 5-day-old Wistar rats were prepared according to interface technique (46). After dissection of the frontal pole of the hemispheres and the cerebellum, the brains were cut into 350 μm thick horizontal slices on a vibratome (Leica Microsystems, Wetzlar, Germany). For each experiment, three slices were transferred into culture plate insert membranes (BD Biosciences, San Jose, CA, USA) and thereafter into six-well culture dishes (BD Biosciences) containing 1.2 ml culture medium as described in detail by Eyüpoglu et al. (63). One day after preparation, the culture medium was changed and OHSCs were exposed to the test compound for 48 h and snap-frozen in liquid nitrogen, as described previously (63). Hippocampal specimens were derived from two patients submitted to epilepsy surgery at the Erlangen Epilepsy Center. The patients suffered from drug-resistant epilepsy and were scheduled for hippocampal resection. VPA treatment was stopped several weeks prior to surgery. For scientific use of tissue specimens, informed consent was obtained from each patient with the approval of the local Ethics Committee of the University Hospital of...
Erlangen. Surgical specimens were prepared and processed as described for rat OHSCs.

Evaluation of intracellular ROS

Intracellular ROS levels were estimated using the ROS-sensitive H$_2$-DCFDA probe as described (31). Following incubation with 10 µM H$_2$-DCFDA for 30 min, cells were washed twice with PBS and scraped into water. The fluorescence of H$_2$-DCFDA-stained cells was measured with a spectrofluorimeter (excitation wavelength 493 nm, emission wavelength 527 nm). Fatty acids were dissolved in ethanol and added to the medium for 24 h.

Measurement of oxidative lesion markers: GSA, AASA, CML, CEL and MDAL

GSA, AASA, CML, CEL and MDAL concentrations in total proteins from spinal cord homogenates or human fibroblasts were measured by gas chromatography/mass spectrometry (GC/MS), essentially as previously reported (31). The amounts of products were expressed as the ratio of micromole of GSA, AASA, CML, CEL or MDAL/mol of lysine.

Fatty acid determination

Total cellular fatty acids were analyzed by electrospray ionization mass spectroscopy method described by Valianpour et al. (2).

Synthesis of C26:1ω9

C26:1ω9 was synthesized from commercially available C24:1ω9 using established methods.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using RNeasy Kit (Qiagen) and quantitative PCR experiments were performed according to the manufacturer’s instructions (LightCycler, Roche Diagnostics). PCRs were carried out with 36B4 (also called RPL0) used as a standard control gene. Primers used are in Supplementary Material, Table S1. Data are given as mean ± SEM.

Pilot trial with VPA

Five patients with X-ALD were enrolled after assessing normal liver transaminase activities. Four of the patients were affected with demyelinating cerebral adrenoleukodystrophy at ages between 7 and 18 years old. The fifth patient was affected with pure AMN and Addison’s disease. Laboratory testing, including a basic chemistry profile, complete blood count with platelets, transaminases, carnitine profile, amylase, lipase and trough VPA levels, was performed at baseline and every 4 weeks following initiation. Dosing of VPA (Depakine), calculated at 40 mg/kg/day, was typical of that used in epilepsy patients (15–50 mg/kg/day). Patients reported no side effects related to VPA administration and compliance with the treatment was checked by monitoring the serum concentrations of VPA. All treated patients achieved serum concentrations within the range considered therapeutic for epilepsy (70–100 mg/l). Capsules were administered once daily during a 6-month period. Blood samples were taken on the day of starting the treatment ($t = 0$), 3 months ($t = 3$) and 6 months later ($t = 6$). Informed and written consent was obtained from all patients or their legal representatives and studies were approved by the local Ethics Committee of Hôpital Saint Vincent de Paul, Paris.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

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peroxisomal ABC half transporter ALDP functions as a homodimer and accepts acyl-CoA esters. 

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