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Lovastatin and sodium phenylacetate normalize the levels of very long chain fatty acids in skin fibroblasts of X- adrenoleukodystrophy

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Abstract The present study underlines the importance of lovastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, and the sodium salt of phenylacetic acid (NaPA), an inhibitor of mevalonate pyrophosphate decarboxylase, in normalizing the pathognomonic accumulation of saturated very long chain fatty acids (VLCFA) in cultured skin fibroblasts of X-adrenoleukodystrophy (X-ALD) in which the ALD gene is either mutated or deleted. Lovastatin or NaPA alone or in combination stimulated the β-oxidation of lignoceric acid ($C_{24:0}$) and normalized the elevated levels of VLCFA in skin fibroblasts of X-ALD. Ability of lovastatin and NaPA to normalize the pathognomonic accumulation of VLCFA in skin fibroblasts of X-ALD may identify these drugs as possible therapeutics for X-ALD.

Key words: Lovastatin; Phenylacetate; Adrenoleukodystrophy; Very long chain fatty acid; Beta-oxidation

1. Introduction

X-linked adrenoleukodystrophy (X-ALD), an inherited peroxisomal disorder, is characterized by progressive demyelination and adrenal insufficiency. It is the most common peroxisomal disorder affecting between 1/15 000 and 1/20 000 boys with the manifestation of different degrees of neurological disability. Although X-ALD presents as various clinical phenotypes, including childhood X-ALD, adrenomyeloneuropathy (AMN), and Addison's disease, all forms of X-ALD are associated with the pathognomonic accumulation of saturated very long chain fatty acids (VLCFA) as a constituent of cholesterol esters, phospholipids and gangliosides [1,2]. Due to the presence of similar concentration of VLCFA in plasma and as well as in fibroblasts of X-ALD, fibroblasts are generally used for both prenatal and postnatal diagnosis of the disease [1,2].

A number of laboratories, including ours, showed that β-oxidation of VLCFA is a peroxisomal function [3–7]. The deficient activity of lignoceroyl-CoA ligase as compared to the normal oxidation of lignoceroyl-CoA in purified peroxisomes isolated from fibroblasts of X-ALD suggested that the abnormality in the oxidation of VLCFA may be due to deficient activity of lignoceroyl-CoA ligase required for the activation of lignoceric acid to lignoceroyl-CoA [4,6]. While these metabolic studies indicated lignoceroyl-CoA ligase gene as a X-ALD gene, positional cloning studies led to the identification of a gene that encodes a protein (ALDP), with significant homology with the ATP-binding cassette (ABC) of the superfamily of transporters [8]. The normalization of fatty acids in X-ALD cells following transfection of the X-ALD gene [9] supports a role for ALDP in fatty acid metabolism; however, the precise function of ALDP in the metabolism of VLCFA is not known at present.

Similar to other genetic diseases affecting the central nervous system, gene therapy in X-ALD does not seem to be a real option in the near future and in the absence of such a treatment a number of therapeutic applications have been investigated [1,10]. Adrenal insufficiency associated with X-ALD responds readily to steroid replacement therapy; however, there is as yet no proven remedy for the neurological disability [10]. Two forms of treatment are currently under investigation. The dietary therapy with 'Lorenzo's oil' does normalize the plasma levels of VLCFA, but it does not seem to improve the clinical status of the X-ALD patients [10–12]. Bone marrow transplantation also appears to be of only limited value because of the complexity of the protocol and the insignificant improvement in the clinical status of the patient [10].

The studies described in this article demonstrate that lovastatin and sodium phenylacetate (NaPA), inhibitors of the mevalonate pathway, normalize the levels of VLCFA in skin fibroblasts of X-ALD by increasing the peroxisomal activity for β-oxidation of VLCFA. In light of the fact that these compounds also inhibit the induction of proinflammatory cytokines and nitric oxide synthase in astrocytes and microglia [13], we propose that these drugs may have therapeutic potential in correction of the metabolic defect and inhibition of the neuroinflammatory disease process in X-ALD.

2. Materials and methods

2.1. Reagents

DMEM, bovine calf serum and Hanks' buffered salt solution (HBSS) were from Gibco. [1-^{14}C]Lignoceric acid was synthesized by treatment of *n*-tricosanoyl bromide with K^{14}CN as described previously [14].

2.2. Enzyme assay for β-oxidation of lignoceric acid

The enzyme activity of [1-^{14}C]lignoceric acid β-oxidation to acetate was measured in intact cells suspended in HBSS. Briefly, the reaction mixture in 0.25 ml of HBSS contained 50–60 g of protein and 6 μM [1-^{14}C]lignoceric acid. Fatty acids were solubilized with α-cyclodextrin and β-oxidation of [1-^{14}C]lignoceric acid was carried out as described previously [3,6].

2.3. Measurement of VLCFA in fibroblasts

Fatty acid methyl ester (FAME) was prepared as described previously by Lepage and Roy [15] with modifications. Fibroblast cells, suspended in HBSS, were disrupted by sonication to form a homogeneous solution. An aliquot (200 μl) of this solution was transferred to a glass tube and 5 μg heptacosanoic (27:0) acid was added as internal

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standard and lipids were extracted by Folch partition. Fatty acids were transesterified with acetyl chloride (200 μl) in the presence of methanol and benzene (4:1) for 2 h at 100°C. The solution was cooled down to room temperature followed by addition of 5 ml 6% potassium carbonate solution at ice-cooled temperature. Isolation and purification of FAME were carried out as detailed by Dacremont et al. [16]. Purified FAME, suspended in chloroform, was analyzed by gas chromatograph GC-15A attached with chromatopac C-R3A integrator from Shimadzu Corporation.

2.4. Preparation of post-nuclear membrane and Western blot analysis

The membranes were prepared as described previously [17]. Briefly, the post-nuclear fraction was diluted with an ice-cold solution of 0.1 M sodium carbonate, 30 mM iodoacetamide, pH 11.5. After 30 min of incubation at 4°C, the membranes were sedimented by ultracentrifugation. The sedimented membranes were electrophoresed in 7.5% sodium dodecylsulfate-polyacrylamide gel, transferred to PVDF membranes and immunoblotted with antibodies against ALDP as described [17].

2.5. RNA isolation and Northern blot analysis

Cultured skin fibroblasts were taken out from culture flasks directly by adding Ultraspec-II RNA reagent (Biotecx) and total RNA was isolated according to the manufacturer's protocol. 20 μg of RNA from each sample was electrophoretically resolved on 1.2% denaturing formaldehyde-agarose gel, transferred to nylon membrane, and cross-linked using UV Stratalinker (Stratagene, USA). Full length ALDP cDNA was kindly provided by Dr. Patrick Aubourg, INSERM, Hospital Saint-Vincent-de-Paul, Paris, France. ^{32}P-labeled cDNA probes were prepared according to the instructions provided with Ready-To-Go DNA labeling kit (Pharmacia Biotech). Northern blot analysis was performed essentially as described for Express Hyb Hybridization solution (Clontech) at 68°C. GAPDH cDNA probe was used as standard for comparing hybridization signals.

3. Results

3.1. Inhibitors of mevalonate pathway stimulate the β-oxidation of lignoceric acid in X-ALD fibroblasts

First, we studied the effect of mevalonate inhibitors (lovastatin, mevastatin and NaPA) on the β-oxidation of lignoceric acid in control human skin fibroblasts. It is apparent from Table 1 that lovastatin, mevastatin and NaPA stimulated the β-oxidation of lignoceric acid in control human skin fibroblasts. Since the β-oxidation of lignoceric acid is impaired in X-ALD patients, we studied the effect of these compounds on lignoceric acid β-oxidation in cultured skin fibroblasts of X-ALD. Similar to control fibroblasts, these compounds also stimulated lignoceric acid β-oxidation in X-ALD fibroblasts (Fig. 1). Both lovastatin (Fig. 1A) and NaPA (Fig. 1B) stimulated lignoceric acid β-oxidation in X-ALD fibroblasts in a dose-dependent manner. The highest dose of lovastatin found to stimulate lignoceric acid β-oxidation (by 70%) was 5 μM whereas the highest dose of NaPA found to stimulate lignoceric acid β-oxidation (by 40%) was 5 mM. However, a greater degree of stimulation (more than two-fold) was observed by a combination of lovastatin and NaPA even at a dose lower than the one used individually (Fig. 1B). Higher doses of lovastatin (10–20 μM) or NaPA (10–20 mM) were cytotoxic to the X-ALD fibroblasts and did not result in further significant stimulation (data not shown). In the cell fatty acids are oxidized by mitochondrial and peroxisomal β-oxidation enzyme systems. We examined the effect of etomoxir, an inhibitor of mitochondrial β-oxidation, on the β-oxidation of fatty acids [18]. Etomoxir had no effect on lovastatin- or NaPA-

Table 1
Lovastatin and NaPA stimulate the β-oxidation of lignoceric acid in control human skin fibroblasts

Treatment	Lignoceric acid β-oxidation (pmol/h/mg protein)
Control	570.2 ± 52.3
Lovastatin (5 μM)	945.7 ± 105.6
Mevastatin (5 μM)	889.6 ± 78.4
NaPA (5 mM)	826.2 ± 87.2

Cells were treated for 72 h in serum-containing DMEM with the listed reagents; β-oxidation of lignoceric acid was measured as described in Section 2. Medium was replaced after every 24 h with the addition of fresh reagents. Data are mean ± S.D. of three different experiments.

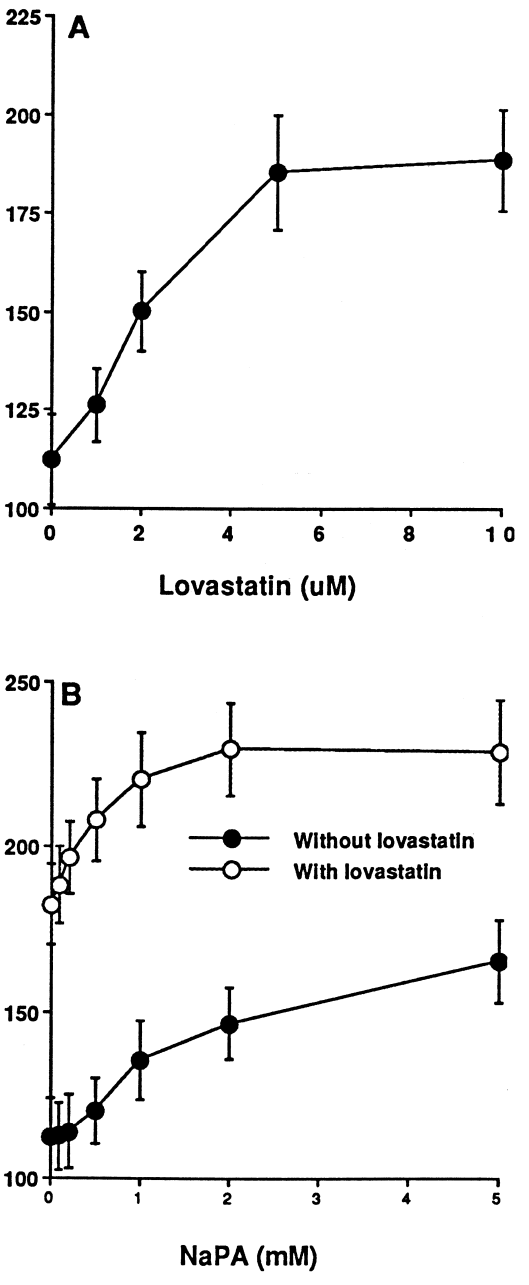

Fig. 1. Lovastatin and NaPA stimulate the β-oxidation of lignoceric acid in cultured skin fibroblasts of X-ALD in a dose-dependent manner. Cells were incubated in serum-containing DMEM with different concentrations of lovastatin (A) or NaPA in the presence or absence of 2 μM lovastatin (B). After every 24 h, medium was replaced with the addition of fresh reagents. Lignoceric acid β-oxidation was measured after 72 h in cell suspension as mentioned in Section 2. Values are mean ± S.D. of three different experiments.

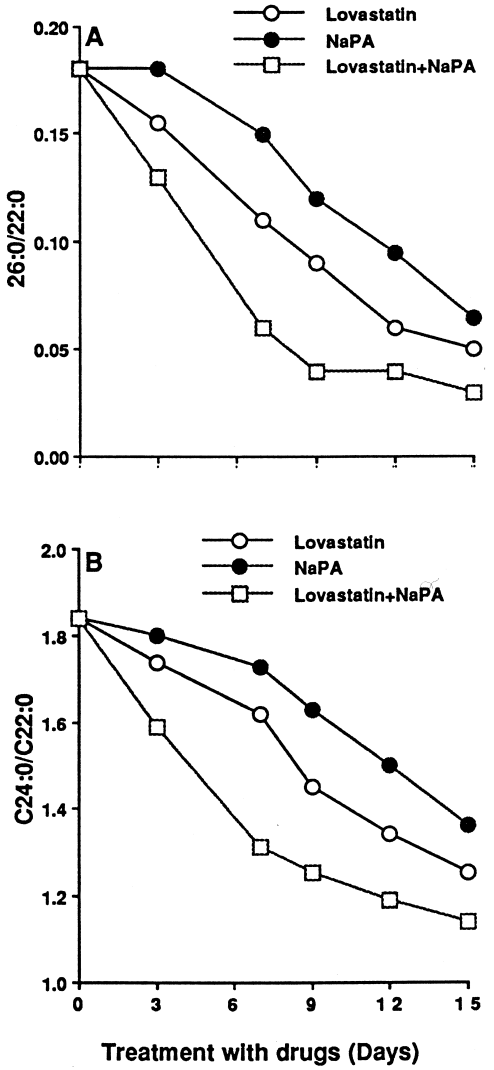

Fig. 2. Time-dependent effect of lovastatin and NaPA on the ratios of VLCFA ($C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$) in cultured skin fibroblasts of X-ALD. Cells were incubated in serum-containing DMEM with 5 μM lovastatin, 5 mM NaPA or the combination of 2 μM lovastatin and 2 mM NaPA for different days, and the ratios of $C_{26:0}/C_{22:0}$ (A) and $C_{24:0}/C_{22:0}$ (B) were measured as described in Section 2. Values are mean of two different experiments.

mediated stimulation of lignoceric acid β-oxidation (data not shown) suggesting that the observed stimulation of lignoceric acid β-oxidation was a peroxisomal function.

3.2. *Modulation of cellular content of VLCFA in X-ALD fibroblasts by lovastatin and NaPA*

Since mevalonate inhibitors increased β-oxidation of lignoceric acid in control as well as X-ALD fibroblasts, we examined the effect of these compounds on the in situ levels of VLCFA in X-ALD fibroblasts. Treatment of X-ALD fibroblasts with 5 μM of lovastatin for different time periods (days) resulted in a time-dependent decrease in the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ as shown in Fig. 2. Within 12–15 days of treatment, the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in X-ALD fibroblasts decreased to the normal level. Similar to lovastatin, NaPA also lowered the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in X-ALD fibroblasts almost to the normal level after 15 days of treatment. However, consistent with the higher degree of stimulation of lignoceric acid β-oxidation by a combination of lovastatin and NaPA, the same combination lowered the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ to normal levels within 7 days (Fig. 2). This decrease in the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ was also associated with a decrease in the absolute amounts of $C_{24:0}$ and $C_{26:0}$ whereas no significant change was observed in the levels of $C_{22:0}$ (behenoic acid) (data not shown).

3.3. *Normalization of the levels of VLCFA by lovastatin or NaPA in different X-ALD cells with or without deletion of the X-ALD gene*

Although the precise function of ALDP, X-ALD gene product, in the metabolism of VLCFA is not known at the present time, however, accumulation of VLCFA in X-ALD cells with loss or mutations in ALDP and their normalization following transfection of cDNA for ALDP indicate a role of ALDP in the metabolism of VLCFA [9]. Therefore, we next attempted to examine whether lovastatin or NaPA were able to lower the levels of VLCFA in X-ALD fibroblast cell lines with mutation or deletion of the X-ALD gene. The status of ALDP mRNA or protein (Fig. 3) and the rate of β-oxidation of lignoceric acid (Table 2) in different X-ALD fibroblasts indicates that ALDS2, ALDS3 and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene, whereas ALDS5 and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene. It is apparent from Table 2 that the treatment of X-ALD fibroblasts with lovastatin or NaPA or a combination of these stimulated the β-oxidation of lignoceric acid (55–80%) and normalized the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ suggesting that these drugs are capable of lowering

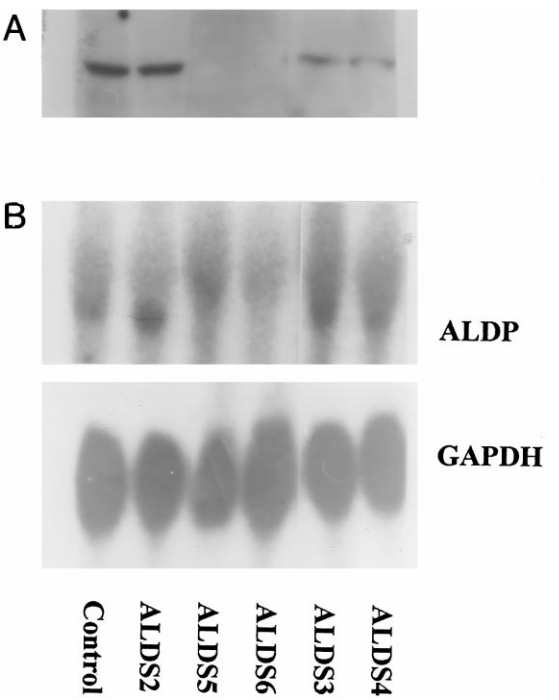

Fig. 3. Expression of ALDP mRNA and protein in different skin fibroblasts of X-ALD. Western blot analysis (A) of post-nuclear membrane fraction with antibodies against ALDP and Northern blot analysis for ALDP mRNA (B) were carried out as described in Section 2. ALDS2, ALDS3 and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene, whereas ALDS5 and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene.

Table 2
Effect of lovastatin and NaPA on (A) β-oxidation of lignoceric acid and (B) the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in cultured skin fibroblasts of X-ALD

Cell line	Lignoceric acid β-oxidation (pmol/h/mg protein)					
	Control	Lovastatin	NaPA	Lovastatin+NaPA		
A.						
ALDS2	142.7 ± 15.7	223.5 ± 24.1	202.5 ± 17.4	274.6 ± 30.5		
ALDS5	154.2 ± 14.2	248.2 ± 26.2	211.5 ± 22.6	296.2 ± 25.6		
ALDS6	132.4 ± 15.9	218.3 ± 19.8	189.7 ± 21.2	250.1 ± 28.3		
ALDS3	122.3 ± 11.7	201.3 ± 22.3	183.2 ± 17.3	248.6 ± 29.6		
ALDS4	118.5 ± 12.6	192.8 ± 20.5	178.9 ± 18.3	238.7 ± 21.1		
	$C_{26:0}/C_{22:0}$			$C_{24:0}/C_{22:0}$		
	Control	Lovastatin	Lovastatin+NaPA	Control	Lovastatin	Lovastatin+NaPA
B.						
ALDS2	0.17 ± 0.022	0.049 ± 0.01	0.04 ± 0.008	1.84 ± 0.25	1.25 ± 0.15	1.14 ± 0.15
ALDS5	0.18 ± 0.025	0.055 ± 0.008	0.04 ± 0.007	1.94 ± 0.29	1.28 ± 0.21	1.18 ± 0.12
ALDS6	0.22 ± 0.034	0.058 ± 0.01	0.045 ± 0.008	2.01 ± 0.3	1.31 ± 0.18	1.21 ± 0.14
ALDS3	0.16 ± 0.024	0.045 ± 0.06	0.03 ± 0.005	1.88 ± 0.21	1.26 ± 0.16	1.19 ± 0.25
ALDS4	0.19 ± 0.028	0.052 ± 0.07	0.036 ± 0.006	1.96 ± 0.23	1.29 ± 0.02	1.22 ± 0.15

Cells were incubated in serum-containing DMEM with 5 μM lovastatin, 5 mM NaPA or the combination of 2 μM lovastatin and 2 mM NaPA for 15 days, and the β-oxidation of lignoceric acid (A) and the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ (B) were measured as described in Section 2. Results are mean ± S.D. of three different experiments. ALDS2, ALDS3 and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene, whereas ALDS5 and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene.

the level of VLCFA in X-ALD fibroblasts to the normal levels, irrespective of mutation or deletion of the X-ALD gene, the candidate gene for X-ALD.

4. Discussion

In the current work we provide evidence for the possible therapeutic intervention against pathognomonic accumulation of VLCFA in X-ALD. Despite mutation or deletion of the X-ALD gene, lovastatin and NaPA lowered the cellular content of VLCFA to normal levels in different skin fibroblasts of X-ALD. The detailed mechanism leading to the decrease in the accumulation of VLCFA in X-ALD fibroblasts is not known, but is likely through the stimulation of peroxisomal β-oxidation.

The pathogenetic mechanisms of X-ALD are poorly understood. At present it is not known that how the accumulation of VLCFA as a result of an inherited defect makes the myelin sheath unstable and causes demyelination. Recent documentation of the presence of immunoreactive tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) in astrocytes and microglia of X-ALD brain has suggested the involvement of these cytokines in immunopathology of X-ALD and aligned X-ALD with multiple sclerosis (MS), the most common immune-mediated demyelinating disease of the CNS in man [19–21]. In spite of a significant progress on X-ALD research since 1972, a drug therapy to lower the elevation of VLCFA as well as to stop the neurological deterioration in X-ALD patients is not yet available. Addition of monoenoic fatty acid (e.g. oleic acid) to cultured skin fibroblasts of X-ALD patients causes a reduction of saturated VLCFA presumably by competition for the same chain elongation enzyme. [10]. Treatment of X-ALD patients with trioleate resulted in 50% reduction of VLCFA. Subsequent treatment of X-ALD patients with a mixture of trioleate and trieruciate (popularly known as Lorenzo's oil) also led to a decrease in plasma levels of VLCFA [10–12]. Unfortunately, the clinical efficacy has been unsatisfactory since no proof of favorable effects has been observed by attenuation of the myelinolytic inflammation in X-ALD patients [10]. Moreover, a recent study has demonstrated that exogenous addition of unsaturated VLCFA induces the production of superoxide, a highly reactive oxygen radical, by human neutrophils [22]. Since cerebral demyelination of X-ALD is associated with a large infiltration of phagocytic cells to the site of the lesion [20], treatment with unsaturated fatty acids may even be toxic to X-ALD patients.

Recently we have observed that lovastatin and NaPA inhibit the induction of nitric oxide synthase and proinflammatory cytokines (TNF-α, IL-1β and IL-6) in rat primary astrocytes, microglia and macrophages [13] suggesting that these drugs, alone or in combination, may represent a possible avenue of research for therapeutics directed against cytokine- and NO-mediated brain disorders, particularly in demyelinating conditions. Lovastatin and NaPA have already been approved for medication/drug trials for human diseases. Therefore, normalization of VLCFA by lovastatin and NaPA in X-ALD fibroblasts indicate that these drugs may be of therapeutic value in lowering the levels of VLCFA and ameliorating the myelinolytic inflammation in X-ALD patients.

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