HDAC inhibitor SAHA normalizes the levels of VLCFAs in human skin fibroblasts from X-ALD patients and downregulates the expression of proinflammatory cytokines in Abcd1/2-silenced mouse astrocytes

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Abstract X-adrenoleukodystrophy (X-ALD) is a peroxisomal metabolic disorder caused by mutations in the ABCD1 gene encoding the peroxisomal ABC transporter adrenoleukodystrophy protein (ALDP). The consistent metabolic abnormality in all forms of X-ALD is an inherited defect in the peroxisomal β-oxidation of very long chain FAs (VLCFAs >C22:0) and the resultant pathognomonic accumulation of VLCFA. The accumulation of VLCFA leads to a neuroinflammatory disease process associated with demyelination of the cerebral white matter. The present study underlines the importance of a potent histone deacetylase (HDAC) inhibitor, suberoylanilide hydroxamic acid (SAHA) in inducing the expression of ABCD2 [adrenoleukodystrophy-related protein (ALDRP)], and normalizing the peroxisomal β-oxidation, as well as the saturated and monounsaturated VLCFAs in cultured human skin fibroblasts of X-ALD patients. The expression of ELOVL1, the single elongase catalyzing the synthesis of both saturated VLCFA (C26:0) and monounsaturated VLCFA (C26:1), was also reduced by SAHA treatment. In addition, using Abcd1/Abcd2-silenced mouse primary astrocytes, we also examined the effects of SAHA in VLCFA-induced inflammatory response. HDAC treatment decreased the inflammatory response as expression of inducible nitric oxide synthase, inflammatory cytokine, and activation of NF-kB in Abcd1/Abcd2-silenced mouse primary astrocytes was reduced. These observations indicate that SAHA corrects both the metabolic disease of VLCFA as well as secondary inflammatory disease; therefore, it may be an ideal drug candidate to be tested for X-ALD therapy in humans. — Singh, J., M. Khan, and I. Singh. HDAC inhibitor SAHA normalizes the levels of VLCFAs in human skin fibroblasts from X-ALD patients and downregulates the expression of proinflammatory cytokines in Abcd1/2-silenced mouse astrocytes.

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Supplementary key words peroxisome • very long chain FA • glia • nitric oxide • cytokine • suberoylanilide hydroxamic acid • X-adrenoleukodystrophy

X-adrenoleukodystrophy (X-ALD) is the most common peroxisomal disorder, with an incidence of approximately 1:17,000 (1, 2). It is a postnatal progressive demyelinating disorder that primarily affects nervous system white matter and axons, the adrenal cortex, and testis (3–5). The biochemical signature of X-ALD is increased levels of saturated straight-chain very long chain FAs (VLCFAs; >C22:0). VLCFA accumulates in all tissues and lipid classes; however, the degree of accumulation is higher in the cholesterol ester and sphingolipid fractions of brain white matter and adrenal cortex (6). The elevation of VLCFAs is the consequence of reduced VLCFA peroxisomal β-oxidation (7) and/or increased activity of FA elongases (8, 9). The ALD gene (ABCD1), identified by positional cloning (10), encodes a protein that is related to the peroxisomal ATP binding cassette (ABCD) transmembrane transporter proteins (11, 12). The function of the adrenoleukodystrophy protein (ALDP) and its role in VLCFA metabolism and in the pathogenesis of X-ALD is not well understood at present. However, the VLCFA, especially C26:0, has been documented to cause metabolic alterations leading to membrane perturbation, redox imbalance (13), and changes in membrane lipid composition (14–18), as well as the induction of inflammatory mediators in cultured astrocytes (19).

Abbreviations: ALDP, adrenoleukodystrophy protein; ALDRP, adrenoleukodystrophy-related protein; AMN, adrenomyeloneuropathy; BRB, blood-brain barrier; CALD, cerebral adrenoleukodystrophy; FAME, FA methyl ester; HDAC, histone deacetylase; iNOS, inducible nitric oxide synthase; 5-LOX, 5-lipoxygenase; PA, phenylacetate; PBA, phenylbutyrate; ROS, reactive oxygen species; SAHA, suberoylanilide hydroxamic acid; TNF-α, tumor necrosis factor-α; X-ALD, X-adrenoleukodystrophy; VLCFA, very long chain FA.

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At present, there is no satisfactory therapy for X-ALD. Reduction of VLCFA may be an ideal approach, but then none of the reported agents significantly decrease the levels of VLCFA in human brain. Other approaches, such as hematopoietic stem cell transplantation and lentiviral-vector-associated gene therapy, have shown clinical benefits (20). They, however, are effective only in patients with the earliest stage of cerebral adrenoleukodystrophy (cALD). Thus, finding a treatment for X-ALD remains a challenge. Recent studies on neuroinflammation using histone deacetylase (HDAC) inhibitors show potential to ameliorate metabolic defects in neurological disorders, including (21, 22) compromised gene metabolic disorders, by the activation of the overlapping function of the redundant gene. Pharmacological therapy for X-ALD remains attractive because of its potential to be effective pre- as well as postnatally. Various lines of evidence suggest that pharmacological induction of ABCD2 and/or ABCD3 may represent a therapeutic strategy for X-ALD. ABCD2, the closest homolog of ABCD1 (23), and ABCD3 (24) can compensate for β-oxidation defects in X-ALD fibroblasts when overexpressed (25). Furthermore, the biochemical abnormalities found in Abcd1-knockout mice can be restored by overexpression of Abcd2 (26).

A key role of protein lysine acetylation in metabolic regulation was recently shown, in which virtually every enzyme of the FA oxidation pathways was found to be acetylated (27). HDAC-inhibitory activity (28) of 4-phenyl butyrate (4-PBA) was associated with induction of ABCD2 expression in Abcd1-knockout mice. This induction resulted in reduced levels of VLCFA in brain and adrenal glands (29). Surprisingly, no ABCD2 induction or VLCFA correction was observed in adrenomyeloneuropathy (AMN) patients (30) with 4-PBA therapy. Valproic acid, a nonselective HDAC inhibitor, was recently shown to induce ABCD2 expression and correction of oxidative damage in a mouse X-ALD model and in peripheral blood mononuclear cells from X-ALD patients (31). The levels of C26:0 however, remained unchanged (31). Previous studies from our laboratory have shown that lovastatin, an inhibitor of HMG-CoA reductase and sodium phenyl acetate NAPA, can enhance VLCFA β-oxidation and reduce VLCFA levels in human skin fibroblasts (32) and lymphoblasts (33) from X-ALD patients. Lovastatin also lowered the plasma levels of VLCFA in X-ALD patients (34) and decreased the production of nitric oxide in X-ALD lymphoblasts (33). A number of other compounds, including 4-PBA (29), fenofibrate (25), and testosterone metabolites (35), have been shown to have the ability to lower VLCFA levels in X-ALD fibroblasts. However, none of the compounds to date have shown the ability to halt neurodegenerative progression.

Perturbation in acetylation homeostasis is emerging as a central event in the pathogenesis of neurodegeneration (36). Hence, recent studies have indicated that HDAC inhibitors might prove useful in treatment of such neurodegenerative disorders as Huntington’s Disease (37–39), spinal muscular atrophy (40), amyotrophic lateral sclerosis (41–43), and experimental autoimmune encephalomyelitis (44). Amelioration of neurodegenerative conditions such as oxidative stress (45), and preservation of white matter structure and function (46) by HDAC inhibitors strongly point toward acetylation-dependent mechanisms in demyelination and neurodegeneration.

Although treatment of cancer has been the primary target for the clinical development of HDAC inhibitors, administration of HDAC inhibitors has also shown beneficial effects in some noncancer disorders, such as sickle cell anemia, muscular dystrophy, neurodegenerative diseases, and inflammatory disorders (47). Among the various classes tested, suberoylanilide hydroxamic acid (SAHA) was reported as the most promising therapeutic agent for treatment of spinal muscular atrophy (SMA) due to its ability to substantially increase survival motor neuron (SMN) protein levels at low micromolar concentration and completely inhibit HDAC activity.

The present study demonstrates that treatment with the potent and selective HDAC inhibitor SAHA (48, 49) normalized the levels of VLCFA in skin fibroblasts from X-ALD patients by increasing the peroxisomal β-oxidation activity. SAHA also inhibited the induction of proinflammatory cytokines and inducible nitric oxide synthase (iNOS) in astrocytes silenced for Abcd1/2. Because SAHA crosses the blood-brain barrier (BBB), increases acetylation in brain (39), and reduces neuroinflammation, we propose that this drug may have therapeutic potential to ameliorate the X-ALD disease process.

MATERIALS AND METHODS

Reagents

DMEM and HBSS were purchased from Invitrogen Life Technologies; FBS was purchased from BioAbChem, Inc. (Ladson, SC). ALDP antibody was from Chemicon International, Inc. (Temecula, CA). Adrenoleukodystrophy-related protein (ALDRP) antibody was custom-made from ANASPEC against the mouse 20-residue c-terminal sequence: 722 CKILGEDSVLKTIQTPEKTS 741. 5-Lipoxygenase (5-LOX) antibody was purchased from Cayman Chemical (Ann Arbor, MI). Na’K’ATPase antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ECL and nitrocellulose membranes were purchased from Amersham Biosciences. FA methyl ester (FAME) standards were obtained from Supelco (Bellefonte, PA). [1-14C]linoleic acid was prepared as described previously (50). [1-14C]palmitic acid and 125I-labeled protein A were obtained from ICN (Cleveland, OH).

Cell culture

Fibroblasts. Human skin fibroblasts derived from normal (control; GM03348), X-ALD (GM04932, GM04934, and GM04904), and AMN (GM07531) patients, and were obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research (ccr.coriell.org/). The fibroblasts were cultured in DMEM containing 10% FBS and antibiotic/antimycotic.

Astrocytes. C57BL6 mice from Jackson Laboratory (Bar Harbor, ME) were maintained at the Medical University of...
A pool of three siRNAs for human ABCD2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and was used to transfect X-ALD fibroblasts at final concentration of 2 μM. Forty-eight hours after silencing for ABCD2, cells were treated with SAHA for 3 days with fresh addition of medium/SAHA every day.

FA β-oxidation

The peroxisomal oxidation of FAs in control, X-ALD fibroblasts, and SAHA-treated X-ALD fibroblasts was determined in 6-well plates as described previously (19). β-Oxidation of FAs to acetate (water-soluble product) was determined using [1-14C]-labeled FAs as substrate (C24:0, lignoceric acid, or C16:0, palmitic acid) (American Radiolabeled Co.; St. Louis, MO), as described previously (52). Cells grown in parallel in the same plate were used to determine the protein present in the assays. Experiments were performed in triplicate.

Lipid extraction and FA analysis

Total lipids were extracted from control and treated cells as described previously (53). The FAMEs were analyzed by GC (Shimadzu chromatograph GC-15A attached to a Shimadzu chromatopac C-R3A integrator) using a fused silica capillary column (25 M 007 series methyl silicone, 0.25 mm internal diameter, 0.25 μm film thickness) from Quadrex Corporation (Woodbridge, CT) in a gas
cDNA synthesis and real-time PCR

Following total RNA extraction using TRIzol (Invitrogen) per the manufacturer’s protocol, single-stranded cDNA was synthesized from total RNA as described previously (19). Real-time PCR was conducted using the Bio-Rad iCycler (iCycler iQ Multi-Color Real Time PCR Detection System; Bio-Rad). The primer sets for use were designed (Oligoperfect™ designer, Invitrogen) and synthesized from Integrated DNA Technologies (Coralville, IA). The primer sequences for mouse tumor necrosis factor-α (TNF-α) were: FP 5′-ctt gct ctt aac gtt tgg cg-3′; RP 5′-gct gaa gtc gtt gaa gga gaa cat cat cc-3′; glyceraldehyde-3-phosphate dehydrogenase FP, 5′-ctt gcc ccc acc ccc aat gta tcc gtt gg-3′; RP-5′-gga aga gca act act gct ggt ggt gga gtc-3′.

RESULTS

SAHA induces the mRNA expression of ABCD2 and ABCD3 in a concentration-and time-dependent manner in control normal human skin fibroblasts

It has been reported that ABCD3/PMP70 (54) or ABCD2/ALDRP (25), two close ABCD1/ALDP homologs, can compensate for the activity of ABCD1/ALDP. Because the overexpression of ABCD2/ALDRP resulted in the complete correction of VLCFA β-oxidation in X-ALD fibroblasts (29) and the prevention of clinical symptoms in Abcd1 knockout mice (26), ABCD2/ALDR is an attractive candidate gene for pharmacological gene therapy. Therefore, we first studied the effect of SAHA on ABCD2 and ABCD3 expression in normal control human skin fibroblasts. The fibroblasts were treated with SAHA with varying doses and durations and the gene expressions for ABCD2 and ABCD3 were analyzed by RT-PCR. SAHA significantly induced the expression of both ABCD2 (P < 0.001) and ABCD3 (P < 0.001) in a dose- and time-dependent manner (Fig. 1). Although ABCD2 expression was induced 7.2-fold (Fig. 1A), there was a 5.7-fold induction of ABCD3 expression (Fig. 1B) at the maximum dose of 5 μM SAHA. SAHA treatment for 3 days was sufficient to induce 7.2- and 5.7-fold induction of ABCD2 (P < 0.001) and ABCD3 (P < 0.001) gene expressions, respectively (Fig. 1C, D). No significant increase in the expression of ABCD2 or ABCD3 was observed at higher doses of SAHA (7.5–10 μM). There was an insignificant increase in expression of ABCD2 and
SAHA induces ABCD2 and ABCD3 mRNA expression and protein levels in X-ALD human fibroblasts

To assess the therapeutic potential of SAHA for the treatment of X-ALD patients, we cultured human X-ALD fibroblasts in the presence of SAHA for 3 days and quantified its effect on ABCD2 mRNA expression by RT-PCR. Treatment with SAHA resulted in a dose-dependent increase in ABCD2 mRNA expression in four different X-ALD cell lines (Fig. 3). The highest dose of SAHA (5 μM) increased the ABCD2 mRNA expression by 11.8- (Fig. 3A), 6.0- (Fig. 3B), 2.8- (Fig. 3C), and 3.88- (Fig. 3D) fold in 3 days in the respective cell lines. Much like ABCD2, we observed a maximum of 10-, 3.92-, 2.77-, and 3.45-fold increase in ABCD3 mRNA expression in cell lines following treatment with 5 μM SAHA for 3 days (Fig. 4).

Western blots using antibodies against ALDRP and PMP70 were performed on carbonate membranes (integral membrane proteins) obtained from control normal and X-ALD fibroblasts suggests that SAHA treatment has an overall enhancing effect on FA oxidation.
HDAC inhibition lowers VLCFA in human X-ALD fibroblasts.

Fig. 6B. Palmitic acid β-oxidation activity was also increased in a dose-dependent manner (43.5% to 51%) following SAHA (0.5–5 μM) treatment for 3 days (Fig. 6A). The induction of lignoceric acid and palmitic acid β-oxidation by SAHA suggests that it affects both mitochondrial and peroxisomal β-oxidation.

Fig. 7. Transient silencing with ABCD2 siRNA did not result in any further inhibition of lignoceric (Fig. 7B) or palmitic acid (Fig. 7A) β-oxidation activity. However, no increase in lignoceric acid β-oxidation activity was seen when the cultures were treated with SAHA. There was still a statistically significant increase (P < 0.05) in palmitic acid β-oxidation activity.

SAHA reduces the levels of very long chain FAs in X-ALD human fibroblasts

Levels of total hexacosanoic acid (C26:0) and the ratios of C26:0/C22:0 or C24:0/C22:0 are widely used for...
the diagnosis of X-ALD and other peroxisomal disorders. Although the precise function of ALDP in the metabolism of VLCFA is not known at the present time, 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. Also, overexpression of ABCD2 leads to reduced VLCFA accumulation in cultured fibroblasts from X-ALD patients (29). Therefore, we determined whether induction of ABCD2 mRNA expression and protein levels with SAHA correlates with the reduction of VLCFA levels. C26:0 levels were significantly higher (9-fold, \( P < 0.001 \)) in human X-ALD fibroblasts as compared with control human fibroblasts (Fig. 8A). Treatment of X-ALD fibroblasts with SAHA (5 \( \mu \)M) for 3 days significantly reduced (37%, \( P < 0.002 \)) the levels of VLCFA (C26:0).

In addition to saturated VLCFA, X-ALD fibroblasts have significantly higher levels of monounsaturated FAs (C26:1), indicating that \( \beta \) oxidation of C26:1 is also reduced in X-ALD fibroblasts and the plasma and brain of X-ALD patients (59, 60). We detected significantly higher (1.3-fold, \( P < 0.02 \)) levels of C26:1 in X-ALD fibroblasts compared with control human fibroblasts (Fig. 8B). Treatment of human X-ALD fibroblasts with SAHA also resulted in a significant decrease of 47.6% \( (P < 0.001) \) and 71.4% \( (P < 0.001) \) in the levels of C26:1 in a dose- and time-dependent manner.

SAHA decreases ELOVL1 expression in X-ALD fibroblasts

Elongases are responsible for the initial step in the elongation of specific acyl-CoA FAs with different chain lengths.

Seven types of ELOVLs have been identified in mammals, of which two (ELOVL1 and ELOVL3) have chain length specificity toward VLCFA (61, 62). There was no significant difference in the levels of ELOVL1 and ELOVL3 in control and X-ALD fibroblasts (Fig. 9A, B) as reported previously (63), indicating that availability of substrate is the limiting factor for their elongation. Knockdown of ELOVL1 in human X-ALD fibroblasts significantly lowers the levels of C26:0 (63). SAHA treatment of control and X-ALD human fibroblasts significantly decreased [81.55% \( (P < 0.001) \) for control and 63.35% \( (P < 0.001) \) for X-ALD fibroblasts at a 5 \( \mu \)M dose] the ELOVL1 expression. However, the expression of ELOVL3 was significantly increased after SAHA treatment. The results highlight the dual effect of SAHA on induction of \( \beta \)-oxidation activity and inhibition of VLCFA elongation, ultimately resulting in a net effect of lowering the levels of both saturated and unsaturated VLCFAs.

SAHA inhibits proinflammatory cytokines and reduces oxidative stress in Abcd1/2-silenced mouse astrocytes

Accumulation of VLCFA in X-ALD patients leads to secondary injury of inflammatory demyelination due to a marked increase in the activation of microglia and astrocytes, and thus the production of proinflammatory cytokines (TNF-\( \alpha \) and IL-1\( \beta \)) (64, 65). Expression of TNF-\( \alpha \) and iNOS was higher in inflammatory areas compared with normal areas of cALD brain (66). Therapeutic reduction of the levels of VLCFA correlates with decreased expression of proinflammatory cytokines (19). Our laboratory has recently reported the accumulation of VLCFA and increased production of ROS and proinflammatory cytokines (iNOS, TNF-\( \alpha \), and IL-1\( \beta \)) in Abcd1/2-silenced mouse primary astrocytes (19). Expression of iNOS and TNF-\( \alpha \) was significantly increased in Abcd1/2-silenced mouse astrocytes. Therefore, we evaluated the effect of SAHA on ROS and proinflammatory cytokines in Abcd1/2-silenced mouse primary astrocytes (Fig. 10). SAHA significantly \( (P < 0.001) \) inhibited ROS generation in Abcd1/2 silenced mouse astrocytes (Fig. 10A). Treatment with SAHA also downregulated \( (P < 0.001) \) the expression of iNOS and TNF-\( \alpha \) (both at mRNA and protein levels) in Abcd1/2-silenced mouse astrocytes (Fig. 10B–E).

A growing body of evidence suggests the role of certain immunomodulatory leukotrienes (67) in the signaling cascade of inflammatory gene expression (18). 5-LOX expression was increased in Abcd1/2-silenced mouse astrocytes, which was significantly decreased by treatment with SAHA (Fig. 10D). Mechanisms of Abcd1/2 silencing-induced proinflammatory response involve activation of NF-\( \kappa \)B (19). Western blots showed increased levels of p65 protein in Abcd1/2-silenced mouse primary astrocytes (Fig. 10D). The level of p65 in the nucleus of Abcd1/2-silenced astrocytes was significantly reduced by SAHA. Because SAHA crosses the BBB (39), downregulates inflammatory mediators, and corrects abnormality of VLCFA, it may have the potential for X-ALD therapy.
Pharmacological therapy for a genetic disease is aimed at upregulating redundant genes to compensate for biochemical defects. Because the accumulation of VLCFA triggers an inflammatory response associated with progressive demyelination in X-ALD (19), a decrease in the VLCFA content may be beneficial for the patients. However, the other ALDP-related transporter gene, ABCD2, is not mutated in X-ALD patients, and the X-ALD phenotype is independent of the ABCD2 genotype (68). Our recent observation of an inverse correlation between the VLCFA content and the expression of Abcd2/ALDRP and a greater accumulation of VLCFAs following silencing for both Abcd1 and Abcd2 in mouse primary astrocyte cultures (19), suggests that Abcd1 and Abcd2 have overlapping functions in the metabolism of VLCFA and related compounds. Accordingly, Abcd1 and Abcd2 double-knockout mice accumulate higher levels of VLCFA than Abcd1 knockout mice (69), and elevated expression of ALDRP increased the β-oxidation activity and lowered the C26:0 and C24:0 levels (70). Treatment with SAHA increased the levels of ABCD2 and ABCD3 in control and X-ALD fibroblasts. Interestingly, the degree of increase was higher in control fibroblasts than in the X-ALD fibroblasts, possibly due to the dominant-negative effect of ABCD1 mutation/deletion and/or the metabolites on expression of other transporters in X-ALD fibroblasts. Earlier studies from our laboratory (7, 71–74) and others (75) have reported that VLCFAs are preferentially β-oxidized in peroxisomes, whereas oxidation of palmitic acid occurs largely in mitochondria. Treatment with SAHA increased both the lignoceric acid (C24:0) and palmitic acid (C16:0) β-oxidation activities in X-ALD fibroblasts. However, SAHA treatment of human X-ALD fibroblasts silenced for ABCD2 led to increased C16:0 β-oxidation but had no effect on C24:0 β-oxidation, indicating that the restoration of the peroxisomal VLCFA β-oxidation activity in SAHA-treated X-ALD fibroblasts resulted from upregulation of ABCD2 expression. Thus, SAHA-mediated induction of ABCD2 expression and reduction of C26:0 levels should be able to compensate for the lack of ABCD1 function in X-ALD patients.

Recent observations indicate that in addition to saturated (C26:0), monounsaturated (C26:1) VLCFAs also accumulate in X-ALD tissues, implicating both C26:0 and C26:1 in the X-ALD pathology (31). Similar to C26:0 (7), C26:1 is also β-oxidized in peroxisomes, and ABCD2 plays a prominent role in their catabolism (31, 70, 76). Accordingly, X-ALD fibroblasts had higher levels of C26:1, as compared with control normal human skin fibroblasts (Fig. 5B), and treatment with SAHA, at doses that upregulated...
ABCBD2 expression, significantly reduced the C26:1 levels in X-ALD fibroblasts, suggesting a causal relationship between ALDRP expression and levels of monounsaturated VLCFAs. Although the differential substrate specificities of individual ABCD transporters remain speculative, recent studies report overlapping substrate specificities for ALDP and ALDRP (70), especially for saturated and monounsaturated VLCFAs (31, 70, 76). A significant decrease in C26:1 level was observed with SAHA (1 μM), at which there was a relatively small increase (2-fold) in ABCD2 expression, suggesting that higher affinity of C26:1 for ABCD2 may be sufficient for decrease in C26:1 with a relatively small increase in ABCD2, but not sufficient for the transport of saturated VLCFA (C26:0). Second, increased expression of ABCD3 in 1 μM SAHA-treated cells may facilitate the transport and catabolism of monounsaturated VLCFA similar to one observed for PUFA (77).

In addition to the abnormality in peroxisomal VLCFA β-oxidation (74), the observed enhanced FA chain elongation activity (8) may also contribute to increased accumulation of VLCFA in X-ALD. To date, seven FA elongases (ELOVL1-7) have been identified and characterized in mammals (78, 79). Of these, two elongases (ELOVL1 and ELOVL3) have chain length specificity toward VLCFA (31, 63) and would be the most attractive candidate elongases to play a role in enhanced FA chain elongation in X-ALD.

Treatment with SAHA decreased the expression of ELOVL1 in control as well as in X-ALD human skin fibroblasts. In contrast to ELOVL1, ELOVL3 expression was significantly increased (31). A recent study reporting reduced elongation of C22:0 to C26:0 and reduced levels of C26:0 in X-ALD fibroblasts following silencing of ELOVL1 indicates a role for ELOVL1 in VLCFA elongation (63). Therefore, the effect of SAHA on lowering the C26:0 and C26:1 levels in X-ALD fibroblasts is a net effect of decreased chain elongation (decreased ELOVL1 expression), increased ABCD2 expression, and increased β-oxidation activity (Figs. 6B and 8A).

The VLCFA-induced secondary inflammatory response is believed to participate in the neurodegeneration in X-ALD (19). Therefore, for effective therapy, pharmacological agents need to cross the BBB to reduce the accumulated VLCFA in the brain and prevent the progression of neurological symptoms in X-ALD. SAHA has been reported to cross the BBB in different models (39), making it an attractive candidate for X-ALD therapy. Previous studies from our laboratory have documented that lowering of VLCFA leads to inhibition of the inflammatory response (19). Abcd1/2 silencing of wild-type mouse primary astrocytes induced a proinflammatory response (iNOS and...
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proinflammatory cytokines) (19), which was mediated by the activation of NF-κB and 5-LOX and increased levels of ROS (13). SAHA treatment of Abcd1/2-silenced mouse astrocytes reduced the expressions of iNOS and TNF-α (mRNA and protein), suppressed NF-κB activation, and reduced ROS production. The observed increased activation of lipoxidative enzymes in X-ALD brain (18) and in Abcd1/2-silenced mouse astrocytes (19) suggests the role of these proinflammatory mediators in the pathobiology of X-ALD. SAHA treatment also reduced the activation of 5-LOX in Abcd1/2-silenced mouse astrocytes. Our observation of inhibition of NF-κB activation is consistent with studies from other laboratories reporting inhibition of proinflammatory mediators and NF-κB activity by SAHA in glial cell cultures (80) and in mouse brain (81). The observed inhibition of NF-κB in the brain (81) and protection against ischemic injury (46) in animals treated with SAHA document that SAHA is a good candidate drug for neurological disorders.

Given the recent observation of the key role of protein acetylation in FA metabolism (27), as well as the significance of altered gene expression in disease pathogenesis, a great deal of effort has been directed to the development of chemical inhibitors of HDACs as therapeutic drugs (82). Studies with phenylacetate (PA) from our laboratory (83) and with PBA from other laboratories (29) have shown that these compounds (PBA and PA) have very low potency, insofar as millimolar amounts of these...
compounds were required to observe their effects on β-oxidation and levels of VLCFA in X-ALD cells. Second, the effects of PBA were not long-lasting; the initially decreased VLCFA levels later returned to pretreatment levels in long-term studies (84). In a recent report, published while this manuscript was under review, a millimolar concentration of PBA was required for lowering the VLCFA levels in cultured oligodendrocytes differentiated from pluripotent stem cells of X-ALD patients (85). Furthermore, PBA/PA are nonselective inhibitors of HDAC and have been reported to inhibit the mevalonate pathway (86). Compare-
vatively, SAHA is a potent and specific HDAC inhibitor (the only known inhibitor of HDAC11) and is effective at micromolar concentrations (approximately 1000-fold difference). A recent report documents the therapeutic potential of SAHA in mouse models of progressive neurodegenerative frontotemporal dementia (87). In the neuro muscular disease SMN, SAHA had a significantly better outcome than butyrate or valproic acid (VPA) (88). Additionally, SAHA has been shown to provide a favorable outcome in a variety of animal models of inflammatory disease conditions including LPS-induced endotoxemia (89), lupus (90), graft-versus-host disease (91), ischemia (46, 81), TLR activation (92), septic shock (93), and inflammatory hyperalgesia (94). Moreover, SAHA is presently in numerous clinical trials (95); more than 100 clinical trials with SAHA are in progress at the National Institutes of Health (http://www.clinicaltrials.gov). An oral preparation of SAHA (200–600 mg) was safely administered chronically (up to 37 months) and has shown good bioavailability, linear pharmacokinetics, and inhibition of HDAC activity in Phase 1 trials (96) and in patients with hematologic malig-
nancies (97). Furthermore, SAHA was recently approved by the Food and Drug Administration for treatment of cutaneous T-cell lymphoma. Pediatric Phase 1 trials have shown that SAHA (at 230 mg/m²/day) is well-tolerated in children with solid tumors (98). The in vivo dose of SAHA (50 mg/kg) showing protection in animal models of inflammation translates to 6 mg/kg (total dose of 120 mg/day or 150 mg/m² for a 20 kg human child), which is much lower than the maximum dose administered safely in pediatric patients (98). In summary, (Fig. 11), our observations describe upregulation of peroxisomal β-oxidation activity and inhibition of chain elongation, and thus correction of VLCFA levels in X-ALD cells, as well as inhibition of inflammatory response in ABCD1/2-silenced astrocytes by SAHA, indicating that SAHA is a potential candidate drug for correction of the metabolic defect as well as the secondary neuroinflammatory disease in X-ALD.

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