Molecular biology and pharmacogenetics of x-linked adrenoleukodystrophy
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Citation for published version (APA):

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Chapter 6

Gene redundancy and pharmacological gene therapy: Implications for X-linked adrenoleukodystrophy


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Adapted from Gene redundancy and pharmacological gene therapy: Implications for X-linked Adrenoleukodystrophy

Abstract

As more functional redundancy in mammalian cells is discovered, enhanced expression of genes involved in alternative pathways may become an effective form of gene therapy. X-linked adrenoleukodystrophy (X-ALD) is a peroxisomal disorder with impaired very-long-chain fatty acid metabolism. The \textit{ALD} gene encodes a peroxisomal membrane protein (ALDP) that is part of a small family of related peroxisomal membrane proteins. We show that 4-phenylbutyrate treatment of cells from both X-ALD patients and X-ALD knockout mice results in decreased VLCFA levels and increased VLCFA $\beta$-oxidation; increased expression of the peroxisomal protein ALDRP; and induction of peroxisome proliferation. We also demonstrate that ALDP and ALDRP are functionally related, by ALDRP cDNA complementation of X-ALD fibroblasts. Finally, we demonstrate the \textit{in vivo} efficacy of dietary 4-phenylbutyrate treatment through its production of a substantial reduction of very-long-chain fatty acid levels in the brain and adrenal glands of X-ALD mice.

Introduction

Functional gene redundancy and/or redundant metabolic pathways may be a fundamental aspect of vertebrate evolution.\cite{1} The observation that null mutations in mouse models of human disease created by targeted gene disruption often have no relevant clinical phenotype or a more subtle one than predicted has been explained in part by genetic redundancy.\cite{2} Redundant genes that can completely or partially substitute for each other are candidates for an approach to gene therapy for genetic disease that is based on increased expression of an endogenous gene rather than the introduction of a normal copy of the defective gene by transgenesis. This approach avoids the complications inherent in transgene gene therapy, such as targeting expression to the appropriate tissue and the immunosurveillance of tissues expressing the new transgene. In principle, enhanced gene expression could be accomplished \textit{in utero}, allowing for early intervention. Initial exploration of pharmacologic induction of redundant genes in a clinical setting involved the stimulation of fetal hemoglobin by 5-azacytidine, hydroxyurea, sodium butyrate and sodium 4-phenylbutyrate (4PBA) to ameliorate the effects of adult hemoglobinopathies.\cite{3-6} Also, it has recently been suggested that such an approach might be useful in Duchenne muscular dystrophy by increasing the expression of utrophin, a protein that is structurally similar to dystrophin, the abnormal protein in
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this genetic disorder. As more functional gene redundancy is recognized, increased expression of a related gene could become a general approach to the treatment of genetic disease. X-linked adrenoleukodystrophy (X-ALD: McKusick No. 300100) is a candidate for such pharmacological gene therapy.

X-ALD is associated with defective peroxisomal β-oxidation of saturated very-long-chain fatty acids (VLCFA) and reduced activity of peroxisomal VLCF-acyl CoA synthetase. It affects mainly central and peripheral myelin, the adrenal cortex and the testis. X-ALD shows a highly variable clinical phenotype including a rapidly progressive childhood cerebral form (CCALD), with inflammatory cerebral demyelination; a milder adult form, adrenomyeloneuropathy (AMN) that is slowly progressive and with initial symptoms limited to the spinal cord and peripheral nerves of the limbs; and a form (Addison-only) in which there is adrenal insufficiency without neurologic involvement. All forms of X-ALD segregate in the same families and arise from identical mutations including null mutations.

The gene for X-ALD, identified by positional cloning, encodes a peroxisomal membrane protein (ALDP) with a predicted molecular mass of 83 kDa. Based on sequence homology, it belongs to the ATP-binding cassette (ABC) superfamily of transmembrane transporters, with the structure of a half-transporter. Although peroxisomal VLCF-acyl CoA synthetase activity is impaired in X-ALD, mutational analysis and complementation studies have shown that ALDP and not that for VLCF-acyl CoA synthetase is responsible for X-ALD.

There are three additional mammalian peroxisomal membrane ABC half-transporters that are closely related by nucleic acid and protein sequence: ALDRP (ALDPL1), an ALDP related protein; PMP70, a 70-kDa protein; and PMP69 (P70R), a 69-kDa protein. The function(s) of the peroxisomal ABC half-transporters and their interaction with VLCF-acyl CoA synthetase is unknown, but their considerable sequence similarity indicates that they might have related and/or overlapping function(s) in peroxisomal fatty acid metabolism. This is supported by the observations that X-ALD cells lacking ALDP have a residual activity for VLCFA β-oxidation, which could result from one or more of the other peroxisomal ABC half-transporters; and that PMP70 overexpression partially restores VLCFA β-oxidation in X-ALD fibroblasts, indicating that other peroxisomal ABC half-transporters can, at least in part, substitute for the absence of ALDP. In addition, the high level of identity between ALDP and ALDRP has led to speculation that these proteins might be functionally related.

We have generated a mouse model for X-ALD by targeted gene disruption. The X-ALD mouse has elevated levels of VLCFA in tissues that resemble the characteristic biochemical defect in X-ALD patients. Thus, the in vivo efficacy of
treatment can be monitored by determination of its effect on VLCFA levels in the tissues, brain and adrenal gland, the organs most affected in X-ALD.

At present, no completely satisfactory therapy for X-ALD is available. Some success has been achieved with bone marrow transplantation.30,31 ‘Lorenzo’s oil’, a dietary therapy, depresses plasma and liver levels of VLCFA within a month; however, it has no effect on the clinical course of the disease, perhaps because erucic acid, the active ingredient of ‘Lorenzo’s oil’, does not get into the brain.32,33 Thus, new approaches to therapy of X-ALD are warranted.

We explored the possibility of pharmacological gene therapy for X-ALD by treating cell lines derived from X-ALD patients and from our X-ALD mouse model with 4-phenylbutyrate (4PBA). 4PBA has been used for many years in the treatment of patients with urea cycle disorders with few, if any, side effects.34 Although details of their mode(s) of action are unclear, 4PBA and other butyrate derivatives seem to increase expression of certain target genes.35-38 Fenofibrate, a compound structurally related to 4PBA, also increases expression of ALDRP and PMP70, but not ALDP in rats.39 Treatment of nasal epithelia cells from cystic fibrosis patients who are homozygous or heterozygous for the del-F508-CFTR mutation with 4PBA results in rescue of del-F508-CFTR from premature degradation in the endoplasmatic reticulum. It is now believed that del-F508-CFTR protein biosynthesis is enhanced by 4PBA because of altered regulation of protein folding by a chaperone in the endoplasmatic reticulum.40,41 In X-ALD, 70% of mutations result in unstable ALDP.18,42,43 Thus, 4PBA could similarly rescue unstable ALDP from premature degradation. In rodents, exposure to compounds related to 4PBA, like clofibrate and fenofibrate, induces peroxisome proliferation.44 Although classic mammalian peroxisome proliferators are generally only effective in rodents, indirect evidence indicates that 4PBA might cause peroxisome proliferation in human cells.45 Because X-ALD patients have residual VLCFA β-oxidation activity, an increase in the number of peroxisomes could increase VLCFA β-oxidation. In addition, in vivo efficacy has been explored by determination of VLCFA levels in X-ALD mice after exposure to dietary 4PBA.

Materials and Methods

Cell culture and 4PBA treatment. Cell lines derived from X-ALD patients and X-ALD mice were grown in MEM (fibroblasts) or RPMI (lymphoblastoid) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml) and glutamine (2 mM). On day 0, cells were divided into two separate tissue culture flasks, and 4PBA (5 mM final concentration, diluted from a 0.5 M stock solution in PBS, pH 7.6: Mediatech, Grand Island, New York) was added to
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one flask. Cells in the second flask were grown without drug for the same length of time and served as controls. The media were changed every 3-4 days. X-ALD patient cell lines with the following mutations were included in this study: missense (R591Q, S606L, A626T, R660W), nonsense (Q645X), an in-frame amino acid deletion (E291del), an identical frame shift (fs E471) in two independent cell lines, one from a patient with CCALD and one from a patient with the Addison-only phenotype, and a large deletion in the COOH-terminal half of ALDP (exon 7-10del). The phenotypes included: CCALD, AMN and Addison-only. Two of the X-ALD cell lines have detectable ALDP (R591Q, S606L), whereas all of the other cell lines have no detectable ALDP.

In vivo delivery of 4PBA to X-ALD mice. 4PBA was delivered to X-ALD mice in their regular chow and water. 4PBA chow was prepared by Bio-Serv (Holton Industries, Frenchtown, New Jersey). 7.5 gram of 4PBA (Buphenyl; provided by Ucyclyd Pharma, Glen Burnie, Maryland) was mixed into each kilogram of Purina Rodent Diet 5001 (Bio-Serv; Holton Industries, Frenchtown, New Jersey) before they were shaped into half-inch pellets. 4PBA chow in the form of half-inch pellets that had not been autoclaved was used to feed X-ALD mice. In addition, the water for the X-ALD mice on the 4PBA diet contained 10 grams of 4PBA per liter. The daily 4PBA uptake was estimated to be 70 mg of 4PBA, based on 4 grams of food and 4 ml of water intake per day. Mice were killed after four or six weeks on the 4PBA diet. Brains and adrenal glands from normal, X-ALD and X-ALD/4PBA mice were collected for VLCFA analysis as described.

Biochemical measurements. Tissue culture cells were grown in the presence or absence of 5 mM 4PBA, collected from tissue culture flasks with trypsin, washed twice with PBS and subjected to biochemical analysis. For VLCFA measurements, total lipids were extracted, converted to methyl esters, purified by TLC, and subjected to capillary GC analysis as described. Duplicate assays were set up independently and were assayed on different days. C24:0 β-oxidation activity of human and mouse fibroblasts and human lymphoblastoid cells was determined by measuring their capacity to degrade [1-14C]-C24:0 fatty acid (American Radiolabeled Chemicals, St Louis, Missouri) to water-soluble products as described. Phytanic acid oxidation was measured as described. Cells were incubated with [2,3-3H]-phytanic acid for 24 hours, and the release of [3H]-H2O to the aqueous medium was determined (activity in pmol/hr/mg protein). The statistical significance of measured biochemical differences between untreated and 4PBA treated X-ALD cell lines was determined by the two-tailed Student’s t-test. For transfection studies with recombinant expression vector (pcDNA3) alone or with vector containing cDNA for PMP70, ALDP or ALDRP, human X-ALD fibroblasts were transformed with SV40 T antigen as described.

Semi-quantitative RT-PCR analysis. Total RNA from tissue culture cells (10^6-10^7 cells/sample) was isolated using TRIzol Reagent™ (Life Technologies), treated with DNase I (100-200 units in 40 mM HCl, 10 mM NaCl, 6 mM MgCl2, pH 7.3), incubated at 37°C for 30 minutes, extracted with phenol/chloroform, and precipitated with isopropanol. cDNA was generated using MMLV reverse transcriptase (RT) (Life Technologies). PCR was done in the linear range of amplification (determined for each primer pair-cDNA combination). For each cDNA sample, a ‘cocktail’ was prepared, containing PCR buffer (Promega), 200 μM of each dNTP, 1.5 mM MgCl2, 250 ng cDNA, 0.25 U of Taq polymerase (Promega) and 5% DMSO. The ‘cocktail’ was divided into separate tubes containing cDNA specific primers.
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Primers. The PCR primers used are listed below. In parentheses, h indicates human and m, mouse; this is followed by the gene name, product size and annealing temperature. For primer sequences, F is forward and R is reverse. (hALD, 249, 64°C) F: 5'-GGA GCT GGT GGC AGA GGA-3', R: 5'-ACA GCC ACC ATG AGC AGG-3'; (mALD, 526, 59°C) F: 5'-CCT ATC ATC ACA CCC ACT GGA GAG-3', R: 5'-GCA GTG CGA TAC CAG CAT CTT TG-3'; (hPMP70, 405, 60°C) F: 5'-TTT GAA GTT CGA TCT GGG-3', R: 5'-ATC CAA AAT GCC AAA CTG GG-3'; (mPMP70, 405, 60°C) F: 5'-TTT GAA GTT CGA TCT GGG-3', R: 5'-ATC CAA AAT GCC AAA CTG GG-3'; (hALDR, 312, 59°C) F: 5'-GCT GTC AGC ATT GAT GTC GAA GGA-3', R: 5'-TTA AGA TGT CTC ATC-3'; (mALDR, 711, 57°C) F: 5'-TGG TGG CTT CCA GGC TAA ACT TC-3', R: 5'-CCG AGT CTT CCC CCAGAA TTT TG-3'; (hPMP69, 319, 60°C) F: 5'-TGT GAG CTG ACC CTT CAC CCG CTT CAC C-3', R: 5'-CCA CGC GGC GGC CTT CAC CCG CTT CAC C-3'; (hAOX, 405, 60°C) F: 5'-TGC CCA GTG ACC ATG ACC AA-3', R: 5'-TGA ACA GGT TCC ACA GGC CTT CAC CCG CTT CAC C-3'; (mAOX, 247, 60°C) F: 5'-TCA CGC ACC CTG TGG ATG GTA GTC-3', R: 5'-CCA ACA GGT GCC ACC ACA AAA TTG ACC ACC-3'; (hPPARα, 347, 60°C) F: 5'-AGA GCC GTT ATT GTT CAG CAA GAT GGT ATG-3', R: 5'-GGA GCC GGA TCT TTA CAG AAG ATG ACC-3'; (mPPARα, 268, 60°C) F: 5'-AGA GCC GTA GCT CGG TGA GG-3', R: 5'-GGA GCC CTT ACA GCA GCC TTC ACA GCA GCC TTC AGC-3'; (hPexlla, 658, 55°C) F: 5'-CCC GTC GAC GCA ATG ACC CAC AGC CAA CTG CAG CAA CTG CAG CCA-3', R: 5'-CCC GTC GAC GCA ATG ACC CAC AGC CAA CTG CAG CAA CTG CAG CCA-3'; (mPexlla, 720, 55°C) F: 5'-CCC GTC GAC GCA ATG ACC CAC AGC CAA CTG CAG CAA CTG CAG CCA-3', R: 5'-CCC GTC GAC GCA ATG ACC CAC AGC CAA CTG CAG CAA CTG CAG CCA-3'; (ß-Actin, 460, 62°C) F: 5'-TCT GCA GAA TGG TAA GCT CGG TAA-3', R: 5'-TCT GCA GAA TGG TAA GCT CGG TAA-3'; (GAPDH, 528, 62°C) F: 5'-AGA GCC ATG GGG CAG ATG ACC CAA GAA ACC-3', R: 5'-AGA GCC ATG GGG CAG ATG ACC CAA GAA ACC-3'.

Western blot analysis. Tissue culture cells were scraped from tissue culture dishes using a ‘policeman’, into cold PBS solution and protease inhibitor cocktail (Boehringer). Samples were mixed (1:1) with 2x sample buffer. Total protein samples were fractionated by SDS-PAGE using the method of Laemmli. Proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, Massachusetts) for 2 hours at 24 V. Immunoblot analysis used antibodies against ß-actin (Sigma), mouse ALDP and mouse ALDRP (anti-ALDP and anti-ALDRP were a kind gift from Dr. P. Aubourg) and human SCP2 (SCP2 antibody was a kind gift from Dr. J. Strauss). Enhanced chemiluminescence (SuperSignal Substrate; Pierce, Rockford, Illinois) was used to detect bound antibody.

Immunofluorescence and peroxisome counting. Human skin fibroblasts were grown on cover slips in the presence or absence of 5 mM 4PBA, and indirect immunofluorescence was done as described. The anti-SKL (PTS1) antibody was a kind gift from Dr. S. Gould and the anti-Pex14p antibody was a kind gift from Dr. S. Mihalik. To count peroxisomes, we photographed immunofluorescence-stained cells after focusing on the cell nucleus, and determined the peroxisome number per cell with the aid of a colony counter after projecting the slides to enlarge the image.

Results

4PBA corrects VLCFA levels in cultured X-ALD cells

We treated human primary fibroblasts and lymphoblastoid cells (EBV-transformed lymphocytes) derived from X-ALD patients with 1 mM to 7.5 mM 4PBA and compared the results to those obtained with untreated X-ALD cells and cells from
normal individuals. The genotypes of the X-ALD patients included missense, nonsense, frame shift and deletion mutations and all of the main clinical phenotypes. We found that between the concentrations of 1 mM and 5 mM 4PBA, there was a dose dependent decrease in VLCFA levels, whereas concentrations exceeding 7.5 mM were toxic for both human and mouse primary fibroblasts (data not shown). We also analyzed VLCFA levels in primary fibroblasts from normal mice and X-ALD mice that have no detectable ALDP. We determined the effect of 5 mM 4PBA on C24:0 and C26:0 levels in human fibroblasts, human lymphoblastoid cells and mouse fibroblasts (Table 1). After two days of treatment, a time-dependent reduction in VLCFA levels in human and mouse X-ALD cell lines was observed. In human lymphoblastoid cells and mouse fibroblasts, C24:0 and C26:0 levels were normalized within 10 days of treatment. In human X-ALD fibroblasts, C24:0 levels were normalized within 10 days, whereas C26:0 levels were reduced by 47% after 22 days. The results obtained were independent of genotype or phenotype. The difference in C26:0 response between human and mouse fibroblasts may reflect the greater initial elevation in human (0.46% of total fatty acids) compared with mouse (0.30% of total fatty acids) fibroblasts, rather than a difference in response to 4PBA (these percentages are the average of those listed for untreated cell lines in Table 1).

4PBA increases VLCFA (C24:0) β-oxidation

To determine if the reduction in VLCFA is caused by an increase in their degradation, we measured C24:0 β-oxidation in mouse and human X-ALD primary fibroblasts and human X-ALD lymphoblastoid cells after treatment with 4PBA (Table 2). Exposure to 5 mM 4PBA for 6 days resulted in a twofold to threefold increase in C24:0 β-oxidation in all X-ALD cell lines tested. Human fibroblasts C24:0 β-oxidation increased from 25% to 48% of normal; human lymphoblastoid cells, from 18% to 53% of normal; and mouse fibroblasts, from 15% to 29% of normal. Although exposure to 4PBA did not increase β-oxidation of C24:0 to wild-type levels, the twofold to threefold increase seems to be sufficient to normalize C24:0 levels in all cell types tested, indicating a direct correlation between the decrease in VLCFA levels and the increase in VLCFA degradation. Furthermore, the increase in C24:0 β-oxidation was identical in mouse and human X-ALD primary fibroblasts, indicating an equivalent response to 4PBA. There are several possible mechanisms that could account for the action of 4PBA in X-ALD.
To assess the effect of 4PBA on VLCFA levels, we compared results obtained from treated cells with those obtained from untreated cells grown in parallel. The time of 4PBA treatment varied (Exposure). VLCFA levels were calculated as a weight percentage of the total fatty acids in the cells. Percentages are the average of multiple assays (n) in mixed cell lines from X-ALD patients with different mutations and different phenotypes. Two of the X-ALD cell lines have detectable ALDP; all of the other cell lines have no detectable ALDP. There were no detectable differences in response to 4PBA among the various cell lines. Mouse values are for individual cell lines from different X-ALD mice with no detectable ALDP. (a) The percent correction was calculated relative to the difference in the values observed in untreated X-ALD cells (0%) and the values observed in normal cells (100%). Correction exceeding 100% indicate VLCFA levels that are lower than those observed in corresponding normal cells. P values were calculated using the two-tailed Student’s t-test.

### Table 1: Effect of 4PBA on VLCFA (C24:0 and C26:0) levels in cultured cell lines

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>n</th>
<th>Exposure (days)</th>
<th>Untreated (percent of total fatty acids)</th>
<th>Treated (percent of total fatty acids)</th>
<th>P value</th>
<th>Percent Correction*</th>
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<tbody>
<tr>
<td>Human Primary Fibroblasts C24:0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>X-ALD</td>
<td>6</td>
<td>1</td>
<td>2.62 ± 0.40</td>
<td>2.65 ± 0.36</td>
<td>0.988</td>
<td>-1</td>
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<td>X-ALD</td>
<td>4</td>
<td>6</td>
<td>2.48 ± 0.67</td>
<td>1.82 ± 0.28</td>
<td>0.122</td>
<td>81</td>
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<tr>
<td>X-ALD</td>
<td>10</td>
<td>12</td>
<td>2.11 ± 0.33</td>
<td>1.43 ± 0.34</td>
<td>&lt;0.001</td>
<td>155</td>
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<td>X-ALD</td>
<td>7</td>
<td>16</td>
<td>2.22 ± 0.12</td>
<td>1.69 ± 0.27</td>
<td>0.001</td>
<td>96</td>
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<tr>
<td>X-ALD</td>
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<td>22</td>
<td>2.20 ± 0.23</td>
<td>1.52 ± 0.14</td>
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<td>1.67 ± 0.45</td>
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<td>Human Lymphoblastoid Cells C24:0</td>
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<tr>
<td>X-ALD</td>
<td>21</td>
<td>6</td>
<td>1.03 ± 0.19</td>
<td>0.65 ± 0.17</td>
<td>&lt;0.001</td>
<td>126</td>
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<tr>
<td>X-ALD</td>
<td>5</td>
<td>10</td>
<td>1.04 ± 0.20</td>
<td>0.54 ± 0.16</td>
<td>0.004</td>
<td>161</td>
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<tr>
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<td>8</td>
<td></td>
<td>0.73 ± 0.22</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Human Lymphoblastoid Cells C26:0</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>X-ALD</td>
<td>21</td>
<td>6</td>
<td>0.21 ± 0.07</td>
<td>0.11 ± 0.05</td>
<td>&lt;0.001</td>
<td>71</td>
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<tr>
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<td>10</td>
<td>0.26 ± 0.13</td>
<td>0.09 ± 0.05</td>
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<td>0.07 ± 0.03</td>
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<tr>
<td>Mouse Primary Fibroblasts C24:0</td>
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<tr>
<td>X-ALD</td>
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<td>5</td>
<td>1.88 ± 0.08</td>
<td>1.10 ± 0.15</td>
<td>0.002</td>
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<td>9</td>
<td>1.84 ± 0.10</td>
<td>0.87 ± 0.04</td>
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<td>1.20 ± 0.40</td>
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<td>Mouse Primary Fibroblasts C26:0</td>
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<tr>
<td>X-ALD</td>
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<td>5</td>
<td>0.30 ± 0.08</td>
<td>0.07 ± 0.03</td>
<td>0.005</td>
<td>92</td>
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<tr>
<td>X-ALD</td>
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<td>0.30 ± 0.06</td>
<td>0.05 ± 0.01</td>
<td>0.002</td>
<td>100</td>
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<tr>
<td>Normal</td>
<td>7</td>
<td></td>
<td>0.05 ± 0.02</td>
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Table 2: The effect of 4PBA on C24:0 β-oxidation.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>n</th>
<th>Untreated</th>
<th>Treated</th>
<th>P value</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ALD human primary fibroblasts</td>
<td>15</td>
<td>0.20 ± 0.07</td>
<td>0.39 ± 0.07</td>
<td>&lt; 2e-07</td>
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<tr>
<td>X-ALD human lymphoblastoid cells</td>
<td>8</td>
<td>0.06 ± 0.01</td>
<td>0.18 ± 0.03</td>
<td>&lt; 3e-06</td>
<td>2.92</td>
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<tr>
<td>X-ALD mouse primary fibroblasts</td>
<td>6</td>
<td>0.15 ± 0.02</td>
<td>0.28 ± 0.03</td>
<td>&lt; 9e-05</td>
<td>1.87</td>
</tr>
<tr>
<td>Normal human primary fibroblasts</td>
<td>13</td>
<td>0.81 ± 0.14</td>
<td>nd</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Normal human lymphoblastoid cells</td>
<td>7</td>
<td>0.36 ± 0.06</td>
<td>nd</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Normal mouse primary fibroblasts</td>
<td>3</td>
<td>0.97 ± 0.15</td>
<td>nd</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

Cells were grown in the presence or absence of 5 mM 4PBA for 6 days. C24:0 β-oxidation activities in treated cells were compared with those in untreated control cells. Values reported are mean specific activity in nmol/hr/mg protein. Equal amounts of protein (200-300 µg) from treated and untreated X-ALD cells were assayed. A 6-day exposure to 5 mM 4PBA resulted in about a twofold increase in human and mouse X-ALD fibroblasts, and about a threefold increase in the C24:0 β-oxidation in human lymphoblastoid cells. nd, not done; na, not applicable.

Effect of 4PBA on ALDP stability

The response to 4PBA (Table 1) was independent of the X-ALD genotype; cell lines assayed included a deletion of the four C-terminal exons and cell lines derived from patients with different X-ALD phenotypes. In addition, human X-ALD primary fibroblasts with de-stabilizing missense mutations (A626T, R660W) treated with 5 mM 4PBA for up to 10 days did not result in detectable levels of ALDP, as determined by cytological immunofluorescence using anti-ALDP antibody (data not shown). Thus, the mechanism by which 4PBA effects VLCFA metabolism in X-ALD cannot be through stabilization of unstable ALDP, as has been suggested for cystic fibrosis.40,41

Effect of 4PBA on functional homologs of ALDP

To monitor the effect of 4PBA on the expression levels of the family of peroxisomal ABC half-transporter proteins (ALDRP, PMP70 and PMP69), we grew normal and X-ALD primary fibroblasts from human and mouse in the presence or absence of 5 mM 4PBA for 2 days. Semi-quantitative RT-PCR was done on the mRNA isolated from these cells (Fig. 1). Treatment with 4PBA resulted in a substantial increase in ALDR mRNA level in normal and X-ALD primary fibroblasts from both human and mouse. However, exposure to 4PBA had little, if any, effect on the expression levels of either PMP70 or PMP69 in X-ALD or normal primary fibroblasts, or on the expression of ALDP in normal primary fibroblasts from human or mouse. In addition, the expression of acyl-CoA oxidase (AOX), the rate-limiting enzyme in VLCFA β-oxidation,46 was not affected.
Although previous studies have reported that ALDR mRNA was not detectable in fibroblasts by either northern or RT-PCR analyses, those RT-PCR studies used about 66 ng of cDNA; we used about 250 ng of cDNA.

**Figure 1.** RT-PCR analysis of RNA levels in mouse (A) and human (B) primary fibroblasts before (-) and after (+) treatment with 5 mM 4PBA for 2 days. The relative abundance of mRNA for the genes indicated was estimated by semi-quantitative PCR analysis using gene-specific PCR primers. After exposure of both human and mouse fibroblasts to 5 mM 4PBA, the relative abundance of ALDR was substantially increased compared with the RNA levels for the housekeeping genes GAPDH (mouse) or β-actin (human). The mRNA levels of all other genes monitored were unchanged. AOX, acyl-CoA oxidase; nd, not done; bp, size of PCR product.

Because of the inherent variability in quantitation of RT-PCR, we analyzed protein levels in normal and X-ALD mouse primary fibroblasts, both untreated and treated with 4PBA, to measure the increase in ALDRP expression. The relative ALDRP levels in both normal and X-ALD fibroblasts and ALDP in normal fibroblasts were determined by comparing their abundance with that of the protein of the housekeeping gene β-actin in the same samples after densitometric analysis of western blots (Fig. 2). In agreement with the RT-PCR results, treatment with 4PBA did not alter the relative level of ALDP in normal mouse fibroblasts. However, ALDRP levels were increased in X-ALD fibroblasts (by 3.4-fold) but not in normal fibroblasts, even though mRNA levels were increased in both. These findings indicate that ALDP and ALDRP may occupy equivalent domains within the peroxisomal membrane. This would agree with the observation that ALDP and ALDRP are generally expressed in different cell types.⁴⁷ Although ALDRP was not
increased in normal fibroblasts after 10 days of exposure to 4PBA (data not shown), it is possible that even longer exposures would result in increased levels of ALDRP in normal fibroblasts due to turnover of ALDP.

Figure 2. (A) Western blot analysis of mouse primary fibroblasts before (-) and after (+) treatment with 5 mM 4PBA for 5 days. nd, not detectable. (B) The relative abundance of ALDRP or ALDP was compared with the levels for the housekeeping protein β-actin present in the same preparations, by laser scanning densitometric analysis of blots in a. Open bars, untreated cells; Hatched bars, treated cells.

Figure 3. Peroxisomal ABC half-transporter complementation of C24:0 β-oxidation. SV40 T antigen transformed human X-ALD fibroblasts were transfected with recombinant expression vector (pcDNA3) alone or with vector containing cDNA for PMP70, ALDP or ALDRP (hatched bars). The rates of C24:0 β-oxidation observed in the transfected cells were corrected for the fraction of cells expressing the transgene, as determined by IMF staining of the transgene. The adjusted rates were compared to rates of C24:0 β-oxidation determined in transformed fibroblasts from normal individuals (filled bar). The increase in C24:0 β-oxidation after expression of ALDRP cDNA provides an explanation for the normalization of C24:0 levels in X-ALD cells after treatment with 4PBA. Duplicate measurements were done for each transfection. The indicated values are the mean and standard deviation for pcDNA3, n = 6; ALDP, n = 5; ALDRP, n = 4; and normal, n = 5. PMP70, n = 1.
ALDR cDNA complementation restores C24:0 β-oxidation

We previously demonstrated that expression of either ALDP or PMP70 cDNA in X-ALD fibroblasts increased C24:0 β-oxidation. To determine if increased ALDR expression could account for increased C24:0 β-oxidation and the restoration of C24:0 levels following 4PBA treatment, we overexpressed ALDRP cDNA in cultured transformed fibroblasts from X-ALD patients. We analyzed the resulting C24:0 β-oxidation activity in X-ALD fibroblasts complemented with vector (pcDNA3), PMP70, ALDP or ALDRP cDNA (Fig. 3). Over-expression of each of the peroxisomal ABC half-transporters tested increased C24:0 β-oxidation, and both ALDRP and ALDP resulted in activity near that observed in transformed normal fibroblasts. Thus, increased expression of ALDR after exposure of X-ALD cells to 4PBA provides an explanation for the reduction of VLCFA levels.

Effect of 4PBA on peroxisome proliferation

Although human cells generally do not respond to agents known to function as peroxisome proliferators in other species, it has been suggested that 4PBA may function as a peroxisome proliferator in humans. To determine if 4PBA induces peroxisome proliferation, we initially examined untreated and treated human X-ALD primary fibroblasts. Peroxisomes were made visible with immunofluorescence using a polyclonal antibody to the C-terminal tripeptide (SKL) peroxisomal targeting signal 1 (PTS1), which is present in most peroxisomal matrix proteins. Cells were assayed after 5, 8 and 10 days of exposure to 5 mM 4PBA. As seen by immunofluorescence, there was an approximately twofold increase in peroxisome number after 8 days of treatment (Fig. 4). We counted the peroxisomes after 10 days of treatment (Table 3). Slides were scanned visually and cells categorized as having low, medium or high numbers of peroxisomes. For each cell line, the number of peroxisomes is the mean of three ‘low’, four ‘medium’ and three ‘high’ cells (Table 3). There was no difference between control cells and X-ALD cells in either the number of peroxisomes per cell or in the response to 4PBA. The combined results from five X-ALD cell lines and four control cell lines were 416 ± 27 peroxisomes per cell before exposure to 4PBA and 1014 ± 108 after exposure. The 2.4-fold increase in the number of peroxisomes per cell was statistical significant (P < 0.0000001, two-tailed Student’s t-test). Peroxisome numbers obtained with an antibody against a peroxisomal membrane protein (Pex14p) gave similar results (data not shown).
Figure 4. Peroxisomal staining in human X-ALD primary fibroblasts. Punctate staining peroxisomes in human X-ALD primary fibroblasts were visualized using immunofluorescence analysis with a polyclonal antibody against the C-terminal tripeptide (SKL) peroxisomal targeting signal 1 (present in most peroxisomal matrix proteins) before (A) and after (B) 10-day treatment with 5 mM 4PBA. Counting the number of peroxisomes per cell indicated a 2.4-fold increase ($P < 0.0000001$) after treatment.

Table 3: Effect of 4PBA on peroxisome number in human primary skin fibroblasts

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$n$</th>
<th>Untreated</th>
<th>Treated</th>
<th>$P$-value</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal GM5565</td>
<td>10</td>
<td>425 ± 144</td>
<td>961 ± 383</td>
<td>&lt; 0.0008</td>
<td>2.3</td>
</tr>
<tr>
<td>normal GM5659C</td>
<td>10</td>
<td>398 ± 158</td>
<td>907 ± 270</td>
<td>&lt; 0.0001</td>
<td>2.3</td>
</tr>
<tr>
<td>normal GM5757A</td>
<td>10</td>
<td>423 ± 138</td>
<td>1035 ± 338</td>
<td>&lt; 0.00009</td>
<td>2.4</td>
</tr>
<tr>
<td>normal GM5756</td>
<td>10</td>
<td>373 ± 156</td>
<td>852 ± 271</td>
<td>&lt; 0.0004</td>
<td>2.3</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>405 ± 24</td>
<td>939 ± 78</td>
<td>&lt; 0.001</td>
<td>2.3</td>
</tr>
<tr>
<td>X-ALD (A626T)</td>
<td>10</td>
<td>400 ± 130</td>
<td>968 ± 349</td>
<td>&lt; 0.0002</td>
<td>2.4</td>
</tr>
<tr>
<td>X-ALD (R591Q)</td>
<td>10</td>
<td>419 ± 198</td>
<td>1159 ± 363</td>
<td>&lt; 0.00005</td>
<td>2.8</td>
</tr>
<tr>
<td>X-ALD (fs E471)</td>
<td>10</td>
<td>459 ± 108</td>
<td>992 ± 344</td>
<td>&lt; 0.0003</td>
<td>2.2</td>
</tr>
<tr>
<td>X-ALD (fs E471)</td>
<td>10</td>
<td>398 ± 122</td>
<td>1089 ± 365</td>
<td>&lt; 0.00005</td>
<td>2.7</td>
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<tr>
<td>X-ALD (exon7-10del)</td>
<td>10</td>
<td>449 ± 138</td>
<td>1164 ± 378</td>
<td>&lt; 0.00005</td>
<td>2.6</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>425 ± 28</td>
<td>1074 ± 92</td>
<td>&lt; 0.0002</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Peroxisome numbers in human control and X-ALD primary skin fibroblasts before and after 10 day exposure to 5 mM 4PBA resulted in an increase of approximately 2.4-fold in peroxisomes per cell. The X-ALD mutations are indicated in parentheses for each cell line. The Fs E471 mutation was present in two independent cell lines.

To determine if the increase in peroxisome number resulted in an increase in peroxisome function, we examined peroxisomal components that are not affected in X-ALD: phytanic acid oxidation, and levels of the peroxisomal matrix protein, sterol carrier protein 2 (SCP2). Phytanic acid oxidation occurs exclusively in the peroxisomal matrix, $^{49}$ and can therefore serve as a marker for peroxisomal function. Treatment of either X-ALD or normal human primary fibroblasts resulted in a
A twofold increase in phytanic acid oxidation, in agreement with the increased number of peroxisomes estimated by immunofluorescence (Fig. 5a). SCP2 is a 15-kDa protein located exclusively in the peroxisomal matrix. We determined its abundance in human X-ALD fibroblasts by western blot analysis before and after 5 and 10 days of exposure to 5 mM 4PBA. There was a significant increase in the amount of SCP2, again consistent with an approximately twofold induction of peroxisomes (Fig. 5b). Because peroxisomes in X-ALD cells have a residual VLCFA β-oxidation activity that is independent of ALDP, 4PBA stimulation of peroxisome proliferation increases an alternative metabolic pathway in addition to a functionally redundant gene.

Figure 5. Peroxisome function after treatment with 4PBA. (A) α-Oxidation of phytanic acid in normal and X-ALD human primary fibroblasts before (filled bars) and after (hatched bars) treatment with 5 mM 4PBA for 10 days. The increase in phytanic acid oxidation activity was significant (P < 0.0015 (n = 4) for X-ALD primary fibroblasts and P < 0.013 (n = 3) for normal primary fibroblasts). (B) Protein levels of SCP2 (the peroxisomal matrix-specific sterol carrier protein 2) were determined in human X-ALD primary fibroblasts after exposure to 4PBA for 0, 5 or 10 days, using western blot and densitometric analysis. The relative amount of SCP2 protein was compared with the amount of protein for the housekeeping gene β-actin. Exposure to 4PBA resulted in a substantial (approximately threefold) increase in SCP2, consistent with the increase in peroxisome numbers.

To analyze the mechanism of 4PBA-induced peroxisome proliferation in human cells, we examined the expression of Pex11α, the inducible regulator of peroxisome abundance; peroxisome proliferator-activated receptor α (PPARα), the receptor for peroxisome proliferators; and AOX, a gene whose expression in rodents is considerably increased during peroxisome proliferation (Fig. 6). Pex11 has been shown to be upregulated during peroxisome proliferation in yeast, and in rodents, Pex11α is variably expressed in different tissues and
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shows increased expression in rat liver after treatment with peroxisome proliferators (S. Gould, unpublished observations). By RT-PCR analysis, Pex11α transcripts were undetectable in normal or X-ALD mouse fibroblasts either before or after 10 day exposure to 5 mM 4PBA (data not shown). In contrast, treatment of human fibroblasts resulted in a substantial increase in Pex11α expression after 2 days.

Figure 6. RT-PCR analysis of RNA levels in mouse primary fibroblasts after 2 days of treatment with 5 mM 4PBA (A) and in human primary fibroblasts after 2 days (B) or 10 days (C) of treatment with 5 mM 4PBA. The relative abundance of RNA for the genes indicated was estimated by semi-quantitative PCR analysis using genespecific PCR primers. Compared with the RNA levels for the housekeeping gene GAPDH (mouse) or β-actin (human), AOX was unchanged in both mouse and human primary fibroblasts treated with 4PBA, whereas PPARα RNA was increased in mouse fibroblasts but not in human fibroblasts. Pex11α expression was substantially increased in human fibroblasts after two days.

In rodents PPARα expression is increased by peroxisomal proliferators. Semi-quantitative RT-PCR analysis of PPARα expression showed that its expression is increased in mouse X-ALD fibroblasts as early as 2 days after treatment, but its expression in human X-ALD fibroblasts is unchanged, even after 10 days of treatment, when peroxisome proliferation is already observed. Thus, although the peroxisome proliferation response in mice may be, at least in part, through the documented PPARα pathway, the response in human cells seems to be through a newly discovered pathway that may involve Pex11α. We also examined the
expression of AOX which in rodents is also increased by most peroxisome proliferators. AOX expression in both mouse and human fibroblasts was unchanged after 2 days of exposure to 5 mM 4PBA and remained unchanged in human fibroblasts after 10 days exposure. This again indicates a newly discovered pathway for 4PBA-induced peroxisome proliferation in humans and perhaps in rodents. AOX has been identified as the rate-limiting step in VLCFA β-oxidation. Treatment with 4PBA increases VLCFA β-oxidation in X-ALD cells, but not to the levels observed in normal cells. Thus, the lack of increased AOX expression after 4PBA treatment will not limit the restoration of VLCFA β-oxidation to at least normal levels.

![Figure 7](image)

**Figure 7.** In vivo efficacy of 4PBA. VLCFA analyses of mouse brains and adrenal glands from control mice (black bars; \( n = 4 \) for brain and \( n = 6 \) for adrenal gland), untreated X-ALD mice (grey bars; \( n = 4 \) for brain and \( n = 7 \) for adrenal gland), and X-ALD mice treated with 4PBA for 4 weeks (right hatched bars; \( n = 2 \)) or 6 weeks (left hatched bars; \( n = 2 \)). Dietary 4PBA treatment for 4 weeks resulted in a substantial reduction in both brain and adrenal gland VLCFA levels. Treatment with 4PBA for 6 weeks resulted in complete correction of \( C_{24:0} \) and a 78% correction of \( C_{26:0} \) in X-ALD mice brain, and 90% and 85% corrections of \( C_{24:0} \) and \( C_{26:0} \), respectively, in X-ALD mice adrenal glands.

**In vivo efficacy of 4PBA**

To measure the *in vivo* efficacy of 4PBA, we supplemented mouse chow and water with 4PBA. X-ALD mice were on the 4PBA diet for 4 and 6 weeks. We determined the effect of 4PBA on VLCFA levels in mouse brain and adrenal glands was determined (Fig. 7). Four weeks of dietary 4PBA treatment resulted in substantial reduction of \( C_{24:0} \) and \( C_{26:0} \) levels in both brain and adrenal glands.
Six weeks of dietary 4PBA treatment resulted in complete normalization of brain C24:0 and 80% normalization of brain C26:0 levels, and approximately 90% correction of both C24:0 and C26:0 in the adrenal glands.

Discussion

We have demonstrated that 4PBA decreases VLCFA levels by increasing VLCFA β-oxidation in cultured cells from both X-ALD patients and knockout mice. Although wild-type levels of C24:0 β-oxidation were not achieved, the resultant twofold to threefold increase was sufficient to restore normal C24:0 levels in all cell types tested. A decrease in VLCFA levels in brain, the primary tissue affected in X-ALD, is likely because it has been shown that phenylacetate, the initial product of 4PBA breakdown, can cross the blood-brain barrier in rats. Although we have not yet directly demonstrated that 4PBA can cross the blood-brain barrier in X-ALD mice, the results in Fig. 7 show that 4PBA is effective in lowering VLCFA levels in the brain.

Given the suggestions for 4PBA effects discussed above, these results could be due to increased expression or stabilization of ALDP itself, increased expression of a related peroxisomal ABC half-transporter or increased peroxisome proliferation. The first possibility is ruled out, as the effect is seen in cells from individuals with a variety of mutations, including those with no protein due to partial gene deletions. The other two possibilities, however, could contribute to improved VLCFA metabolism in X-ALD patients.

Our results and previous studies have established that three of the peroxisomal ABC half-transporters are at least partially functionally redundant and thus can correct VLCFA metabolism after overexpression in X-ALD cells. The activities of ALDP and ALDRP are not yet known but the equivalence of their ability to complement the VLCFA metabolic defect in X-ALD cells and their expression in different cell types indicate that they may indeed be functionally redundant.57 The possible functional equivalence of ALDP and ALDRP was suggested on the basis of their high degree of sequence identity.22,23 ALDR has also been proposed as a candidate for the putative X-ALD modifier gene,39 that at least in part determines the clinical phenotype of X-ALD patients.11

Our demonstration of the functional equivalence of ALDP and ALDRP allows for the possibility that high expression of ALDRP in the brains of some X-ALD patients could result in VLCFA levels that are insufficient for induction of
inflammatory demyelination. Whatever the role of ALDR in the manifestation of X-ALD clinical phenotypes, the demonstration of improved VLCFA metabolism in X-ALD cells after 4PBA-induced increased expression of ALDRP makes X-ALD an attractive candidate for pharmacological gene therapy.

Although the first measured response to 4PBA is increased ALDRP expression coincident with increased C24:0 β-oxidation, there is a subsequent increase in peroxisome number as judged by counting immunofluorescence-stained peroxisomes and the increase in abundance of peroxisomal proteins and activity of metabolic pathways. This is the first direct demonstration of a peroxisome proliferator that is effective in human cells. In mouse, but not human cells, there is an increase in the expression of PPARα coincident with the increase in peroxisomes. Known peroxisomal proliferators in rodents stimulate expression of both PPARα and AOX. The lack of increased AOX expression in both mouse and human cells after 4PBA treatment indicates that the response of AOX may be independent of the PPARα response. Thus, in mouse cells 4PBA may induce peroxisomal proliferation through a mechanism that is common to other peroxisomal proliferators. In human cells, however, neither PPARα nor AOX expression are increased during peroxisome proliferation. Thus, it may be that the 4PBA effect on peroxisome proliferation in humans involves an as-yet undescribed mechanism. Because peroxisomes in cells from X-ALD patients retain between 10 and 35% of wild-type VLCFA β-oxidation activity, that is not dependent on ALDP, increased numbers of peroxisomes could also contribute to improved VLCFA metabolism after treatment with 4PBA. Because 4PBA has been shown to affect gene expression in vivo, the molecular in vitro response documented here probably will be obtained in vivo and accounts for the effect of 4PBA on VLCFA levels in X-ALD mice. Thus, this therapy has the potential to ameliorate or prevent the severe cerebral inflammatory demyelinating X-ALD phenotype. Overexpression of peroxisomal membrane ABC half-transporters is capable of suppressing peroxisomal membrane defects due to mutations in unrelated genes. Thus, 4PBA treatment may have a favorable effect on several peroxisome biogenesis disorders.

Acknowledgements
We thank H. Moser for his advise and continuing interest in this study, and S. Brusilow for discussions. We thank A. Liu, S. Wang, S. Bergin, S. Khangoora and D. Gordon for technical assistance. For review of the manuscript, we thank H. Moser, S. Brusilow, S. Gould, D. Valle and P. Zeitlin. This work was supported by grants from the National Institutes of Health, HD10981, HD24061, DK51149, and GM07814, the Myelin Project and the United Leukodystrophy Foundation.
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