

MINI-SYMPOSIUM: X-Linked Adrenoleukodystrophy

Current and Future Pharmacological Treatment Strategies in X-Linked AdrenoleukodystrophyJohannes Berger, PhD¹; Aurora Pujol, MD, PhD²⁻⁴; Patrick Aubourg, MD, PhD^{5,6}; Sonja Forss-Petter, PhD¹¹ Center for Brain Research, Medical University of Vienna, Vienna, Austria.² Neurometabolic Disease Lab and Institut de Neuropatologia, IDIBELL, Hospitalet de Llobregat, ³ ICREA, ⁴ CIBERER, Barcelona, Spain.⁵ INSERM UMR745, University Paris-Descartes, ⁶ Department of Pediatric Endocrinology and Neurology, Hospital Saint-Vincent de Paul, Paris, France.**Keywords**4-phenylbutyrate, *ABCD1*, *ABCD2*, antioxidant, histone deacetylase inhibitor, Lorenzo's oil, lovastatin, therapy, valproic acid, X-ALD.**Corresponding author:**Johannes Berger, PhD, Center for Brain Research, Medical University Vienna, Spitalgasse 4, A-1090 Vienna, Austria (E-mail: johannes.berger@meduniwien.ac.at)

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Abstract

Mutations in the *ABCD1* gene cause the clinical spectrum of the neurometabolic disorder X-linked adrenoleukodystrophy/adrenomyeloneuropathy (X-ALD/AMN). Currently, the most efficient therapeutic opportunity for patients with the cerebral form of X-ALD is hematopoietic stem cell transplantation and possibly gene therapy of autologous hematopoietic stem cells. Both treatments, however, are only accessible to a subset of X-ALD patients, mainly because of the lack of markers that can predict the onset of cerebral demyelination. Moreover, for female or male X-ALD patients with AMN, currently only unsatisfying therapeutic opportunities are available. Thus, this review focuses on current and urgently needed future pharmacological therapies. The treatment of adrenal and gonadal insufficiency is well established, whereas applications of immunomodulatory and immunosuppressive drugs have failed to prevent progression of cerebral neuroinflammation. The use of Lorenzo's oil and the inefficacy of lovastatin to normalize very-long-chain fatty acids in clinical trials as well as currently experimental and therefore possible future therapeutic strategies are reviewed. The latter include pharmacological gene therapy mediated by targeted upregulation of *ABCD2*, the closest homolog of *ABCD1*, antioxidative drug treatment, small molecule histone deacetylase inhibitors such as butyrates and valproic acid, and other neuroprotective attempts.

INTRODUCTION

X-linked adrenoleukodystrophy (X-ALD) is a peroxisomal neurodegenerative disorder with a very broad clinical spectrum ranging from the most severe childhood cerebral form to late-onset adrenomyeloneuropathy (AMN) (8, 70). The fundamental X-ALD symptoms that presumably all male patients would develop, provided they survive long enough, are those of a slowly progressive paraparesis, caused by a distal axonopathy that affects most severely the dorsal columns and the corticospinal tracts in the spinal cord, and represent the major neurological deficit in AMN (80). The mean age of onset of AMN is 28 ± 9 years but initial clinical presentation varies from late adolescence to the fifth decade, and occasionally occurs even later. Female patients (heterozygous carriers, as *ABCD1* is located on the X-chromosome) experience a similar myelopathy, but milder and of later onset. About 65% of all male X-ALD patients will develop a rapidly progressive, inflammatory, demyelinating cerebral form (cALD) of X-ALD either in childhood or adulthood. The occurrence of cerebral demyelination in heterozygous women is exceptional. There are two common

periods for the onset of cerebral inflammatory ALD: the most frequent one between 4 and 12 years of age, with a peak around 7–8 years; and a less frequent one between 20 and 45 years of age, with a peak around 30 years. For untreated patients with childhood cerebral X-ALD, the 5-year survival rate (from the time of onset of first symptoms) was 59% with considerable variation in individual survival times (59). In some cases the demyelinating process can spontaneously stop without further progression ("arrested" cerebral variant) when it is not associated with disruption of blood–brain barrier at brain magnetic resonance imaging (MRI). Primary adrenocortical insufficiency is present in approximately 80% of males with cerebral involvement and approximately 50% of men with AMN, but in only 1% of women who are heterozygous for X-ALD (59).

The primary cause of the entire clinical spectrum is an inherited mutation (or in only 5% a *de novo* mutation) in the *ABCD1* gene encoding the peroxisomal protein ATP-binding cassette (ABC)-transporter D1, in the past referred to as the adrenoleukodystrophy protein (ALDP). The same mutation can give rise to all different clinical variations even within the same nuclear family (6). Genetic

and environmental factors are suggested to modulate the clinical outcome of the disease. If X-ALD is suspected in a male patient, or in the term of a newborn screening, the investigation of very-long-chain fatty acids (VLCFAs) will lead to a clear diagnosis; the clinical manifestation, however, cannot be predicted. Thus, it is critically important to monitor brain MRI every 6 months in presymptomatic male patients between 3 and 12 years of age and yearly after that up to 45 years. The inability to predict a future clinical course represents a major problem concerning the choice of appropriate therapy as well as the evaluation of the efficacy of clinical trials for novel medication in X-ALD. The problem is boosted by the fact that X-ALD is a rare disease with an incidence (hemizygous males plus heterozygous females) of 1 in 16:800 worldwide (9) and, thus, all clinical trials in X-ALD have been conducted with very low numbers of patients.

Although the entire clinical spectrum of the disease is initiated by mutations in the *ABCD1* gene, the pathomechanisms for demyelination, the inflammatory process, the axonal degeneration and the adrenal insufficiency clearly differ, and thus different therapeutic strategies must be considered for individual symptoms. Hematopoietic stem cell therapy (HSCT), for example, is clearly beneficial against the cerebral form of X-ALD, when performed in a relatively early phase of inflammatory progression, but it is still not known whether it would be beneficial against AMN symptoms. In this review, we did not include allogenic HSCT (78) or autologous HSC gene therapy (14), as these approaches are reviewed in a separate chapter of this mini-symposium (15); here we will focus on different pharmacological interventions for the various forms of X-ALD and on future strategies and perspectives for therapies in X-ALD.

TREATMENT OF ADRENAL AND GONADAL INSUFFICIENCY

Approximately 70% of male X-ALD patients develop primary adrenocortical insufficiency, often before the onset of neurological symptoms. Thus, all male patients should be monitored for adrenal insufficiency with plasma adrenocorticotropic hormone (ACTH) levels and if the results are ambiguous, with the ACTH stimulation test (72). Isolated measurements of plasma cortisol levels are insufficient and may lead to the false conclusion (70). The 8:00 am plasma cortisol level may be normal even when it is unresponsive to ACTH stimulation and the ACTH level is more than 1000 picogram/ml (normal <70). In affected patients, adrenal hormone replacement therapy is mandatory and effective. Glucocorticoid dose requirements are generally the same as those used for other forms of primary adrenal insufficiency. Not all patients require mineralocorticoid replacement. Hormone replacement therapy does not influence the development or progression of neurological symptoms (69).

Males with clinical manifestation of hypogonadism that are associated with a low serum testosterone concentration should receive androgens. Impotence, in most instances, is caused by spinal cord involvement or neuropathy, rather than testosterone deficiency (70).

Interestingly, many endocrine cell populations express relatively high levels of *ABCD1* protein, especially those that also produce proopiomelanocortin (POMC), the precursor of ACTH and several other peptide hormones (44). Moreover, the POMC-derived pep-

tides β -lipotropin und β -endorphin colocalize with peroxisomes in cells that express *ABCD1*, but the peroxisomal localization of these peptide hormones was not affected in tissues from X-ALD patients (Höftberger *et al* unpub. obs.). Whether the cellular changes that are caused by *ABCD1*-deficiency in X-ALD patients influence synthesis rate, processing or secretion of POMC-derived peptides, remains to be investigated, and may add a further level of complexity to the endocrinological aspects of X-ALD pathology, which are usually attributed to local toxicity of accumulated VLCFAs.

LORENZO'S OIL

Although the levels of VLCFAs in serum or cultured fibroblasts of X-ALD patients appear indistinguishable among patients affected by different disease severity, the *in vivo* amount of VLCFAs in particular cell types or lipid classes, such as gangliosides and other complex lipids, might play a crucial role in the mechanism underlying AMN and cerebral inflammatory ALD. In good agreement with this hypothesis, Asheuer *et al* (3) reported higher C26:0 levels in normal appearing white matter of patients with the childhood cerebral phenotype than in those with AMN. There is also growing evidence that oxidative stress contributes to the pathogenesis of X-ALD and that excess of VLCFAs plays a role in this process (29, 41, 81). Thus, there is a good rationale for therapies aiming at lowering VLCFAs in the central nervous system (CNS). The reduction of VLCFAs might be beneficial for all different clinical manifestations of X-ALD; the targeted molecular mechanism, however, might differ, for example, autoantigenic activity via CD1-mediated presentation of gangliosides and other lipids containing VLCFAs in the inflammatory process, vs. VLCFA as a primary cause of oxidative stress in the susceptible long cortico-spinal tracts in AMN.

Oral administration of "Lorenzo's oil," a 4:1 mixture of glyceryl trioleate and glyceryl trierucate, combined with moderate reduction of fat in the diet, normalizes or significantly lowers the levels of VLCFA in plasma within 4 weeks (88), but its clinical efficacy and the clinical indications for its use have been controversial for more than 15 years. Based on several open clinical trials including asymptomatic and symptomatic patients, it was concluded that there is no clinically relevant benefit from dietary treatment with Lorenzo's oil (4, 103). The treatment could neither ameliorate nor arrest the rapid progression of neurological symptoms in cerebral variants of X-ALD, and asymptomatic patients have developed the severe, rapidly progressive, inflammatory demyelination (eg, (4, 103)).

However, a study involving 89 asymptomatic X-ALD patients with normal brain MRI and a mean age of 4.7 years at enrollment and a follow-up period of 6.9 ± 2.8 years suggests that substantial and consistent reduction of plasma C26:0 levels led to a twofold or greater reduction in the risk of developing the childhood cerebral form of X-ALD (71). There was a moderate reduction of platelet count in approximately 30% of patients, but this could be managed by adjustment of Lorenzo's oil dosage. The dietary therapy requires a careful supervision by a multidisciplinary team including a nutritionist to ensure that the VLCFA levels are lowered and that nutritional balance is maintained.

Köhler and Sokolowski conducted an open study on 45 men with pure AMN (53). The patients were followed for an average period of 6.3 years (range 2–12.8 years), during which 22 patients (48%) remained stable. In 84% of patients with disease progression, the

progression during therapy was significantly slower than it had been during the pretreatment period. Unfortunately, it is not possible to draw definitive conclusions because of the open study design, the relatively small number of treated patients, and marked variability of AMN progression on an individual basis. Furthermore, the findings are in contradiction to earlier studies (4, 103). Lorenzo's oil teaches a lesson about how raising early hope in patients creates ethical issues that prevent double-blind studies, which in combination with the lack of knowledge concerning the molecular mechanism of a disease and the relatively low patient numbers, have led to a situation where the efficacy of a therapy is still unknown even after 20 years of use in probably more than 500 patients.

FAILURE OF IMMUNOMODULATORS AND IMMUNOSUPPRESSIVE DRUGS

As the rapid progression of X-ALD is associated with brain inflammation, reduction of this response could be of therapeutic benefit. Unfortunately, so far all studies attempting immunosuppressive or immunomodulating therapies have not been successful. Severe immunosuppression with cyclophosphamide failed to prevent neurological progression in a patient with adrenoleukodystrophy (98) and in four out of five patients in a later investigation (75). Interferon (IFN)- β can suppress the synthesis of IFN- γ and tumor necrosis factor (TNF)- α , both of which have been potentially implicated in the pathogenesis of X-ALD. Eight patients with cerebral X-ALD were treated with Lorenzo's oil (glycerol-trioleate/glycerol-trierucate) in combination with subcutaneous injections of IFN- β 1a, but the treatment did not affect the clinical progression (52).

Intravenous immunoglobulins (IVIGs) were reported to be helpful in one patient with adolescent cerebral ALD (65) but without clear benefit in 11 out of 12 patients in a second investigation (67) and without clinical benefit in a third study involving 12 childhood cerebral X-ALD patients under Lorenzo's oil, of which six received IVIGs and six did not (12). An anecdotal plasma exchange did not alter the course of the disease (74). However, when combined with dextran sulphate absorption of VLCFA it improved clinical performance and well-being in one patient (5). In addition, several open trials, each performed in 20–25 children with cerebral X-ALD, applying IVIG therapy or immunosuppressive drugs, like cyclosporine or mitoxantrone, showed no effect (P. Aubourg, unpub. obs.). Based on initial, promising results in multiple sclerosis (MS), Tysabri (NatalizumAb; monoclonal antibody therapy directed against integrin alpha-4 adhesion molecules on activated white blood cells, preventing their entry into the CNS) was tried in two X-ALD patients that were too advanced for HSCT, but without success (Jutta Gärtner and Robert Steinfield, pers. comm.).

Despite some resemblance between the inflammatory lesion in cerebral X-ALD and some subtypes of MS, the failure of all these immunomodulatory/suppressive therapies likely reflect that the physiopathogenesis of neuro-inflammation in X-ALD is in fact completely different, and not just that cALD progresses more rapidly than the typical relapsing-remitting forms of MS. The mononuclear phagocytic cell response in cerebral X-ALD shows a distinct pattern, suggesting that the inflammatory reaction trails behind rather than leads the tissue injury (91). Microglial apoptosis in perilesional white matter appears to be an early event in lesion

evolution and thus may represent a therapeutic target in X-ALD patients with cerebral demyelination (21).

INEFFICACY OF LOVASTATIN TO NORMALIZE VLCFA

Modulation of cellular cholesterol by either cholesterol depletion (107) or treatment with the cholesterol-lowering drug lovastatin (95) normalizes VLCFA accumulation in primary cultured human X-ALD fibroblasts. On the other hand, high cholesterol levels could increase levels of VLCFA in cultured human fibroblasts from X-ALD patients or controls (108). *Abcd1*-deficient X-ALD mice (26) show an increased plasma cholesterol level, comparable with the level observed in high cholesterol-fed wild-type controls. In contrast to the wild-type mice, the plasma cholesterol level could not be further increased by high dietary cholesterol in the *Abcd1*-deficient mice (108). However, neither treatment with lovastatin (112), nor simvastatin (13), nor lovastatin combined with colestipol (a cholesterol-lowering bile acid sequestrant) (108) normalized the levels of VLCFA in tissues of the X-ALD mice. Only in one study, where isolated brain white matter was analyzed (in contrast to whole brain in the other studies) after 3 weeks of lovastatin treatment, was a reduction of VLCFA accumulation reported in the X-ALD mouse model (48).

In two clinical trials, involving 7 and 12 X-ALD patients with different phenotypes that were treated with 20–40 mg lovastatin per day for up to 12 months, reduced levels of VLCFA in plasma were reported (77, 95). A different study of six children with X-ALD, treated for 3 months with 0.2–1 mg simvastatin/kg body weight/day, failed to observe a reduction in plasma VLCFA (105). A recently reported randomized, double-blind, placebo-controlled, crossover trial, including 14 AMN patients and comparing lovastatin at a dose of 40 mg once daily with placebo (22), did observe a slight decrease of VLCFA levels in plasma after 8 weeks. But even after reduction, C26:0 remained above the control level and was no longer significant at 22 weeks. The decrease in VLCFA was considered to be a non-specific result of the decrease in the level of LDL cholesterol. C26:0 levels in peripheral-blood lymphocytes, erythrocytes and in the LDL lipoprotein fraction were not altered after the treatment. It was concluded that lovastatin should not be prescribed as a therapy to lower levels of VLCFA in patients with X-ALD (22). These studies did not report any clinical benefit for the neurological symptoms.

PHARMACOLOGICAL INDUCTION OF THE REDUNDANT GENE *ABCD2* AS POSSIBLE THERAPEUTIC APPROACH

Mutations in the *ABCD1* gene encoding the peroxisomal ABC-transporter ABCD1 is the initial cause of X-ALD regardless of clinical variant. ABCD1 is a half ABC-transporter, forming predominantly homodimers (36, 42), but may also form heterodimers with other peroxisomal ABC-transporters (58, 96, 100). There are at least three peroxisomal ABC-transporters. Whereas the peroxisomal localization of ABCD1, ABCD2 (ALDRP, adrenoleukodystrophy-related protein) and ABCD3 (PMP70, peroxisomal membrane protein 70 kD) is undisputed, a recent study doubted the peroxisomal localization of ABCD4 (P70R/PMP69), the fourth member of the ABCD transporter family (45). ABCD2,

the closest homolog of ALDP shares 66% amino acid identity (43) and when overexpressed in cultured human fibroblast cell lines from X-ALD patients, it can normalize the peroxisomal β -oxidation and prevent accumulation of VLCFAs (25, 76). Moreover, when murine ABCD2 protein is ubiquitously overexpressed from a transgene under the control of the β -actin promoter in *Abcd1*-deficient mice, it can normalize VLCFA levels in the target tissues (brain, spinal cord and adrenals) and rescue late-onset motor coordination defects (85). Moreover, partially overlapping functions of these transporters regarding accumulation of VLCFA and β -oxidation of VLCFA *ex vivo* have been reported (30). Also early signs of oxidative stress in spinal cord of the mouse model were counteracted by the transgene (31). As ABCD2 protein can substitute for the lack of ABCD1 *in vitro* and *in vivo*, it is strongly suggested that these two ABC-transporters have similar and overlapping substrate specificities. The different, rather complementary expression patterns of murine and human ABCD1 and ABCD2 (7, 55, 101) and the level of ABCD2 expression in critical cell types could be the reason why ABCD2 cannot compensate for the loss of ABCD1 in X-ALD patients. Furthermore, the two genes are regulated in opposite directions under some potentially disease-relevant conditions, for example, in response to cholesterol-loading of human peripheral blood monocytes (51). We have also demonstrated that ABCD2 is not mutated in X-ALD patients and that the X-ALD phenotype is independent of the ABCD2 genotype (60).

Thus, pharmacological induction of ABCD2 should be able to compensate for the lack of functional ABCD1 in X-ALD patients, if the induction is sufficiently strong and targets disease relevant cell types. Two main questions considering the induction of ABCD2 as therapeutic approach in X-ALD are: (i) which cell types have to be targeted for the pharmacological induction of ABCD2?; and (ii) is it necessary that a compound passes the blood–brain barrier? HSCT can arrest the demyelinating process when performed at an early stage of inflammation. None of the successfully treated patients developed newly emerging neuroinflammation and cerebral demyelination at later time points, after more than 20 years of follow-up (78). Probably perivascular macrophages and resting microglia are the main cell populations that continually renew from bone marrow hematopoietic stem cells and/or myeloid progenitors in the brain. The success of the hematopoietic stem cell gene therapy (14) suggests that only a limited number of microglia cells needs in fact to be corrected to arrest the demyelinating process. At 24–30 months after *ex vivo* correction of the patient's own CD34⁺ cells using a lentiviral-based ABCD1 vector and re-infusion into the patient, Cartier and coworkers (14) detected ALDP in 9%–14% of granulocytes, monocytes, and T and B lymphocytes. Although one cannot completely exclude a selective advantage of corrected microglia, this percentage of correction was sufficient to arrest the inflammatory demyelinating process in two patients. The clinical benefit could also be caused by the overexpression of ALDP in these cells. Thus, in our view, induction of ABCD2 in the macrophage/microglia population represents an attractive therapeutic target concerning the inflammatory phenotype. In addition, it is possible that the compound does not have to pass the blood–brain barrier in order to be effective. Possibly, cells initially treated in the periphery can migrate into the brain or secrete some peroxisome-derived, trans-acting metabolite with beneficial effects in the CNS. For example, in a cre-lox mouse model the lack of peroxi-

somes exclusively in the liver (albumin/ α -fetoprotein-cre \times Pex5-loxP) leads to abnormalities in brain development (54).

The concept of targeted therapeutic induction of ABCD2 has initiated intense investigations aiming to modulate the rodent and human ABCD2 gene expression. Early studies had indicated that 4-phenylbutyrate (47), discussed in the following in the context of histone deacetylase (HDAC) inhibitors, and fibrates activate ABCD2. Fenofibrate and other related compounds with peroxisome proliferating activity also strongly upregulated ABCD2 mRNA levels in liver and intestine (but not in brain) of rats and mice (1, 2, 7, 27). Also highly potent, specific peroxisome-proliferator activated receptor α (PPAR α) agonists (GW7647, GW6867 and tetradecylthioacetic acid) induced *Abcd2* in mouse liver and adrenals (86). This response to fibrates was abolished in PPAR α knockout mice. But no functional response element (PPRE) could be identified in the *Abcd2* gene in any of these studies, leading to the hypothesis that the PPAR α agonist-mediated effect is indirect (7, 27, 86). Because a PPAR α -dependent increase in the sterol regulatory element-binding protein (SREBP) 2 mRNA level could be demonstrated in the liver of fenofibrate treatment mice, we hypothesized that the PPAR α agonist-mediated induction of *Abcd2* expression is indirect by SREBP2 (86).

Detailed promoter analyses demonstrated that ABCD2 expression is induced upon sterol-depletion in cultured human and murine monocytes as well as in human primary fibroblasts. Reporter gene studies, site-directed mutagenesis and gel shift assays have identified a functional sterol regulatory element (SRE) as a key regulatory element in the promoter of ABCD2 (107, 109). This finding provides a link between ABCD2 expression and possible positive effects of cholesterol-lowering compounds such as lovastatin, which leads to maturation and nuclear translocation of the transcription factor SREBP and subsequently to activation of ABCD2 expression and reduced VLCFA accumulation. As described earlier, apparently lovastatin (or related statins) alone is not sufficient to normalize VLCFAs in human X-ALD patients or in the murine X-ALD model. This might be related to the complex context of the SRE motif in the ABCD2 promoter.

The SRE motif overlaps with a direct repeat (DR4) element, which can bind several nuclear receptors that form heterodimers with retinoid X receptor (RXR), such as liver X receptor (LXR) α (109) or thyroid hormone receptor (TR) α and β (28, 110). The RXR agonist *cis*-retinoic acid induces transcription of endogenous ABCD2 in human NT2 cells (101) and of the human and rodent ABCD2 promoter in transfected cells (83); however, in these studies a direct interaction with a specific promoter element was not demonstrated. Whereas RXR agonists can further induce SREBP-mediated activation of the promoter in reporter gene assays (J. Berger, unpub. obs.), LXR α agonists counteract the SREBP-induced response of ABCD2 (109). Thus, in a scenario where oxidized sterols, the natural ligands for LXR, would down-regulate ABCD2 expression, an LXR α selective antagonist could represent a possible therapeutic intervention. Both TR α and TR β bind the DR4 motif in the ABCD2 promoter, but whereas thyroid hormone-liganded TR α activates the gene, a repressive response seen by unliganded TR β appears to be relieved by hormone-binding, especially in the context of SREBP-activated ABCD2 expression (110). Thyromimetics (GC-1 and CGS 23425) specific for TR β 1 were recently shown to induce ABCD2 in human HepG2 cells as well as in primary X-ALD fibroblasts (33). Thus, under-

standing the relationship between the transcriptional activation mediated by SREBP and a complex network of DR 4-binding transcription factors, including TR α , TR β , LXR α and RXR α , will help to select and test more specific agonists or antagonists for a safe yet effective modulation of *ABCD2* expression in X-ALD patients.

For preclinical proof-of-concept, promising compounds can be tested in cultured macrophage/microglial cell lines and primary monocytes/macrophages, in *ABCD1*-deficient cells in culture, and *in vivo* in the X-ALD mouse model. Of some concern for the pharmacological induction of *ABCD2* in X-ALD patients, was a possible dominant negative effect of certain *ABCD1* mutations, resulting in stable inactive protein when forming heterodimers with the induced *ABCD2* protein (102). However, accumulating evidence indicate that *ABCD1* and *ABCD2* predominantly form homodimers (36, 42) making this argument less important for pharmacological gene therapy, but it may still indicate some caution for the gene therapy of autologous hematopoietic stem cells using viral vectors in X-ALD.

HDAC INHIBITORS AS POTENTIAL THERAPEUTIC AGENT

The net effect of most transcription factors that bind specific gene regulatory motifs and act as activators or repressors of gene expression, is the recruitment of histone acetyl transferases (HAT) or HDACs, respectively, usually via additional layers of protein-protein interactions with coactivators or corepressors. HDACs remove the acetyl groups from the lysine residues of core histones leading to locally condensed, transcriptionally silenced chromatin. Blocking of this activity results in hyperacetylation and thus increased transcription rates at the affected promoter. This family of enzymes is now also appropriately being referred to as lysine deacetylases (KDACs) to include their function on non-histone, lysine-containing targets (16), which among others include transcription factors (e.g. NF κ B, p53, Sp1) and their co-regulators.

In higher eukaryotes, 10 different "classical" HDAC proteins (HDAC1–10) are grouped either into class I or II (reviewed in (17)) and are all inhibited by trichostatin A. Class I HDACs are widely expressed and are normally considered constitutively nuclear proteins, whereas classes II HDACs show more tissue specificity and shuttle between the nucleus and the cytoplasm (66). Nuclear export of HDAC1 has recently been implicated in pathological demyelinating conditions and may be essential for the onset of axonal damage, as it was identified as a critical event for impaired mitochondrial transport in damaged neurons (50).

Small-molecule HDAC inhibitors interfere with class I and II HDAC activity by binding to the Zn-containing catalytic domain and can achieve significant biological effects in preclinical models of cancer (66), but also in neurodegenerative diseases like Huntington's, Parkinson's or Alzheimer's disease (38). The most potent group is hydroxamic acids, such as trichostatin A and the second generation drug vorinostat (SAHA) that has been used with success in preclinical and clinical trials. Small organic acids, such as phenylbutyrate and valproic acid (VPA), have more favorable toxicity profiles but much lower potency, because of rapid absorption in the gastrointestinal tract after oral administration and to albumin and other plasma proteins (93). Because of its anticonvulsant effects, VPA is widely used in treatment of epilepsy.

Application of HDAC inhibitors have been used to target induction of specific genes or relied on global effects on transcription. Some examples of pharmacological treatment strategies for genetic diseases include: the induction of fetal hemoglobin in beta-globin disorders (18), the upregulation of utrophin in dystrophin-deficient muscle cells in Duchenne's muscular dystrophy (49), the stimulation of survival of motorneuron 2 (*SMN2*) transcription and the correction of *SMN2* mRNA splicing by HDAC inhibitors as a potential therapy for spinal muscular atrophy (11, 37). HDAC inhibitors have also been identified as candidate drugs for treatment of several neurodegenerative disorders, including amyotrophic lateral sclerosis, Huntington's, Parkinson's and Kennedy's disease, where perturbation of histone acetylation homeostasis and transcriptional dysregulation of disease-modifying genes clearly play a role in the pathomechanism, reviewed in (38, 46). Several recent studies also indicate that HDAC inhibitors can have an immunomodulatory effect on regulatory T cells, reviewed in (106), and on glial inflammatory responses (23).

4-Phenylbutyrate (4-PBA)

Initial studies with cultured cells showed that small organic acids, such as phenylacetate (94), or its prodrug 4-PBA (47) could reduce the level of VLCFA in X-ALD fibroblasts, which was attributed to increased peroxisomal β -oxidation rates. Kemp and coworkers also showed increased levels of *ABCD2* mRNA and peroxisome proliferation in cultured human and murine primary fibroblasts (both wild-type and X-ALD cells) after 4-PBA treatment. In *Abcd1*-deficient, but not wild-type, mouse fibroblasts, also the level of ALDR protein increased. Most importantly, in a preclinical trial, a substantial reduction of VLCFA (C26:0 and C24:0) was achieved in the brain and adrenal glands of *Abcd1*-deficient mice after 4 and 6 weeks of dietary 4-PBA treatment (47). The article did not show any results on gene expression or peroxisome numbers in the treated mice. However, it was later reported that long-term continuous administration of 4-PBA in *Abcd1*-deficient mice leads to a reduced drug response (tachyphylaxis) and a return to pretreatment levels of VLCFA (63).

The effect of 4-PBA on peroxisome proliferation was considered atypical, because it did not involve the induction *PPAR α* and *acyl-CoA oxidase (Acox)* gene expression that typically accompany peroxisome proliferation. Instead, peroxisome biogenesis factor 11 (*PEX11 α*) was selectively induced together with *ABCD2* (47). It was subsequently shown in *Pex11 α (-/-)* mice that hepatic peroxisome proliferation in response to 4-PBA requires *PEX11 α* but is independent of *PPAR α* (57), and in *Ppar α (-/-)* cells, the activation of *Abcd2* by 4-PBA was unaffected (35). Further studies applying 4-PBA considered the inhibition of HDAC activity, as the mode of induction of *ABCD2* and *PEX11 α* expression and peroxisome proliferation (35, 63, 64). Direct comparisons with the highly potent HDAC inhibitor trichostatin A, showed increased mitochondrial and peroxisomal β -oxidation after application of either drug to cultured fibroblasts, but only 4-PBA induced expression of *ABCD2* (63, 64). In human X-ALD fibroblasts, the effect of 4-PBA was described to first increase mitochondrial β -oxidation of long-chain fatty acids, followed by increased peroxisomal VLCFA β -oxidation, which preceded the upregulation of *ABCD2* mRNA levels. After 8 weeks of oral 4-PBA treatment of *Abcd1*-deficient mice, VLCFA

levels in post-nuclear supernatants of liver tissue were partially reduced by both 4-PBA and tricostatin A, without increased rates of peroxisomal or mitochondrial β -oxidation (64). The effect on VLCFA levels in brain or adrenal glands was not addressed here; but ultrastructural studies of the adrenal glands in 1-year-old *Abcd1*-deficient mice, which had received 4-PBA for 1 month, revealed less structural abnormalities of mitochondria than untreated mice (64).

In another study (35), where 4-PBA was applied to rats orally, long-term (6–9 weeks) or short-term (by i.p. injections for several days), to study acute effects on gene expression, showed peroxisome proliferation and increased amounts of ABCD2 and ACOX mRNA in liver, but unaltered levels in brain (and several peripheral tissues including kidney, spleen and testes). In oligodendroglial and astrocytic cell lines and primary cultures, *Abcd2* but not *Acox* was induced by 4-PBA and butyrate.

Gondcaille and coworkers also identified key elements for the 4-PBA-dependent induction of the rat *Abcd2* promoter using reporter assays in transfected COS-7 cells and DNA binding assays with hepatic nuclear extracts. The authors showed that these GC1 box and CCAAT motifs in the immediate upstream region of the *Abcd2* gene bind their cognate transcription factors, Sp1/Sp3 and NF-Y, respectively, and that HDAC1 is recruited to the GC1 box. For full induction, both elements appears to be required, suggesting a synergistic effect, possibly involving recruitment of HAT activity for gene activation (35). Studies of these interactions *in vivo* will be required to further substantiate this interesting observation. It also remains to resolve whether such recruitment and modulation of HAT activity to antagonize HDACs represents a direct effect on histones and chromatin remodelling or involves acetylation of lysine residues of other protein targets, for example, leading to modified activity Sp1/Sp3 (99).

Clinical applications of 4-PBA and arginine butyrate in human X-ALD patients

Based on clinical applications of 4-PBA for induction of the fetal *globin* gene in human beta-globin disorders and the initial, promising effects of 4-PBA in the mouse model of X-ALD (47), 4-PBA was given orally to seven adult AMN patients in a preliminary clinical study (Raymond, Watkins, Moser, unpub. obs., reviewed in (70)) for two periods of 6 weeks, interrupted by 2 weeks off medication. Although magnet resonance studies of the brain, done in one patient 30 min after administration of 4-PBA, had demonstrated the drug in brain white matter and cerebrospinal fluid, no substantial alterations of VLCFA β -oxidation in white blood cells or of VLCFA levels in plasma, red cells or white blood cells were noted; only C22:0 and C24:0 in platelets were reduced. Analysis of ABCD2 mRNA levels in white blood cells from three of the patients showed no significant change.

Possible complicating factors that contributed to the poor response were suggested to be the short half-life of 4-PBA, which *in vivo* is quickly metabolized in the liver, and tachyphylaxis. These effects might be counteracted by applying a different (interval) treatment regime. Other pharmacological agents that are functionally and/or structurally related to 4-PBA, but with more long-lasting pharmacokinetic properties, were suggested to be tested for their ability to correct the accumulation of VLCFA in X-ALD fibroblasts (63). To circumvent the problems associated with the

large amount of medication needed with oral 4-PBA, an intermittent pulsed regime of intravenous arginine butyrate was developed for treatment of hemoglobinopathies. A case report describes an open trial of intravenous infusion of arginine butyrate during 4 months, with intermittent days off medication, in one patient with childhood cerebral X-ALD (62). The treatment was well tolerated and quickly led to a substantial decrease in plasma VLCFA, showing that this class of compounds can reduce VLCFA levels also in human patients. However, in this patient, the neurological deterioration progressed during treatment, possibly related to the fact that the inflammatory process in the CNS was already underway at the start of the trial. Further clinical studies would be needed to determine effective dosage and interval, and whether neurological degeneration can be prevented with an earlier onset of treatment.

Valproic acid (VPA)

The short-chain fatty acid VPA is a class I-selective HDAC inhibitor with anticonvulsant effects and thus widespread clinical use in treatment of epilepsy. It is well tolerated and passes the blood–brain barrier. VPA provides neuroprotective effects *in vitro* and clinical improvement in spinal muscular atrophy patients (38). Exposure to VPA induces extensive changes in the transcriptome. More than a thousand genes were identified by microarray analysis as being VPA-responsive in the liver of mice after subchronic oral treatment (56); interestingly, among many genes involved in cholesterol and fatty acid metabolism, including *Abcd2*, *Pex11a*, *Acox*, and several elongases (Elovl5) were noticed. Of interest for neurodegenerative aspects, also in a study of primary cultures of rat cortical neurons *Abcd2* emerged as responsive (strongly upregulated) to VPA (32).

In a recent study, Pujol and coworkers evaluated the neuroprotective potential of VPA in X-ALD, particularly targeting AMN (31). The authors showed that VPA induces expression of ABCD2 in human X-ALD fibroblasts via inhibition of HDAC, and that oxidative lesions to proteins are significantly reduced by VPA. Thus, it was expected that VPA should reduce VLCFA levels as well. However, whereas C26:0 was not significantly altered, C26:1 was partially reduced in human X-ALD fibroblast. In the spinal cord of X-ALD mice, overexpression of ABCD2 corrects VLCFA levels and the late-onset neurological phenotype (85) and in mice with targeted deletions of both *Abcd1* and *Abcd2*, overexpression of murine ABCD2 protein prevents oxidative damage. Although VPA induced *Abcd2* expression in hippocampal slice cultures from rat and mouse (wild-type and *Abcd1*-deficient), as well as in human cortical slices, the accumulation of saturated (C26:0) VLCFA could not be rescued by VPA in *Abcd1* or *Abcd1/Abcd2* double-deficient slice cultures. Only mono-unsaturated (C26:1) was partially reduced in the single but not in the double-knockout preparations. As a possible explanation for the discrepancy between induction of ABCD2 and the lack of effect on saturated VLCFA levels, it could be demonstrated that the mRNA levels of several elongase genes (*ELOVL3*, *ELOVL4* and *ELOVL5*) are elevated by VPA in human X-ALD fibroblast (31). As the corresponding enzymes are involved in chain-length elongation during fatty acid synthesis, their increased expression could generate more VLCFA and, thus, counteract the increase in degradation recruited by elevated ABCD2.

Clinical trial of VPA in human X-ALD patients

Because VPA is metabolized very quickly in the liver of mice, no preclinical study was carried out in the mouse model. Instead, as VPA is a well-established and well-tolerated medication, a small clinical trial was performed in five X-ALD patients (31). VPA (Depakine) was administered orally (40 mg/kg/day) for 6 months without side effects. The ABCD2 mRNA levels were slightly increased above baseline in peripheral blood monocytes after 6 months, but the elevated concentrations of VLCFA (both C26:0 and C26:1 ω 9) were not corrected. However, the markers for oxidative damage were completely normalized or strongly reduced at the end of treatment. These results suggest that VPA induces antioxidant effects in X-ALD, which could be of significant benefit especially for patients with AMN (31).

ANTIOXIDANT STRATEGIES

A wealth of human data consistently supports the idea that oxidative stress occurs and is a constant feature of aging and neurodegenerative disease. Some recent evidence even suggests that this phenomenon is an early event and may play a role in the pathogenesis of several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease or amyotrophic lateral sclerosis (90, 113).

Evidence of oxidative damage in X-ALD has been reported in post mortem brain samples from patients with cerebral X-ALD, which show induction of iNOS and nitrotyrosine in astrocytes and microglia from the affected white matter (34), and also increased immunoreactivity to markers of lipoxidative damage (81). Additional data collected in the plasma of X-ALD patients using the thiobarbituric acid test indicate that oxidative stress might not be restricted to affected white matter in the brain, but might be a more general process, and could be used as a disease biomarker (104). In the X-ALD mouse model that mimics mild AMN, time course experiments have provided compelling evidence of oxidative damage of proteins very early in life, at 3 months of age, long before the neuropathological and neurological signs of disease occur at 18–20 months (29). Thus, oxidative damage could be linked to both the initiation and the progression of neurodegeneration in X-ALD. The origin of this oxidative damage is being intensively studied. Current evidence points to the excessive production of free radicals caused by the accumulation of hexacosanoic acid, and to an impaired response of the antioxidant defense system(s) to oxidative insults (29, 82).

In spite of promising epidemiological data, antioxidant therapies have been applied to several neurodegenerative conditions with limited success. Pitfalls include: (i) endogenous levels of antioxidants in the treated patients were not controlled, and this is of importance as patients might react differently to antioxidants based on baseline levels; (ii) the *in vivo* antioxidant effects of the drug and patients compliance were not monitored (39); (iii) the causality link between oxidative stress and disease was not precisely established; (iv) trials were performed in patients with extensive pathologies, where preventive actions might have been too late; (v) the doses used might be wrong—there is some evidence to date that lower doses and/or mixtures of antioxidants might have more benefits than higher doses of single agents (10, 89).

Antioxidants seem in general, better at decreasing oxidative damage in rodent models of disease, and simultaneously beneficially affecting disease progression, than they do in humans (40). However, in diseases in which oxidative stress is well established as the primary cause, these type of approaches might be of interest. A good example is Friedreich Ataxia, caused by depletion of frataxin, a mitochondrial protein implicated in the iron-sulfur metabolism and production of mitochondrial reactive oxygen species (ROS). Idebenone helped in a mouse model and has also improved cardiac hypertrophy in patients at high doses (79, 92).

Our studies in the last years on X-ALD physiopathogenesis using the mouse models of the disease have yielded a strong rationale to support preclinical studies focusing on improving oxidative damage in X-ALD. Moreover, we can use highly sensitive and quantitative markers of oxidative lesions, which were identified first in the murine model and then validated in X-ALD patients' lymphoblasts, to faithfully monitor the beneficial effects of antioxidants (31). These markers have been used to uncover an antioxidant effect of VPA in X-ALD patients, a drug that might combine HDAC inhibitory properties with antioxidant effects and, thus, be of benefit for X-ALD patients.

Recently, we have identified a combination of antioxidants that is able to reduce ROS production *in vitro* and to normalize oxidative lesions to proteins *in vivo* in spinal cords of X-ALD mice (Lopez-Erauskin J, Glia 57, S100, 2009). Work in progress will tell us whether this correction correlates with an arrest or prevention of neurodegeneration in this mouse model. A successful result of the preclinical trial with antioxidants would deliver the proof-of-concept on the contributing vs. causative role of oxidative stress in X-ALD pathogenesis. Extensive experimental data concerning the intracellular sources of ROS, the organelles and enzymatic systems involved, and the cell types responsible for the generation and homeostasis of ROS in the X-ALD murine model are required before a rational strategy with reasonable odds of success might be proposed to patients. Effectiveness of this therapeutic strategy will likely apply only to AMN patients, and at best for patients at very early stage of demyelination.

ADDITIONAL GENERAL NEUROPROTECTIVE EFFORTS

Insulin-like growth factor-1 (IGF-1) and neurotrophin-3 (NT-3)

Addressing features of axonopathy and related destabilization of myelin in the CNS in X-ALD, a recent study in mice was carried to evaluate the potential therapeutic benefit of the growth factors IGF-1 and NT-3 (61). Recombinant adeno-associated viral vector (serotype 6), engineered to produce either IGF-1, NT-3 or enhanced green fluorescent protein (EGFP, as a negative control), were administered into the cerebrospinal fluid of *Abcd1/Abcd2* double-deficient mice, a model for X-ALD (85), for a continuous source of these growth factors. Mice with targeted inactivation of both *Abcd1* and *Abcd2* develop late-onset AMN-like myelopathy and axonal degeneration in the spinal cord, months before axonopathy and motor behavior-deficits are detected in *Abcd1* single mutant mice (24, 84, 85) and were selected for this trial in order to allow evaluation of long-term therapeutic efficacy on disease progression. After injection of the vectors at 17 months of age, the

expected transduction of ependymal and leptomeningeal cells was demonstrated and 20 weeks after treatment, IGF-1 and NT-3 were still detectable by ELISA in the CSF (61). Motor performance was tested every second week over a 4-month-period; before injection, the motor performance of the double-mutant mice had already started to deteriorate when compared with the wild-type controls, and in the EGFP-treated "X-ALD" mice it continually worsened, whereas the motor behavior of mice treated with either IGF-1 or NT-3 vector improved over time to the levels of untreated wild-type controls. Twenty weeks after treatment, the extent of myelination in the spinal cord, as revealed by morphometric analysis of immunohistochemical staining for proteolipid protein (PLP), was indistinguishable in untreated wild-type and "X-ALD" mice treated with neurotrophic factors, whereas the EGFP-treated control mice showed severe loss of myelin (61).

Thus, this long-lasting, gene therapy-based delivery of trophic support for oligodendrocytes showed a protective effect against the demyelination process and appeared to arrest progression of the disease. Although in this study sufficiently large numbers of mice/group were analyzed, no untreated mutant mice or mock (ie, EGFP)-treated wild-type mice were used as controls. Therefore, neither any effects from the procedure or the vector (expressing EGFP) alone nor the range of possible improvement can be assessed. Also, the limited histopathological analysis does not address therapeutic effects on other neurodegenerative features, like axonopathy or microglia activation. Nevertheless, this approach delivers hope for therapeutic intervention aiming to limit primary loss of oligodendrocytes and myelin.

Modified cobratoxin

As Immunokine had been tried in several patients with MS and AMN under non-placebo controlled conditions with initial encouraging results, a study was designed to elucidate whether Immunokine, can improve clinical symptoms in eight AMN patients (mean age 46.7 ± 15 years) with spastic paraparesis that caused walking difficulties. Immunokine is a peptide derived from Thailand cobra venom. It is a detoxified α -toxin, binding to nicotinic acetylcholine receptors at neuromuscular junctions and in neuronal synapses of the brain. Modified cobratoxin was given orally in a double-blind, randomized, crossover (after 3 months) study without significant clinical benefit in the AMN patients (73).

IMPROVED METHODOLOGY FOR EVALUATION OF DISEASE PROGRESSION AND THERAPEUTIC EFFICACY

Considerable efforts have been invested in the last years to develop methods and techniques to permit a more rapid evaluation of therapeutic interventions. Eichler *et al* (19) have shown that MR spectroscopy might be predictive of clinical outcome and may be further substantiated by using additional new imaging approaches, such as diffusion tensor imaging and magnetization transfer (MT) MRI. Hopefully the combination of these imaging techniques, combined with clinical data, will facilitate greatly the assessment of therapeutic interventions in the cerebral forms of X-ALD in children and in adults (20). Quantitative tests of sensory and motor functions (68), together with MT MRI of the cervical spinal cord

(97), should make a more rapid evaluation of the efficacy of potential therapeutics possible also for AMN patients. However, the predictive value of even more sophisticated MRI techniques is limited and there is still an urgent need for a biological markers that can predict disease evolution. The identification of modifier genes will hopefully be of additional predictive value for an early estimation of future clinical manifestation in X-ALD. Thus, on the one hand, the increasing knowledge of the molecular mechanisms underlying the different clinical manifestations of X-ALD has lead to possible novel therapeutic approaches and, on the other hand, has established systems that should allow efficient and rapid evaluation of the efficacy of upcoming novel therapeutic approaches.

HEAD TRAUMA AS RISK FACTOR FOR CONVERSION TO CEREBRAL X-ALD

A report by Raymond and colleagues on five patients (87), as well as other reports (summarized in (87)), and own observations in two cases (P. Aubourg, unpub. obs.) disclose head trauma as one environmental risk factor able to trigger the rapidly progressive inflammatory demyelination in AMN patients. The initiation of demyelination in some of these cases appears at the same area as the original contusion (87, 111, and P. Aubourg, unpub. obs.). Thus, head trauma represents one of several potential environmental factors that can trigger the cerebral phenotype in those X-ALD patients, who are genetically at risk for cALD. Other environmental factors could possibly be viral infections. Thus, in addition to the obligate inherited *ABCD1* mutation and the accumulation of pathogenic lipids, modifier genes will likely add to a predisposition for cerebral inflammatory phenotype that is finally triggered by environmental factors. A more detailed knowledge of the environmental factors and modifier genes involved in X-ALD, not only add to the understanding of the molecular mechanism underlying different manifestations of X-ALD, but might also open additional therapeutic opportunities.

CONCLUDING REMARKS

The adrenal insufficiency can be treated using steroid replacement therapy. For boys or adolescents who show early evidence of inflammatory cerebral demyelination, allogeneic HSCT (78) can provide long-term benefit against the inflammatory demyelination. Autologous HSC gene therapy (14) may likely become a therapeutic option for patients without HLA-matched donors and adult patients with cerebral ALD, given the mortality risk (35%) of allogeneic HSCT with full myeloablation. Attempts are taken for HSCT, as well as for autologous HSC gene therapy, towards treatment of patients with early signs of inflammation with increasing age. Unfortunately, for patients in whom inflammatory demyelination has advanced too far for HSCT, all attempts of immunosuppressive or immunomodulating therapies have not been successful. Lorenzo's oil is certainly not the treatment of choice for the inflammatory form of X-ALD, as it has been shown that it cannot prevent conversion to the inflammatory form even when administered before the onset. Neither for male nor female X-ALD patients with AMN-related symptoms, satisfactory treatment opportunities are available. Whether Lorenzo's oil delays or prevents symptoms in some patients cannot be excluded but is not proven.

Concerning novel therapeutic approaches, antioxidative treatments can possibly be tested in the near future and have a good rationale for being beneficial for male or female AMN patients at any age. HDAC inhibitors like VPA, with the added benefit of reducing the oxidative damage in X-ALD cells, provide more generalized neuroprotective effects that have promising therapeutic potential also for X-ALD. The (possibly fortuitous) induction by several HDAC inhibitors of the redundant gene *ABCD2*, adds to the rationale also for the pharmacological induction of *ABCD2*, more directly targeting specific regulatory promoter elements and critical cell types. The identification of compounds that are able to sufficiently induce *ABCD2* in microglia/macrophages, and the clarification whether or not these compounds have to pass the blood-brain barrier are major research topics. If successful, however, both CALD and AMN might be prevented by such treatment. Neurotrophic factors, such as IGF-1 or NT-3, providing trophic support primarily for oligodendroglial cells and axonal maintenance and, possibly indirectly, for neuronal integrity may be considered for AMN. Continuous delivery of such therapeutic factors can be anticipated to require either gene therapy using viral vectors or intrathecal implantation of encapsulated cells genetically engineered to secrete them.

Thus in conclusion, there is hope that powerful new therapies will be developed in the near future. These therapies are urgently needed for a large number of patients, for whom currently no neuroprotective treatments are available. In particular, as some newborn screening programs have already included X-ALD, more patients will be detected before there is tissue damage and thus the future therapy can entirely focus on prevention of damage.

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