Pharmacological treatment based on gene redundancy: A novel therapeutic approach for X-linked adrenoleukodystrophy

J. Berger, S. Forss-Petter, I. Oezen and I. Weinhofer

Center for Brain Research, Medical University of Vienna, Vienna, Austria

Introduction

X-linked adrenoleukodystrophy (X-ALD; OMIM 300100), the most frequent peroxisomal disorder, presents as a severe neurodegenerative disease with widely varying clinical phenotypes. The biochemical characteristic of X-ALD is the accumulation of very long-chain fatty acids (VLCFA), which should normally be degraded by the peroxisomal β-oxidation pathway, in plasma and tissues of X-ALD patients. It is still a mystery how increased VLCFA levels are connected to the pathophysiology of X-ALD, i.e. the loss of myelin on nerve fibers and the destruction of adrenocortical cells. In 1993, the disease-associated gene *ABCD1* (ALD) was identified by Mosser and coworkers using a positional cloning strategy [1] and surprisingly turned out to encode a peroxisomal ATP-binding cassette (ABC) half transporter, the adrenoleukodystrophy protein (ALDP). Now, eleven years later, the function of this protein still remains to be elucidated and no satisfying cure for the fatal course of X-ALD is available. Next to ALDP, three additional mammalian peroxisomal ABC half transporters have been identified: ALDRP, PMP70 and P70R, which share 66, 35 and 25 % identity, respectively, with ALDP and are encoded by the genes *ABCD2*, *ABCD3* and *ABCD4*, respectively. Thus, ALDR is the protein most closely related to ALDP and based on the high degree of amino acid conservation, hope was raised that ALDP and ALDR would have similar or overlapping functions.

Already at that time, a novel strategy for treatment of X-ALD was suggested based on pharmacological stimulation of the related *ABCD2* gene in disease-relevant cell types, in order to increase the amount of ALDRP to compensate for loss of ALDP in X-ALD patients. The principle of this therapeutic approach and the results obtained so far will be the subject of this chapter.

The principle of pharmacological induction of redundant genes as a therapeutic approach

The observation that no overt phenotype is observed in 40 % of all null mutations in mouse models for human diseases created by targeted gene disruption, has been explained, at least in part, by the presence of genes with overlapping functions [2]. These so-called redundant genes can completely or partially substitute for each other and thus, are candidate targets for a pharmacologic approach to treat genetic diseases based on stimulation of expression of the redundant gene in order to normalize the deficient function.

The principle of pharmacologically inducing the expression of redundant genes has already been attempted in the treatment of several diseases. For instance, different categories of drugs, including cytotoxic agents (5-aza-cytidine and hydroxyurea), hematopoietic growth factors (erythropoietin) and butyrate analogs (including 4-phenylbutyrate, PBA), have been shown to induce production of fetal hemoglobin, which is normally not expressed in adulthood, in patients with sickle-cell disease or β-thalassemia, a situation characterized by deficiency in adult haemoglobin [3,4].

In X-ALD the strategy implied is to induce the non-mutated, functional *ABCD2* gene in order to compensate for *ABCD1* deficiency.

The peroxisomal ABC half-transporters ALDP and ALDR as an example of gene redundancy?

The high degree of amino acid identity between ALDP and ALDRP already suggested functional similarity. Several groups, including ours, were able to demonstrate that overexpression of ABCD2 can correct or ameliorate the pathognomonic accumulation of VLCFA in cultured human X-ALD fibroblasts [7-9]. This demonstration of a functional equivalence of ALDP and ALDRP in terms of VLCFA metabolism allowed the speculation that also *in vivo,* stimulation of *ABCD2* expression would be of benefit for X-ALD pathology. In order to test this hypothesis, Pujol and coworkers recently generated transgenic mice overexpressing ABCD2 in a ubiquitous manner using the β-actin promoter. When this transgene was crossed to the *Abcd1*-deficient mouse background, a complete normalization of VLCFA levels in adrenal gland as well as in peripheral and central nervous system was achieved. This correction of the biochemical phenotype could also be correlated with an improvement of the neurologic AMN-like phenotype found in aged *Abcd1*-deficient mice. Thus, these *in vitro* and *in vivo* findings clearly demonstrate that ALDRP has at least overlapping functions with ALDP.

So why is ALDRP then not able to compensate for ALDP deficiency in X-ALD patients? A probable answer to this question might be found in the different expression patterns of human *ABCD1* and *ABCD2* (Fig. 1). *ABCD1* is ubiquitously expressed at a low level with higher amounts found in tissues belonging to the endocrine system such as adrenal gland and testis. In human brain, the *ABCD1* gene was found to be expressed at a lower level and more homogeneously distributed compared with *ABCD2*. A higher level of ABCD1 mRNA was only observed in the pituitary gland. ABCD2 shows a more restricted expression pattern with high levels in brain gray matter (Fig. 1). Interestingly, in tissues where both genes are found to be expressed at higher levels, a rather distinct expression pattern is observed at the cellular level. A good example for these diverse expression patterns of *ABCD1* and *ABCD2* is the adrenal gland. In this organ, *ABCD1* is mainly expressed in the adrenal cortex whereas *ABCD2* expression is found in the adrenal medulla (Please note that in Fig. 1 mRNA of the total adrenal gland was examinated). In general, tissues associated with X-ALD pathology, like the adrenal cortex and testis, contain relatively high levels of *ABCD1* mRNA, but demonstrate relatively low levels of ABCD2 mRNA.

Thus, these complementary expression patterns of *ABCD1* and *ABCD2* in disease relevant tissues might explain the inability of intrinsic ALDR protein to compensate for ALDP deficiency in X-ALD patients [5,6] and makes it plausible that ALDRP present in the correct cell at the correct time would have the potential to alter the fatal course of X-ALD. So, the strategy of this new therapeutic approach for X-ALD is to stimulate pharmacologi-
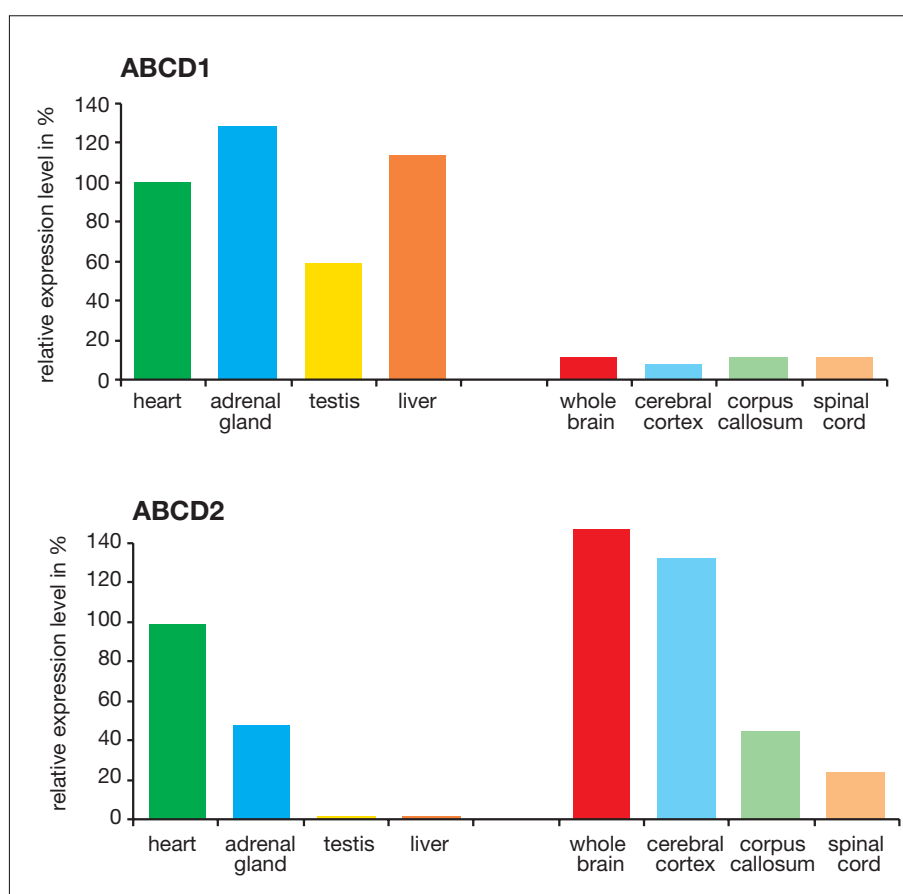

Fig. 1: Gene expression profile of the human ABCD1 and ABCD2 genes.
Comparison of the expression pattern of ABCD1 *and* ABCD2 *in different human tissues. Hybridization of a multiple tissue expression array with ^{32}P-labelled cDNA probes revealed the relative abundance of ABCD1 and ABCD2 mRNAs. The signal intensity of the different tissues was determined by Quantitiy one (Biorad) software and the mRNA abudancy was normalized to the level in heart, which was set to 100 %.*

cally the expression of the related ABCD2 gene in tissues involved in X-ALD pathology, i.e. brain white matter, peripheral nerve, adrenal cortex and testes (Fig. 2).

In summary, these *in vitro* and *in vivo* findings identify *ABCD1* and *ABCD2* as an example of gene redundancy and make *ABCD2* an attractive target for a pharmacological gene therapy approach in X-ALD.

Compounds known to regulate ABCD2 expression

A pharmacological therapy for X-ALD based on induction of the related *ABCD2* gene requires that *in vivo* regulation of *ABCD2* expression is possible. This makes it necessary to characterize the *ABCD2* promoter and to understand its regulation. Several compounds have already been identified to be involved in the transcriptional regulation of the *ABCD2* gene.
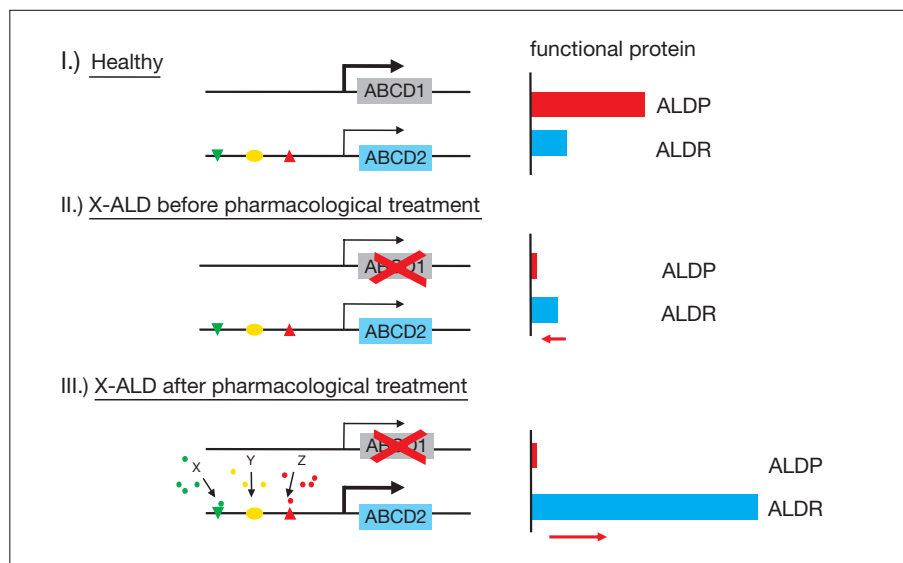

Fig. 2: The principle of pharmacological induction of redundant genes as a therapeutic approach.

ALDR is with 66 % the protein most closely related to ALDP, which is the disease associated protein In X-ALD. Tissues associated with the disease, such as brain white matter, adrenal cortex and testis show high levels of ALDP *but relatively low abundance of* ALDRP. *The new approach comprehends the attempt to pharmacologically stimulate the expression of the ABCD2 gene in tissues involved in the X-ALD pathology to increase the level of ALDR to compensate for functional loss of ALDP.*

a) Cholesterol: sterol regulatory element (SRE)/sterol regulatory element binding protein (SREBP)

ABCD2 expression is induced upon sterol-depletion in cultured human and murine monocytes as well as in human primary fibroblasts [11]. Several genes have been shown to be regulated by cholesterol and each of these sterol sensitive genes was found to contain at least one SRE within the promoter region, through which SREBPs, a family of transcription factors known to control cholesterol and fatty acid synthesis, activate transcription (for a review, see [12]). Indeed, using reporter gene studies, site directed mutagenesis and gel shift assays, a functional SRE could be identified in the human *ABCD2* promoter region, thus providing a closer link between peroxisomes, cholesterol and fatty acid metabolism [11]. This increase in *ABCD2* expression in response to sterol depletion correlated with a normalization of VLCFA levels in human X-ALD fibroblasts, indicating that lowering of cholesterol leads to SREBP maturation, increased ABCD2 expression and reduced VLCFA accumulation [11].

b) 3,5,3'-Tri-iodothyronine (T_3): thyroid hormone responsive element (TRβ)/T_3 receptor β (TRβ), retinoid X receptor (RXR) heterodimer

Computer-based analysis of the human, rat and murine *ABCD2* promoter identified a conserved direct repeat hexameric sequence separated by a 4 nucleotide spacer that fits the thyroid hormone responsive element (TRE) consensus sequence [6,13]. This element was capable of binding a retinoid X receptor α (RXR), T_3 receptor β (TRβ) heterodimer and to mediate T_3 responsiveness of the *ABCD2* gene [14]. Interestingly, the TRE and the SRE are located in close proximity in the *ABCD2* promoter, indeed these two elements overlap by 5 bp (Fig. 3). As TRβ plays an important role in controlling hepatic cholesterol metabolism, a clear link between T_3 and SREBP exists, making the physiological relevance of this finding an interesting target for future research. In cell culture, treatment with T_3 resulted in an induction of *ABCD2* expression in rat oligodendroglial CG4 cells and primary murine *Abcd1*-deficient fibroblasts but not in primary rat astrocytes, suggesting a cell-type specifity of *ABCD2* expression in response to T_3. In murine primary X-ALD fibroblasts, the induction of ABCD2 expression by T_3 treatment correlated with a correction of increased VLCFA levels. *In vivo,* T_3 was found to stimulate *ABCD2* expression in the liver but not in the brain of rats.

c) Peroxisome proliferator receptor α (PPARα) agonists

Dietary treatment of mice or rats with PPARα agonists, e.g. fenofibrate, a hypolipidemic drug used in human medicine, results in a massive induction of hepatic *ABCD2* expression [6,13,15]. This stimulation of murine ABCD2 expression was absent in mice lacking PPARα, a nuclear receptor that activates transcription of genes participating in lipid cata-

bolism including β-oxidation of fatty acids in the peroxisome (for a review, see [16]). PPARα binds to DNA sequences in the promoter region of target genes in the form of a heterodimer, with RXRα as obligate partner. The DNA response elements of these nuclear receptor heterodimers are called peroxisome proliferator response elements (PPREs). Several investigations have failed to demonstrate a functional PPRE or a direct activation of the human or rodent *ABCD2* promoter in a PPARα dependent manner, suggesting that the effect of PPARα-agonists such as fenofibrate on the Abcd2 mRNA level might be indirect.

d) cis-retinoic acid

The human testicular embryonal carcinoma-derived cell line NT2/D1 differentiates along neuroectodermal lineages after exposure to retinoic acid (RA). This RA induced differentiation is characterized by glycolipid changes, induction of homeobox gene clusters and
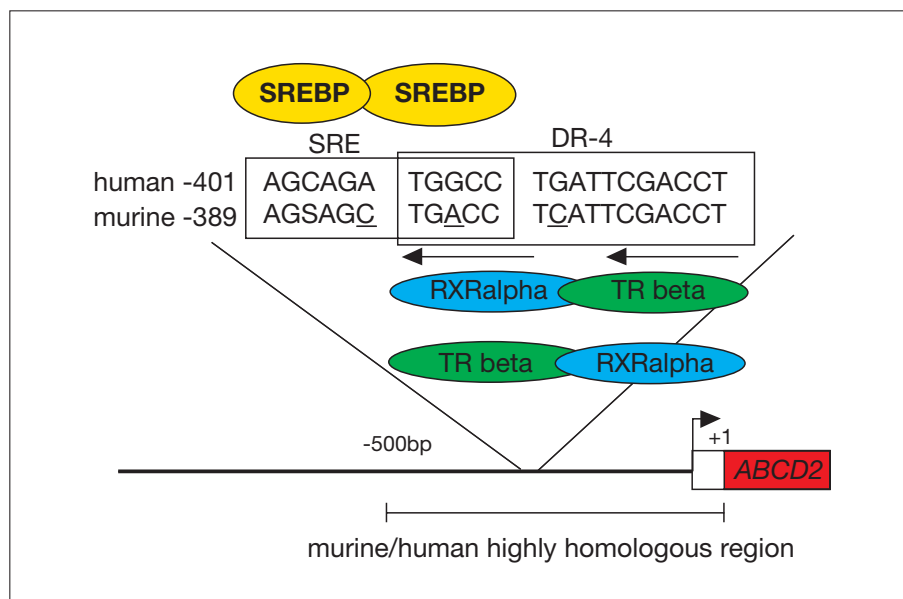

Fig. 3: The SRE and DR-4 motifs of the human and murine ABCD2 promoter. A SRE was identified at nucleotide position -401 to -391 and -389 to -379 with respect to the translation start site of the human and murine ABCD2 genes, respectively. Nucleotides that differ between species are underlined. The SRE sequence overlaps 5 bp with a direct repeat hexameric sequence of the DR-4 type that fits the TRE or LXRE consensus sequence. The DR-4 half-sites are indicated by arrows below the sequence.

appearance of neurons [20,21]. Troffer-Charlier and coworkers demonstrated that in NT2/D1 cells, the expression of the ABCD2 gene was increased after 5-10 day exposure to RA, as measured by RT-PCR analysis [5]. Recently, studies were aimed to investigate whether this effect on ABCD2 gene expression is direct, i. e. due to transactivation of the *ABCD2* promoter by RXRα, or indirect, i. e. due to cell differentiation induced by RA. The results obtained show that either a 1.3 kb human or a 1.0 kb murine promoter fragment of the *ABCD2* gene is sufficient to induce transcription of a reporter gene after cotransfection of COS-7 cells with an expression plasmid encoding RXRα and treatment with cis-RA [22]. These results strongly suggest a direct effect of RXRα on the human and murine *ABCD2* promoter. As RXRα acts as a heterodimer together with other nuclear receptors, a potential dimerization partner could be TRβ.

e) 4-Phenylbutyrate (4-PBA)

The pro-drug 4-PBA is an aromatic fatty acid that has to be converted to sodium phenylacetate by mitochondrial β-oxidation. Among the postulated mechanisms of the effects of 4-PBA on gene expression are alterations in lipid metabolism, regulation of gene expression through DNA hypomethylation and inhibition of histone deacetylase [17-19]. 4-PBA treatment resulted in induction of *ABCD2* expression and peroxisome proliferation in both human and murine X-ALD fibroblasts, which correlated with a correction of VLCFA accumulation. In *Abcd1*-deficient mice, 4-PBA was shown to normalize VLCFA levels in the brain and adrenal gland, however, the effect on *Abcd2* gene expression in these organs was not reported [8]. Moreover, based on cell culture experiments it was speculated that the normalization of VLCFA levels using 4-PBA is an ALDR independent mechanism [26]. Thus, additionally to a direct compensation of the defective ALD protein by the ALDR protein, also ABCD2 independent, currently unknown mechanisms could contribute to a correction of the biochemical defect found in X-ALD. This might open separate opportunities for future treatment strategies but necessarily requires a better understanding of the biochemical function of ALDP and the molecular cause of VLCFA accumulation.

f) Forskolin

Forskolin is a compound that increases the intracellular concentration of the second messenger cyclic AMP (cAMP), which is generated through the enzyme adenylate cyclase as a response to the activation of G protein-coupled receptors. Increased levels of cAMP result in the activation of the protein kinase A (PKA) signaling pathway that has been implicated in a wide range of cellular processes, including transcription, metabolism, cell cycle progression and apoptosis (for a review see [23]). In 1998, Pahan and colleagues showed that substances (forskolin, 8-bromo cAMP and rolipram) that activate PKA by increasing intracellular cAMP levels are able to stimulate peroxisomal β-oxidation of

C24:0 and normalize the accumulation of VLCFA in human X-ALD fibroblasts [24]. Furthermore the investigators demonstrated that the activation of PKA inhibited the fatty acid elongation system. These findings would be in good agreement with recent observations that, at least in tissues of the *Abcd1* deficient mice, the peroxisomal β-oxidation is not defective and to the suggestions that the accumulation of VLCFA rather results from a disturbed elongation of fatty acids; however, also other interpretations are possible as forskolin also stimulates β-oxidation. Recent studies revealed that forskolin is able to stimulate reporter gene expression under the control of 1.3 kb of human or 1.0 kb of murine ABCD2 promoter sequences in human neuroblastoma (IMR32) and adrenocarcinoma NCI-H295 cell lines [22]. The element that mediates the forskolin response on *ABCD2* expression remains to be characterized. Whether the forskolin mediated inhibition of elongation is related to peroxisomes is currently unclear.

Results obtained in preliminary studies involving X-ALD patients and compounds known to stimulate ABCD2 expression

a) 4-PBA

Based on the results obtained in cell culture as well as in X-ALD mice, a preliminary study was conducted to investigate the effect of 4-PBA in seven AMN patients [25]. Patients received a dose of 20 g 4-PBA per day for 6 weeks, then went 2 weeks without and then resumed the medication for another 6 weeks.
Using brain MRI studies, it could be demonstrated that 4-PBA is able to pass the blood-brain barrier, however, measurement of VLCFA levels in plasma and red and white blood cells revealed reduced VLCFA levels only in the latter ones. Analysis of ABCD2 expression in white blood cells from three of these patients showed no difference after 4-PBA administration when compared with untreated controls [25]. A possible explanation for this could be the short half-life (one to two hours) of 4-PBA *in vivo.* As confirmed by long-term studies in ABCD1-deficient mice demonstrating that tachyphylaxis of 4-PBA might be a complicating factor [26]. Thus, several pharmacological agents that are functionally and/or structurally related to 4-PBA are currently being tested for their ability to correct the accumulation of VLCFA in X-ALD fibroblasts [26].

b) PPARα-agonists: clofibrate

The PPARα-agonist clofibrate is a hypolipidemic drug approved for use in human medicine. Treatment of one child with cerebral X-ALD and one asymptomatic child demonstrating ABCD1-deficiency with clofibrate for 12 and 13 months, respectively, resulted in temporarily lowered VLCFA levels [27]. This change appeared to be associated with a reduction in total plasma lipids and there was no alteration in the C26:0/C22:0 or the C24:0/C22:0 ratio, which is typical of X-ALD. In addition, no demonstrable effect on the

clinical status of the patients could be observed. The authors concluded that clofibrate treatment results in redistribution of plasma lipids, but does not have specific effects on plasma VLCFA metabolism.

c) Cholesterol-lowering drugs: lovastatin

It was reported that treatment of human X-ALD fibroblasts with lovastatin, an inhibitor of HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis, resulted in reduced VLCFA accumulation [28]. Our recent finding that ABCD2 expression is induced by SREBP upon cholesterol-depletion provides a molecular link, explaining how cellular cholesterol could affect VLCFA levels [11].

In two small scale clinical trials carried out by Singh and associates, 7 and 12 X-ALD patients, respectively, displaying different phenotypes were treated with lovastatin for up to 12 months. The treatment resulted in successful reduction of total plasma VLCFA and cholesterol levels. Another 3-month trial with the lovastatin-analog simvastatin that involved 6 children with X-ALD could not reproduce a lowering of plasma VLCFA, although plasma cholesterol was successfully lowered [29]. Interestingly, in the two successful studies, lovastatin treatment was accompanied by a low-fat diet [30], whereas in the third study [29] the diet was normal. In conclusion, a multicenter double-blinded placebo-controlled clinical trial would be necessary to evaluate the clinical efficacy of lovastatin on X-ALD pathology.

Conclusion

A functional similarity between ALDP and ALDRP could be established in cell culture studies as well as in the ALDP-deficient mouse model, paving the way to a new therapeutic approach for X-ALD based on a pharmacological stimulation of the ABCD1-related *ABCD2* gene.

A variety of compounds have now been shown to decrease or normalise the amount of VLCFA in fibroblasts of X-ALD patients or in organs of *Abcd1*-deficient mice. It is plausible that this could be achieved through ALDRP by direct compensation of the deficient ALDP transporter as well as through mechanisms acting independently of ALDRP by a more downstream prevention of VLCFA accumulation. We currently do not know whether increased levels of VLCFA are directly connected to inflammation or neuronal degeneration found in X-ALD or whether the accumulation of VLCFA is only a secondary phenomenon useful for diagnosis but not relevant for the pathology of X-ALD. Thus, the highest priority must be given to elucidate ALDP and ALDRP function, also with respect to a pharmacological gene therapy approach for X-ALD based on the direct compensation of *ABCD1*-deficiency by inducing *ABCD2* gene expression.

Currently, stimulation of *ABCD2 in vivo* using pharmacological compounds has only been demonstrated in the liver and adrenal gland, but not in the brain of rodents. Additionally,

species differences in regulation of *ABCD2* gene expression between man and mouse could complicate the direct translation of knowledge from the X-ALD mouse model to X-ALD patients. Thus, it remains to be determined whether induction of *ABCD2* expression in liver or adrenal gland provides more clinical benefit for X-ALD patients than dietary treatment with „Lorenzo's Oil", a 4:1 mixture of glyceryl trioleate and glyceryl trierucate that compete with saturated fatty acids for the microsomal fatty acid elongation system resulting in normalization of VLCFA accumulation in the plasma of X-ALD patients.

Recent findings by Baes and coworkers demonstrate that liver-specific rescue of peroxisome biogenesis in Pex5-deficient mice, an established model system for the Zellweger syndrome in which peroxisomes are absent, resulted in significant correction of the characteristic neuronal migration defect found in this disorder [31]. This clearly indicates that an interplay between liver and brain tissues exists, that might be also of importance for X-ALD pathology and devolpment of possible treatment strategies.

In conclusion, there is still a long way to go and even after elucidating the function of the peroxisomal ABC-transporters ALDP and ALDRP as well as identification of pharmacological compounds able to stimulate *ABCD2* gene expression in disease relevant tissues and cell types in the future, it still has to be clarified whether a stimulation of *ABCD2* in target tissues that would normally not express this ABC transporter could have a negative effect on whole body metabolism. Additionally it needs to determined whether other genes that might be co-induced by these pharmacological compounds could have harmful effects on the patients. These issues will be adequately explored in the near future in order to find an effective treatment for patients with the severe disorder X-ALD.

Acknowledgements

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ADDRESS FOR CORRESPONDENCE

Prof. Dr. Johannes Berger
Center for Brain Research
Medical University of Vienna
Spitalgasse 4
A-1090 Vienna
Austria
Tel.: +43-1-4277-62812
Fax: +43-1-4277-9628
E-Mail: johannes.berger@meduniwien.ac.at