The brain penetrant PPARγ agonist Leriglitazone restores multiple altered pathways in models of X-linked adrenoleukodystrophy

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One Sentence Summary:

Leriglitazone reduces pathologically activated microglia and protects neurons, astrocytes and oligodendrocytes in experimental models of X-ALD.

Abstract:

X-linked adrenoleukodystrophy (X-ALD), a potentially fatal neurometabolic disorder with no effective pharmacological treatment, is characterized by clinical manifestations ranging from progressive spinal cord axonopathy (adrenomyeloneuropathy-AMN) to severe demyelination and neuroinflammation (cerebral ALD-cALD), for which molecular mechanisms are not well known. MIN-102 (INN: leriglitazone) is a recently developed brain penetrant full PPARγ agonist which could modulate multiple biological pathways relevant for neuroinflammatory and neurodegenerative diseases, and particularly for X-ALD. We found that leriglitazone decreased oxidative stress, increased ATP concentration and exerted neuroprotective effects in primary rodent neurons and astrocytes after very long-chain fatty acids-induced toxicity simulating X-ALD. In addition, leriglitazone improved motor function, restored markers of oxidative stress, mitochondrial function and inflammation in spinal cord tissues from AMN mouse models and decreased the neurological disability in the EAE neuroinflammatory mouse model. X-ALD monocyte-derived patient macrophages treated with leriglitazone were less skewed towards an inflammatory phenotype and the adhesion of human X-ALD monocytes to brain endothelial cells decreased after treatment, suggesting the potential of leriglitazone to prevent the progression to pathologically disrupted Blood-Brain Barrier. Leriglitazone increased myelin debris clearance in vitro and increased myelination and oligodendrocyte survival in demyelination-remyelination in vivo models, thus promoting remyelination. Finally, leriglitazone was clinically tested in a Phase 1 study showing CNS target engagement (adiponectin increase) and changes on inflammatory biomarkers in plasma and CSF.

The results of our study support the use of leriglitazone in X-ALD and more generally in other neuroinflammatory and neurodegenerative conditions.

Introduction

X-linked adrenoleukodystrophy (X-ALD, ALD; OMIM 300100) is a rare inherited neurodegenerative disorder that affects the nervous system and the adrenal glands primarily in males. It is the most frequent peroxisomal disorder with an estimated incidence of 1:14,700 hemizygous men and heterozygous women *(1–3)*. The disease is due to mutations in the *ABCD1* gene that result in loss of function of the encoded ALD protein (ALDP, ABCD1), an ATP-binding cassette (ABC) transporter located in the peroxisomal membrane that shuttles very long-chain fatty acids (VLCFA) into the peroxisomes for degradation. Thus, ALDP deficiency results in the accumulation of VLCFA in plasma and tissues, particularly in the brain, peripheral nerves and adrenal glands *(4–7)*.

X-ALD presents with two main phenotypes: adrenomyeloneuropathy (AMN) and cerebral ALD (cALD). AMN is the chronic manifestation of X-ALD and is characterized by slowly progressive adult-onset spinal cord axonopathy with associated demyelination leading to spastic paraparesis *(8)*. Mitochondrial dysfunction, oxidative stress and bioenergetics failure play major roles in the pathogenesis of X-ALD, whereas only a limited inflammatory component is present in AMN.

Approximately 60% of male patients with X-ALD will develop cerebral demyelination and neuroinflammation over a lifetime *(9)*, either in childhood or during adulthood. cALD exhibits a fast and severe progressive cerebral demyelination with disruption of the Blood-Brain Barrier (BBB) and subsequent infiltration of immune cells, mainly monocytes/macrophages and CD8+ T cells, into the brain. Brain inflammatory demyelination results in severe cognitive and neurologic disability leading to a vegetative state within two to five years from onset of clinical symptoms and death *(10)*. Recent studies have shown that a pro-inflammatory state with increased BBB permeability to monocytes precedes overt demyelination. Inflammatory skewing was observed in activated monocyte-derived macrophages from patients with X-ALD *(11–13)* and it has been shown that perilesional ABCD1-deficient astrocytes are abnormally activated likely contributing to BBB alteration and lesion propagation *(14)*. Moreover, selective brain endothelial dysfunction is characterized by activation, loosening of tight junctions and upregulation of adhesion molecules *(11)* resulting in increased adherence and migration of monocytes across the BBB *(15)*. However, ABCD1 deficiency alone does not explain the development of cALD *(16)*. The progression to cALD is caused by the inability to appropriately resolve the inflammatory reaction to ensuing insults by the brain immune system, and can be facilitated by unknown genetic background or external factors such as head trauma or infection *(17, 18)*. Microglial activation and apoptosis have been observed in peri-lesional white matter of cALD patients and could represent an appropriate target for intervention when the first signs of demyelination are detected by brain Magnetic Resonance Imaging (MRI) in patients with X-ALD *(19)*.

To date no effective pharmacological treatments are available to patients with X-ALD. Hematopoietic Stem Cell Transplantation (HSCT) or experimental gene therapy *(20)* can be used to arrest the cerebral form of X-ALD by counteracting activated microglia with differentiated new macrophages/microglia from hematopoietic stem cells. However, delay in diagnosis, lack of appropriate donors and adverse events associated with transplantation make HSCT only available and effective to a minority of patients with cALD *(21)*. In addition, HSCT does not avoid the progression to AMN in a later stage. The advent of the newborn screening for X-ALD *(1)* will offer the opportunity to treat children and adults earlier and in the pre-symptomatic stage. The emerging therapies currently under development aim to target specific altered pathways in X-ALD *(3)*, however a therapy targeting multiple aspects of the pathological cascade could be more effective in halting the complex pathophysiology of X-ALD in its several manifestations and could be more capable of interfering with the progression to the most severe inflammatory cerebral form.

Peroxisome proliferator-activated receptor gamma (PPARγ) agonists act simultaneously on multiple pathways through gene activation or repression and have shown the capacity to induce neuroprotective and restorative effects in several preclinical models of neurodegenerative diseases *(22)* such as Amyotrophic lateral sclerosis *(23)*, Parkinson's disease*(24)*, Friedreich's ataxia *(25)*, Alzheimer's disease *(26)* and AMN *(27)*. PPARγ agonists modulate key genes that counteract oxidative stress, stimulate mitochondrial biogenesis *(28, 29)* and decrease inflammation *(30)* through repressing NF-kB pathway*(31)*. PPARγ engagement can be monitored by measuring adiponectin concentration *(32)*, which is tightly regulated by PPARγ *(33)*.

PPARγ agonists have been tested in clinical trials in several neurodegenerative diseases *(34, 35)* without achieving a clear positive effect *(34–36)*. The lack of efficacy may have resulted from insufficient target exposure in the central nervous system (CNS). Pioglitazone was only effective in relapsing-remitting multiple sclerosis, where the BBB is known to be disrupted *(37)*.

Leriglitazone (5-[[4-[2-[5-(1-hydroxyethyl)pyridin-2-yl]ethoxy]phenyl]methyl]-1,3-thiazolidine-2,4-dione hydrochloride), also known as MIN-102 (Fig. S1A) is the hydrochloride salt of the active metabolite M4 (M-IV) of pioglitazone (Actos, Takeda). Leriglitazone is a PPARγ agonist being developed by Minoryx Therapeutics S.L. for the treatment of X-ALD and of other neurodegenerative diseases due to its adequate BBB penetration, good bioavailability and safety profile.

To gain insights into the potential utility of leriglitazone in X-ALD, and in neurodegenerative diseases in general, we performed several in vitro studies in rodent primary neurons, astrocytes, endothelial cells, oligodendrocytes and microglia. Leriglitazone efficacy in treating X-ALD was further validated in vivo in murine AMN models and in Experimental Autoimmune Encephalomyelitis (EAE) mice, a surrogate model for the neuroinflammatory component of cALD. In order to better understand the mode of action of leriglitazone in potentially preventing early stages of the development of cALD, we used an in vitro model of the BBB, where the brain endothelial permeability was also measured. Additionally, the effect of leriglitazone on the inflammatory component of monocytes/macrophages from patients with X-ALD was measured. Finally, a Phase I pharmacodynamic/pharmacokinetic study in healthy volunteers was completed to confirm inflammatory biomarker changes and target engagement in plasma and CSF in human at concentrations corresponding to preclinical efficacious doses.

Results

1. PPARγ agonism and brain penetration of leriglitazone

The chemical structure of leriglitazone is shown in Fig. S1A. We tested the PPARγ agonist activity of leriglitazone in a transactivation assay and the resulting EC_{50} was 9 μM; leriglitazone did not show PPARα or δ agonist activity (Fig. S1B). Leriglitazone was soluble (up to 300 μM in a sodium phosphate buffer 50mM, pH 7.4), being higher than the parent compound pioglitazone (solubility up to 10 μM) (Table S1).

Leriglitazone showed a good pharmacokinetic profile with a very high bioavailability in mice, rats and dogs (85, 86 and 94%, respectively; Table S2) with a 50% increase in the brain/plasma exposure ratio compared to pioglitazone in mice (Table S3).

The unbound fraction of leriglitazone in plasma varied from 3.6% in humans to 5.1% in rats and 34.6% in mice. In contrast pioglitazone showed <1% plasma free fraction in all species. The unbound fraction of leriglitazone in brain also increased to 9.1 and 13.6%, compared to 1.6 and 1.2% of pioglitazone, in mice and rats, respectively (Table S4).

2. Protective effects of leriglitazone from VLCFA induced toxicity in an in vitro model simulating X-ALD

The main clinical biochemical feature of X-ALD is the highly increased concentration of saturated VLCFA (24 or more carbon atoms, mainly hexacosanoic acid C26:0), both circulating and in different organs *(38)*, particularly in the tissues affected by the pathology, like brain white matter, adrenal cortex and testes *(7)*. Thus, rat neural cultures treated with VLCFA could be used to test the potential neuroprotective effects of leriglitazone in ALD. We first assessed whether leriglitazone reduced VLCFA toxicity in cocultures of spinal cord motor neurons and astrocytes from wild-type rat embryos exposed to C26:0. Immunostaining for neuronal and astrocytic

markers, MAP-2 and GFAP, revealed that C26:0 at 40 μM induced cell death of both motor neurons and astrocytes in agreement with previous findings *(39)* and protected them in a dose-dependent manner (Fig. 1A-C). In addition, mouse neural cultures from *Abcd1* Knockout (*Abcd1* KO) mice confirmed neuroprotection of leriglitazone under VLCFA induced toxicity (Fig. 1D) and the reliability of using wild-type rat cultures treated with VLCFA as a model for X-ALD. In the same experimental paradigm, we observed that leriglitazone protected neuronal mitochondria, as measured by increased concentration of ATP and decreased oxidative stress assessed by immunofluorescence staining with reduction of Methionine sulfoxide reductase and Reactive Oxygen Species (ROS) production (Fig. 1E). Reduced NF-kB pathway activation was indicated by Simple Western Blot immunoassays of IkBα, which showed increased IkBα protein amount after leriglitazone treatment (Fig. 1F). Furthermore, we assessed the concentration of IL1-β, a downstream target of NF-κB, released into the medium and found that leriglitazone countered the inflammatory response by strongly reducing IL1-β (Fig. 1G).

3. Protective effects of leriglitazone in mouse models of AMN

Leriglitazone was studied in both validated *Abcd1* KO *(40)* and *Abcd1/Abcd2* double KO (DKO) mouse models *(41, 42)*. In the *Abcd1* KO mice, treated orally twice daily for seven days with 17, 50 and 125 mg/kg/day leriglitazone, plasma concentrations increased dose dependently (Table S5) and produced a dose related PPARγ engagement, as measured by dose dependent changes in adiponectin concentration in plasma (Fig. S2). Leriglitazone at the tested dose of 50 mg/kg significantly increased the transcription of *Nrf1* and *Sod2,* related to mitochondrial function and oxidative stress respectively (Fig. 2A; $P < 0.001$ and $P < 0.0001$) , and reduced the expression of the pro-inflammatory cytokines genes *Il-1β* and *Tnf-α* (Fig. 2A; $P < 0.001$ and $P < 0.05$) in the spinal cord.

Next, we investigated the inflammatory biomarker changes in the spinal cord in a pilot study showing that 4-month-treatment of *Abcd1/Abcd2* DKO mice with 75 mg/kg/day leriglitazone reverted the increased concentration of Monocyte chemoattractant protein-1 (MCP-1), Eotaxin-1, Stem cell factor (SCF) and Interleukin-1α (IL-1α) (Fig. 2B) and increased the expression of mitochondrial biogenesis (*Pgc1α, Nrf1*) and target engagement (*Fabp4*, *Pparγ*) markers (Fig. S3). VLCFA C26:0-lysoPC accumulation and the C26:0/C22:0 ratio, both known to be elevated in X-ALD *(43)*, were measured in the spinal cord from *Abcd1/Abcd2* DKO mice after the 4 months treatment. In leriglitazone-treated mice the mean ratio C26:0/C22:0 was decreased by 13% and the amount of C26:0-lysoPC- by 30% (Fig. S4). In *the Abcd1/Abcd2* DKO mice, 6-month-treatment with three doses of leriglitazone administered in the feed dose-dependently improved the motor dysfunction changes from baseline (14 months) in the balance beam and rotarod test (Fig. 2C). Amyloid precursor protein (APP), which marks axonal swelling as a sign of axonal degeneration, and MAC-3, a marker of activated microglia were analyzed by immunohistochemistry in spinal cords. At the highest dose, leriglitazone significantly reduced axonal degeneration (Fig. 2D; $P < 0.05$) and decreased microglia activation (Fig. 2D; $P < 0.01$) in the spinal cord white matter of these DKO mice.

4. Brain penetration and protective effects of leriglitazone in models of blood brain barrier mimicking X-ALD conditions

A triple cell co-culture transwell model with human brain microvascular endothelial cells (HBMEC) transfected with *ABCD1* siRNA or without *ABCD1* siRNA (NT siRNA) in the luminal

part (top), astrocytes in the underneath layer and neurons with microglia in the bottom (abluminal compartment) was used to reproduce the BBB in X-ALD (Fig. 3A). Conditions mimicking cALD were induced by challenging the brain endothelium/astrocytes with lipopolysaccharide (LPS) as an inflammatory stimulus and C26:0 in the abluminal compartment. Paracellular permeability of sodium fluorescein (Na-F), was measured at the luminal part and was only mildly altered by 17% in silencing conditions after C26:0 and LPS addition (Fig. 3B).

VLCFA (C26:0) and LPS significantly caused more toxicity to neurons and astrocytes and increased microglia activation in the *ABCD1* silenced (+siABCD1) than in the non-silenced (-siABCD1) endothelium model (Fig. 3C-G:+). Moreover, in the *ABCD1*-silenced model (+siABCD1), the presence of an additional inflammatory stimulus (C26:0+LPS) had a larger induced toxicity compared to single C26:0 treatment (&). Leriglitazone showed protective effects either by increasing neuronal survival (Fig. 3C), neurite outgrowth (Fig. 3D) and astrocyte survival (Fig. 3E) or by decreasing microglia activation (Fig. 3F), both in the presence of C26:0 and C26:0+LPS. At 24 hours supernatants from the abluminal compartment were analyzed confirming that leriglitazone had reached the neuronal culture in both silencing and non-silencing conditions (Table S6).

5. Anti-inflammatory effects of leriglitazone in monocytes/macrophages derived from patients with X-ALD

Monocyte-derived macrophages from patients with X-ALD were used to determine whether leriglitazone was able to prevent or halt inflammatory skewing of X-ALD macrophages. This study confirmed that upon pro-inflammatory LPS activation, X-ALD monocyte-derived macrophages had significantly higher amount of TNF-α mRNA compared to healthy macrophages (Fig. 4A; $P < 0.01$) and showed that leriglitazone significantly decreased TNF-α expression in X-ALD, but not in healthy volunteer monocyte-derived macrophages (Fig. 4B; $P < 0.01$). The anti-inflammatory action of leriglitazone was further confirmed in human monocytic THP-1 cells, where leriglitazone dose-dependently decreased TNF-α release upon LPS-stimulation (Fig. S5).

Adhesion of monocytes to endothelial cells could represent a first crucial step in the development of cALD. Leriglitazone attenuated ABCD1 related-brain endothelial dysfunction by decreasing adhesion of THP-1 cells to *ABCD1*-silenced HBMECs (Fig. 4C). These results were replicated with human peripheral blood mononuclear cells (PBMCs): the increased adhesion of fresh control PBMCs to HBMECs following *ABCD1*-silencing was normalized by the treatment of PBMCs with leriglitazone for 2 hours (Fig. 4D).

6. Protective effects of leriglitazone against demyelination and/or enhancement of remyelination in vitro and in vivo

Microglia play a crucial role for effective myelin debris clearance and oligodendrocyte precursor cells (OPC) activation *(44)*, two processes necessary for remyelination to occur. Thus, we investigated the effect of leriglitazone on the capacity of primary mouse microglia to phagocytose myelin debris labelled with pHrodo. Leriglitazone increased phagocytosis of myelin debris with a more sustained higher percentage of microglia performing phagocytosis under control and inflammatory conditions (LPS treatment) (Fig. 5A), possibly promoting remyelination after the necessary clearance of myelin debris. cALD is characterized by very rapid progression of demyelinating lesions in the brain that may result from abnormalities in the capacity of oligodendrocytes to correctly differentiate and remyelinate. Consequently, we tested whether leriglitazone could promote the survival of OPC and mature oligodendrocytes after exposure to

C26:0 and found that leriglitazone protected OPC and mature oligodendrocytes at the last two highest tested doses (Fig. 5 B).

To confirm the potential of leriglitazone in preventing demyelination and/or enhancing remyelination we used an in vivo cuprizone demyelination mouse model *(45)*. In this study demyelination was achieved after 5 weeks of dietary treatment with 0.2% of cuprizone. Leriglitazone treatment at an equivalent dose of 110mg/kg/day continued until cuprizone treatment ended. Leriglitazone significantly decreased myelin (MBP+) degeneration associated with cuprizone (Fig. 5C; $P < 0.05$) and the oligodendrocyte (Olig2+) population was protected in the leriglitazone treated group (Fig.5C; $P < 0.05$). A second cohort was analyzed after 9-week of intragastric treatment with 75mg/kg/day of leriglitazone (6 weeks with 0.3% cuprizone treatment - plus 3 weeks without cuprizone). At the end of the study the proportion of myelinated axons measured by Electron Microscopy (EM) imaging was higher in the leriglitazone treated group (Fig. 5D; $P < 0.05$).

7. Improvement of the clinical score in the EAE mouse model of neuroinflammation by leriglitazone

The preclinical EAE model for T-cell driven neuroinflammation is a highly reproducible and well-established model used in Multiple Sclerosis (MS) research *(46)*. The development of the EAE disease was analyzed daily by scoring clinical symptoms of mice *(47)* until the end of the study. Treatment with leriglitazone at three different doses (17, 50, 125 mg/kg/day) started at day 5 after immunization. Disease progression was reduced from day 12 after immunization until day 20 for the three doses of leriglitazone compared to the untreated vehicle group (Fig. 5E).

8. Target engagement and biomarker changes induced by leriglitazone in animals and humans

We first verified that the dosing in the *Abcd1* KO mice orally produced a dose-dependent increase in the circulating plasma concentration of leriglitazone (Table S5). Leriglitazone increased plasma adiponectin concentration at the efficacy dose of 50 mg/kg to around 200%, while pioglitazone at the equivalent efficacious dose of 9 mg/kg elevated adiponectin to around 150% (Fig S2).

C_{max} (maximum concentration) and exposure (area under the concentration vs time curve: AUC) increased dose proportionally in plasma after multiple doses of leriglitazone in healthy volunteers. During the MAD (multiple ascending doses) part of the study, C_{max} on the 8^{th} day of administration was 9,630 ng/ml and 17,271 ng/ml and AUC was 147,599 and 296,725 ng*h/ml for doses of 135 and 270 mg respectively. CSF concentrations of leriglitazone on day 8 were 189.7 ng/mL (509nM) and 334 ng/mL (898nM) 4 hours after the last dose of 135 mg and 270 mg, respectively (Table S6).
Even though the volunteers were healthy and inflammatory biomarkers were in the normal range prior to administration of leriglitazone, decreases from baseline (not treated) were observed at the time of highest exposure to leriglitazone (C_{max}) on day 8 for both plasma (Fig. 6A) and CSF (Fig. 6B) in IL-8 (plasma: P=0.1; CSF: P=0.2) ,CXCL10-IP10 (plasma: P<0.05; CSF: P=0.3), IL-6 (plasma: P<0.05, CSF: P=0.2) and MCP-1 (plasma:P=0.06; CSF: P=0.06).

Adiponectin plasma concentration was positively correlated with leriglitazone concentrations (Fig. 6C) confirming PPAR-γ engagement of leriglitazone in humans. Mean plasma concentrations of adiponectin showed a clear increase from baseline after dosing for 8 days with 135 mg and 270

mg leriglitazone respectively and significantly correlated with leriglitazone concentration at Cmax (Fig. 6C; $P<0.0001$). The efficacious exposure range in the preclinical studies, increased adiponectin from 2 to -7-fold (Fig. S2).
Considering all the in vivo results the minimum efficacious dose (MED) was established at 50 mg/kg/day. However, in the DKO and cuprizone models, as the 50 mg/kg/day dose was not tested, the lowest dose showing efficacy was 75 mg/kg/day. The corresponding plasmatic exposures for these doses were estimated to be 109-134 μg·h/ml and 212 μg·h/ml for 50 mg/kg/day and 75 mg/kg/day, respectively. This estimation was based on information from previous PK studies in mice which indicated a good correlation between dose and exposure.

Discussion

Leriglitazone is the main metabolite of pioglitazone and in humans it represents 50-60% of the antidiabetic effect of pioglitazone. Leriglitazone has been characterized as a drug in preclinical species and has been administered to humans at doses that reach sufficient concentrations in the brain to activate the receptor PPARγ. Leriglitazone displays lower PPARγ transactivation than pioglitazone, however it stabilizes the activation function-2 (AF-2) co-activator binding surface of PPARγ and enhances co-activator binding, affording slightly better transcriptional efficacy than pioglitazone *(48)*. Importantly, we found that leriglitazone has superior in vivo brain penetration over pioglitazone (+50%) as well as a higher free fraction in both murine plasma and brain homogenates. Moreover, it shows higher solubility than pioglitazone and it maintains a dose proportionality in AUC and C_{max} at high doses, unlike what is suggested to occur with pioglitazone in patients with type 2 diabetes *(49)*.

The neuroprotective effects of leriglitazone were first studied in primary spinal cord neural cells injured by C26:0, a model that mimics VLCFA toxicity in X-ALD cells *(50)*. The relevance of this in vitro model was further validated assessing the protection in spinal cord motor neuronal cultures from *Abcd1* KO treated with leriglitazone. Remarkably, leriglitazone prevented motor neuron and astrocyte loss, decreased oxidative stress and restored ATP concentration. Furthermore, leriglitazone treatment repressed NF-κB pathway activation through increasing the amount of IKBα, known to inhibit NF-κB by masking the nuclear localization signals, and IL-1β release (Fig. S6A).

Abcd1 KO mice reproduce the VLCFA accumulation characteristic of the human disease *(40)* and the failure in mitochondrial function in the spinal cord of AMN *(51)*. The *Abcd1/Abcd2* DKO additionally lack the ABCD2 transporter, the closest homolog that can partially compensate for the ABCD1 deficiency, and develop an earlier onset of motor impairment than the *Abcd1* KO mice (at 14 vs 20 months) *(52–55)*. This model is more appropriate to study the effects of a drug in ameliorating motor dysfunction. Treatment with pioglitazone showed to halt locomotor disability and axonal damage in the *Abcd1/Abcd2* DKO mice *(27)*. The biomarker analysis in the spinal cord of *Abcd1* KO mice revealed that treatment with leriglitazone restored the expression of genes involved in mitochondria biogenesis, oxidative stress and inflammation. Moreover, microglia activation and axonal degeneration were reduced in the spinal cord white matter of the DKO mice together with a decrease in the ratio C26:0/C22:0 and the amount of C26:0-LysoPC, which was

also reported for pioglitazone *(27)*. Leriglitazone showed dose-dependent efficacy in correcting the motor dysfunction during the disease progression assessed by two different tests, the rotarod and the balance beam, in the *Abcd1/Abcd2* DKO mice. These results provide support that leriglitazone can cross the intact BBB and can reach and protect spinal cord neurons and prevent axonal degeneration and provide the mechanistic basis for preventing motor dysfunction (Fig. S6A). However, although mouse lines with targeted knockout of *Abcd1* and *Abcd1/Abcd2* have provided good models to investigate the pathogenesis of AMN they do not develop the cerebral phenotype of X-ALD *(41, 52–55)*. The EAE model is widely used in MS research and given the similarity of the neuroinflammation component of cALD and certain aspects of MS, it is proposed as a surrogate model for cALD. In this model, leriglitazone improved motor dysfunction when treatment started at 5 days post immunization, before disruption of the BBB occurs *(56)*. Moreover, leriglitazone delayed the onset and alleviated clinical symptoms starting from the lowest dose tested.

Human ABCD1 deficiency leads to an impaired plasticity of X-ALD macrophages and incomplete establishment of anti-inflammatory responses *(13)*. In that regard, leriglitazone reversed the proinflammatory status in monocyte-derived macrophages from AMN patients. In X-ALD, it seems plausible that the development of the severe cerebral phenotype may be enhanced by an immune response that target oligodendrocytes and abnormal myelin with excess of VLCFA, thus resulting in demyelination, reactive gliosis *(57)* and impaired oligodendrocyte differentiation and aberrant immune activation in patients with X-ALD *(58)*. Although oligodendrocytes and axons are the evident targets in cerebral X-ALD, the loss of microglia *(19)* and/or abnormal microglia function may impair the ability to provide neuroprotective factors to deficient oligodendrocytes *(58)*. Injury to oligodendrocytes may be enhanced via an inflammatory response that follows tissue injury and plays an important role by initiating and accelerating the progression of the disease. In that sense, leriglitazone protected oligodendrocytes in vitro after VLCFA injury and increased the phagocytosis of myelin debris by microglia. Moreover, in the in vivo cuprizone demyelination model leriglitazone increased myelination and protected oligodendrocytes. By EM analysis, a more sensitive technique to assess the number of myelinated axons, a higher proportion of myelinated axons was observed in treated animals after three weeks of remyelination. Interestingly, myelination was increased at a dose aligned with the one used in efficacy studies in humans. Altogether, these results suggest a distinctive role of leriglitazone in preventing demyelination and/or promoting remyelination after the necessary clearance of myelin debris (Fig. S6).

ABCD1 mutation is necessary but not sufficient to develop cALD implying that other factors ('hits') modulate the conversion to this phenotype *(1)*. BBB disruption with migration of leukocytes to the brain, predominantly macrophages *(59)*, as indicated by a rim of contrast enhancement on MRI *(60, 61)* and ex-vivo histopathology *(19, 62)*, has for a long time been suggested as crucial to identify disease progression in cALD *(63)*. Moreover, post-mortem brain specimens from patients with cALD display proliferation of white matter microvessels, increased permeability to monocytes *(62)* and altered endothelial tight-junction proteins, adhesion molecules and metalloproteinase expression *(11)*. Thus, increased BBB permeability is a critical element for the transition from AMN to cALD. Disease progression in cALD may be a combination of different "hits" including changes in adhesion molecules and tight junctions in brain endothelium promoting increase in BBB permeability, possibly together with VLCFA accumulation which also causes toxicity in the brain *(50, 64, 65)* and subsequent brain inflammation with macrophage infiltration. We found that leriglitazone reduced monocyte-endothelial cell adhesion in *ABCD1*-

silenced HBMECs exposed to VLCFA and activated with TNF-α. In line with this, it has been shown that endothelial cell dysfunction in X-ALD is associated with activation of NF-kB signalling, a pathway highly regulated by the sirtuin SIRT1 *(66, 67)* and that activation of SIRT1 could have therapeutical potential for peroxisomal disorders *(66, 68)*. In a model mimicking the BBB we found that permeability was not substantially altered with intact ABCD1 expression and leriglitazone was able to penetrate and protected cortical neurons, astrocytes, and decreased microglia activation upon exposure to C26:0 and LPS inflammatory stimulus. On the other hand, when ABCD1 expression was silenced in HBMECs, barrier function was mildly impaired after treatment with VLCFA in the presence of an inflammatory stimulus, which resulted in more leriglitazone reaching the abluminal compartment. This higher net concentration was accompanied by a higher efficacy in preventing neuronal and astrocyte loss and in decreasing microglia activation (Fig. S6B) .

Detectable quantities of leriglitazone and adiponectin in brain and spinal cord of animals and in human CSF confirmed the ability of leriglitazone to penetrate the brain and engage the PPARγ target in the CNS independently of the integrity status of the BBB. The free brain concentrations of leriglitazone reached in animals at the efficacious doses were similar to those obtained in vitro in glial and neuron experiments (50-500 nM). Similar concentrations were reached in the CSF of humans after oral administration. Target CSF concentrations have been used to guide dose selection for Phase 2/3 clinical trials. PPARγ engagement in vivo was confirmed in leriglitazone-treated *Abcd1* KO mice, where adiponectin concentration correlated with leriglitazone plasma concentrations and increased between 100 and 600%. The Phase 1 study in healthy volunteers further confirmed the dose- and concentration-dependent increases of plasma adiponectin with a 200% increase at 135 mg and a 450% increase at 270 mg. Pioglitazone at the highest approved dose of 45 mg/day only induced an approximate 80% increase in plasma adiponectin after 4 months of treatment*(32)*, therefore, it is not possible to achieve sufficient target engagement in the brain within the recommended dose range of pioglitazone.

Furthermore, leriglitazone decreased proinflammatory cytokine concentration in human CSF and according to our data, probably through repressing NFkB activation. These promising results on the effects of leriglitazone on decreasing pro-inflammatory biomarkers in plasma and CSF in a human Phase 1 study warrants its further evaluation in patients. The superior profile of leriglitazone compared to pioglitazone and other known PPARγ agonists and the effects on decreasing neuroinflammation and demyelination, characteristic of cALD, opens the possibility to treat both phenotypes of X-ALD, AMN and cALD (Fig. S6). In fact, a phase 2/3 study is currently ongoing to test the safety and efficacy of leriglitazone on patients with AMN. In the case of cALD, a separate phase 2 study is ongoing treating patients with cALD before they require HSCT with the aim to arrest or slow down disease progression and avoid or delay transplantation. The results from the phase 2/3 trial will also elucidate whether leriglitazone could stop disease progression by preventing or delaying the conversion of AMN into cALD.

Nevertheless, there are some limitations in our study either due to difficulties to get material or non availability of the model that could be addressed in future mechanistic studies, although not crucial for leriglitazone development. First, the use VCLFA treatment as a surrogate model for X-ALD instead of silencing Abcd1 in all cell types or by using primary cells from single *Abcd1* or DKO *Abcd1*/*Abcd2* mouse in all the in vitro experiments could mask some differences inherent to Abcd1 mutation. Secondy, in the adhesion studies, we did not measure an extended profile of expression of tight junctions and adhesion molecules that could potentially be modified by

leriglitazone treatment. X-ALD mouse models mimic some features of the disease but they do not reproduce entirely the disease progression and the neuroinflammatory component of cALD.;the EAE model is a good surrogate model to study the neuroinflammatory component of cALD but is not disease specific. Remyelination capacity of leriglitazone could be further explored in future studies following a temporal profile of the expression of specific markers for X-ALD OPC and mature oligodedroctyes. Finally, it would have been desirable to have a larger sample size to increase the statistical power when analyzing the effects of leriglitazone on healthy volunteers, although testing samples from patients with X-ALD would be more relevant.
In summary, these encouraging results obtained in in vitro and in vivo pre-clinical and healthy volunteers studies suggest that leriglitazone has an improved profile for treating X-ALD, compared to other drugs acting on the same target, including pioglitazone. Alhough PPARγ activation has already been proposed as a treatment for AMN, we are adding here new mechanistic evidence that this approach could be beneficial for all X-ALD clinical manifestations, including cALD. Moreover, although the etiology of X-ALD differs from other neurodegenerative diseases, there are similarities in the pathophysiology of these diseases such as neuronal loss, axonal damage, oxidative stress and mitochondrial dysfunction, which can be exacerbated by neuroinflammation in a vicious cycle involving microglia activation and disruption of the BBB. Hence, leriglitazone treatment might be extended to a broader range of neurodegenerative diseases with a high unmet medical need such as Multiple Sclerosis, Amyotrophic Lateral Sclerosis, Parkinson's Disease or Alzheimer's Disease.

Materials and Methods

Study design

The purpose of this study was to characterize the therapeutic potential of leriglitazone for the treatment of X-ALD and other neurodegenerative and neuroinflammatory conditions. The experiments were done in various models for different purposes including primary cultures of rat brain cells, ABCD1 silencing human brain endothelial cells (HBMEC), the human monocytic cell line THP-1, healthy and AMN human macrophages, mouse models of X-ALD (*Abcd1 KO and Abcd1/Abcd2 DKO)* and demyelinating conditions (cuprizone model and EAE model of Multiple Sclerosis), as well as in samples from a phase 1 study in healthy human volunteers. Table 1 summarizes all the in vivo and in vitro models conducted to test the efficacy of leriglitazone.

Sample sizes for the in vitro studies were determined on the basis of prior results and pilot experiments. In all the in vivo experiments animals were randomly assigned to experimental or treatment groups and caretakers and investigators conducting the experiments were blinded to the treatment allocations. Group sizes were confirmed with power analysis. Further experimental details and protocols of each model, including animal care/handling and the number of biological/technical replicates are in the Supplementary Materials, or in the figure legends.

Data analysis and statistics

All values are expressed as the mean ± SEM. Data analysis and statistics was performed using GraphPad Prism 8 Software (GraphPad Software Inc.). *P* values are reported in the figure legends. Unless otherwise stated, differences in measured variables were assessed by using a two-tailed

Student's t test for single comparisons or one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc correction for multiple-comparison testing when data followed normal distribution. Normality was checked with Shapiro-Wilks test. Results were considered statistically significant at P values < 0.05.

Supplemental materials

Supplementary Materials and methods.

Supplementary figures S1-S6

Supplementary tables S1-S7

Datafile S1

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Acknowledgments:
We thank Noëlle Callizot and Alexandre Henriques from Neuro/Sys, Grahame Kidd from Renovo Neural, Gerhard Zeitler from the Center for Brain Research (Medical University of Vienna) and Bernardo Castellano and Gemma Manich from the Universitat Autònoma de Barcelona for their experimental technical support and Sonia Poli and Delphine Charvin for their insightful comments on the manuscript. **Funding:** The research leading to these results has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 712949 (TECNIOspring PLUS) to AV and from the Agency for Business Competitiveness of the Government of Catalonia and from Retos (RTC-2017-5867-1), Nuclis (RD14-1-O114), CDTI (IDI-20180106) and Enisa Jóvenes Emprendedores 2012 to Minoryx Therapeutics. AV is the recipient of a TECNIOspring plus fellowship. PP received funding from Torres Quevedo (PTQ-13-06015). **Author contributions:** LRP, AV, MM and PP contributed to the conception and design of the study. LRP, AV, MC, ET,, SFP (Fig. 2C,D), IW (Fig. 4A,B), JBA (Fig. 2C,D), , SK (Fig, GP (Fig. 6), SP (Fig. 6), UM (Fig. 6), PM (Fig.4C and D), JBE (Fig.2C,D and Fig.4A,B) and PP contributed to the acquisition and/or analysis of the data. LRP, AV, ET, SFP, IW, JBE, and PP contributed to the drafting of the text and preparing the figures. **Competing interests:** LRP, AV, MC, ET, GP, SP, UM, MM and PP are current or former employees at Minoryx Therapeutics. MM and PP report a patent application (10179126) issued on January 15th 2019 for the use of Thiazolidinedione derivatives in the treatment of CNS disorders. The patent will be owned by those authors. MM is the cofounder of Minoryx Therapeutics which focus on therapies for rare neurodegenerative diseases. SK, PM and JB received a payment for their study proposals to carry out some of the studies shown in this manuscript, which just covered the purchase of the reagents and the time invested. PM and JBE are consultants for Minoryx Therapeutics. Data and materials availability: All data associated with this study are in the paper or the Supplementary Materials. Leriglitazone was provided upon request after a Material Transfer Agreement agreement.

Figures:

Fig. 1. **Leriglitazone is neuroprotective in models of X-ALD. (A)** Survival of rat spinal cord motor neurons and astrocytes **(B)** treated with C26:0 (40µM) with or without increasing doses of leriglitazone (Leri). **(C)** Representative immunofluorescence images of neurons (MAP-2 staining) and astrocytes (GFAP staining) quantified in A are shown below; scale bar 100µM. **(D)** Abcd1 KO neurons were treated with C26:0 without or with increasing doses of Leri and neuronal survival was assessed. **(E)** Oxidative stress (methionine oxidation measurement) and concentration of ATP and ROS production (superoxide) of rat spinal cord motor cultures from A. **(F)** IKBα Simple Western WES™ representative graphs and quantification from spinal cord motor cells from A. **(G)** IL-1β protein concentration of spinal cord motor supernatants from A determined by ELISA; some values were under the detection limit (<LLQQ) and changed to the lowest detected value in the assay. C26:0 condition was compared to vehicle (*) and leriglitazone treatment doses to C26:0 (#); *n*=4-6 (20,000 cells/repeat); One-way ANOVA followed by Fisher's LSD test except for F

and G (Kruskal Wallis and uncorrected Dunn's test).#: $P < 0.05$; **/##: $P < 0.01$; ***/###: $P < 0.001$; ****: $P < 0.0001$.

Fig. 2. Leriglitazone is neuroprotective in in vivo models of AMN. (**A**) *Abcd1* KO mice treated twice daily at different doses of leriglitazone (Leri) for one week. *Nrf1, Sod2, Il-1β* and *Tnf-α* gene expression in the spinal cord normalized to Gapdh, fold-changes from vehicle are shown; *n*=5-11 spinal cord/ treated group; unpaired t-test except for *Il-1β* (Mann-Whitney test) (**B**) Protein concentration of several inflammatory markers (MCP-1, Eotaxin-1, SCF, IL-1α) at 4 months of treatment from DKO spinal cord tissues; *n*= 3-5 spinal cord/ treated group; one-way ANOVA followed by Fisher's LSD test. (**C**) Balance beam and rotarod behavioral tests in DKO mice treated for 6 months with leriglitazone by dietary admixture (correspondence to mg/kg/day is shown) were assessed with 2-month intervals from pretreatment (14 months) until treatment endpoint. Differential scores of the Balance Beam performance and the time (latency, seconds) to fall from an accelerating Rotarod over treatment duration are indicated as the difference (Δx-14) between the values x, at 16, 18, or 20 months and baseline (14 months); *n*=7-15 mice/ treated group; one-way ANOVA followed by Fisher's LSD test. (**D**) Quantification and representative immunohistochemistry images of axonal degeneration (APP-positive axonal swellings, open arrow) and microglia activation (Mac-3-positive nodules) in spinal cord white matter of DKO mice treated with different doses of leriglitazone; *n*=6-13 spinal cord/treated group; one-way ANOVA followed by Fisher's LSD test. DKO control was compared to wild-type control (*) and leriglitazone treatment doses to DKO control or Abcd1 KO (#). */#: $P < 0.05$; **/##: $P < 0.01$; ###: $P < 0.001$ and ####: $P < 0.0001$.

Fig. 3. Brain penetration and protective effects of leriglitazone in a model of BBB mimicking X-ALD conditions. (**A**) Schematic representation of the transwell system used to mimic the BBB with or without silencing *ABCD1* in HBMEC. (**B**) BBB permeability in the experimental setting depicted in A in control (Vehicle) and inflammatory conditions (LPS) together with C26:0 treatment; two-way ANOVA (**C**) Effect of leriglitazone pre-treatment (500nM) on neuron survival, (**D**) neurite outgrowth, (**E**) astrocyte survival and (**F**) microglia activation following exposure to C26:0 and LPS (50ng/ml) for 24h; mean±SD. (**G**) Representative immunofluorescence images of MAP-2 (neuron/neurite), GFAP (astrocyte) and OX-41 (microglia) detection from C; scale bar 100µM; *n*=3-8 (20,000 cells/repeat). C26:0+LPS or C26:0 condition was compared to vehicle (*) each in non silencing (-siABCD1) or silencing (+siABCD1) conditions, leriglitazone (Leri) treatment was compared to their own vehicle controls (#), C26:0+LPS was compared to C26:0 in the +siABCD1 arm (&) and C26:0+LPS was compared in +siABCD1 to -siABCD1 (+) condition; one-way ANOVA was used to compare pairs within +siABCD1 or -siABCD1 conditions and two-way ANOVA was used to compare C26:0+LPS in +siABCD1 to -siABCD1. +/#: $P < 0.05$; **/##: $P < 0.01$; ***/###/+++/&&&: $P < 0.001$ and ****/####/&&&&: $P < 0.0001$.

Fig. 4. Leriglitazone exerts anti-inflammatory effects in monocytes/macrophages derived from patients with X-ALD and decreases adhesion to endothelial cells. **(A)** TNF-α mRNA quantity of monocyte-derived macrophages from healthy volunteers and patients with X-ALD; unpaired test. **(B)** TNF-α expression of monocyte-derived macrophages from healthy volunteers and patients with X-ALD with or without treatment with leriglitazone (1µM); paired t-test. **(C)** THP-1 monocytic cell adhesion to preactivated (TNF-α, 10ng/ml) HBMEC with or without silencing of ABCD1 and after treatment with leriglitazone (1µM) ; *n*=4; one-way ANOVA

followed by Fisher's LSD test . (**D**) PBMC adhesion to TNF-α (10ng/ml) preactivated HBMEC after treatment with leriglitazone (1μM); n=9: one-way ANOVA.*/#: $P < 0.05$; **/##: $P < 0.01$.

Fig. 5. Leriglitazone is neuroprotective in neuroinflammatory disease models. (**A**) Microglia phagocytosis of myelin debris up to 24 h measured by timelapse imaging after treatment with different doses of leriglitazone with or without LPS treatment (50ng/ml) and expressed as the AUC bar graph for all conditions; n=3; mean±SD. #: Leriglitazone (Leri) treatment group comparison of LPS stimulated and unstimulated cells; *: not treated (NT) without LPS compared to LPS treated cells, #: leriglitazone treatment group compared with their corresponding untreated control (without or with LPS stimulation), &: comparison of each treated pair with or without LPS treatment; two-way ANOVA. (**B**) Effects of leriglitazone on survival of rat oligodendrocyte precursor cells (OPC, A2B5+) and mature oligodendrocytes (MAG+) upon VLCFA insult; *n*=5-6 (20,000 cells/repeat); one-way ANOVA. (**C**) Total counts of oligodendrocytes (Olig2+) and Myelin Basic Protein (MBP) intensity in the medial corpus callosum with or without leriglitazone administration (125 mg/Kg/day) after 5 weeks of demyelination with 0.2% cuprizone; *n*=3-7; upaired t-test. (**D**) Number of myelinated axons and representative images (scale bar 10μM) from mice demyelinated for 6 weeks with 0.3% cuprizone and after 3 weeks of remyelination with or without leriglitazone (75 mg/Kg/day) treatment; n=8; Mann-Whitney test. (**E**) Clinical score of naïve mice and mice with MOG peptide induced EAE following treatment with vehicle or leriglitazone at different doses for 15 days; n=8-9; two-way ANOVA followed by Dunnett's test; *: vehicle was compared to naïve mice, #: leriglitazone treatment doses were compared to vehicle. #: $P < 0.05$; ##: $P < 0.01$; ***/###: $P < 0.001$; ****/####/&&&&: $P < 0.0001$.

Fig. 6. **Leriglitazone is safely tolerated in humans and exerts anti-inflammatory properties**. Six healthy volunteers were treated with two different doses of leriglitazone. (**A**) Measurement at baseline and at Cmax of different inflammatory markers in plasma. **(B)** Measurement at baseline and at Cmax of different inflammatory markers in CSF. **(C)** Plasma adiponectin correlation with leriglitazone measurements at C_{max} for the six healthy volunteers treated with two different doses of leriglitazone (401-3:135mg, 501-3: 270mg). #: $P < 0.05$; paired t-test.

Table legend

Table 1. Summary of the in vitro and in vivo studies performed with leriglitazone with doses used and parameters evaluated.

Treatment dose (leriglitazone)	**Experimental model**	**Treatment length**	**Parameters evaluated**	**Results**
3, 10, 30nM	Rat primary spinal cord motor neurons and astrocytes; mouse *Abcd1* KO primary spinal cord motor neurons	1h +24h (with C26:0)	Survival (astrocytes, neurons), ATP, Methionine sulfoxide reductase area, ROS, IKBα, IL-1β	Fig. 1
500nM (luminal); 44-34nM (abluminal)	BBB transwell model: HBMEC, rat primary astrocytes, primary neurons, microglia	1h +24h (with 40μM C26:0)	Brain penetration, survival (neurons, astrocytes), neurite outgrowth, microglia activation	Fig. 3
1μM	Monocyte/macrophages derived from patients with AMN and healthy volunteers	1 week +24h (with LPS 100ng/ml)	TNF-α	Fig. 4A, B
10μM, 100μM	THP-1 human monocytes and HBMEC	24h +5h (with TNF-α 10ng/ml)	Monocyte adhesion to endothelial cells	Fig. 4C
1μM	PBMC healthy volunteers	3h (only with TNF-α 10ng/ml) +2 h (both)	PBMC adhesion to endothelial cells	Fig. 4D
100μM, 50μM, 10μM, 5μM, 1μM	THP-1 human monocytes	1h +4h (with LPS)	TNF-α	Fig. S5
0.5μM, 1μM, 5μM	Pure microglial cell culture	24h ±LPS (50ng/ml)	Phagocytosis of myelin debris	Fig. 5A
3nM,10nM, 30nM, 100nM, 300nM	Primary oligodendroctyes	1h +24h (with 40μM C26:0)	Oligodendrocyte and OPC survival	Fig. 5B
17, 50, and 125 mg/kg/day	*Abcd1* null mice	6 weeks	Adiponectin concentration in plasma samples, plasma concentration of Leriglitazone	Fig. S2, Table S5
50 mg/kg/day	*Abcd1* null mice	1 week	Expression of mitochondrial biogenesis, oxidative stress, and inflammatory markers in spinal cord tissues	Fig. 2A
75 mg/kg/day	*Abcd1*/*Abcd2* double knockout mice	4 months	Expression of mitochondrial biogenesis and target engagement genes, protein concentrations of inflammatory markers, VLCFA ratio C26:O and C26:O-lysoPC amount in spinal cord tissues	Fig. 2B, Fig. S3, Fig. S4
17, 75, and 110 mg/kg/day	*Abcd1*/*Abcd2* double knockout mice	6 months	Motor function (Balance Beam and Rotarod test), axonal degeneration and microglia activation	Fig. 2C, 2D
110 mg/Kg	Demyelination model (Cuprizone 0.2%)	6 weeks	Myelin Basic protein staining and oligodendrocyte density	Fig. 5C
75 mg/kg/day po	Demyelination and Remyelination model (Cuprizone 0.3%)	9 weeks	Myelinated axons (EM)	Fig. 5D
17, 50, and 125 mg/kg/day	Neuroinflammatory and demyelinating mouse model (EAE)	15 days	EAE score	Fig. 5E
135mg/day, 270mg/day	Phase 1	8 days	IL-8, IP-10, IL-6, MCP-1 (plasma and CSF); adiponectin (plasma); Pharmacokinetics of leriglitazone in healthy volunteers.	Fig. 6, Table S7