Identification and in vitro characterization of novel ABCD1 positive functional modulators for the treatment of X-linked adrenoleukodystrophy

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Abstract

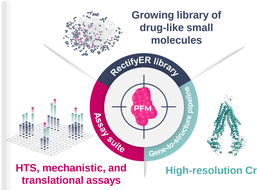
Background
X-linked adrenoleukodystrophy (X-ALD) is caused by loss-of-function mutations in the gene encoding the very-long-chain fatty acid (VLCFA) transporter, ABCD1. Deficient ABCD1 transport leads to pathogenic accumulation of cytoplasmic VLCFAs, prognostic markers for disease progression and severity. We leveraged Rectify's positive functional modulator (PFM) discovery platform to identify compounds that restore ABCD1 function.

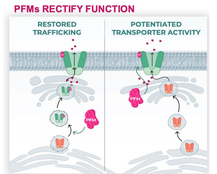
Methods
Rectify's proprietary small molecule library was screened to identify PFMs which increase expression of ABCD1 wild-type and missense variants. Whole exome sequencing of X-ALD patient-derived fibroblasts was used to identify missense variants. We developed a novel quantitative AlphaLISA assay to measure ABCD1 protein. Levels of the lysophosphatidylcholine (LPC) derivative of VLCFA were determined by LC/MS.

Results
Applying the Rectify platform to ABCD1 provided multiple PFM profiles that potently corrected disease-relevant variants. We identified two patient-derived primary fibroblast lines harboring ABCD1 missense mutations that exhibit reduced ABCD1 expression. We demonstrated PFMs correct ABCD1 expression and LPC levels in both missense variant lines, as well as in fibroblasts from two female ABCD1 variant carriers.

Conclusion
These results highlight the applicability of Rectify's platform to discover high-quality PFMs targeting ABCD1. The PFM-mediated increase in ABCD1 expression and function in patient fibroblasts demonstrates the utility of this approach to identify small molecules which address the underlying cause of X-ALD.

Positive Functional Modulators (PFMs) to unlock the therapeutic potential of the ABC proteome

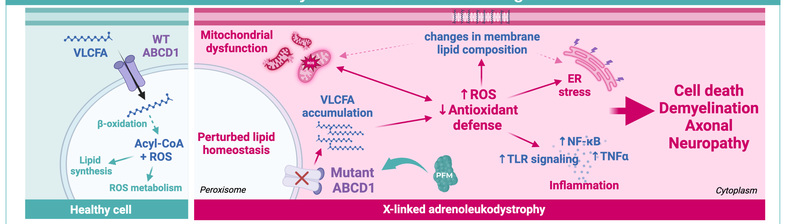




- ATP-binding cassette (ABC) transporters are a superfamily of membrane proteins which transport diverse substrates
- ABC transporters are associated with multiple human diseases:
  - –Etiological causes of **rare monogenic diseases**
  - –Predisposition to symptoms & severity of **complex diseases**
  - –Mechanistic association to pathways implicated **in common diseases**
- Genetic mutations can cause **protein trafficking defects** and/or **substrate transport defects**

- Rectify is leveraging its three-pronged **drug discovery platform** to accelerate discovery of **Positive Functional Modulators (PFMs)** to treat a broad range of human diseases
- PFMs are small molecule compounds that rescue ABC transporter function
  - –**PFM correctors** can rescue mutant protein **trafficking** to reestablish membrane localization
  - –**PFM potentiators** can rescue mutant protein **functional** activity to reestablish substrate transport
  - –PFMs also have potential to **enhance wildtype (WT) ABC transporter biology**

X-ALD arises due to impaired VLCFA transport from the cytoplasm into peroxisomes and is driven by mutations in the ABCD1 gene



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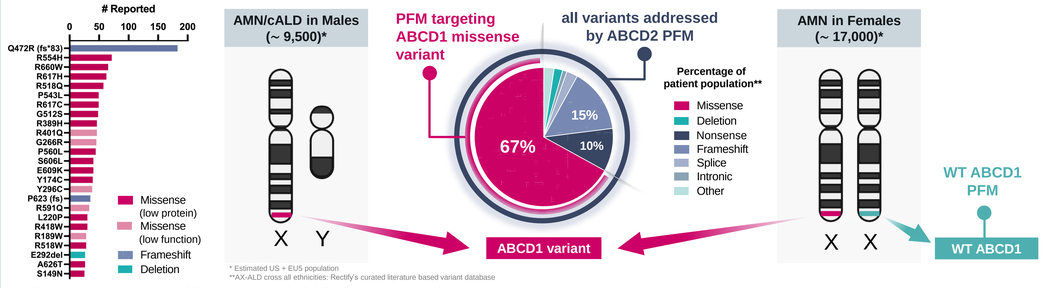
- In a normal physiological state, ABCD1 transports VLCFAs into peroxisomes where they undergo β-oxidation
- In X-ALD, VLCFAs build up in the cytoplasm due to dysfunctional ABCD1, which disrupts the cell's oxidative homeostasis owing to the generation of toxic reactive oxygen species (ROS) and the weakening of antioxidant defenses
- Oxidative stress triggers several harmful processes (mitochondrial degeneration, ER stress, inflammation) ultimately culminating in the X-ALD-associated syndrome of neuronal cell death, demyelination, and axonal neuropathy
- **Rectify is applying its drug discovery platform to target ABCD1, the underlying cause of X-ALD pathology**

Rectify's Platform Offers Multiple Target Paradigms for the X-ALD Mutational Landscape



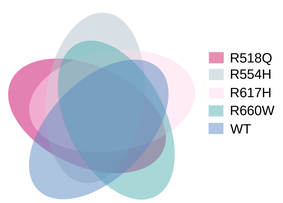
* Estimated US + EU5 population
**AX-ALD cross all ethnicities: Rectify's curated literature based variant database

- Twenty-two out of the top 25 most prevalent ABCD1 mutations are missense mutations (reduced expression or functional defects); two thirds of patients harbor missense mutations, offering an opportunity for pan-variant ABCD1 PFMs to restore ABCD1 function
- An ABCD2 PFM could benefit patients with non-missense ABCD1 mutations, while a WT ABCD1 PFM could enhance the ABCD1 expressed from the healthy allele in female X-ALD patients

Focused screening of the RectifyER library accelerates ABCD1 PFM discovery

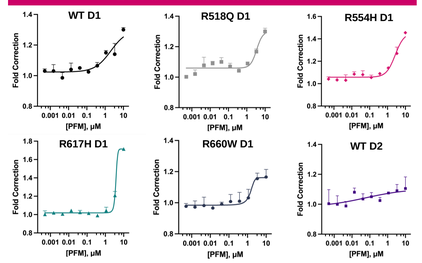
RectifyER Screening

Variant overlap ABCD1 screening hits

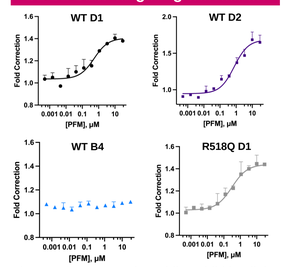


- Proprietary RectifyER library consists of a rich collection of lead-like compounds which rapidly accelerated ABCD1 drug discovery
- RectifyER primary screen in HEK-293 cells delivered hits against ABCD1 WT and four of the most frequently occurring variants at 10-30x higher hit rate compared to a diversity library and enabled identification of hits targeting single or multiple overlapping ABCD1 variants

D1 Pan Variant Correction



D1/D2 Dual-Targeting Correction

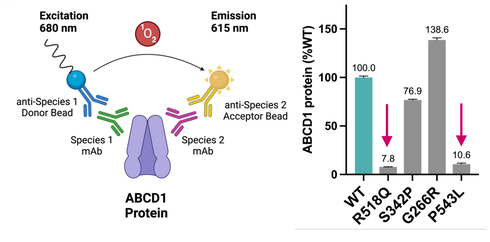


- The left panel is highlighting a PFM profile which exhibits selective pan-variant ABCD1 correction while exhibiting little activity against ABCD2
- In the right panel, a different PFM profile is selective against both WT ABCD1 and WT ABCD2, while showing no observable activity against other ABC transporters

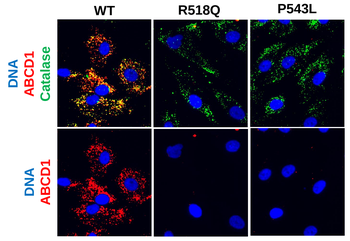
Identifying and characterizing X-ALD patient fibroblasts to enable PFM evaluation

X-ALD patient fibroblast selection funnel

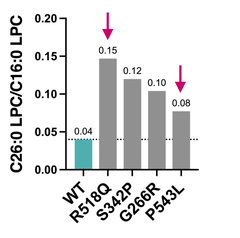
ABCD1 protein - AlphaLISA



ABCD1 - Immunofluorescence



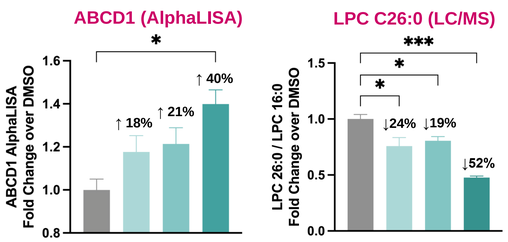
LPC C26:0 levels – LC/MS



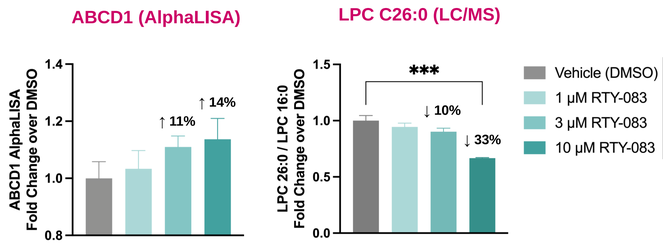
- From 16 ALD patient fibroblast lines, we used whole exome sequencing to identify four lines harboring missense mutations
- In addition to confirming elevated levels of the C26:0 lysophosphatidylcholine (LPC) derivative of VLCFA via LC/MS, we developed a novel ABCD1 AlphaLISA to demonstrate that two out of the four lines (**R518Q** and **P543L**) exhibited **reduced ABCD1 expression**
- The two patient fibroblast lines expressing **R518Q** and **P543L** were prioritized for testing of compound-induced restoration of ABCD1 expression

ABCD1 PFMs correct ABCD1^{P543L} and ABCD1^{R518Q} expression and reduce C26:0 LPC levels in patient fibroblasts

Fibroblast with ABCD1^{P543L}



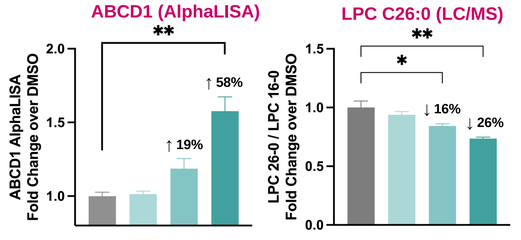
Fibroblast with ABCD1^{R518Q}



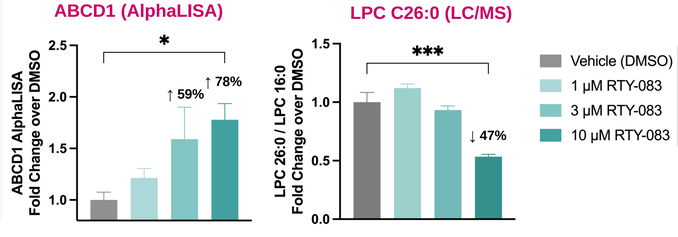
- In patient fibroblast cells harboring R518Q or P543L, RTY-083 dose-dependently **increases ABCD1 protein levels** which suggests RTY-083 is successfully correcting the reduced expression of these variants
- The compound-induced ABCD1 increase is accompanied by a dose-dependent decrease in the LPC C26:0 / LPC 16:0 ratio; this indicates that RTY-083 is **lowering the levels of LPC C26:0**, a key diagnostic X-ALD biomarker for disease progression and severity

PFMs show ABCD1 correction in fibroblasts from mothers of X-ALD patients

Female carrier of unknown genotype



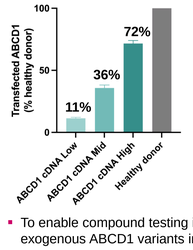
Female carrier of frameshift ABCD1 variant



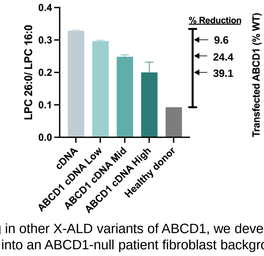
- We also tested whether RTY-083 can correct ABCD1 protein levels and function in fibroblast cells from two female patient carriers of pathogenic ABCD1 variants
- We observed that RTY-083 treatment resulted in a dose-dependent increase in ABCD1 protein levels, as well as a decrease in the LPC C26:0 / LPC 16:0 ratio
- Because the female carrier of the frameshift ABCD1 variant is unlikely to make protein or contribute to function, these results indicate that **RTY-083 acts on WT ABCD1** in addition to the variants that are the only expressed in the male X-ALD fibroblasts

Beyond: ABCD1^{P543L} and ABCD1^{R518Q}: introducing exogenous ABCD1 variants into ABCD1-null X-ALD patient fibroblasts to enable compound testing in more ABCD1 variants

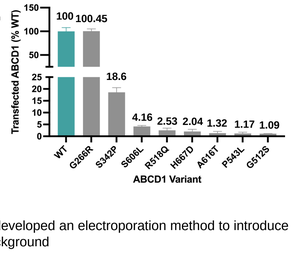
Exogenous ABCD1



↑ABCD1 decreases VLCFA



Exogenous ABCD1 variants



- To enable compound testing in other X-ALD variants of ABCD1, we developed an electroporation method to introduce exogenous ABCD1 variants into an ABCD1-null patient fibroblast background
- Exogenous expression of WT ABCD1 correlates with LPC C26:0 reduction and thus demonstrates proof of mechanism of this approach
- Electroporation of ABCD1 variants reveals protein expression profiles similar to their endogenous profiles across patient fibroblasts, suggesting that the consequence of the variants on protein stability is recapitulated in this system

Conclusions

- Pathogenic mutation of ABCD1 is the etiological cause of X-ALD
- ABCD1 normally functions to transport VLCFAs into the peroxisome for degradation; when ABCD1 is reduced or eliminated by mutation, toxic VLCFAs accumulate in the cytoplasm, leading to cellular toxicity and death
- Our platform's primary screen has delivered multiple PFM profiles which are active against ABCD1 variants, WT ABCD1, and the alternative VLCFA-modulating ABCD2
- PFM testing showed that RTY-083 can correct ABCD1 protein and function in two male X-ALD lines carrying pathogenic ABCD1 variants P543L and R518Q
- Fibroblasts from two female carriers of pathogenic ABCD1 variants also showed increased ABCD1 protein and reduced VLCFA in response to RTY-083
- Our preliminary results indicate that RTY-083 can engage X-ALD variant and non-variant ABCD1 in a manner that reduces toxic VLCFA levels
- Employing studies with both endogenous and exogenous expression of ABCD1 variants, Rectify is continuing to optimize our chemical matter and advance towards therapeutic development