Conclusions (1)

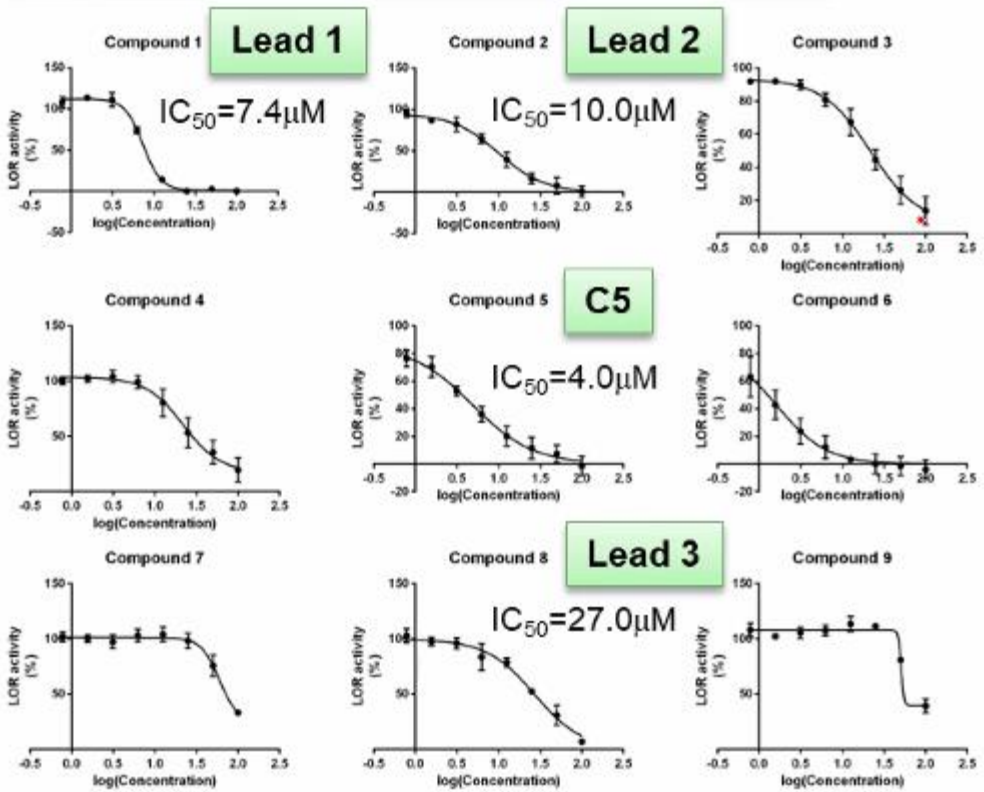
- AASS is the main source of GCDH substrates, not only in the periphery, but also in the brain
- Metabolite accumulation in GA1 does not reach "control" levels upon AASS inactivation
- There are alternative sources of GCDH substrates
  - Hydroxylysine degradation > ubiquitous
  - Tryptophan degradation > liver and kidney
  - Pipecolate pathway > enigmatic, plays likely no significant role
- High lysine exposure of GA1 mice suggests that AASS inactivation is sufficient to prevent neurotoxicity
- Pharmacological inhibition of AASS may represent an attractive strategy to treat GA1

Discovery of LOR inhibitors

- Performed virtual screening using a molecular model of LOR (127 compounds picked) > 1 low affinity hit (IC_{50} = 142 µM) with preliminary SAR
- Performed a HTS for LOR inhibitors
  - ICCB-Longwood Screening Facility (Harvard)
  - Z' generally between 0.7 and 0.8: excellent assay
  - Screened 106,377 molecules (ChemDiv and ChemBridge)
  - 142 hit inhibitors (0.13%)
  - 82 selected for cherry pick based on having "lead-like" properties
  - 39 molecules confirmed (48%), lowest IC_{50} 2µM
  - 10 molecules selected for re-purchase and IC_{50}s matched cherry pick results
  - At least 4 re-purchased molecules had activity in an orthogonal cell-based model
  - Of these: 2 are very promising leads, and 2 are backup structures

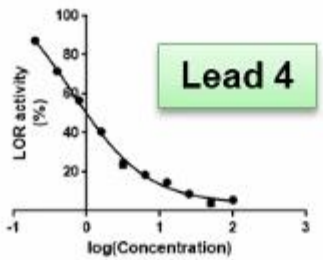
HTS follow up data

IC_{50} for re-purchased compounds



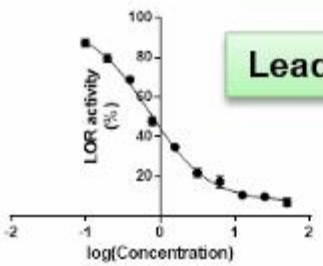
structure-activity relationship (SAR)

SAR-by-catalog
in collaboration with DeVita lab



Analog of **C5**
IC_{50} = 0.62 μM

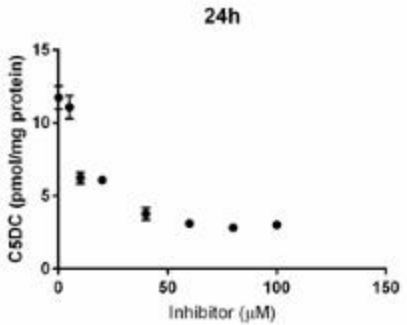
Re-synthesis of this compound



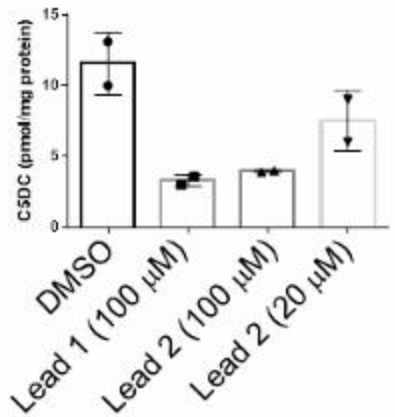
Analog of **C5**
IC_{50} = 0.72 μM

AASS inhibitors decrease C5DC in a GA1 cell line model

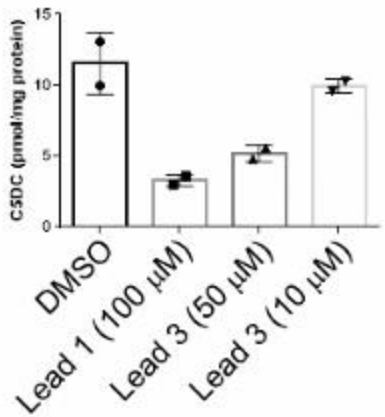
Lead 1



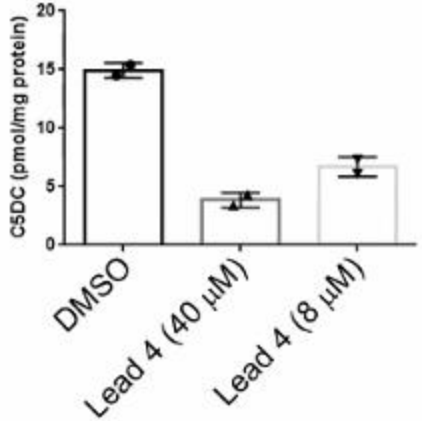
Lead 2



Lead 3



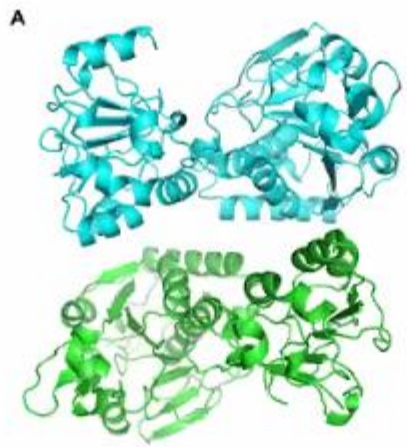
Lead 4



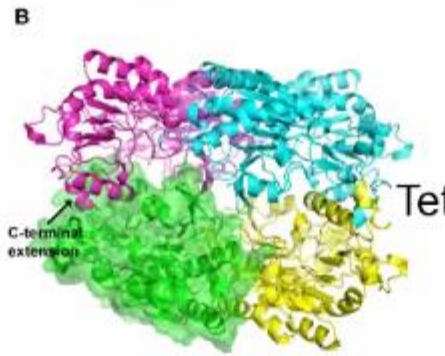
Formal proof that pharmacological inhibition of the LOR domain of AASS can decrease glutarylcarnitine accumulation in a GA1 cell line model

LOR crystal structure

- In collaboration with the Lazarus lab
- First crystal structure of human LOR
  - Identifies the tetrameric interface
  - Explains why the human protein binds NADPH (R267) in contrast to NADH in the yeast ortholog
  - Identifies a C-terminal extension into the adjacent monomer that appears critical for activity
- Co-crystal structures with lead 2 and lead 4.
  - Discovery of a novel inhibitory allosteric site



LOR dimer at 2.2Å



Tetrameric structure

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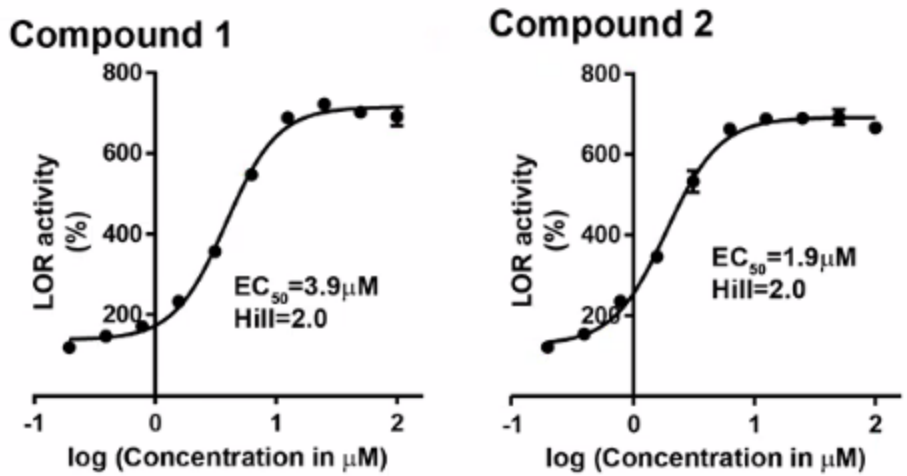
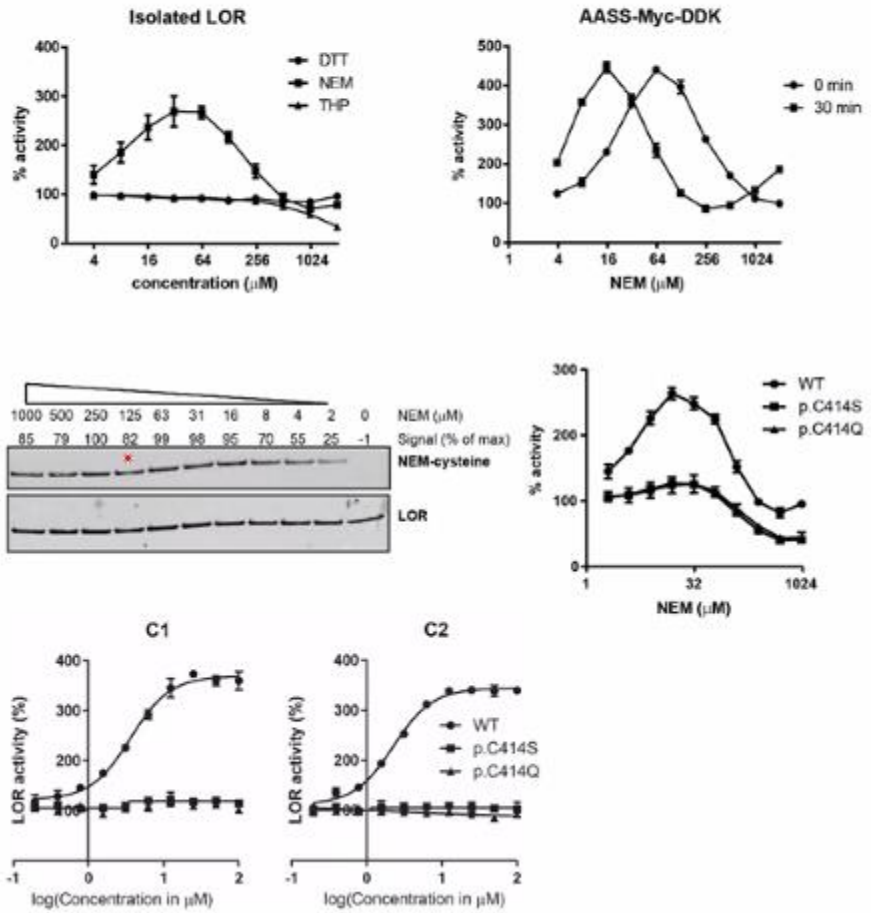
Discovery of LOR activators

- Serendipity: Our HTS for LOR inhibitors also identified many activating compounds
  - ICCB-Longwood Screening Facility (Harvard)
  - Z′ generally between 0.7 and 0.8: excellent assay
  - Screened 106,377 molecules (ChemDiv and ChemBridge)
  - 798 hit activators (0.75%)
  - Hit rate 5.6x higher than for inhibitors
  - 4 molecules selected for re-purchase and all were able to activate LOR
  - EC_{50} for 2 compounds < 10μM



Allosteric regulation of LOR

- LOR uniquely activated by N-ethylmaleimide (NEM), a sulfhydryl alkylating agent
  - Time and concentration dependency
- C414 identified as alkylated using X-ray crystallography
- C414 is necessary for activation
- HTS identified activators appear to bind to the NEM site
- Two distinct allosteric sites, one positive and one negative
  - Endogenous ligands unknown



Leandro et al, 2022, Open Biology

Conclusions (2)

- We identified 4 lead LOR inhibitors that are able to decrease C5DC accumulation in a GA1 cell line model
- Two leads bind to a newly identified allosteric site
- LOR can also be activated through an allosteric mechanism
  - NEM
  - Small molecules
- The positive and negative allosteric regulation of LOR constitutes a new regulatory mechanism for lysine degradation, which may be important for GA1 and PDE-ALDH7A1
- Drug discovery and medicinal chemistry not only lead to new therapies, but also increase our fundamental understanding of the targeted pathway

Ongoing studies and future goals

- Ongoing collaboration with the DeVita and Lazarus labs
- Discover novel brain penetrant small-molecule LOR inhibitors suitable for IND-enabling studies
  - Lead optimization using medicinal chemistry, structural biology and structure-based drug design
  - Mechanism-of-action
  - ADME-PK
  - In vivo biology and efficacy in GA1 mouse model
- Study the allosteric regulation of AASS
  - Identify endogenous ligands
  - Co-crystallization/cryo-EM with activators
  - Regulation of lysine degradation and its impact on GA1/PDE-ALDH7A1
- Explore AASS as a target for PDE
  - Validation in our cell line model using CRISPR-Cas9 genome editing
    > Generate *ALDH7A1*/*AASS* DKO cell lines > metabolomic analyses
  - Validation in a recently reported mouse model

GENERAL ARTICLE

A novel mouse model for pyridoxine-dependent epilepsy due to antiquitin deficiency

Hilal H. Al-Shekaili[1], Terri L. Petkau[2], Izabella Pena[3], Tess C. Lengyell[2], Nanda M. Verhoeven-Duif[4], Jolita Ciapaite[4], Marjolein Bosma[4], Martijn van Faassen[5], Ido P. Kema[5], Gabriella Horvath[6], Colin Ross[7], Elizabeth M. Simpson[1,2], Jan M. Friedman[1,8], Clara van Karnebeek[9,10,11] and Blair R. Leavitt[2,*]

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