Insights into the mechanism of action of structurally targeted allosteric regulators for the treatment of GLB1-related disorders

GAIN THERAPEUTICS

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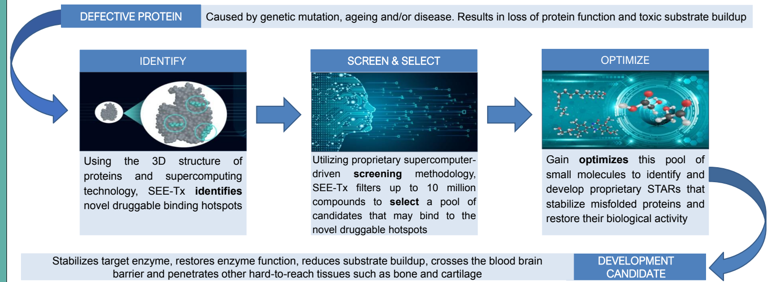
Abstract

GLB1-related disorders due to lysosomal β-galactosidase (β-gal) deficiency comprise two phenotypically distinct lysosomal storage disorders: GM1 gangliosidosis and Morquio B syndrome. There are currently no approved therapies that can prevent or reverse disease progression, thus creating a high unmet medical need for patients with these conditions. Gain Therapeutics has applied its innovative proprietary drug discovery platform, Site-directed Enzyme Enhancement Therapy (SEE-Tx™), to the development of small-molecule structurally targeted allosteric regulators (STARˢ) for the treatment of lysosomal storage disorders. Such STARˢ can allosterically bind and stabilize the target mutant enzymes thus avoiding their degradation and recovering their enzymatic activity. Here we report recent insights into the mechanism of action of lead STARˢ, which has so far shown promising effects in in vitro models of GLB1-related disorders. Indeed, they bind to the target enzyme in a non-inhibitory manner and tend to increase its delivery to the lysosome presumably by rescuing it from early degradation in the endoplasmic reticulum. Most importantly, they enhance both enzymatic activity and substrate depletion in in-vitro cell-based assays. All together this data supports and validates the application of SEE-Tx™ as an innovative drug discovery platform for the identification of allosteric regulators for the treatment of GLB1-related disorders.

SEE-Tx™ Drug Discovery Platform



Identification of a new allosteric binding site

- The published human GLB1 3D structure obtained by X-ray crystallography and refined to 1.8 Å resolution was used (PDB ID: 3thc).
- Molecular dynamics simulations of the protein in organic-aqueous solvent mixtures (**MDmix**) reveal a druggable cavity.
- MDmix was also used to identify key interaction sites (binding hot spots), which were used as pharmacophoric restraints to guide docking, and to explore the conformational flexibility of the binding site.

3D structure of the GLB1 monomer

Hit ID by Virtual Screening

- A virtual collection of >6 million commercially-available compounds were evaluated computationally with the docking program **rDock** using the standard scoring function, pharmacophoric restraints and a high-throughput protocol.
- Best scoring compounds were visually inspected and 80 were selected based on the plausibility of the binding mode and chemical diversity considerations.
- Screening by DSF afforded 3 hits (**4% hit rate**). Hit validation was based on SAR-by-catalogue, which provided 6 additional active compounds (14% hit rate).

STARˢ Specifically Bind β-Gal

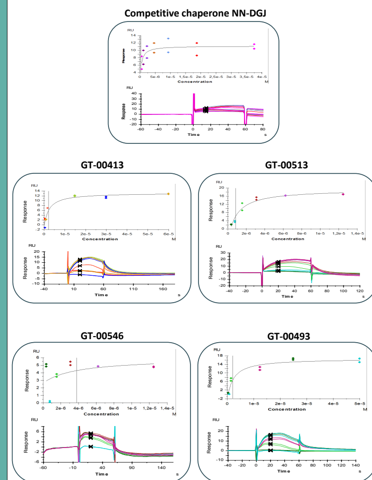


Fig 1. STARˢ bind and stabilize β-galactosidase.
The SPR technique detects changes in the refractive index on a sensor's surface due to mass variations. These changes are used to measure the biomolecular interaction between purified β-gal and STARˢ. Data shows clear evidence of direct binding. Similar affinity obtained among compounds with a range of Kd 1-4 µM. Data is expressed as mean ± SD (*n*=2).

STARˢ Do Not Inhibit β-Gal

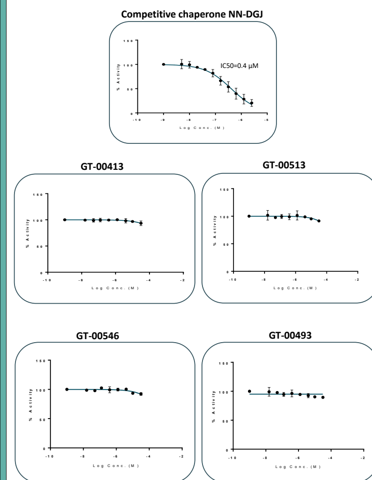


Fig 2. STARˢ do not inhibit β-galactosidase.
Cell lysates from WT fibroblasts are incubated with compounds at several doses and the samples are assayed for β-gal activity using resorufin beta D-galactopyranoside. STARˢ have no inhibitory activity at concentrations as high as 33.3 µM whereas the competitive chaperone NN-DGJ has an IC50 of 0.4 µM. Data is expressed as mean ± SD (*n*=2).

STARˢ Increase β-Gal Maturation and Delivery To The Lysosome

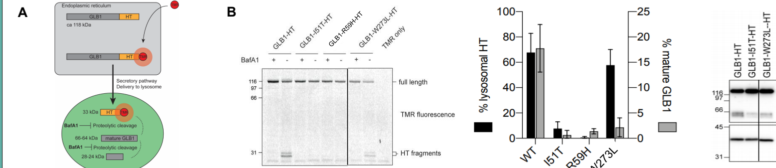


Fig 3. Characterization of wild-type (WT) and mutant β-gal in transfected HEK293 cell lines using Halo-Tag constructs.
A. Schematic representation of the Halo-Tag (HT) assay. **B.** Left panel: HT cleavage as measure for lysosomal delivery of disease-linked β-gal mutants. Fluorescent gel showing TMR signal from GLB1-HT variants expressed for 2 days in HEK293 cells in presence or absence of 50 nM BafA1 for the last 17 h. Right panel: HEK293 transfected with HA-tagged β-gal mutants. Cells were then lysed and collected samples were subjected to SDS-PAGE and subsequent western blot analysis with anti-HA (GLB1) or anti-GAPDH antibodies. WT β-gal is delivered to the lysosome and is stable; I51T and R59H mutants are retained in the endoplasmic reticulum (ER) whilst W273L mutant is delivered to the lysosome but it is not stable, therefore it is degraded.

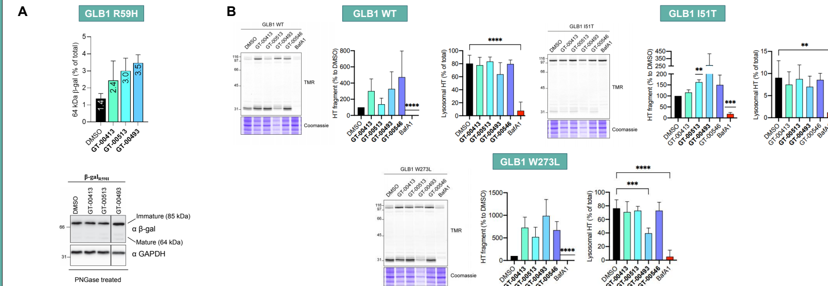


Fig 4. Effect of STARˢ on protein maturation and lysosomal delivery of WT and mutant β-gal.
A. HEK293 transfected with GLB1 R59H-HA were treated with STARˢ at 25 µM for 17h. Cells were then lysed and collected samples were subjected to SDS-PAGE and subsequent western blot analysis with anti-HA or anti-GAPDH antibodies. Quantification of mature GLB1 (64 kDa) and non-mature GLB1 (85 kDa) was performed. Data is presented as mean ± SD (*n*=3). **B.** HEK293 cells were transfected with WT, I51T or W273L GLB1-HaloTag and were treated with the indicate compounds (25 µM) and a fluorescent Halo ligand for 17h. Once the protein reaches the lysosome, the tag is cleaved off β-gal but is resistant to lysosomal hydrolases, thus enabling the detection of a 31 kDa fluorescent fragment that corresponds to the protein that reached the lysosomes. Lysosomal fragment is measured by western blot. Data is presented as mean ± SD (*n*=3). Statistical analysis was performed with one-way ANOVA followed by Dunnett's multiple comparison test. **p<0.01, ***p<0.001, ****p<0.0001.

STARˢ Enhance β-Gal Activity And Decrease Substrate Accumulation

Treatment (25 µM)	Canine p.R60H/p.R60H (equivalent to human R59H)	p.W273L/p.W273L	p.R201C/p.R201C	p.W161X/p.R208C
Untreated	**1.0** (*n*=24) (0.61% ± 0.3)	**1.0** (*n*=3) (8.3% ± 3.7)	**1.0** (*n*=3) (3.2% ± 2.0)	**1.0** (*n*=2) (0.3% ± 0.1)
GT-00413	**1.2 ± 0.1** (*n*=18)	**1.2 ± 0.1** (*n*=3)	**1.2 ± 0.3** (*n*=3)	**0.8 ± 1.4** (*n*=2)
GT-00493	**1.2 ± 0.2** (*n*=18)	**1.2 ± 0.5** (*n*=3)	**1.4 ± 0.4** (*n*=3)	**1.8 ± 0.6** (*n*=2)
GT-00513	**1.2 ± 0.2** (*n*=18)	**1.2 ± 0.2** (*n*=3)	**1.3 ± 0.1** (*n*=3)	**1.3 ± 0.1** (*n*=2)
GT-00546	**1.2 ± 0.1** (*n*=14)	**1.1 ± 0.2** (*n*=3)	**1.3 ± 0.5** (*n*=3)	**4.5** (*n*=1*)

Fig 5. STARˢ enhance β-gal activity in canine and patient-derived fibroblasts.
Canine and patient-derived fibroblasts were treated for 4 days with STARˢ. β-gal activity was measured using resorufin beta-D-galactopyranoside. Fold increase mean (treated cells/untreated cells) ± SD of β-gal activity is indicated in bold. For untreated samples % of WT activity (residual activity) ± SD is also reported in brackets. **n*=1 results are considered preliminary.

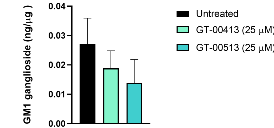


Fig 6. STARˢ tend to decrease GM1 ganglioside accumulation in canine fibroblasts.
p.R60H/p.R60H canine fibroblasts (Coriell GM11473) were treated with 25 µM of GT-00413 or GT-00513 in triplicates. Cells were harvested at day 4 and samples were analysed for GM1 ganglioside quantification using MS/MS. Data is expressed as mean ± SD (*n*=1, preliminary results).

Conclusions

SEE-Tx™ is a fast and cost-effective drug discovery approach for the identification of structurally targeted allosteric regulators (STARˢ).

The orally bioavailable as well as brain and bone penetrant allosteric β-gal regulators can:

- specifically bind to the target enzyme in a non-inhibitory way;
- increase protein maturation and its delivery to the lysosome;
- enhance enzymatic activity in a panel of fibroblast cell lines;
- decrease substrate accumulation in canine fibroblasts.

All together, the allosteric regulators identified with the proprietary SEE-Tx™ drug discovery platform support the restoration of key biological activities found to be impaired in GLB1-related disorders, thus warranting further developments towards the clinic.