Uncovering key steps for GALK1 allosteric inhibtion

Shi Yuan Ng* (s.y.ng3@newcastle.ac.uk), Jake D. Baulch, Arnaud Baslé, Sabrina R. Mackinnon, Wyatt W. Yue, Paola Lanzoni-Mangutchi

Biosciences Institute, The Medical School, Newcastle University, Newcastle upon Tyne, UK

What is galactosaemia

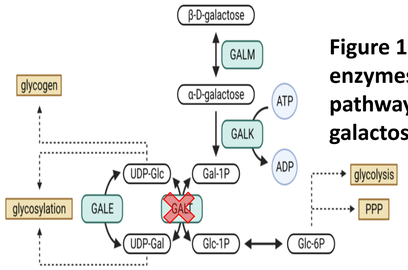


Figure 1: Defective enzymes in Leloir pathway that can cause galactosaemia[1].

- Classic galactosaemia is a rare metabolic disorder caused by defective GALT enzyme in the Leloir pathway, leading to the accumulation of neurotoxic Gal-1-P.
- GALK1 is a kinase upstream of GALT in the pathway, converting Galactose into Gal-1-P.
- By inhibiting GALK1, this could prevent the accumulation of Gal-1-P. Therefore, this project is focusing on allosteric inhibition of GALK1

Project overview

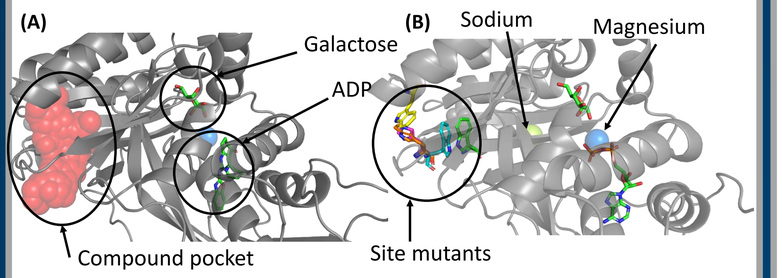


Figure 2: Site mutations are based on the compound pocket on GALK1 alloseric site.

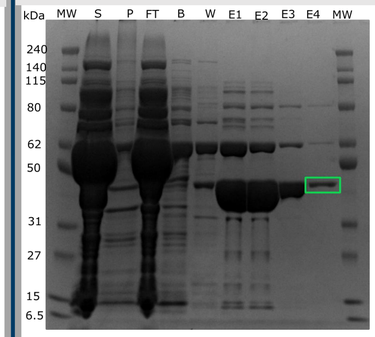
(A) Compounds identified (Red spheres) previously that could affect the kinase function of GALK1 through an allosteric site[2]

(B) AlphaFold model prediction of site mutants with ADP. Cyan: L213W, Orange: L218W, Green: V220W, Yellow: Y300W, Magenta: L218H

- **Background:** Previously, compounds binding on the allosteric site have shown GALK1 inhibition.
- **Hypothesis:** Site mutations that mimic the presence of the compounds in the allosteric site would cause a self-inhibitor effect.
- **Questions:**
- 1 – Functional effects (Kinase Glo assay)
- 2 – Protein stability (NanoDSF)
- 3 – Protein structure (macromolecular crystallography)

1. Homolak J, Babić Perhoč A, Virag D, Knezovic A, Osmanovic J, Salkovic-Petrisic M., BioEssays. 2024 Feb;46(2):2300061
2. Mackinnon SR, Krojer T, Foster WR, Diaz-Saez L, Tang M, Huber KVM, et al. ACS Chemical Biology. 2021;16(4):586-95.
3. Liu L, Tang M, Pragani R, Whitby FG, Zhang Y-q, Balakrishnan B, et al. J. Med. Chem. 2021;64(18):13551-71.

GALK1 variants were purified successfully



- Site mutants were expressed in *E. coli* and had a N-terminal 6x His-tag
- The His-tag attached to nickel, allowing the the first step of purification.

Figure 3: SDS-PAGE gel showing GALK1 site mutant purified by nickel affinity chromatography.
MW: PrimeStep Prestained broad range, S = Soluble fraction, P: pellet fraction, FT: Flow through, B:binding fraction (10 mM imidazole), W; Washing fraction (40 mM imidazole fraction), E: elution fraction (250 mM imidazole fraction) The green box identifies the presence of GALK1 site mutant

Allosteric site mutants keep galactose conversion activity

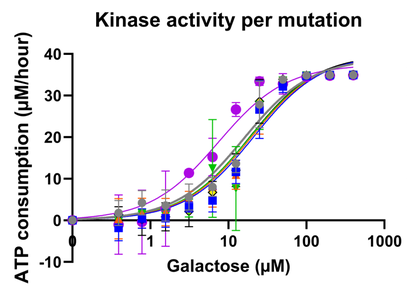


Figure 4: The kinase activity of each variant assayed with Kinase Glo activity assay. X-axis is log scaled

GALK1 variant	V_{max} (μM)	K_m (μM)
WT	39.06±1.41	15.57±2.25
L213W	39.97±1.75	20.90±3.45
L218W	39.78±1.76	19.49±3.30
V220W	39.62±2.43	18.46±4.38
Y300W	39.95±1.78	18.63±3.21
L218H	37.78±1.54	7.406±1.37

Table 1: Enzymatic parameters of each construct

- Kinase Glo reagent uses the remaining ATP after the GALK1 reaction, giving signal proportional to the amount of ATP. The higher the signal, the lower the kinase activity.
- All the site mutants showed a similar enzymatic parameters in comparison to the wild type except L218H, showing a K_m which was about half of the WT.

Mild structural destabilisation on site mutants

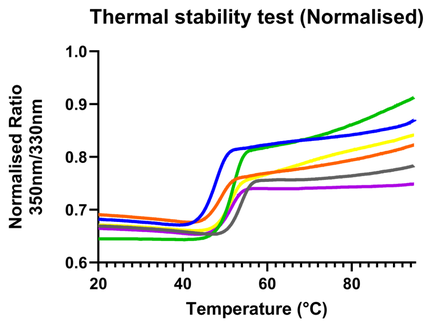


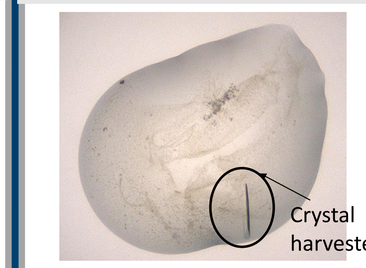
Figure 5: Normalised data of Thermal stability test from NanoDSF

GALK1 variant	T_m (°C)	Std Dev.
WT	53.6	0.0025
L213W	47.6	0.0057
L218W	48.9	0.0009
V220W	51.7	0.0107
Y300W	51.2	0.0037
L218H	51.2	0.0016

Table 2: Melting temperature with average standard deviation for each construct.

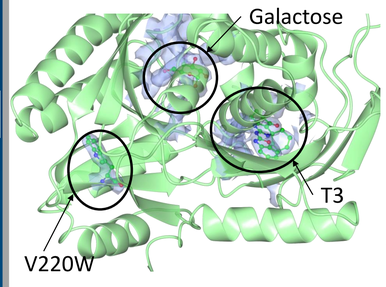
- Melting points (T_m) were determined using the fluorescence ratio from inside and outside of Tryptophan over a temperature gradient.
- L213W and L218W showed a decrease in T_m, suggesting an impact on the thermal stability of the site mutants in comparison to the wild type.

Structure and confirmation of mutation



- V220W variant crystallised and were harvested
- Protein concentration: 15.3 mg/mL
- Temperature: 20 °C

Figure 6: The formation of $GALK1_{V220W}$ crystal harvested after 12 days.



- All variants were crystallised, but only 4 diffracted to high resolution.
- Here shows the crystal structure and results of V220W
- Electorn density at V220W confirms the site mutation.
- The structure was refined to R/R_{free} : 0.25/0.29

Figure 7: Refined crystal structure of V220W with electron density (blue) shaded around the site of mutant, galactose and T3 (ATP analogue).

AlphaFold prediction is consistent with the crystal model

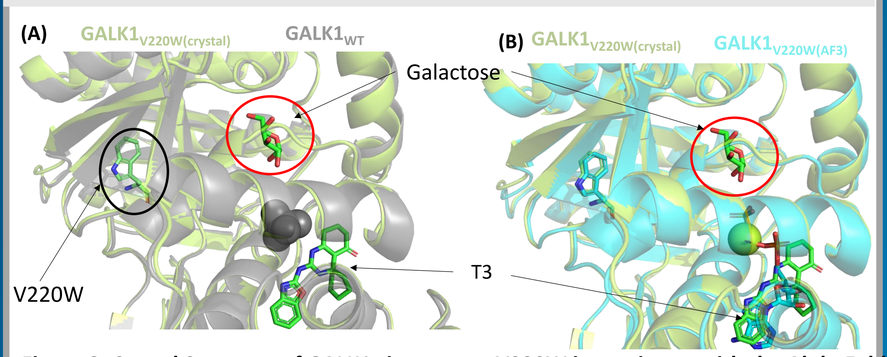


Figure 8: Crystal Structure of GALK1 site mutant V220W is consistent with the AlphaFold prediction

(A) Structure alignment of the WT crystal structure which was diffracted at home and the V220W crystal structure refined.

(B) Structure alignment of the AlphaFold predicted V220W structure and the V220W crystal structure refined.

- The crystal structure is consistent with the AlphaFold predicted structure.
- There is no major difference between the site mutant crystal structure and that of the wild type.

Conclusions and future perspective

- Although the Kinase Glo activity assay did not suggest a decrease in all mutants, the protein stability test could indicate a potential in inhibiting GALK1 through its allosteric site.
- Different mutations can be combined in the future to investigate more in its effect on the kinase activity.