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THE DEVELOPMENT AND CHARACTERISATION OF CELLULAR ASSAYS FOR MEASURING THE EFFECTS OF LIMK1 INHIBITORS

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Introduction

The LIM Kinase family: LIMK1 and LIMK2 are dual-specificity kinases that regulate actin-filament dynamics through the phosphorylation of actin depolymerizing factor (ADF) and cofilin (CFL)[1,2]. LIMK1 is implicated downstream of Rho-ROCK, Rac-PAK1 /Cdc42 signalling pathways[3]. The phosphorylation of CFL (on Ser3) inactivates the protein and ultimately prevents the binding and severing of filamentous actin. Dysregulation of these signalling cascades alters the actin-filament dynamics [4,5], which has been shown to drive several diverse pathological processes including in many cancers and the intellectual disorder, Fragile X Syndrome (FXS)[6].

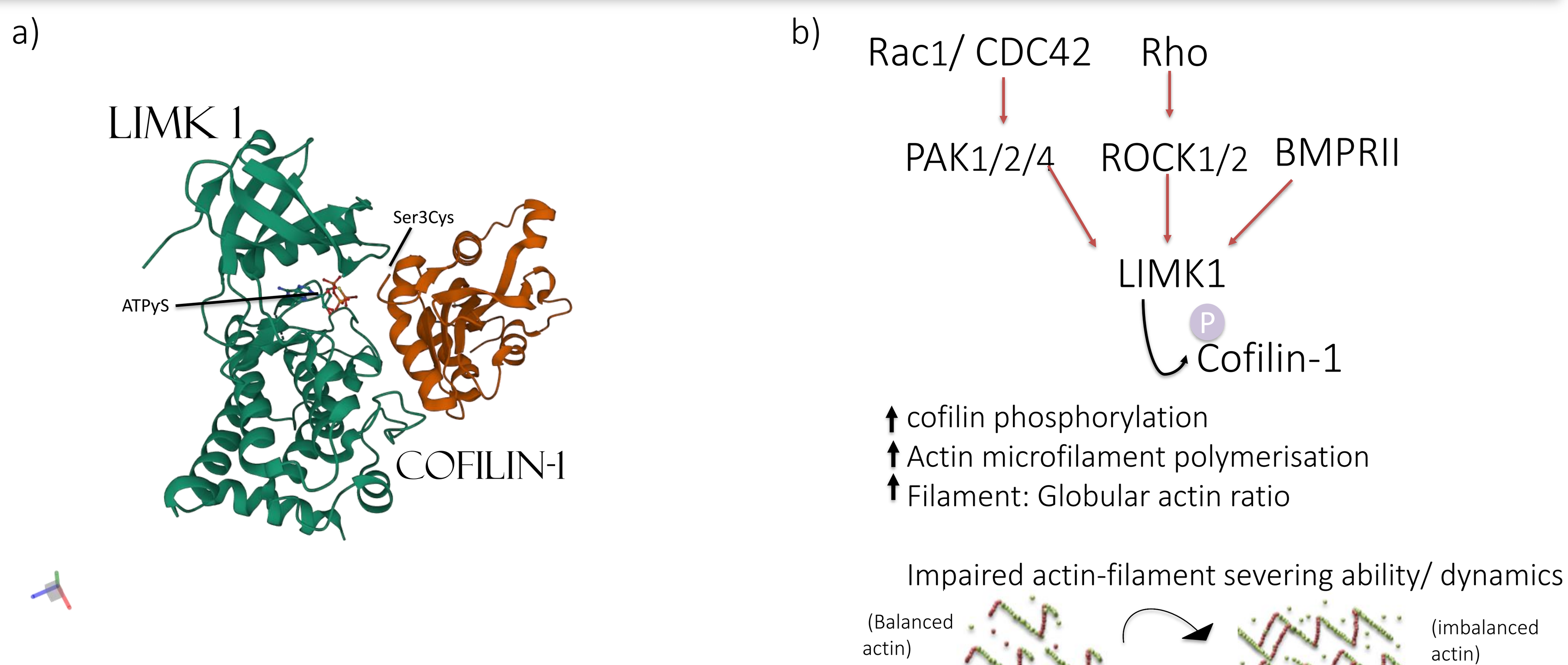

Figure 1. a) Crystalised structure of LIMK1-ATP-CFL1 complex (PDB: 5L6W) **b) Schematic of LIMK1 signalling pathways and dysregulation of actin-filament dynamics.**

Fragile X Syndrome

- An intellectual disorder caused by an expansion of a CGG repeat sequence in the promotor region of *FMR1* gene, resulting in loss of the protein product FMRP.
- FMRP regulates the Rac/Rho/BMPR2 pathways, therefore loss of FMRP leads to dysregulated signalling and thus impaired actin-filament dynamics.
- Abnormalities of the synapse dendritic morphology and function.
- Pharmacologic inhibition of LIMK1 in cellular and mice models have rescued spine morphology and alleviates symptoms associated with FXS[7,8].

Acute myeloid leukaemia (AML)

- Most common form of Leukaemia in adults (~25% cases).
- Aggressive malignancy of the white blood cells – genetic alterations disrupt the maturation of the myeloid blast cell, so they remain in an immature, undifferentiated, proliferative state.
- Survival rate is low with 80-90% of older patients experiencing relapse within a year of treatment and overall, 5-year survival of less than 15%.
- High levels of LIMK1 activity correlates with poor patient survival and elevation in p-CFL was paralleled by enhanced migration and drug resistance[9].
- AML cell lines identified as sensitive to LIMK1 compounds.

Aims & Objectives

- Develop reliable cellular assays – secondary to the primary: biochemical RapidFire.
- In Vitro and Ex vivo assessment for the optimization of novel LIMK1 inhibitors.

Development of LIMK1 inhibitors (Chemists) → Biochemical profile of novel & tool LIMK1 inhibitors → Cellular target engagement (NanoBRET assay) → Proof of mechanism (AlphaLISA assay /target specific assays) → Ex vivo application (Target engagement & proof of mechanism)

Overview of Cellular Methods

- **NanoBret Target Engagement Kinase assay –**
Assess compound binding ability and engagement with LIMK1/2 in HEK-293.
- **AlphaLISA SureFire technology –**
Detect and quantify the functional target p-cofilin in SH-SY5Y cell model and patient –derived stem cells.
- **Western blotting –**
SH-SY5Y cell lysate and mice brain slices.
- **Nitroblue tetrazolium test (NBT) –**
Used for indication of cell differentiation.

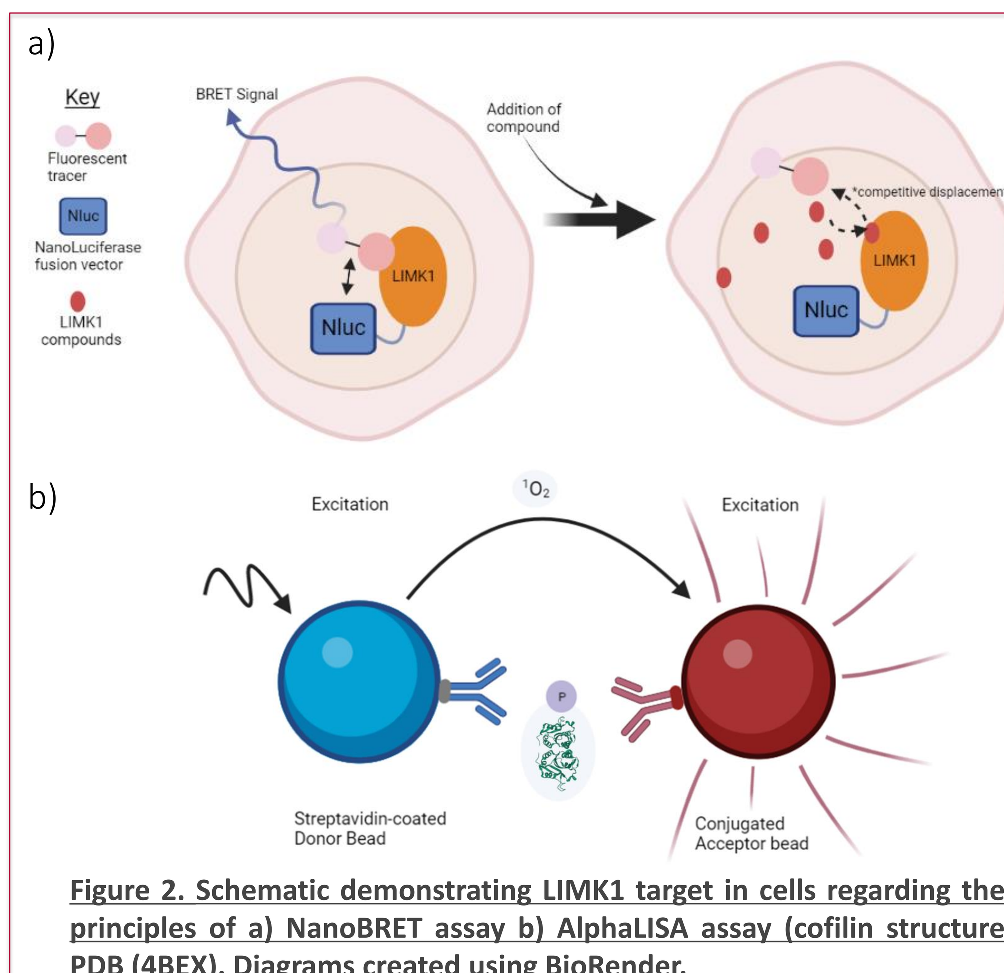

Figure 2. Schematic demonstrating LIMK1 target in cells regarding the principles of a) NanoBRET assay b) AlphaLISA assay (cofilin structure PDB (4BEX). Diagrams created using BioRender.

Results

Novel and Tool compounds were profiled using cell-based assays to first address whether they can inhibit intracellular LIMKs and then assess the functional consequences, such as inhibition of p-cofilin. Here, a lead inhibitor MDI-compound I and the tool inhibitor LIMKi3 are demonstrated following compound screening.

- Both MDI-compound I and LIMKi3 are cell-permeable and demonstrate good target engagement. LIMKi3 is particularly selective towards LIMK2 (Figure 3a).
- Both LIMKi3 and MDI-compound I demonstrated similar potency in SH-SY5Y cell line towards a reduction in p-CFL by both AlphaLISA and western blot analysis (Figure 3b&c). In addition, LIMK1 and cofilin maintain consistent expression across treatments.

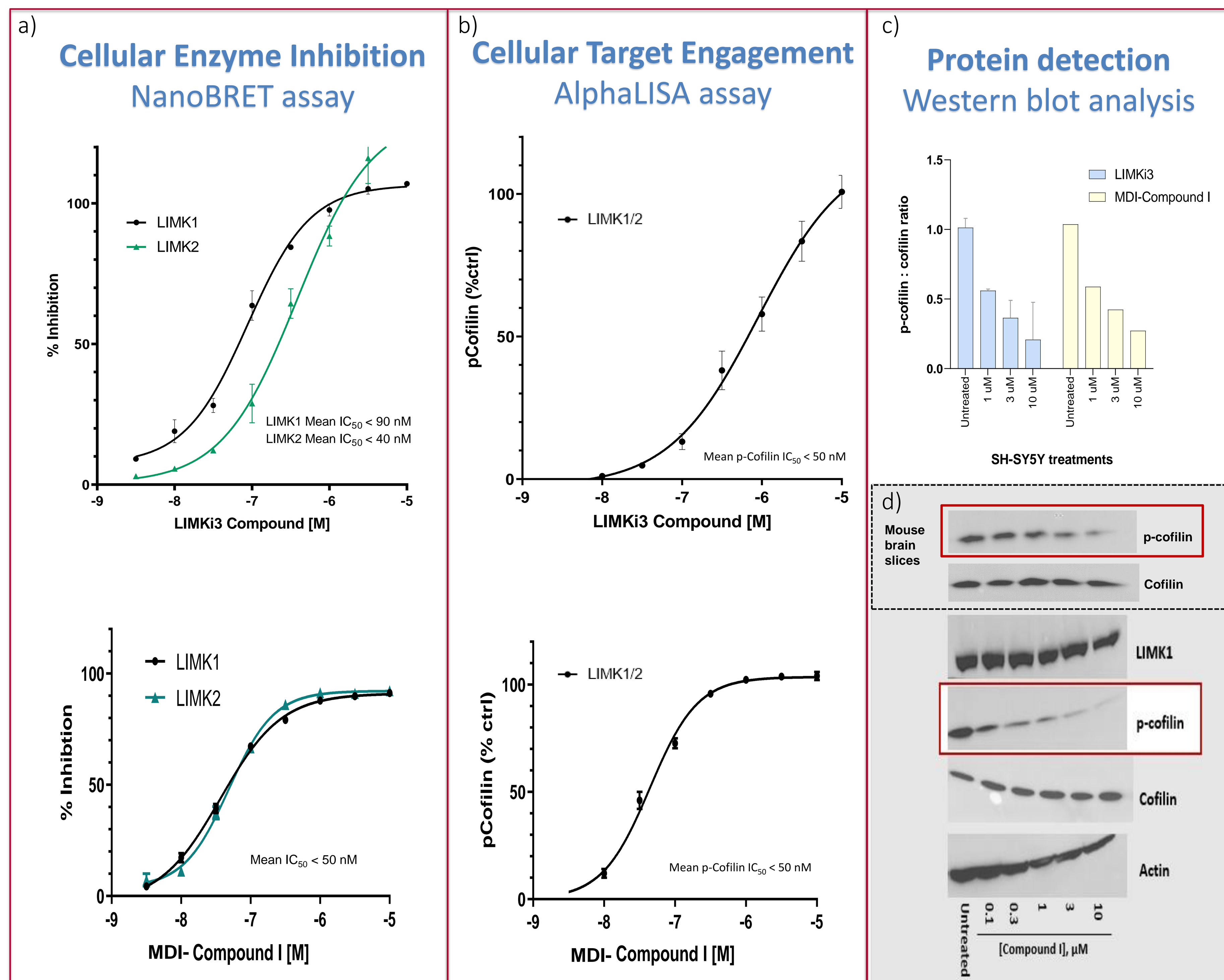

Figure 3. Concentration response curves following treatment (top concentration 10µM) with LIMKi3 or MDI-compound I in SH-SY5Y cells. a) shows response curves generated by NanoBRET fluorescence reading for both inhibition of LIMK1 and LIMK2. b) shows response curves generated by AlphaLISA luminescence technology for p-CFL levels. c) Representative semi-quantification of treated and untreated SH-SY5Y cells showing p-cofilin/cofilin ratio and d) representative blots of LIMK1, p-CFL and CFL protein abundance in both SH-SY5Y cell lysate and mouse brain tissue samples.

Oncology

The MOLM-13 cell line, representative of AML malignancy, was treated with MDI-compound at various concentrations to assess whether induction of differentiation can occur. A NBT test was used to assess the state of the cells following treatment (Figure 4).

- MOLM-13 cells expressed high levels of NBT positive cells following treatment, indicative of cell differentiation.

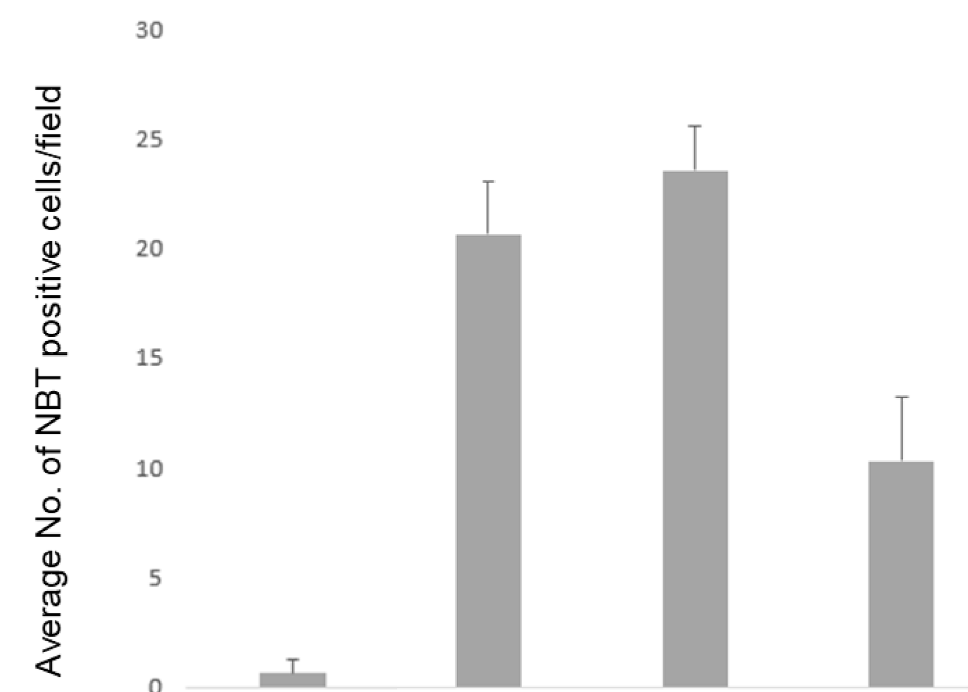

Figure 4. Quantification of myelopid blast differentiation in MOLM13 cell line following treatment with MDI-compound I.

Conclusions

- The biochemical and functional aspects of LIMK1 inhibition can be effectively assessed with reliable cell-based technology.
- LIMK1 inhibitors demonstrate promising pharmakinetic profiles – potent inhibition, good target engagement, functional effects and no observed toxicity to healthy cells.
- Limitations to FXS application, namely with crossing the blood-brain barrier.
- Compounds demonstrate multi-purpose potential for treatment in disease areas such as AML.

References

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