Apparent Potency of Small Molecule Inhibitors is Influenced By the Choice of Substrate in LIMK Biochemical In-Vitro Kinase Activity Assays

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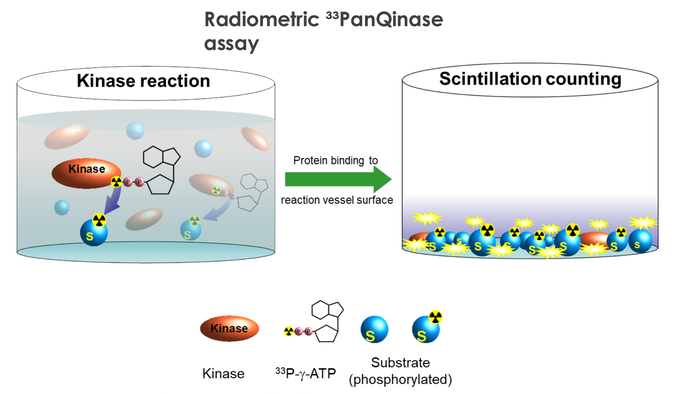
Introduction

Protein kinases are essential for the regulation of many biological processes, e.g. proliferation, differentiation, migration, and apoptosis. Deregulated kinase activity is observed in tumor cells and altered activity of specific kinases is essential for development and progression of cancer and other diseases. Consequently, the regulation of protein kinase activity became a prime target for therapeutic intervention. Intensive research resulted in the development of many small molecule kinase inhibitors (SMI) being clinically approved as targeted therapies. Functional biochemical in-vitro assays are an essential requirement to assess candidate SMIs effects on target kinase activity for drug development. Limited information may be obtained by monitoring direct binding of kinase and compound, while in-vitro kinase activity assays yield more comprehensive data. However, data relevance of both approaches depend on how well the assay setup reflects the mode-of-action of the compounds. Recombinant proteins and generic substrates are broadly used in early development phase protein kinase assays due to consideration of cost and technical feasibility. While some generic substrates are highly artificial and may be considered as phosphate group acceptors only, they have successfully been used for development of numerous clinically approved SMIs. Other kinases are not compatible with generic substrates and require less artificial substrates or even their respective physiological in-vivo substrate for relevant in-vitro activity.

We compared biochemical in-vitro kinase activity assays for the LIM kinase, using either a generic substrate or the published physiological substrate, CFL1.
Several published LIMK inhibitors displayed significant differences in the relative potency when tested with generic or physiological substrate.

Generic substrates for biochemical in-vitro kinase activity assays have been applied successfully in early preclinical drug development in the past. However, our data indicate, that the choice of substrate should be considered carefully, as selecting a more physiologically relevant substrate might significantly increase the relevance and therefore value of the results obtained from early-stage compound development.

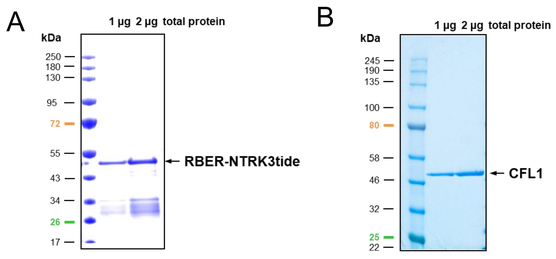


The radiometric, ScintiPlate™ based **33PanQinase** assay setup was used to determine the in-vitro kinase activity of LIMK2 using either recombinant CFL1 or the generic RBER-NTRK3tide as substrates. Kinase and substrate were incubated in presence of ATP containing ^{33}P-γ-ATP as tracer and increasing concentrations of respective LIMK2 kinase inhibitors. After reaction stop, all proteins are immobilized on the reaction vessel surface and the incorporated radioactivity is measured by scintillation counting.

Results

LIMK2 substrates RBER-NTRK3tide and CFL1

Recombinant RBER-NTRK3tide and human CFL1 were expressed in and purified from E.coli by GST-affinity chromatography. RBER-NTRK3tide was identified as suitable substrate for LIMK2 by screening a panel of generic broad-spectrum kinase in-vitro substrates, while CFL1 was selected based on available literature data for LIM kinases.



Generic protein consisting of an N-terminal GST-tag, a fragment of the human retinoblastoma protein RB1, amino acids S_{773}-K_{928} (NP_000312.2) followed by 11 Arg residues and a peptide sequence (VYSTDYYRLFNPS), aa V_{704}-S_{716} of human NTRK3 protein (NP_001012338.1)
MW: 47450 Da

CFL1, full length, amino acids M_1-L_{166} (as in NCBI/Protein entry NP_005498.1), N-terminal GST-HIS6 fusion protein
MW: 47007 Da

Fig. 1: Recombinant RBER-NTRK3tide (A) and CFL1 (B) purified from E.coli by GST-affinity chromatography.
Coomassie stained SDS-PAGE of 1µg and 2 µg of each recombinant protein

Phosphorylation of RBER-NTRK3tide and CFL1 by LIMK2

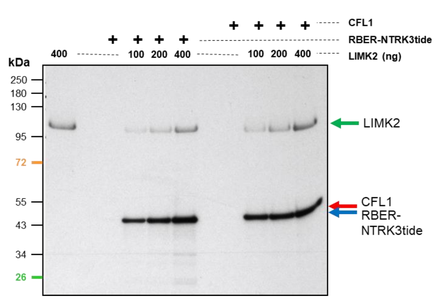


Fig. 2: Recombinant RBER-NTRK3tide and CFL1 (4 µg/lane) were incubated with increasing amounts of recombinant LIMK2 in presence of radiolabelled ^{33}P-γ-ATP. The proteins were analyzed by SDS-PAGE and the gel was exposed to X-ray film to detect radioactive protein bands (5 hours exposure time).

Phosphorylation of CFL1 by LIMK2 in the 33PanQinase assay

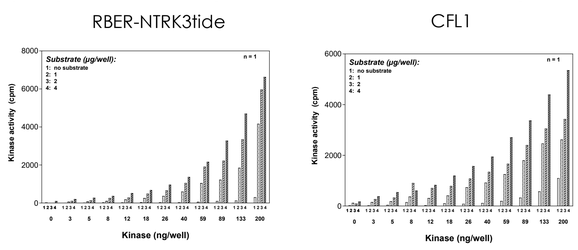
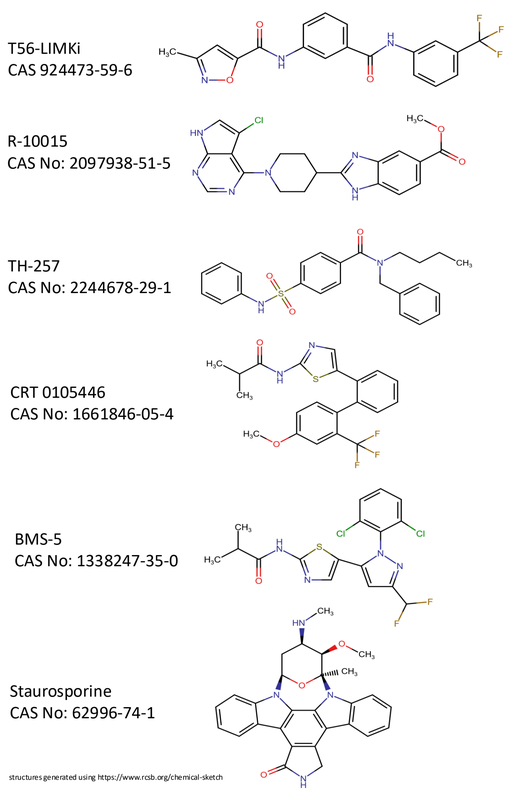


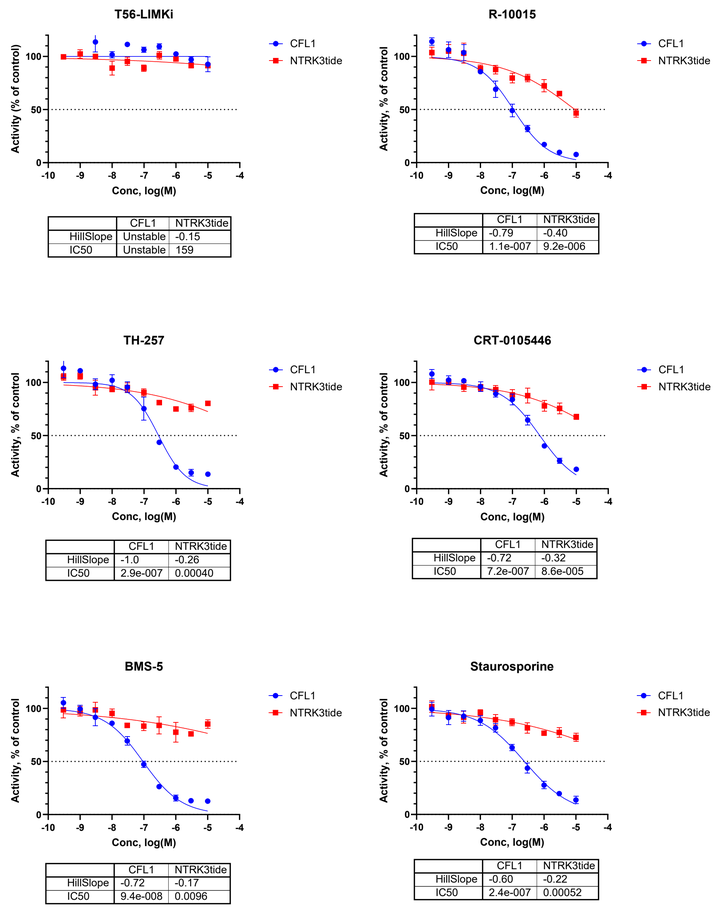
Fig. 3: RBER-NTRK3tide and CFL1 were incubated with increasing amounts of LIMK2. Radioactivity transferred to the respective substrates was determined by scintillation counting.

Compounds tested for LIMK2 inhibition with substrates RBER-NTRK3tide and CFL1



Kinome Profiling result for BMS-5 specificity

LIMK2 IC_{50} determination for six small molecule kinase inhibitors



	CFL1	NTRK3tide
HillSlope	Unstable	-0.15
IC50	Unstable	159

	CFL1	NTRK3tide
HillSlope	-0.79	-0.40
IC50	1.1e-007	9.2e-006

	CFL1	NTRK3tide
HillSlope	-1.0	-0.26
IC50	2.9e-007	0.00040

	CFL1	NTRK3tide
HillSlope	-0.72	-0.32
IC50	7.2e-007	8.6e-005

	CFL1	NTRK3tide
HillSlope	-0.72	-0.17
IC50	9.4e-008	0.0096

	CFL1	NTRK3tide
HillSlope	-0.60	-0.22
IC50	2.4e-007	0.00052

Fig. 5: Potency of LIMK2 inhibition was tested for 5 LIMK specific inhibitors and one broad spectrum kinase inhibitor with two substrates.
LIMK2 [13 nM] was incubated with RBER-NTRK3tide (80 µg/ml) and CFL1(80 µg/ml) in presence of radiolabelled ^{33}P-γ-ATP, ATP concentration corresponding to the apparent (assay specific) K_M[ATP] for LIMK2 of 0.1 µM. In-vitro activities were determined in presence of increasing concentrations of the respective inhibitory compound (10-point semi-logarithmic dilution steps), each concentration step determined in triplicates.

Fig. 4: Specificity of BMS-5 for LIM kinases
BMS-5 was profiled at a concentration of 1E-06M against a panel of 355 human wt kinases in an in-vitro kinase activity assay (Reaction Biology Wild Type 355 Panel) to determine its specificity.
BMS was found to inhibit 1.7% of the 355 kinases tested by more than 50%, including LIMK1 and LIMK2 (tested with substrates CFL2 and CFL1 respectively).

Summary

- LIMK2 phosphorylates the generic protein substrate RBER-NTRK3tide comparably well as the physiological protein substrate CFL1
- Phosphorylation signal and biochemical parameters in the ^{33}PanQinase assay are comparable for CFL1and the generic substrate RBER-NTRK3tide
- In direct comparison, significant effects on their respective potency were detected for four LIMK2 specific and one broad spectrum inhibitor. One LIMK2 inhibitor (T56-LIMKi) did not show inhibitory activity with either substrate
- The potency of the tested LIMK2 small molecule inhibitors was dependent on the used in-vitro substrate. Published LIMK2 inhibitors (e.g. BMS-5) exhibited inhibitory potential only with substrate CFL1 but not with the equally well phosphorylated substrate RBER-NTRK3tide

Literature:
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