Comparative Biochemical Evaluation of Small-Molecule Inhibitor Efficacy on CDK/Cyclin Complexes Across Diverse Mammalian Species

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Introduction

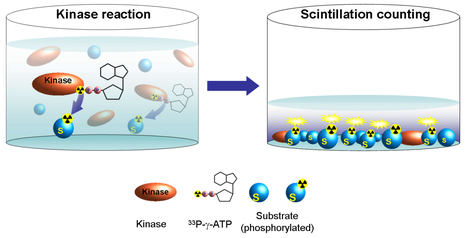
Cyclin-dependent kinases (CDKs) and their associated cyclins are central regulators of cell cycle progression and transcriptional control. Dysregulation of CDK/Cyclin complexes is a hallmark of many cancers, driving uncontrolled proliferation and tumor development. Consequently, these complexes have become critical targets in oncology, with CDK4/6 inhibitors already established as a cornerstone in the treatment of e.g. hormone receptor-positive breast cancer and under investigation for other malignancies. Early preclinical development of CDK inhibitors relies on biochemical screening assays to identify small molecules capable of inhibiting CDK/Cyclin activity. These assays, frequently based on kinase activity measurements, enable high-throughput evaluation of compound libraries and provide essential insights into inhibitor potency and selectivity. These approaches are mandatory for rational drug design, guiding the optimization of lead compounds toward improved efficacy and reduced off-target effects.

Despite their obvious limitations in predicting cellular and in vivo responses, these early screening strategies are instrumental in shaping the current generation of CDK- and other kinase-targeted therapeutics. In subsequent development stages, cellular and in vivo model systems are employed to validate inhibitor activity and assess pharmacodynamics and toxicity. Many of these models are based on non-human mammalian species, such as murine or primate systems. These studies are essential for bridging the gap between biochemical screening and clinical application, ensuring that candidate molecules demonstrate efficacy and safety before entering human trials.

However, despite the importance of such data, it is rarely assessed in early biochemical screening whether results from human and non-human assays are consistent.
Such early biochemical evaluation of potential differences in the effects of drug candidates on the kinase target from different species could generate valuable insights to select the most relevant cellular or in-vivo model systems for advanced drug-development.

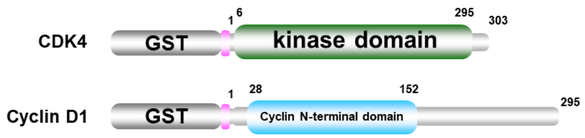
Here, we present comparative biochemical data for late-stage development or already approved CDK inhibitors tested against CDK/Cyclin complexes from human, rat, mouse, dog and primate origin, focusing on CDK4/CycD1. Notably, differential inhibitory potency was observed for several compounds, including palbociclib, which showed an approximately ten-fold difference in CDK4/CycD1 inhibition between human and murine enzymes.

Principle of the assay



Final assay conditions:
70 mM HEPES pH 7.5
3 mM MgCl2
3 mM MnCl2
3 µM Na-orthovanadate
1.2 mM DTT
1 % DMSO
[ATP]=KM (app.) of respective kinase substrate/ kinase at variable concentration

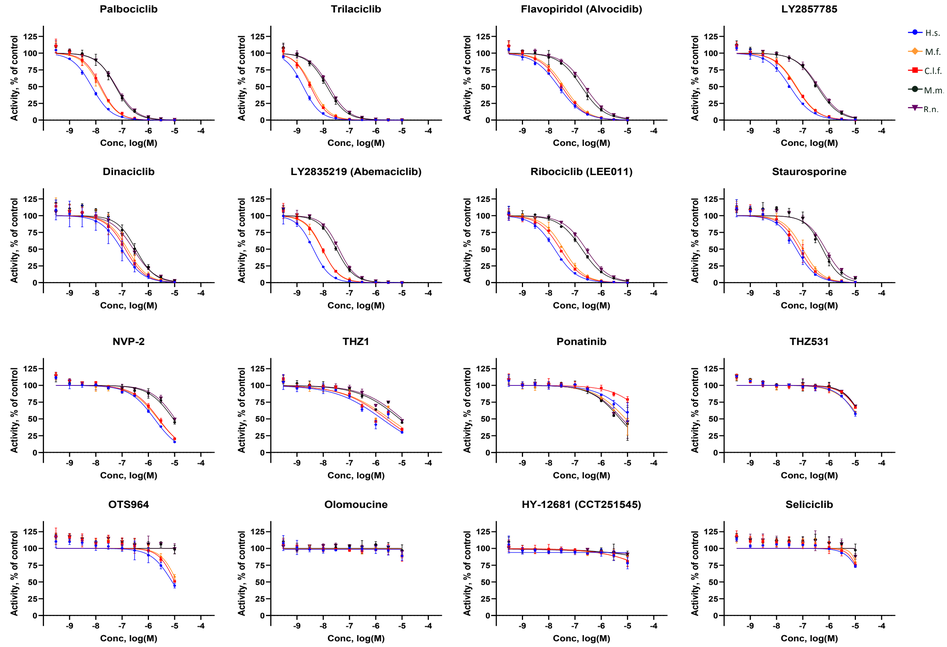
The radiometric, ScintiPlate™-based **^{33}PanQinase™** assay setup was used to determine the in-vitro activity of CDK4-CycD1 complexes from various species.
Kinase and substrate were incubated in presence of ATP containing ^{33}P-g-ATP as tracer.
After reaction stop, proteins were immobilised on the reaction vessel surface and the incorporated radioactivity was measured by scintillation counting.

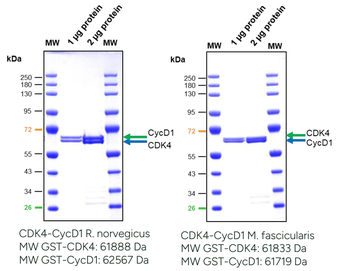


Schematic overview of recombinant CDK4 and its associated Cyclin D1.
CDK4-CycD1 complexes were produced by recombinant expression in insect cells using the Baculo Virus Expression System. Complexes were purified under identical, native conditions. All recombinant CDK4-CycD1 complexes from the different mammalian species were constructed identically with an N-terminal GST-tag/protease cleavage site.
Amino acid positioning according to UniProt, human sequence, accession numbers P11802/P24385

Results

IC50 values for 16 small molecule inhibitors using CDK4-CycD1 complexes from 5 mammalian species



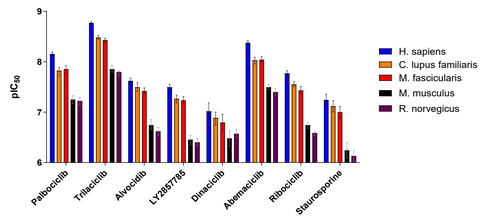


CDK4-CycD1 R. norvegicus
MW GST-CDK4: 61888 Da
MW GST-CycD1: 62567 Da

CDK4-CycD1 M. fascicularis
MW GST-CDK4: 61833 Da
MW GST-CycD1: 61719 Da

Figure 1. Recombinant proteins. The ORFs of CDK4 and Cyclin D1 of H. sapiens, M. fascicularis, C. lupus familiaris, M. musculus and R. norvegicus were co-expressed as N-terminally GST-tagged proteins using the Baculo Virus Expression System (BVES). Recombinant proteins were purified using GST-affinity chromatography under native conditions. Purity of all preparations was comparable and in the range of >90% by densitometric analysis of a 2 µg sample. Example protein preparations shown for CDK4-CycD1 of R. norvegicus and M. fascicularis species origin.

pIC$_{50}$ values for CDK4-CycD1 from different mammalian species



Compound	H. sapiens IC50 (M)	C. lupus familiaris IC50 (M)	M. fascicularis IC50 (M)	M. musculus IC50 (M)	R. norvegicus IC50 (M)
OTS964	8.1E-06	1.0E-05	>1E-05	>1E-05	>1E-05
THZ531	>1E-05	>1E-05	>1E-05	>1E-05	>1E-05
NVP-2	1.7E-06	2.5E-06	2.3E-06	8.9E-06	>1E-05
Trilaciclib	1.7E-09	3.3E-09	3.7E-09	1.4E-08	1.6E-08
Alvocidib	2.4E-08	3.2E-08	3.8E-08	1.8E-07	2.4E-07
LY2857785	3.2E-08	5.4E-08	5.8E-08	3.5E-07	4.0E-07
Palbociclib	7.0E-09	1.5E-08	1.4E-08	5.6E-08	6.0E-08
Seliciclib	>1E-05	>1E-05	>1E-05	>1E-05	>1E-05
Dinaciclib	9.6E-08	1.3E-07	1.6E-07	3.3E-07	2.7E-07
Olomoucine	>1E-05	>1E-05	>1E-05	>1E-05	>1E-05
Abemaciclib	4.2E-09	9.3E-09	9.1E-09	3.2E-08	4.0E-08
Ribociclib	1.7E-08	2.8E-08	3.7E-08	1.8E-07	2.6E-07
HY-12681	>1E-05	>1E-05	>1E-05	>1E-05	>1E-05
THZ1	1.7E-06	2.5E-06	3.4E-06	8.2E-06	1.0E-05
Ponatinib	>1E-05	>1E-05	8.5E-06	5.2E-06	6.2E-06
Staurosporine	5.7E-08	7.6E-08	1.0E-07	5.7E-07	7.4E-07

Figure 2. Comparison of the potency of selected small molecule inhibitors. pIC$_{50}$ values were used for better visualization of the differences in inhibition potency. Error bars represent the 95% confidence interval of the IC$_{50}$ calculation result generated by GraphPad Prism software 11.0.0.

Figure 3. A radiometric protein kinase activity assay (^{33}PanQinase™ Activity Assay) was used for measuring the kinase activity of the five CDK4-CycD1 complexes. All kinase assays were performed in 96-well ScintiPlates™ from Revvity in a 50 ml reaction volume. The reaction cocktail was pipetted in four steps in the following order:

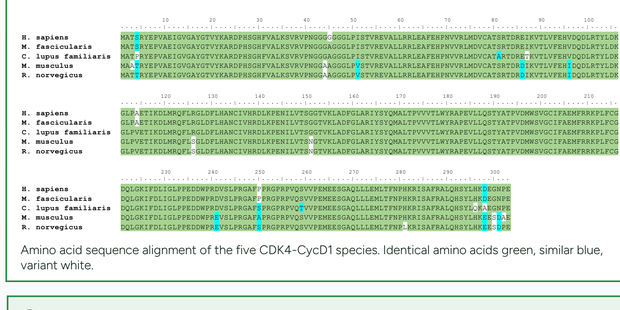
- 25 ml of assay buffer (standard buffer/[g-33P]-ATP)
- 10 ml of ATP solution (in H2O)
- 5 ml of test compound (in 10 % DMSO)
- 10 ml of enzyme/substrate mixture

The assay contained 70 mM HEPES-NaOH pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 mM Na-orthovanadate, 1.2 mM DTT, 50 µg/ml PEG_{20000}, 3 µM ATP (corresponding to the apparent ATP-K_m of CDK4-CycD1), [g-^{33}P]-ATP (approx. 5 x 10^5 cpm per reaction as tracer), 12 nM CDK4-CycD1, and 27.5 µM substrate (RBER-CHKtide).
IC$_{50}$ value calculation was performed with GraphPad Prism software 11.0.0 using model non-linear fit, variable slope, least squares fit, top fixed at 100%, bottom fixed at 0%, n=2.

Sequence Alignments

Cyclin D1

CDK4



Amino acid sequence alignment of the five CDK4-CycD1 species. Identical amino acids green, similar blue, variant white.

Summary

- 16 small molecule inhibitors with activity towards CDKs have been tested against CDK4-CycD1 complexes from 5 mammalian species
- Species dependent differential inhibition potency of up to one order of magnitude was observed for several inhibitors
- Inhibitors showed lowest and similar potency towards rat and mouse CDK4-CycD1
- Human complexes were most potently inhibited
- Dog and monkey complexes showed mostly similar or slightly lower sensitivity compared to human complexes

Conclusion

- Potency of small molecule inhibitors may differ relevantly for the same enzyme from different species. Careful evaluation of model systems in later stages of drug development should take such differences into consideration

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