IC_{50} profiling against 320 protein kinases: Improving the accuracy of kinase inhibitor selectivity testing

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Introduction

Protein kinases belong to one of the largest families of evolutionarily related proteins. The human genome encodes more than 500 kinases. Due to the structural similarity, especially within the ATP-binding site, many kinase inhibitors show limited selectivity. Still, sufficient selectivity within the human kinases is critical e.g. to reduce the risk of adverse side effects during treatment.

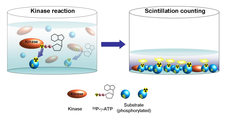
Therefore, measuring and improving the selectivity of a compound within the kinome is of pivotal importance during drug discovery and optimization phase in the development of therapeutically relevant kinase inhibitors.

Broad profiling of kinase inhibitors in biochemical activity assays of several hundred kinases is nowadays well established. Usually, kinase profiling is done using one or two concentrations of a test compound and measurement of the relative inhibition of the kinase activity compared to a high and low control.

However, due to the limited dynamic range of this approach, and the challenge of selecting the most appropriate compound concentration, this profiling approach oftengives limited information with respect to the potency of compounds against on- and off-target kinases.

We set up an IC50 kinase profiling approach that consists of measuring the impact of a compound on the activity of 320 human protein kinases at six different concentrations with the ^{33}PanQinase™ assay format. Here, we present data showing the effect of compound concentration on the selectivity score in traditional profiling settings. IC50 kinase profiles of different approved and clinical stage kinase inhibitors demonstrate that an IC50 based profiling allows a more accurate determination of selectivity of a compound based on the comparison of the IC50 values against the on-target kinases in relation to the IC50 values of the off-target kinases providing significantly improved guidance in the further optimization of the test compound.

^{33}PanQinase™: Principle of the assay



The assay is based on radiolabelled ^{33}P-γ-ATP. Kinase and substrate are incubated in presence of ATP containing ^{33}P-γ-ATP as tracer in 96 well FlashPlates (Perkin Elmer). After stopping the kinase reaction, the proteins are immobilized to the plate surface and bound radioactivity is quantified by scintillation counting.

Assay Procedure:

All assay steps are performed using a robotic pipetting system 96 well FlashPlate per well:

- 10 µl ATP/ ^{33}P-γ-ATP mix
- 25 µl 2.5x assay buffer
- 5 µl compound in 10% DMSO
- 10 µl kinase-substrate mix
- - mix on a plate shaker
- - incubate for 60 min at 30°C
- - stop reaction with 50 µl 2% (v/v) H_3PO_4
- mix on a plate shaker
- wash 2 times with 200 µl 0.9% (w/v) NaCl
- count dry plate with a scintillation counter

Final assay conditions:

- 70 mM HEPES pH 7.5
- 3 mM $MgCl_2$
- 3 mM $MnCl_2$
- 3 µM Na-orthovanadate
- 1.2 mM DTT
- 1 % (v/v) DMSO
- ATP*, substrate and kinase at variable concentration

**: [ATP]: apparent K_m[ATP] of the respective kinase*

Final assay conditions were modified for those kinases requiring specific cofactors for kinase activity.

Kinase and substrate combinations are selected for optimal results under the assay conditions described above. Kinase concentration is typically 1-40 nM (assuming 100% pure and active protein preparations), substrate concentration is 10-30 times higher than the kinase concentration.

Results

A: Selectivity of Crizotinib and Dinaciclib at six concentrations

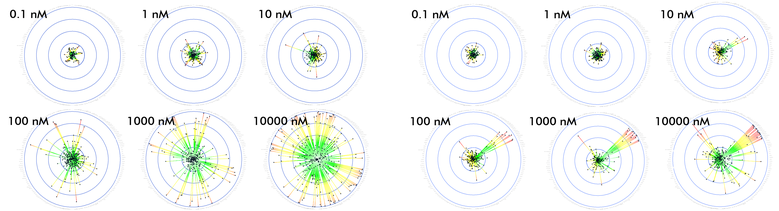
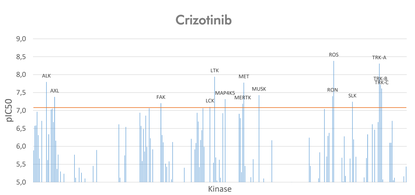
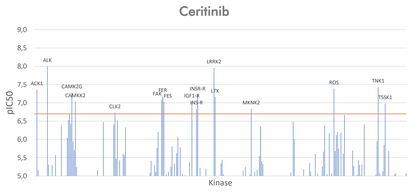


Figure A: 320 kinases were tested with Crizotinib (left) and Dinaciblib (right) in 6 concentrations. For each compound concentration, the percent inhibition was determined for the respective kinase relative to the non-inhibited positive control. The circles represent from the inside 25, 50, 75, and 100 % inhibition.

B: Selectivity of three compounds based on IC_{50} values





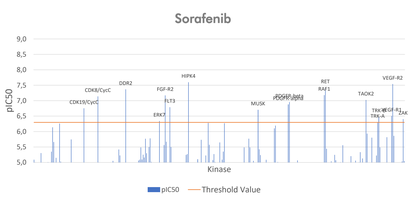


Figure B: IC_{50} values were determined for compound Crizotinib, Ceritinib, and Sorafenib by determining the residual activities in the presence of 6 increasing, logarithmically diluted concentrations of the respective compounds. From these six values IC_{50} values were calculated using Graphpad Prism 5.04. The mathematical model used was "Sigmoidal response (variable slope)" with parameters "top" fixed at 100 % and "bottom" at 0 %. pIC_{50} (negative decimal logarithm of IC_{50} values) were depicted in the graphs for better visualization of the range of the compound potency. An arbitrary threshold value 20 fold below the pIC_{50} of the most sensitive kinase was set to select kinases showing significant inhibition by the respective compound. Red circles indicate the primary target of the respective compound.

C: Sorafenib selectivity scores at single concentrations relative to VEGF-R2 inhibition

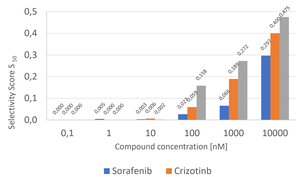


Figure C: Selectivity scores were determined for Sorafenib, Crizotinib and Ponatinib for a panel of 320 protein kinases at different compound concentrations based on residual activities. Selectivity scores were calculated using the formula:

(count of data points < 50 %) / (total number of data points).

D: Selectivity scores relative to target IC_{50} value

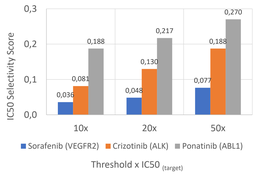


Figure D: Selectivity scores were determined for Sorafenib, Crizotinib and Ponatinib using a panel of 320 protein kinases based on IC_{50} values calculated from six different compound concentrations in the assay. Selectivity scores were compared with different threshold values of 10/20/50 fold of the target IC_{50} value based on the calculation:

count of IC_{50} values < threshold) / total number of IC_{50} values determined

E: Reproducibility of pIC_{50} profiles

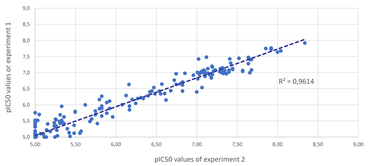


Figure E: The pIC50 profile of compound Ponatinib was determined twice in independent experiments and the resulting pIC50 values for each kinase were compared

Summary & Conclusion

1. Apparent kinase inhibitor selectivity depends on the compound concentration used to measure percental inhibition of kinases
2. Accuracy and reproducibility of profiling are limited by the dynamic range and assay variability of single compound concentration measurement.
3. IC_{50} selectivity profiling based on six compound concentrations represents a significant improvement to assess differences in the potency of a given compound and allows a more precise judgment of the selectivity of protein kinase inhibitors