

A multiple assay strategy for kinase inhibitor screening

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Aim of the study:

Using a recently performed high-throughput screen for inhibitors of a Receptor Tyrosine Kinase (RTK) as an example, our aim was to investigate whether a combined biochemical/cellular assay cascade delivers suitable information for lead identification.

Conclusions:

- Both biochemical assays had excellent Z' -values (>0.7) and IC_{50} -values, which correlated well.
- Reference compounds had comparable IC_{50} -values in all three assays
- However, only a small overlap between the biochemical and cellular RTK inhibition was found (only 7 % of the 350 tested compounds delivered an IC_{50} in the cellular RTK assay).
- Potential explanations: Compounds do not penetrate the cell (CaCo prediction points towards this hypothesis)
- Different substrates in the biochemical and cellular tests (peptide substrate vs. autophosphorylation in the cell)

Materials and methods

	Alphascreen	HTRF
RTK	0.5ng	0.5ng
Compound	10 μ M	10 μ M
Incubation	30 min	30 min
Poly(GluTyr)-biotin	1nM	3nM
ATP	3 μ M	3 μ M
Incubation	30 min	30 min
Detection P-Tyr100 kit [5 μ g/ml]		PT66-Eu 5ng/SA-XI ^{ent} 3nM
Incubation	16 hours	
Analysis Fusion (Perkin Elmer)		Rubystar (BMG)

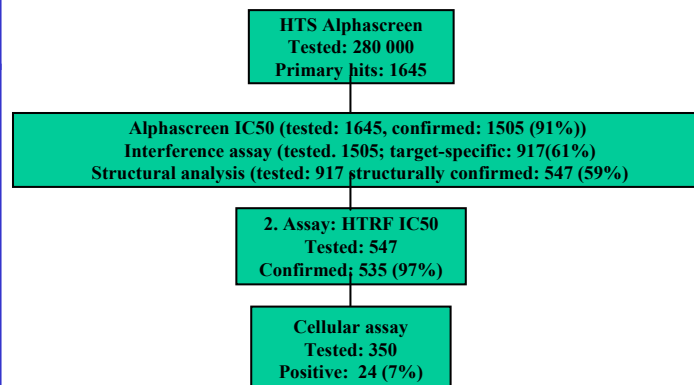
Alphascreen interference assay

Streptavidin-donor beads	6 μ g/ml
Compound	10 μ M
Incubation	30 min
Biotin-acceptor beads	6 μ g/ml
Incubation	16 hours

Cellular phosphorylation assay

3500 cells/well in black 96-well plate
6 hours incubation
Serum starvation for 16 hours
Compound addition (4 hours)
Growth factor stimulation (5 min.)
Cell fixation, permeabilization
Anti-p-RTK (rabbit) antibody for 16 hours
wash, add anti-rabbit antibody-Alex488; 1 hour
wash, add Syto64 (30min), wash, read on Acumen Explorer

Screening flow



Assay cascade from HTS to cellular compound activity. Numbers show the tested and confirmed compounds in the individual assays.

Results I

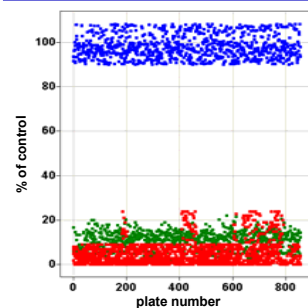


Fig1: High-throughput screen for RTK inhibitors using Alphascreen assay format. 280 000 compounds were tested @10 μ M concentration which results in 1645 primary hits (0.6%). Shown are the normalized values (control, standard, selected actives) over the tested plates; overall Z' value was 0.78.

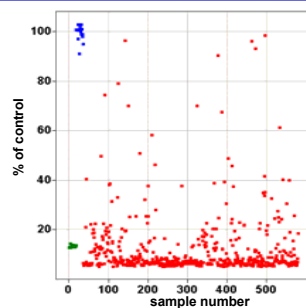


Fig.2: Results of the HTRF RTK assay. 545 compounds (target-specific and structurally confirmed) were tested in the HTRF RTK assay. 537 of them were found to be active. Shown are the normalized values (control, standard, samples); overall Z' value was 0.8.

Results II

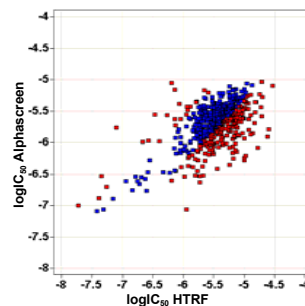


Fig.3: Correlation of $logIC_{50}$ values obtained by Alphascreen and HTRF RTK assay. Shown are the results of those 537 compounds which were active in both assay formats. Blue compounds showed the best correlation ($n=350$; $r^2=0.8$) and were selected for cellular testing.

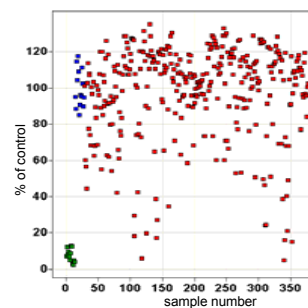


Fig. 5 Results of the cellular RTK assay. Shown are the normalized results of those 350 compounds which showed the best correlation between biochemical assays. (Control, standard, samples); overall Z' value was 0.65.

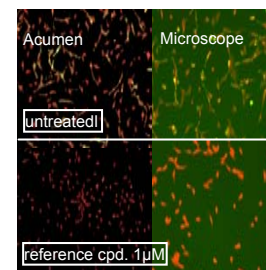


Fig. 4 Cellular RTK assay. The cellular RTK assay was run on our recently installed Acumen Explorer. RTK autophosphorylation is detected in the green-, whole cellular staining in the red channel. Explorer- and microscope view (both connected via the Acumen „locator“).

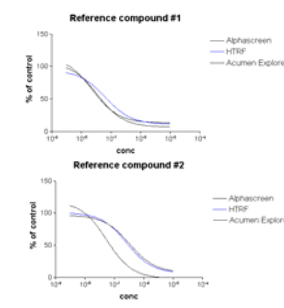


Fig. 6: Activities of reference compounds in all three assays. Both reference compounds show highly comparable activities (IC_{50} values in the nanomolar ranges) in both the biochemical and cellular assays. Compound no.2 has a slightly higher activity in the cellular assay.