CELLULAR PHOSPHORYLATION ASSAYS - IMPORTANT TOOLS FOR THE CHARACTERIZATION OF PROTEIN KINASE INHIBITORS

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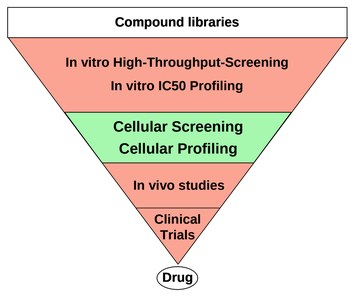
Introduction

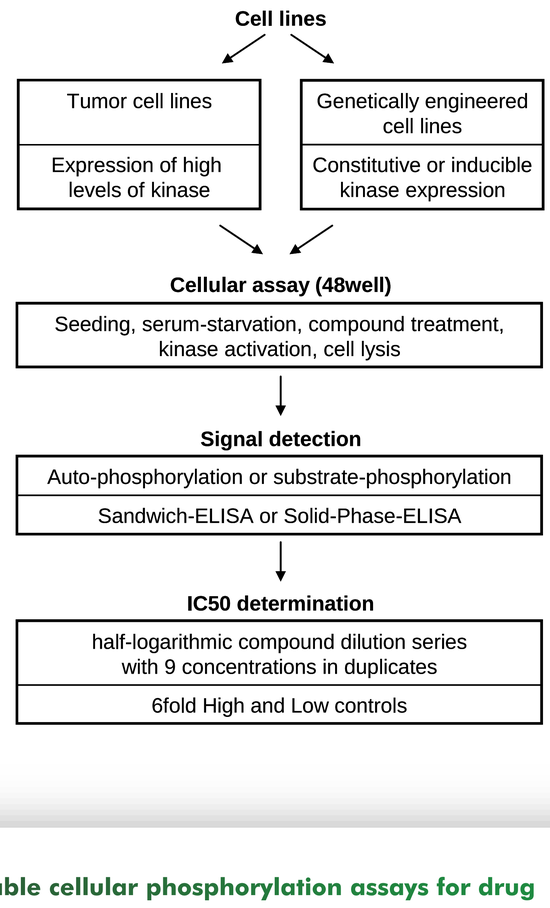
Today more than 30 protein kinases of the human kinome were validated as causal molecular targets in cancer diseases. Therefore a main focus of drug development is the identification of specific protein kinase inhibitors. In order to find compounds which are able to inhibit one or more of the known target kinases libraries of thousands to millions of substances based on in silico drug design, combinatorial chemistry or fractionation of natural products were analyzed by in vitro High-Throughput-Screening (HTS) with recombinant target kinases. The selectivity of hits from HTS could be explored by in vitro IC50 Profiling with a broad panel of protein kinases.

Compounds showing an interesting inhibition profile should be further analyzed by cellular screening for their inhibitory characteristics concerning the target kinases in a cellular background and finally the determination of cellular inhibition profiles helps to decide which compounds will be selected for time-and-cost-intensive in vivo studies.



Here we present cellular results of the drug development of inhibitors of the receptor tyrosine kinase FLT3. These assays are a part of the Reaction Biology technology platform which is offered as fee-for-service for drug discovery.

Concept of the establishment of a cellular phosphorylation assay

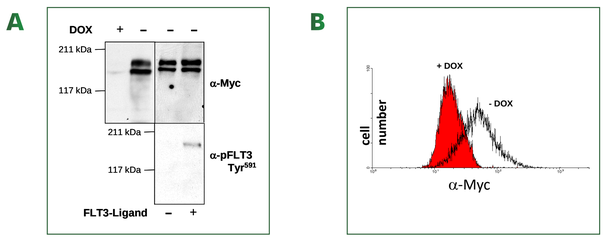


Available cellular phosphorylation assays for drug discovery at Reaction Biology:

Kinase	Cell line	Transfected	Type of Kinase	Origin: Kinase/Cell line
Available as Fee-for-Service:				
AKT1	Rat1-myrAKT1	yes	Ser/Thr Kinase	human/rat
Aurora-B	HT-29	no	Ser/Thr Kinase	human/human
B-RAF-VE	Rat1-B-RAF-VE	yes	Ser/Thr Kinase	human/rat
EGF-R	A431	no	RTK	human/human
EPHB4	MEF-EPHB4	yes	RTK	human/mouse
ERBB2	NIH3T3-ERBB2	yes	RTK	human/mouse
FAK	MEF-FAK	yes	Non-receptor TK	human/mouse
FLT3-wt	MEF-FLT3-wt	yes	RTK	human/mouse
IGF1-R	MEF-IGF1-R	yes	RTK	human/mouse
PDGFR-beta	NIH3T3	no	RTK	mouse/mouse
TIE2	CHO-TIE2	yes	RTK	human/hamster
VEGF-R2	HUE	no	RTK	human/human
Offered soon:				
c-MET	MKN-45	no	RTK	human/human
c-Src	MEF-cSrc	yes	Non-receptor TK	human/mouse
FLT3-D835Y	MEF-FLT3-D835Y	yes	RTK	human/mouse
FLT3-ITD	MEF-FLT3-ITD	yes	RTK	human/mouse
MEK1	Rat1-B-RAF-VE	yes	Ser/Thr Kinase	mouse/rat

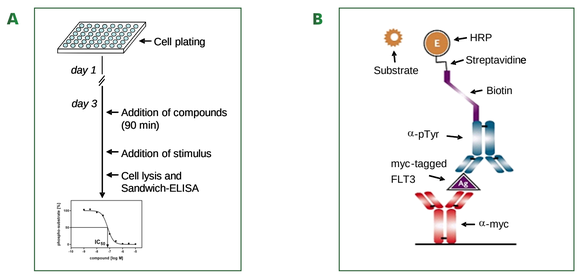
Results

01. Generating a cell line inducibly expressing myc-tagged receptor tyrosine kinase FLT3



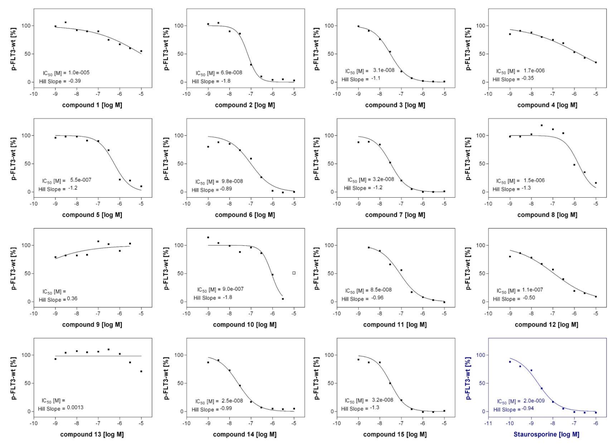
A murine embryonal fibroblast cell line was stably transfected with human myc-tagged FLT3, expressed under the control of a tet-regulator. In the absence of Doxycylin the cells express homogenously high levels of myc-tagged FLT3 analyzed by Western Blot (A) and FACS (B). Autophosphorylation of FLT3-Tyr591 could be activated by stimulation with FLT3-Ligand (A).

02. Establishment of a cellular assay in 48 well format and measurement of FLT3 autophosphorylation



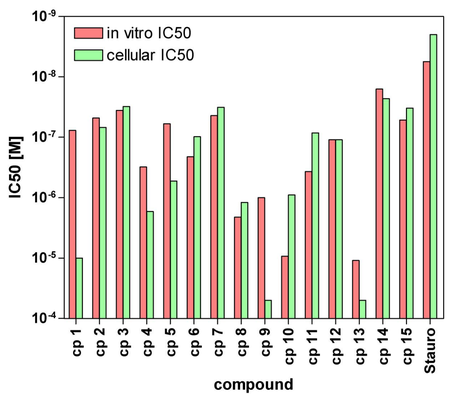
The development of a cellular FLT3 assay was performed in a 48 well format. Different assay parameters like cell-density, ligand-concentration and stimulation-time were optimized for assay robustness with regard to FLT3 autophosphorylation of unstimulated vs. ligand-stimulated cells. (A) Schematic overview of the assay procedure. (B) Autophosphorylation of FLT3 is measured via sandwich-ELISA.

03. Cellular Screening of FLT3 inhibitors



16 compounds were analyzed with the cellular 48-well FLT3 assay. IC50 values were calculated by a non-linear regression with a sigmoidal dose-response with variable slope (GraphPadPRISM®4.03 software). Mean values of each duplicate were presented as %-values of non-pretreated but ligand-stimulated cells.

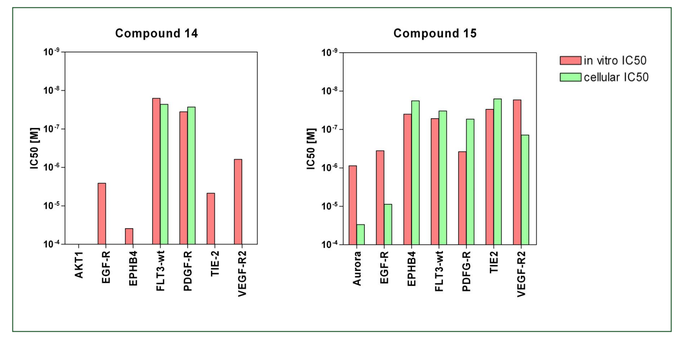
04. Comparison of in vitro vs. cellular IC50 values of FLT3 inhibitors



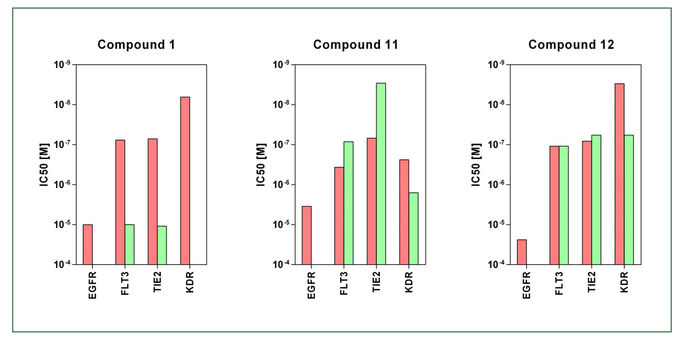
The majority of the compounds showed cellular IC50 similar to the respective in vitro IC50 value. However, the in vitro inhibitory potency of some compounds differed significantly to the cellular inhibitory potency (e.g. cp 1, cp 9 and cp 10).

05. Cellular Profiling of FLT3 inhibitors

In a cellular context the selectivity profile of a compound can dramatically change.



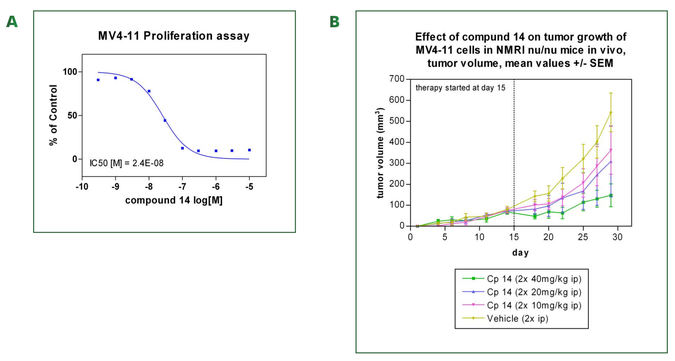
The inhibitory potency of two compounds were analyzed with 7 different protein kinases. Compound 14 shows a higher selectivity for the two target kinases in the cells where the cellular profile of compound 15 is very similar to the in vitro one.



Inhibitory profiles of three compounds with 4 kinases. Compound 1 lost almost all of its high inhibitory potency against the three target kinases in the cells, where compound 11 shows a mono-specific cellular profile versus a multi-specific in vitro profile. By contrast, the selectivity profile of compound 12 changed from a mono-specific in vitro profile towards a multi-target cellular profile.

These data reveals the impact of cellular profiling for the determination of the selectivity profile of a protein kinase inhibitor.

06. Inhibition of FLT3-dependent cell proliferation in vitro and in vivo



Compound 14 with a high inhibitory potency at recombinant and cellular FLT3 shows also a strong inhibition of FLT3-dependent cell proliferation and tumor growth of the human leukemia cell line MV4-11. (A) 72h alamar blue assay of compound 14. All data points represent mean values of duplicates and were presented as %-values of non-treated cells. 0% is equal to no cells. (B) MV4-11 cell were transplanted s.c. into nude mice at day 0. From day 15 mice were treated twice per day i.p. with different amounts of compound 14 or with vehicle alone as control.

Conclusions

- Cellular phosphorylation assays of 17 protein kinases with high relevance in cancer diseases were established at Reaction Biology.
- The established assays facilitate the examination of the inhibitory potency of compounds in a cellular context.
- The cellular selectivity profile of a compound can dramatically diverge from the profile determined in vitro.
- A compound showing high inhibitory potency and high selectivity in cellular phosphorylation assays was able to inhibit target kinase dependent tumor growth in vivo.

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