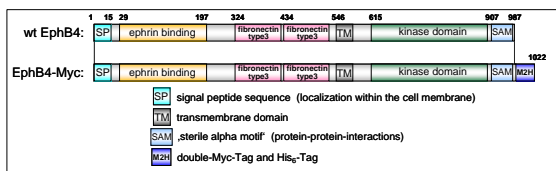




Introduction

The receptor tyrosine kinase EphB4, a member of the large family of Eph (erythropoietin-producing hepatoma amplified sequence) receptors, and its ligand ephrin-B2 are involved in tumor angiogenesis and up-regulation of EphB4 has been shown for different kinds of cancer with partial correlation of EphB4 expression and tumor malignancy. The important role of EphB4 in tumor growth led to many efforts in identification of pharmaceutical relevant compounds which are able to inhibit the kinase activity of EphB4. A pivotal step in the development of new drug candidates is the validation of positive „hits“ from in vitro High-Throughput-Screenings in suitable cellular test systems.



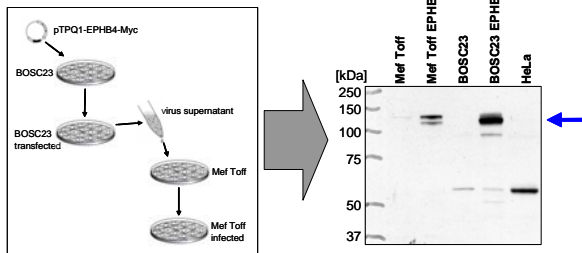
Domain structure of the human wt and myc-tagged EphB4 gene product. The kinase domain is located on the cytoplasmatic side. Amino acid positions are given above the gene product.

Aim of this study

The development of a cellular test system that allows the characterization of compounds inhibiting the receptor tyrosine kinase EphB4.

Results

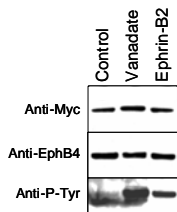
1. Construction of a stable transfected cell line expressing inducible human EphB4 by retroviral transduction



The packaging cell line BOSC23 was transfected with full-length myc-tagged human EphB4 cloned in a retroviral expression vector downstream of the tet-operon. Isolated virus supernatant was used to stably transfect an embryonic mouse fibroblast cell line expressing the tet-repressable transactivator. The retroviral transduction was checked via Western blotting with the myc-antibody 9E10 using lysate from HeLa cells as positive control for the anti-myc staining. Successfully transduced cells were selected by treatment with Puromycine and single-cell clones were isolated via limited dilution.

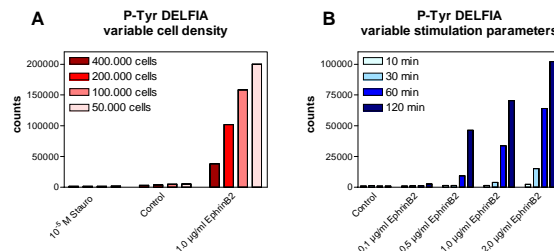
2. Stimulation of the auto-phosphorylation of cellular EphB4

Isolated clones expressing inducible EphB4-Myc were cultured for 24 h in growth-medium and than serum-starved overnight. The cells were either not stimulated, stimulated with orthovanadate or stimulated with mEphrin-B2/Fc chimera. EphB4 receptor auto-phosphorylation was analyzed via Western blotting with the phospho-tyrosine antibody P-Tyr100 (Cell Signaling Technology).



3. Development of a cellular EphB4 assay

In order to obtain a good assay range with regard to EphB4 auto-phosphorylation of unstimulated vs. ligand-stimulated cells different assay parameters like cell-density, ligand-concentration and stimulation-time were optimized. For optimization the assay was initially performed in a 24 well format and later adjusted to 48 well format. Cells were cultured over night, incubated with or without inhibitor, stimulated with mEphrin-B2/Fc chimera and at last lysated with lysis-buffer. Cellular EphB4 was bound via the c-terminal myc-tag on anti-myc 9E10 coated microtiter plates and auto-phosphorylation was detected by ELISA or DELFIA with a phospho-tyrosine specific antibody.



In Fig.A the cells were grown in growth-medium, either incubated with Staurosporine for 90 min at 37°C, non-treated or stimulated with mEphrinB2/Fc for 60 min at 4°C. In Fig.B 100.000 cells were serum-starved over night and stimulated with mEphrinB2/Fc at 4°C.

4. Assay layout for the determination of cellular IC₅₀ values

	compound 1				compound 2			
	1	2	3	4	5	6	7	8
A	low control: 10 ⁵ M Stauro	1e-5	3e-6	1e-6	high control: EphrinB2 1% DMSO	1e-5	3e-6	1e-6
B		1e-5	3e-6	1e-6		1e-5	3e-6	1e-6
C		3e-7	1e-7	3e-8		3e-7	1e-7	3e-8
D		3e-7	1e-7	3e-8		3e-7	1e-7	3e-8
E		1e-8	3e-9	1e-9		1e-8	3e-9	1e-9
F		1e-8	3e-9	1e-9		1e-8	3e-9	1e-9

An assay layout in the 48 well format allows cellular IC₅₀ value determination of two compounds per assay plate. The compounds were applied in a half-logarithmic dilution series and each inhibitor concentration was measured twice. In order to determine the quality of the assay the Z'-factor was calculated using unstimulated cells as low and stimulated cells in absence of inhibitor as high control. For the low control the cells were treated with a high concentration of Staurosporine to reduce background auto-phosphorylation of the EphB4 kinase. Since the compound dilution series is made with 100% DMSO and applied in a rate of 1:100, 1% DMSO was added to the high control.

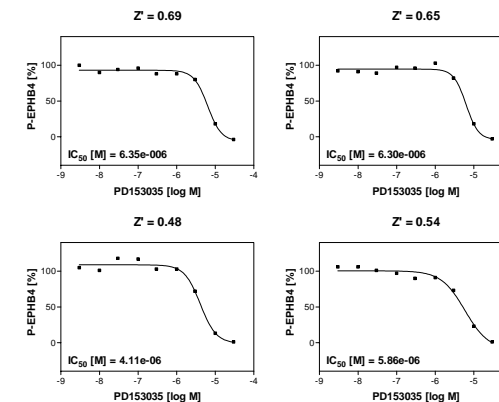
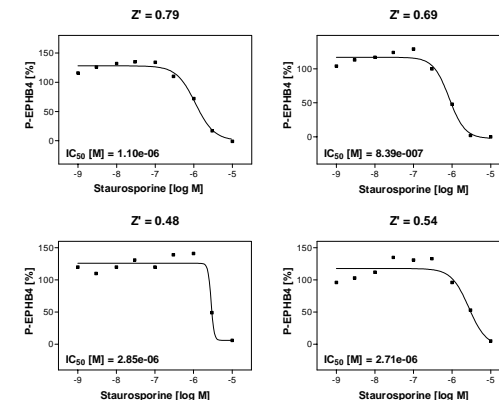
Conclusions

- a cellular test system for the characterization of EphB4 inhibitors was successfully established.
- the functionality of the test system was proved by reproducible determinations of IC₅₀ values of the inhibitors Staurosporine and PD153053 with high Z'-factors indicating the reliability of the results.
- this cellular test system can be used to characterize compounds for their ability to inhibit the kinase activity of EphB4 (for further details see www.proqinase.com).

Acknowledgement

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5. Determination of cellular EphB4 IC₅₀ values of Staurosporine and PD153053



The developed cellular EphB4 test system was validated by measurement of IC₅₀ values of Staurosporine, a broad range protein kinase inhibitor, and PD153053 an inhibitor of EGF receptor kinase (known to inhibit also EphB4) in four independent experiments. Levels of auto-phosphorylation are presented in percentage of the signal of high control. The IC₅₀ values were calculated by a non linear regression of all data points using the GraphPad-PRISM® Software.

6. Summary of cellular EphB4 IC₅₀ value determinations

	Z'-factor	IC ₅₀ (Stauro)	IC ₅₀ (PD153053)
experiment 1	0.79	1.10e-06 M	5.65e-06 M
experiment 2	0.69	0.84e-06 M	6.35e-06 M
experiment 3	0.48	2.85e-06 M	4.11e-06 M
experiment 4	0.54	2.71e-06 M	5.86e-06 M
mean value	0.63	1.88e-06 M	5.49e-06 M

Four independent experiments with the protein kinase inhibitors Staurosporine and PD153053 resulted in reproducible cellular EphB4 IC₅₀ values of about 1.9 and 5.5 μM. The Z'-factors varied from 0.48 to 0.79 with a mean value of 0.63 indicating the high reliability of this cellular test system.