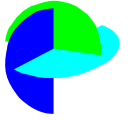


# Development of a novel cellular test system for the characterization of inhibitors of the receptor tyrosine kinase FLT3



## Introduction

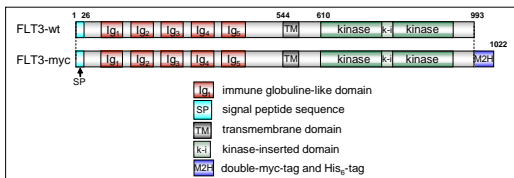
The receptor tyrosine kinase FLT3 (FMS-like tyrosine kinase 3), also known as FLK2 (fetal liver kinase 2), belongs to the 'class III receptor family' like PDGFR-alpha/beta, CSF-1R and c-KIT. These membrane-bound receptors consist of an extracellular domain with 5 immune globuline-like domains and a split intracellular tyrosine kinase domain. FLT3 is activated by binding of the FLT3-Ligand resulting in receptor dimerization. It is mainly expressed in early myeloid and lymphoid progenitor cells and plays an important role in normal haematopoiesis. In haematopoietic malignancies FLT3 expression is found in 70-90% of all cases of acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL). Furthermore, in some of these cases (~30% of AML and ~3% of ALL) activating internal tandem duplications (ITDs) or point mutations within the FLT3 gene were identified. Recently several compounds which inhibit FLT3 kinase activity entered clinical trials.

A pivotal step for the development of new therapeutic FLT3 inhibitors is their characterization in suitable cellular test systems, which should have the following characteristics:

1. A strong expression of the FLT3 protein for the developing of a robust assay for cellular screening in 48 well format.
2. An inducible expression of the receptor tyrosine kinase for the characterization of its tumorigenic potential in vitro and in vivo.
3. In order to study the selectivity of new FLT3 inhibitors for the three different forms of FLT3 (wt, ITD and point mutation), the cellular test systems should have the same cellular genetic background.

## Aim of this study

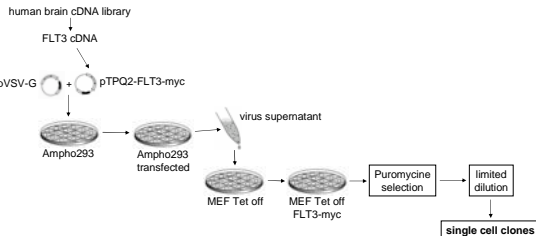
The aim of this study was the development of a cellular test system that allows the identification and characterization of compounds inhibiting the human wild type receptor tyrosine kinase FLT3. For this study following myc-tagged FLT3 construct was used:



Domain structure of the human wt and myc-tagged FLT3 gene product. Amino acid positions are given above the gene product. The split kinase domain is located on the cytoplasmic side.

## Methods and Results

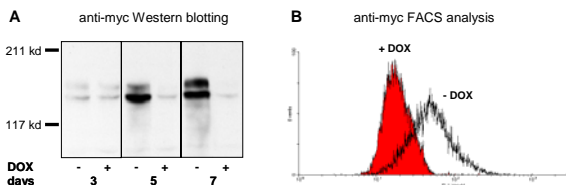
### 1. Construction of a stably transfected cell line expressing Tet-regulated human wild type FLT3 by retroviral transduction



Wild type FLT3 cDNA was isolated via PCR from a human brain cDNA library and cloned with a c-terminal myc-tag in a retroviral expression vector downstream of the tet-operon. The packaging cell line Ampho293 was cotransfected with the FLT3 construct and the pVSV-G vector. Isolated virus supernatant was used to stably transfect an embryonic mouse fibroblast cell line expressing the tet-repressible transactivator. Successfully transduced cells were selected by treatment with Puromycin and single-cell clones were isolated via limited dilution.

### 2. Tet-regulated expression of FLT3-myc in the MEF Tet off FLT3-myc clone #19.9

The tet-regulated expression of a single cell clone was analyzed via Western blotting and FACS analysis during Doxycycline (DOX) depletion. After 7 days the FLT3-myc expression was completely recovered.

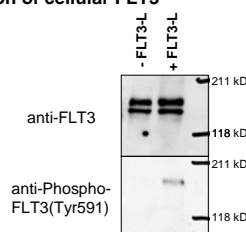


A culture of DOX repressed MEF Tet off FLT3-myc #19.9 cells was continued with or without DOX in the medium. (A) 3, 5 and 7 days after depletion of DOX the expression of FLT3-myc was analyzed via Western blotting with an anti-myc antibody. (B) Cells grown with or without DOX for 7 days were also analyzed via FACS analysis with the same anti-myc antibody.

### 3. Ligand-induced auto-phosphorylation of cellular FLT3

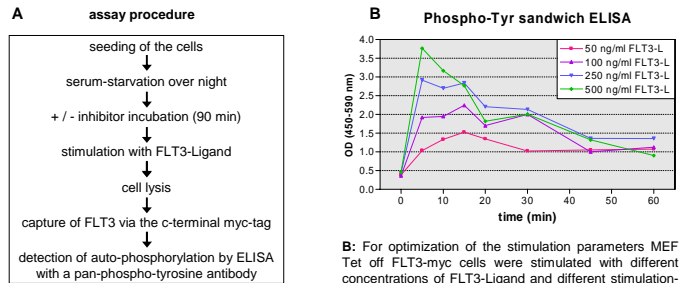
MEF Tet off FLT3-myc cells were cultured for 24 h in growth-medium, serum-starved overnight and stimulated +/- FLT3-Ligand. Auto-phosphorylation of FLT3 was analyzed via Western blotting with a phospho-FLT3-specific antibody (Cell Signaling Technology) recognizing phosphorylated Tyr591 within the FLT3 auto-phosphorylation site.

Ligand binding led to auto-phosphorylation of the upper 150 kDa band of FLT3 which is known to be represented on the cell surface.



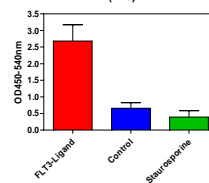
## 4. Development of a cellular FLT3 assay

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 auto-phosphorylation of unstimulated vs. ligand-stimulated cells.



**B:** For optimization of the stimulation parameters MEF Tet off FLT3-myc cells were stimulated with different concentrations of FLT3-Ligand and different stimulation-times at 37°C. FLT3 auto-phosphorylation was measured via a phospho-tyrosine sandwich ELISA. The peak stimulation was reached with a FLT3-Ligand concentration of 500 ng/ml.

### C Phospho-Tyr Sandwich ELISA (N=6)

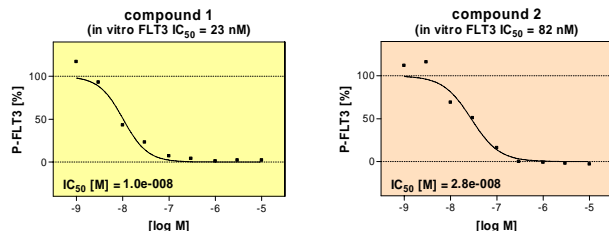


**C:** Background FLT3 auto-phosphorylation of unstimulated cells (assay 'low control') can be reduced with the broad range kinase inhibitor Staurosporine.

MEF Tet off FLT3-myc cells were stimulated with 250 ng/ml FLT3-Ligand for 5 min, untreated or incubated 90 min with Staurosporine with N=6 for each condition.

## 5. Determination of cellular FLT3 IC<sub>50</sub> values of two compounds

The established cellular test system was used to determine cellular FLT3 IC<sub>50</sub> values of two compounds from preclinical drug development, selected based on their in vitro IC<sub>50</sub> values (compound 1: 23 nM and compound 2: 82 nM).



The compounds were applied in a half-logarithmic dilution series with 9 concentrations from 1 nM to 10 µM and each inhibitor concentration was measured in duplicates. Levels of auto-phosphorylation are presented in percentage of non-pretreated but FLT3-Ligand stimulated cells ('high control'). The IC<sub>50</sub> values were calculated by a non linear regression of all data points using the GraphPad-PRISM® 4.0 software. Compound 1 showed a cellular IC<sub>50</sub> of 10 nM and compound 2 of 28 nM.

wild type FLT3 IC<sub>50</sub> values of compound 1 and 2:

	in vitro IC <sub>50</sub>	cellular IC <sub>50</sub>
compound 1	23 nM	10 nM
compound 2	82 nM	28 nM

## Conclusions

- an embryonic mouse fibroblast cell line stably expressing tet-regulated human wild type FLT3 was constructed.
- a cellular assay for the determination of IC<sub>50</sub> values of FLT3 inhibitors was successfully established.
- the functionality of the cellular test system was proofed by determination of IC<sub>50</sub> values of two inhibitors of FLT3.

## Outlook

- the development of three cellular test systems for wt, ITD and point mutated FLT3 in the identical cell background (MEF tet off) will allow the generation of selectivity profiles.
- the inducible expression of the different FLT3 forms could be used to analyze their tumorigenic potential in vivo.

## Acknowledgement

This work was supported by the European Commission (Contract No. LSHB-CT-2004-503467)