Application of biochemical and cellular activity assays for the characterization of inhibitors targeting disease-relevant mutants of the EGF receptor

Totzke, F.; Feger, D.; Weber; T.; Siedentopf, O.; Birkle, M.; Pathe, M; Ehlert, J.E., and Kubbutat, M.

ProQinase GmbH, Engesserstrasse 4, 79108 Freiburg, Germany

Introduction

The occurrence of resistance mutations upon treatment with kinase inhibitors is a common challenge in the clinical application of kinase inhibitors. Development of next-generation kinases inhibitors targeting treatment-induced resistant mutants became a successful approach in cancer therapies, especially in non-small cell lung cancer (NSCLC). Modulation of the potency of an inhibitor against a wild type or mutant form towards an inhibitor targeting a different mutant form might have a significant impact on the overall selectivity towards other kinases.

Here we show a comparative analysis of approved EGFR inhibitors (table 1) of four generations with respect to their biochemical and cellular potency against different EGFR mutants as well as their selectivity against the human kinome.

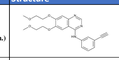
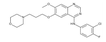
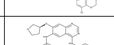
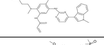
Table 1: Overview of selected EGFR mutation found in NSCLC

(Ref 1-3)

Mutation	Function	Frequency in NSCLC
L853	activating	41%
d746 - 750 d747 - 749 d752 - 759	activating	44%
G718S	activating	6%
L861Q	activating	rare
P753S	activation	very rare
T790M	Resistence mutation	
C797S	Resistence mutation	
L718Q	Resistence mutation	

Table 2: Overview of EGFR inhibitors used in the study

(Ref. 4 modified)

Drug	Structure	Binding mode
Erlotinib (1st Gen. EGFR inh.)		reversible, active conf
Gefitinib (1st Gen. EGFR inh.)		reversible, active conf.
Lapatinib (dual EGFR/HER2)		reversible, inactive conf.
Afatinib (2nd Gen. EGFR inh.)		irreversible (ATP-bdg site; C797))
Osimertinib (3rd Gen. EGFR inh.)		irreversible (ATP-bdg site; C797))
Brigatinib (4th Gen. EGFR inh.)		reversible (ATP-bdg site)

Biochemical EGFR mutant selectivity

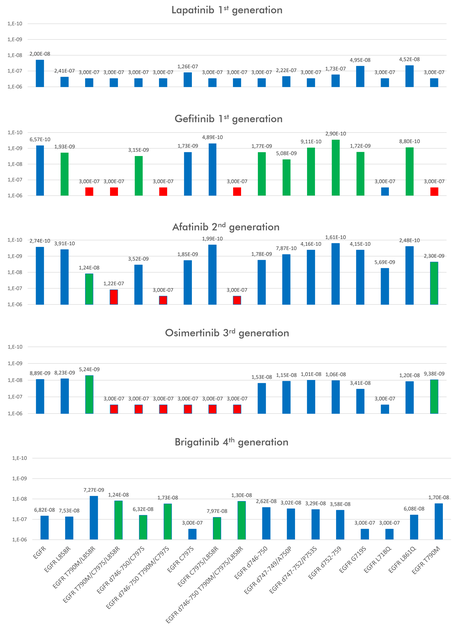


Fig. 1: Biochemical IC50 profiling of different generations of EGFR inhibitors shows mutant specific gain and loss of resistance

Lapatinib, a dual EGFR/HER2 inhibitor approved for the treatment of breast cancer, shows significant potency against wild type EGFR.

Gefinitib (a 1st generation inhibitor for treatment of NSCLC) shows additional potency against activating mutations (green bars). In patients treated with Gefitinib resistance occurs most frequently via mutation T790M (red bars).

2nd and 3rd generation covalent inhibitors **Afatinib** and **Osimertinib** overcome T790M resistence at least in the context of initial activating mutations (green bars). Afatinib as well as Osimertinib function as covalent inhibitors addressing C797 residue of EGFR, therefore the mutation of C797 results in resistance to these two inhibitors (red bars).

4th generation inhibitor **Brigatinib** (reversible inhibitor) overcomes resistance based on C797 mutation in the context of activating mutations and resistence mutation T790M and C797S (green bars).

Methods: EGFR inhibitors were tested in the EGFR IC50 profiler at ten semilog concentations ($3E^{-07}$M – $3E^{-12}$M) using the radiometric ^{33}PanQinase™ assay. IC50 values are depicted in M.

Cellular EGFR mutant selectivity

Fig. 2: EGFR inhibitor testing in the cellular phosphorylation assay confirms biochemical potency

Cellular phosphorylation assay was performed in Rat1 cells expressing a designed transmembrane domain fused to the intracellular domain human EGFR. EGFR mutant and wild-type expression results in constitutive, ligand-independent receptor tyrosine autophosphorylation. Rat1 cells were treated with three different EGFR inhibitors (90 min at 37°C) in serum-free medium with eight concentrations (semilog dilution $1E^{-05}$ – $3E^{-09}$M). Receptor autophosphorylation was assessed via sandwich-ELISA using a kinase-specific capture antibody and an anti-phosphotyrosine detection antibody. IC50 values are depicted in M.

For details of biochemical assays see figure 1.

Kinome selectivity

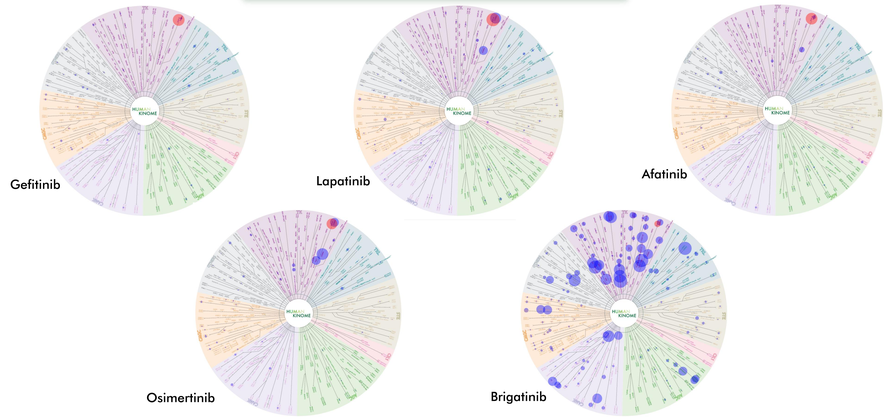


Fig. 3: Gain of potency against C797S mutant results in reduced kinome selectivity of Brigatinib

To normalize the kinome profile on compound potency against EGFR L858R mutant, the concentrations of 5 different EGFR inhibitors was adjusted for profiling on 320 wild type kinases according to previously generated IC50 values against EGFR L858R mutant. Inhibition (in %) of EGFR L858R at the selected profiling concentration (in nM) gave similar results for all compounds tested: Gefitinib 7nM/73%; Lapatinib 200nM/55%; Afatinib 2nM/ 75%; Osimertinib 50nM/ 75%; Brigatinib 100nM/ 42%, data not shown). Circle diameter reflect % inhibition (wild type EGFR is marked in red).
Compounds have been subjected to ^{33}PanQinase™ wild type profiler assay in which the effect on the activity on 320 wild type kinases are measured in a radiometric flash plate assay.

Summary

- Biochemical IC50 profiling of a kinase mutant panel allows the detection of clear differences of compounds targeting the wild type and the mutants form of a target kinase.
- Differences in the potency of inhibitors targeting a specific mutation can be detected in the context of single or multiple mutations.
- The cellular kinase activity phosphorylation assay based on stable transfection of mutant kinases is a valuable tools to compare efficacy of compounds against different mutants of a given target kinases.
- Gearing the selectivity of a kinase inhibitor towards a higher potency against a particular mutation can have significant impact on the overall kinome selectivity.

References

1. Yoneda et al (2019) J UOEH 2 153-163
2. Graham R (2018) Arch Pathol Lab Med 142, 163 -167
3. Yu et al (2013) Clin Cancer Res 19(8), 2240 - 2247
4. Roskowski R. Jr. (2020) Pharmacol Res. 152

Contact Information

Michael Kubbutat, PhD

ProQinase/Reaction Biology
Engesserstr. 4
Freiburg, 79108, Germany

+49-761-769996-0
m.kubbutat@proqinase.com
www.ProQinase.com