Functional analysis of mutant receptor tyrosine kinases involved in cancer pathogenesis

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Abbrevations

μg	10^{-6} Gram
μl	10^{-6} Litre
μM	10^{-6} Mole
AKT	v-akt murine thymoma viral oncogene homolog
ALK	anaplastic lymphoma kinase
AML	acute myeloid leukemia
APS	ammoniumpersulfate
bp	base pair
BCR-ABL	breakpoint cluster region-abelson
cDNA	complementary DNA
c-KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CML	chronic myeloid leukemia
CMML	chronic myelomonocytic leukemia
DMEM	Dulbecco's modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleoside-5'-triphosphate
E.coli	*Escherichiacoli*
EDTA	Ethylene diamino tetraacetic acid
EGFR/ ERBB1/ HER1	epidermal growth factor receptor
ERBB2/ HER2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ENU	N-ethyl-N-nitrosourea
ERK1/2	Extracellular regulated MAP kinase ½
FCS	fetal calf serum
FL	FLT-3 receptor ligand
FLT3	FMS-like tyrosine kinase 3
GIST	Gastrointestinal stromal tumor
IL3	interleukin-3
JAK2	Janus kinase 2
kD	kilo Dalton
mM	10^{-3} Mole
MAPK	mitogen activated kinase-like protein
MEK1/2	MAP kinase-ERK kinase
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PDK1	3'-phosphoinositide-dependent protein kinase 1
PDGFRalpha	platelet-derived growth factor receptor, alpha polypeptide
PIP_2	phosphotidylinositolbisphosphate
PI3K	Phosphotidylinositol 3 kinase
PKC	Protein kinase C
PLCgamma	phospholipase C gamma
RAF	v-raf murine sarcoma viral oncogene homolog
RAS	rat sarcoma viral oncogene homolog
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SH2	src homology 2
SCF	stem cell factor
STAT	signal transducer and actovator of transcription
TKD	tyrosine kinase domain

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1. Introduction

1.1. Targeting oncogenic mutations in receptor tyrosine kinases – an overview

Identification of oncogenic mutations in tyrosine kinases and demonstration of their role in cancer development led to testing of small molecule inhibitors that specifically target mutated kinases[1-4]. The first success was seen with the development of imatinib (Gleevec) which inhibits BCR-ABL tyrosine kinase activity for the treatment of chronic myeloid leukemia (CML)[5-8]. These findings were extended to the use of imatinib for targeting other mutated tyrosine kinases such as c-KIT (systemic mastocytosis and gastrointestinal stromal tumors) and PDGFRalpha (hyper eosinophilic syndrome)[9-13]. Eventually many kinases were found to be mutated in various cancers such as ALK (anaplastic large cell lymphoma and medulloblastoma), FLT-3 (acute myeloid leukemia), EGFR (non small cell lung cancer) or JAK2 (polycythemia vera)[14-17]. This led to the development of novel drugs in the last decade that inhibit specific mutated targets thus holding promise to treat cancer[18].

Development of a targeted drug involves several steps including identification of genetic lesions in cancer patients, establishment of appropriate *in vitro* and *in vivo* models to demonstrate the oncogenic nature of mutations, design and synthesis of drugs that specifically inhibit mutated protein, testing of inhibitors for their efficacy, and translating those findings to the treatment of cancer patients[19]. The search for novel mutations as oncogenic events continue so is the development of new inhibitors that target them[20]. Few examples include PKC412 (FLT-3), gefitinib (EGFR), erlotinib (EGFR), and lapatinib (EGFR and ERBB2)[21-24]. Additionally, multikinase inhibitors (sunitinib and sorafenib) that target more than one kinase showed significant promise in the treatment of some solid cancers[25, 26]. However, development of drug resistance is a frequent phenomenon mainly due to additional mutations acquired by the tumor cells[27, 28]. For example, CML patients display imatinib resistance in advanced stages of the disease due to secondary mutations in the kinase domain of BCR-ABL[29, 30]. This led to the development of new inhibitors that are able to overcome secondary drug resistance[31, 32]. Nilotinib, dasatinib and bosutinib are next generation ABL inhibitors which can inhibit most of the imatinib resistant kinase domain mutants of BCR-ABL[33-35]. Development of drug resistance due to acquisition of kinase domain mutations upon inhibitor treatment was shown with other kinase-inhibitor pairs as

well (EGFR-gefitinib, KIT-imatinib, PDGFRalpha-imatinib and FLT3-PKC412)[36-38]. Thus, the story of targeted inhibitor treatment continues to evolve with new findings offering significant challenges on the path towards superior cancer treatment.

1.2. Receptor tyrosine kinases (RTKs)

Receptor tyrosine kinases are membrane proteins constituted by an extracellular ligand binding domain, a transmembrane domain, an intracellular juxtamembrane domain and a kinase domain[39]. RTKs act as signal transmitters from extracellular region in to the cell thus influencing cellular physiology, in response to environmental conditions[40]. There are several families of RTKs and the classification is based on sequence homology (Figure 1)[39]. As shown in figure 1, RTKs may differ significantly in the ligand binding region that determines the specificity towards particular ligands. Most RTK families have a single intact kinase domain whereas the kinases of the PDGFR and the Flt family of receptors possess a split-kinase domain. The signaling potency of RTKs stems primarily from their kinase activity and is thus tightly regulated under normal physiological conditions.

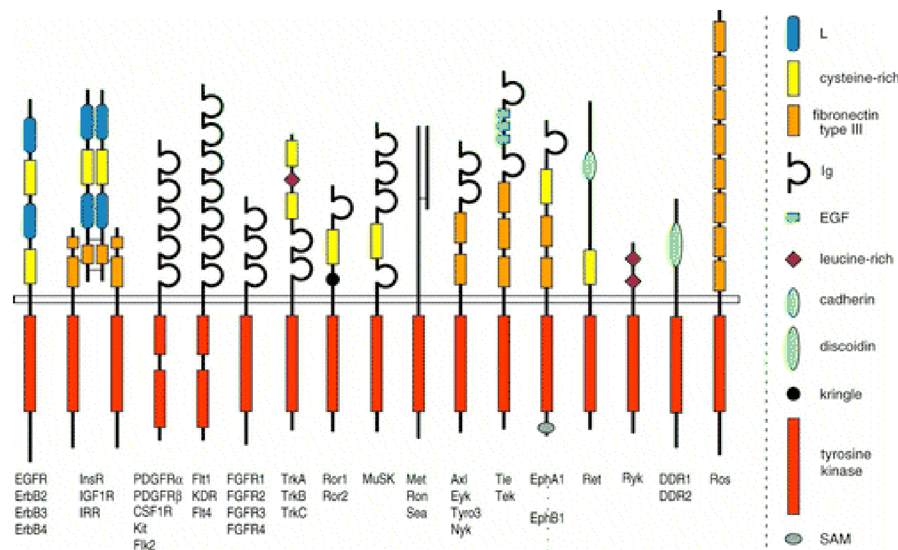


Figure 1. Representation of RTK families.
Several RTK families are represented based on the structural similarities within the members of a family. Respective domains in kinases are indicated. Representive kinases from each family is shown. Different RTK domains are illustrated (Figure adapted from Hubbard *et al*.[39]).

1.3. Regulation of RTK activity and cell signaling

The kinase activity of RTKs is tightly regulated and is activated upon ligand binding to the extracellular domain (Figure 2)[41]. Ligand binding leading to the activation of RTKs is regulated by various mechanisms[42, 43]. For example, certain receptors dimerize upon ligand binding leading to the activation of kinase while other RTKs exist as preformed inactive dimers whose kinase is activated upon ligand binding[43]. Auto-inhibition mechanisms exist to keep intracellular kinase inactive in the absence of a stimulating ligand[41]. Upon ligand binding, autoinhibited kinase becomes active and phosphorylates itself (autophosphorylation) and it's substrates (transphosphorylation) (Figure 2)[41, 44]. Activation of RTKs upon ligand stimulation leads to further activation of downstream pathways such as PI3K-AKT, JAK-STAT or RAS-RAF-MAPK [43, 45].

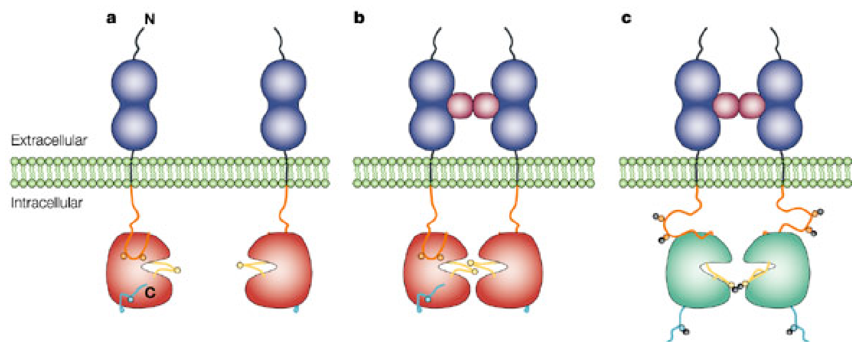


Figure 2. Activation of receptor tyrosine kinase (RTK).
Monomeric RTKs exist in inactive form (a). Upon ligand binding RTKs dimerize (b) leading to the activation of the kinase (c) resulting in auto- and trans-phosphorylation (Figure adapted from Hubbard *et al.*[41]).

The conformation of an RTK kinase domain can vary between fully inactive ("off" state) and fully active ("on") states[46]. The importance of tight regulation of RTK's activity is evident from their implication in several cancers[27]. Multiple layers of regulatory mechanisms involving distinct domains of RTK's exist to prevent kinase activation[46]. This is evident from the constitutive activation due to mutations in the extracellular domain (eg. EGFR), juxtamembrane region (eg. FLT3) or kinase domain (eg. ERBB2).

1.3.1. Structural features of kinase domain

The kinase domain of RTKs contains an amino-terminal (N-terminal) lobe and a carboxy-terminal (C-terminal) lobe[46]. While the amino-terminal lobe is rich in β-sheets, the carboxy-terminal lobe is primarily α-helical. The N-terminal and the C-terminal lobes are connected by a hinge which binds the adenine of the ATP. A deep cleft (active site or catalytic cleft) between the two lobes is the site of ATP binding. The α-helix of the N-lobe is called control- or C-helix. A conserved glutamic acid residue of the C-helix forms a salt bridge with the side chain of a buried lysine that is important for proper orientation of phosphates and facilitates phosphoryl transfer reactions. The glycine-rich phosphate-loop (P-loop) also contacts the phosphates of ATP. A bulky tyrosine or phenylalanine of the P-loop shields the active site from solvent thus facilitating the kinase reaction. Upon phosphorylation, the A-loop (activation loop) transforms into an extended conformation aiding substrate binding. The highly conserved DFG motif coordinates the binding of ATP to the hinge region. A typical kinase reaction involving various regions of the kinase domain is schematically shown in Figure 3[47].

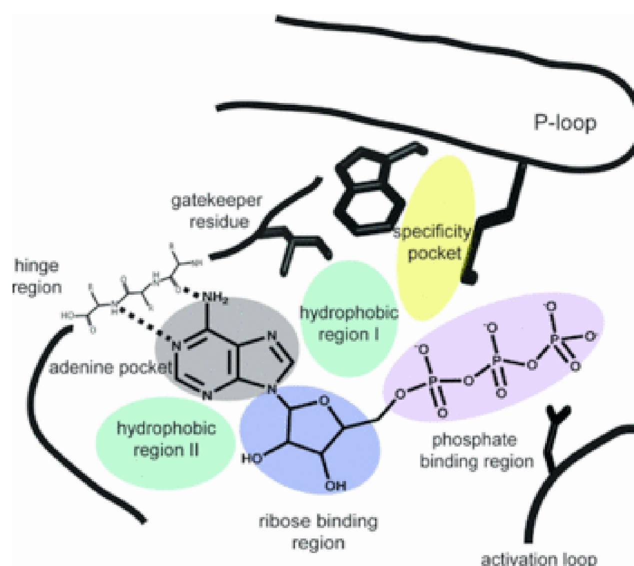


Figure 3. Schematic representation of a kinase domain with bound ATP (Figure adapted from Williams *et al.*)[47].
The catalytic domain of RTKs consists of an N-terminal lobe and a C-terminal lobe with an ATP-binding site situated between these domains. Both the N- and C-terminal lobes were connected by hinge region. Phosphorylation of the activation loop leads to stabilization of active kinase form. Gatekeeper residue within the ATP binding site controls the size of purine binding site and regulates the accessibility of substrate to the hydrophobic pocket. P-loop (phosphate-binding loop) forms the ceiling of kinase domain and interacts with the phosphate groups of the nucleotide and the Mg2+ ion.

Even though there is a great resemblance among active conformations of many kinases, their inactive conformations differ a lot depending on the kind of autoinhibitory mechanism[39, 46].

1.3.2. Cellular signaling mediated by the activated RTKs

Receptor oligomerization upon ligand binding increases the local concentration of kinases leading to transphosphorylation of tyrosines in the activation loop of the catalytic domain[40]. Upon tyrosine phosphorylation, the activation loop adopts an "open" configuration giving access to ATP and to substrates thus enabling phosphorylation of receptor itself (autophosphorylation) and of substrate proteins (transphosphorylation)[40]. A typical kinase reaction involves the transfer of a gamma ATP to reactive hydroxy group on a substrate (Figure 4)[43]. This is facilitated by the loss of autoinhibition imposed by different conformational constraints, and binding of ATP as well as proper orientation of the substrate[46].

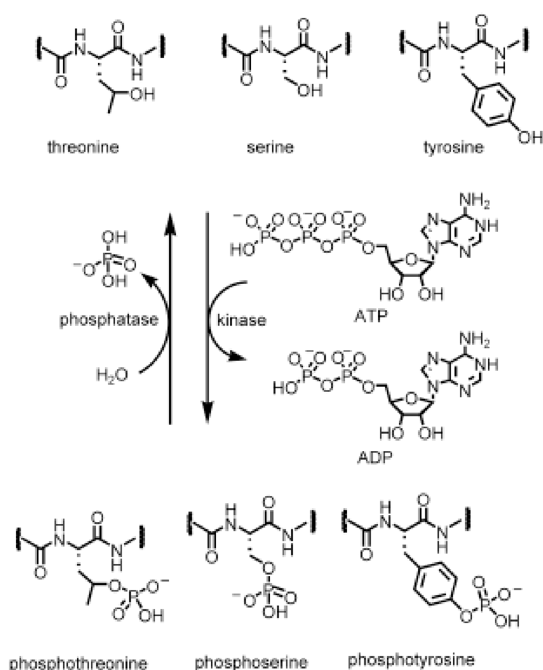


Figure 4. Mechanism of reversible protein phosphorylation (adapted from Bialy *et al.*)[48].

The autophosphorylated sites on RTK serve as binding sites for several signaling proteins with distinct modules such as SH2 and PTB domains[40, 43, 49-51]. These proteins also serve as

adaptors and recruit further downstream molecules resulting in a signaling complex[52]. One such adaptor is Grb2 which is complexed to Sos protein that serves as an activator of Ras[53]. Activated Ras recruits Raf kinase to the membrane which activates MEK1 and MEK2[53, 54]. MEK1 and MEK2 inturn activate ERK1 and ERK2 which then dimerize and translocate into the nucleus and phosphorylate transcription factors (Ras/Raf/MAP Kinase pathway) (Figure 5)[55]. Additionally, SH2 domain containing enzyme PLCgamma is also recruited to activated RTK which hydrolyzes PIP_2 to PIP_3 and diacylglycerol which in turn activates PKC family of kinases and Ca^{2+}/calmodulin-dependent protein kinases (PLCgamma pathway)[40, 55]. PI-3 kinase complex is also recruited to the activated RTK via its p85 subunit thus phosphorylating PIP_2 to PIP_3[56]. PH-domain containing protein AKT binds to PIP3 and gets phosphorylated by another PH-domain containing protein PDK1[40]. Activated AKT phosphorylates various substrates that are part of several survival and anti-apoptotic signaling processes (PI-3K/AKT pathway) (Figure 5)[57]. RTKs also activate JAK family of kinases which phosphorylate STAT proteins (Figure 5)[40]. STAT family of proteins either homodimerize or heterodimerize upon phosphorylation and translocate into the nucleus where they regulate gene expression (JAK/STAT pathway) (Figure 5)[58]. Thus, a myriad of proliferative and survival pathways are activated by kinase active RTKs upon ligand stimulation (Figure 5)[59].

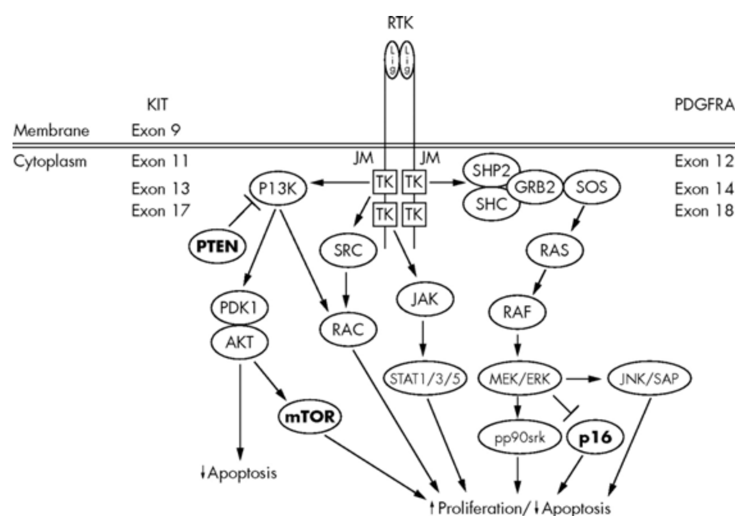


Figure 5. Schematic illustration of signaling pathways activated by RTKs[59].
Activation of various proliferation and anti-apoptic pathways by activated RTK dimers upon ligand binding. Activating mutations in the exons of KIT and PDGFRA were indicated. RTK = Receptor tyrosine kinase, Lig = ligand, JM = Juxtamembrane region, TK = Tyrosine kinase.

Attenuation of kinase activity and downstream signaling is also an important step and is achieved by the action of phosphatases, receptor endocytosis and lysosomal degradation, and by negative feedback mechanisms[40]. Negative regulators of RTK signaling involves inhibitory phosphorylation of MAPKs, upregulation of transcriptional repressors of MAPKs and targeting the proteins of JAK/STAT pathway for degradation by SOCS1[60].

1.4. Activating mutations in RTKs

Enhanced activation of RTKs in the absence of stimulating ligands is possible either due to over expression of the proteins or due to genetic mutations[61]. Activating mutations disrupt autoinhibitory mechanisms thus conferring constitutive kinase activity even in the absence of stimulatory ligands[44, 62, 63]. This results in deregulated activation of key pro-survival and proliferation signaling pathways resulting in cancer[64]. It is reasonable to assume that the success of cancer treatment by targeting a particular mutated kinase depends on to which extent the cancer cell is dependent on the oncogene for survival (oncogene addiction)[65-67]. Eventhough tumors accumulate multiple genetic lesions, they may require a single activated gene or signaling pathway for maintenance. Thus, the phenomenon of oncogene addiction can be explained in a setting where oncogene inactivation results in the death of tumor cells but not of normal cells[68]. However, the mechanism by which the phenotypic outcome upon oncogene inactivation is achieved remains elusive[68].

Genetic mutations reported in RTKs include insertions, deletions and point mutations in the key regulatory regions of the kinase including extracellular domain, juxtamembrane domain and the intracellular kinase domain [61]. For example, mutations in the extracellular domain that were reported in patients include a) deletions (EGFR in glioblastoma patients)[69], b) in-frame insertions (c-KIT in GIST patients)[70], and point mutations (EGFR in glioblastoma patients)[71]. In addition, mutations in the extra cellular domain were also reported in RET and TrkA kinases as well[72, 73]. Apart from extracellular domain, mutations in the juxtamembrane domain of FLT3, PDGFR alpha and KIT were shown to be activating[15, 74-76]. Another important class of activating RTK mutations were reported in the kinase domain of FLT3, KIT, PDGFR, EGFR and ERBB2[16, 77-82].

1.4.1. Mutations in the FLT3 receptor are reported in AML patients

FLT3 (FMS-like tyrosine kinase 3) (alternate names: fetal liver kinase-2, FLK-2 and stem cell kinase 1, STK-1) is a class III RTK (other members of the family include c-KIT, c-FMS and PDGFR) with an extracellular Ig-like ligand binding domain, a juxtamembrane domain and an intracellular split kinase domain[83-85]. The human *FLT3* gene is mapped to chromosome 13q12 and encodes 993 aminoacids[86]. Stimulation with FLT ligand (FL) induces dimerization resulting in FLT3 activation as evident by the autophosphorylation as well as the transphosphorylation of substrate proteins[87]. Phosphorylation of FLT3 followed by the internalization of FLT3L–FLT3-phosphate complex is rapid and degraded by-products are seen as early as 20 minutes after stimulation[86]. FLT3 ligand levels are low in healthy individuals but are elevated in patients who have low white-blood-cell counts secondary to either haematopoietic disease or chemotherapy indicating that the systemic release of FLT3L may contribute to haematopoiesis[86]. Thus, availability of FLT3 ligand determines the kinase activity of FLT3 receptor[86]. It has been proposed that the regulated activation of FLT3 receptor by FLT3 ligand may involve both autocrine and paracrine mechanism[86]. Together with SCF (Stem Cell Factor) and IL-3 (interleukin-3), FL was shown to induce proliferation in multiple cell types of the hematopoietic lineage[88]. In the absence of other growth factors, FLT3 ligand induces monocytic differentiation of early hematopoietic progenitors[86]. In combination with growth factors FLT3 ligand activates the proliferation of primitive and more committed myeloid progenitor cells[86]. Together with IL-7 and IL-11, FLT3 ligand stimulates differentiation of uncommitted mouse hematopoietic progenitor cells[86].

The most common genetic alterations in AML were reported in the FLT3 receptor [89]. FLT3 receptor mutations are more prevalent in adult AML patients than in paediatric AML patients[86]. In-frame internal tandem duplications (FLT3-ITDs) of varying length in the juxta membrane region were reported in approximately one third of AML patients, while point mutations in the activation loop of the tyrosine kinase domain (FLT3-TKD or FLT3-D835Y) were found in 8% to 12% of AML patients (Figure 6)[84, 89]. Additionally, point mutations in the FLT3 juxtamembrane region were also reported in AML patients[74]. A high mutant-to-wild–type ratio was shown to result in a decreased survival in FLT3-ITD positive patients[77, 90].

Constitutive activation upon ligand independent dimerization of FLT3-ITD and downstream signaling pathways was shown *in vitro*[91-93]. Both FLT3-ITD and FLT3-TKD were shown to be activating and can transform hematopoietic cell lines to cytokine independent growth (Figure 6)[77, 94, 95]. Interestingly, signaling differences between the two classes of FLT3 mutations were shown recently[96]. FLT3-ITD but not FLT3-TKD was shown to activate STAT5 signaling pathway in myeloid cells[96].

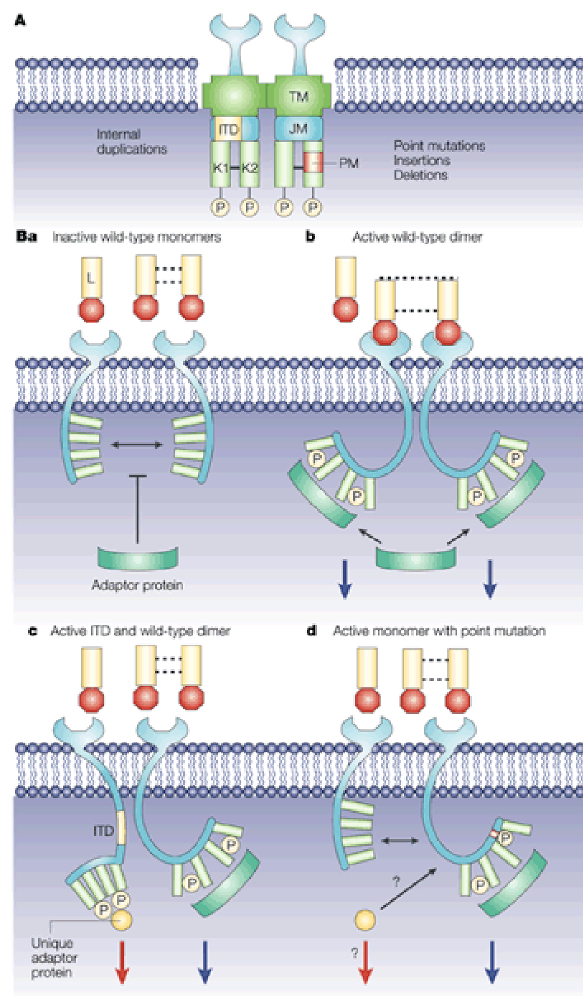


Figure 6. Mechanism of activation of wild type and mutant FLT3 receptors.
Diagrammatic representation of FLT3 mutations reported in AML (Figure adapted from Stirewalt *et al.*[86]). (A) Internal tandem duplication (ITD) in juxtamembrane (JM) region of FLT3 kinase is the most frequent FLT3 mutation reported in AML. Point mutation (PM) in the split kinase domain (K1 and K2) was also reported in AML patients. (B) Mechanism of activation of mutated FLT3 receptors is shown. Inactive wild type FLT3 monomers (a) dimerize upon ligand (L) binding to form kinase active dimers (b). Mutant FLT3 (c and d) kinases are constitutively active and don't require stimulation by FLT3 ligand.

Activating FLT3 mutants also induce disease in murine bone marrow transplantation models[21]. However, FLT3-ITD and FLT3-D835Y were shown to induce distinct phenotypes upon transplantation of transduced bone marrow[97]. While FLT3-ITD induces a myeloproliferative disease in bone marrow transplantation model, FLT3-TKD induces an oligoclonal lymphoid disorder[97]. A knock-in mouse model for FLT3-ITD was recently reported to develop myeloproliferative disease resembling human CMML (Chronic Myelomonocytic Leukemia)[98]. Despite these models, so far there is no report where FLT3 mutation alone causes AML suggesting that a second hit (additional oncogenic event) is needed for AML onset[86]. Given the high frequency of mutations in AML, FLT3 is an attractive therapeutic target. Several kinase inhibitors like herbimycin A, AG1295, AG1296, CEP-701 and PKC412 have shown significant activity against FLT3 mutants both *in vitro* and in murine bone marrow transplantation models[21, 95, 99-103]. However, TKI resistant mutations in the FLT3 kinase domain were recently reported in a cell based screen[104]. Thus, alternate inhibitors need to be developed for superior efficacy compared to the existing FLT3 inhibitors against TKI-resistant FLT3 mutations[105-109].

1.4.2. EGFR kinase domain mutations are reported in NSCLC patients

EGFR/ERBB1/HER1 is a prototypical member of ERBB family of RTKs which include ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4 kinases[110]. While ERBB2 has no known ligand, ERBB3 is kinase defective[110]. Several ligands were shown to activate one or more of the ERBB family members by inducing either homo- or heterodimerization resulting in activation of downstream signaling processes whose strength depends on the dimerization partners (Figure 7)[111, 112]. The signaling network involving ERBB receptors can be viewed as multilayered process with an input layer (comprising the ligands and their receptors), a signal-processing layer (includes adaptor proteins as well as intermediate kinases and activated transcription factors) and an output layer (physiological effects depending on cellular context and ligand-receptor pairs) (Figure 7)[111, 113]. Overexpression of either ERBB receptors or their ligands were implicated in various cancers[114-117].

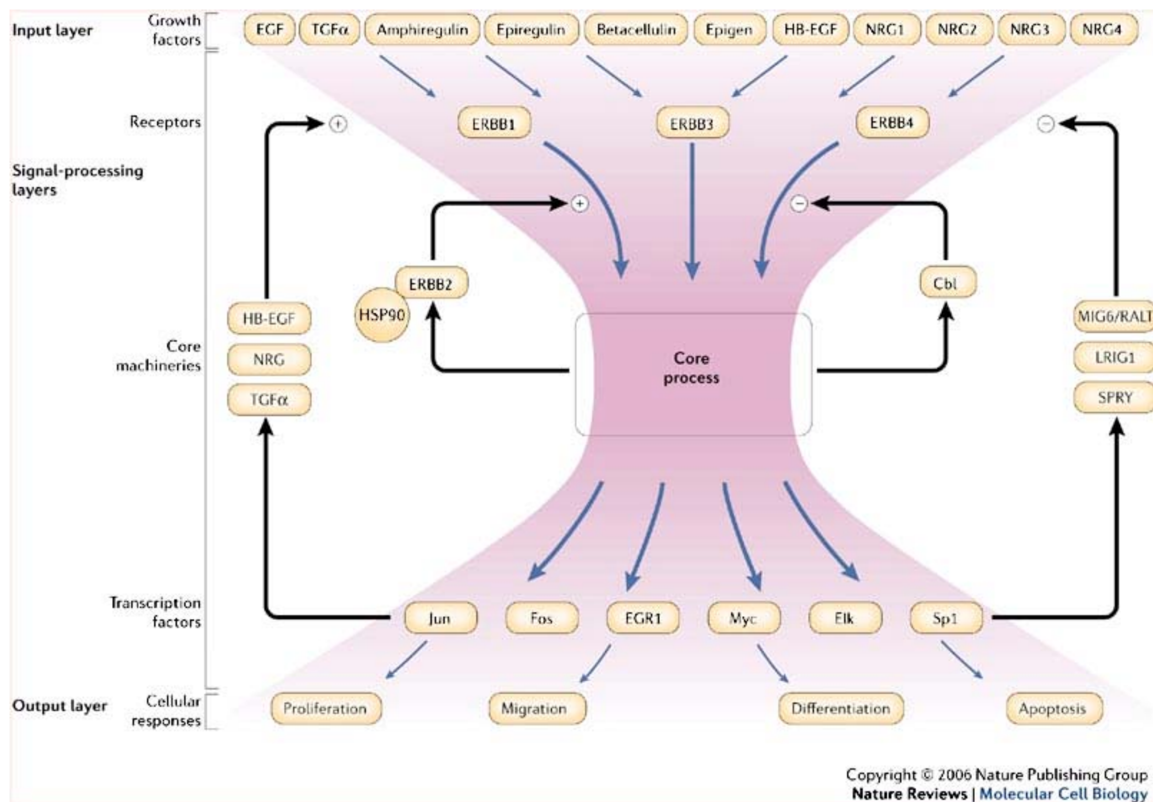


Figure 7. Multilayered signaling cascades in ERBB network (adapted from Citri A *et al.*[113]).
The input layer involves the binding of ligands to the extracellular domain of the corresponding receptor(s). The signal processing layer involves multiple cellular processes ranging from signal amplification to feed back loops while the output layer involves the execution of cellular processes.

Epidermal growth factor receptor (EGFR) is a 170 kD RTK with an extracellular ligand binding domain, a transmembrane domain and an intracellular kinase domain with distinct N- and C-lobes[118, 119]. Mice lacking EGFR show defective epithelial development and survive only for a short period after birth[120]. EGFR ligands (EGF and TGF alpha) are type I integral membrane proteins which are cleaved to mature growth factors[121]. Upon ligand binding, the extracellular domain shifts from dimer-incompatible conformation to a dimer-compatible conformation[118]. This leads to the formation of stable dimers of EGF:EGFR complexes in the extracellular milieu[122]. This results in the relaxation of autoinhibition thus enhancing the autokinase activity leading to phosphorylation of several tyrosines in the carboxy terminal tail[122]. The critical role of the juxtamembrane region and the C-terminal tail in autoinhibition was also reported recently[123, 124]. Moreover, the kinase domains of EGFR form asymmetric dimers: C-lobe of one monomer (activator) docking on the N-lobe

of another monomer (activated)[125].Thus, EGFR displays unique mechanisms of autoinhibition and kinase activation compared to other RTKs. Constitutive activation of EGFR (in the absence of ligand stimulation) due to gene/protein overexpression was shown to be an oncogenic event in several cancers making it an ideal target for EGFR inhibitor treatment[126]. Moreover, EGFR kinase domain mutations were recently reported in a subset of lung cancer.

Lung cancer can be broadly classified into either small-cell lung cancer (SCLC, comprising 20% of lung cancers), or non-small-cell lung cancer (NSCLC, comprising 80% of lung cancers)[127]. NSCLC includes adenocarcinoma, bronchioloalveolar carcinoma, squamous carcinoma, anaplastic carcinoma and large-cell carcinoma subtypes[127]. NSCLC is a relatively aggressive disease with a median survival, if left untreated, of approximately 4-5 months after diagnosis. EGFR kinase domain mutations are predominantly reported in nearly 10% of NSCLC and rarely in SCLC, ovarian, colorectal, and pancreatic cancers. The majority of EGFR mutations reported in NSCLC patients are somatic even though germline mutations have been reported[16, 128, 129]. Moreover, the percentage of NSCLC patients with mutated EGFR varies depending on the population studied[130]. EGFR activating mutations are more common in female patients, of east asian descent, non smokers and with adenocarcinoma subtype[131].

Significant positive clinical responses were seen recently in NSCLC (non-small-cell lung cancer) patients treated with EGFR inhibitors like gefitinib or erlotinib[132, 133]. Interestingly, activating mutations in the EGFR kinase domain were shown to sensitize a subset of NSCLC patients to EGFR inhibitor treatment[16, 134, 135]. In addition, several recent studies demonstrate that NSCLC patients with EGFR mutations show significant increase in overall survival upon gefitinib/erlotinib treatment[136-139]. Moreover, patients with K-ras mutations (nearly 20% of NSCLC) almost never harbored EGFR mutations, and do not respond to erlotinib treatment[127].

Initial studies with the EGFR mutant NSCLC cell line (H3255) demonstrated that there is a significant difference in the biology between wildtype and EGFR mutant lung cancer cell lines. Gefitinib treatment caused cell cycle arrest in EGFR wildtype NSCLC cell lines and apoptosis in EGFR mutant NSCLC cell lines[140]. Moreover, mutant EGFR is more sensitive to phosphorylation inhibition by gefitinib treatment than the wildtype EGFR[134]. In contrast to these findings, no correlation between EGFR mutation status and overall survival was

shown in a study which also reported novel mutations in the EGFR kinase domain[141]. Subsequent studies also reported additional novel mutations in the EGFR kinase domain[142, 143]. Interesitingly, distinct EGFR kinase domain mutations showed differential responses to inhibitor treatment[144]. Moreover, secondary gefitinib resistance due to an additional kinase domain mutation was also reported in NSCLC patients[145]. The concepts of differential drug response and secondary drug resistance are not new and were previously shown with different RTKs[30, 95, 146]. Such studies indicate that it is important to functionally characterize every mutation and test them for their sensitivity towards EGFR kinase inhibitors.

All the EGFR kinase domain mutations reported in NSCLC localize in exons 18, 19, 20 and 21 and can be classified into three groups [127, 147, 148]: A) In-frame exon 19 deletions (involving L747-A750 residues) constitute class I, B) point mutations constitute class II and C) in-frame insertions in exon 20 constitute class III mutations[148]. Of these, exon 19 deletions and an exon 21 point mutation (L858R) are the most frequent genetic alterations accounting up to 85% of the reported EGFR mutations in NSCLC patients[127, 148]. Other mutations include in-frame duplications in exon 20 and point mutations affecting G719 and L861[127]. The activating nature and drug sensitivity towards EGFR inhibitors was established only for the most frequent mutations and the role of less frequent mutations is not yet known[149-151]. Thus, it is important to biochemically characterize and test the less frequent mutations for their sensitivity towards EGFR inhibitors.

1.4.3. Mutations in the ERBB2 kinase are reported in solid cancers

ERBB2/HER2 (185 kDa) is a RTK belonging to the EGFR family (ERBB family) and its amplification or overexpression was reported in nearly 30% of breast cancers[152, 153]. The rodent ortholog of ERBB2, Neu was identified as an oncoprotein from mutagen treated rats[154, 155]. ERBB2 lacks a known ligand (orphan receptor) but is a preferred dimerizing partner for other members of the ERBB family[156]. ERBB heterodimers containing ERBB2 have enhanced signaling potency compared to ERBB homodimers due to reduced rate of ligand dissociation (Figure 8)[111, 157].

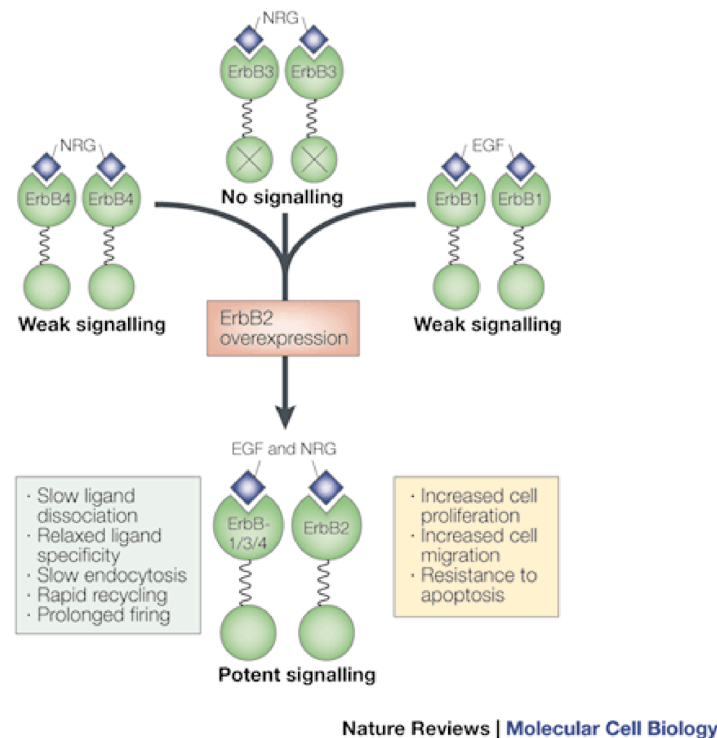


Figure 8. ERBB2 will form potent signaling complex with other ERBB members (Figure adapted from Yarden Y *et al.*[111]).
ERBB2 is the preferred dimerization parter for other members of the ERBB family leading to stronger signaling compared to homodimers.

Constitutive activation of ERBB2 (in the absence of a ligand) induces cell transformation and tumor growth[158]. Additionally, transgenic mouse expressing *Neu* allele develops mammary tumor phenotype[159-164]. The role of *ERBB2* gene amplification or protein overexpression is significant in breast cancer and a recent report showed its correlation with poor prognosis[165]. *ERBB2* amplification was also associated with the resistance to both chemotherapy and hormonal therapy[166-168]. Re-replication, unequal exchange, episome excision, and the breakage-fusion-bridge (BFB) cycle are the proposed mechanisms to explain *ERBB2* gene amplification in cancer[169-171]. The expression of ERBB2 is also transcriptionally regulated by several factors[172-176].

A genetic polymorphism resulting in the substitution of I655 by V655 of ERBB2 was shown to increase susceptibility to breast cancer[177]. Additionally, another polymorphism I654V in tandem with I655V was shown to be associated with increased risk of familial breast cancer[178]. On the contrary, several studies have shown that there is no correlation or

inverse correlation between ERBB2 polymorphism and the incidence of breast cancer risk[179, 180]. However, there is no experimental and biochemical basis (kinase activity and transformation ability) to explain the discrepancies between these different studies. Moreover, the role of ERBB2 polymorphisms in predicting the clinical response of breast cancer patients towards ERBB2 inhibitor treatment is unknown. More recently, mutations in the ERBB2 kinase domain were also reported in several cancers[181-186]. For example, the ERBB2 mutation ins774 (AYVM) identified in lung cancer confers drug resistance towards EGFR inhibitors[187, 188]. However, the properties of other ERBB2 mutations were not studied. Thus, it is important to study the role of genetic variants (both polymorphisms and mutations) in ERBB2 kinase activity, transformation ability and drug sensitivity.

1.5. Targeted therapy of cancer and drug resistance

Personalized cancer medicine is a clinical strategy in which a set of cancer patients are selected for appropriate therapy based on defined clinical features or biomarkers[28]. It is based on the fact that the outcome of a drug treatment varies significantly within patient populations[28]. Thus, a detailed understanding of factors that influence treatment outcome is needed to select patient subsets for a particular therapeutic strategy. In the recent past, deregulated activity of kinases are shown to have significant role in several cancers making them attractive targets for personalized therapy. The development of ATP-competitive inhibitors to target oncogenic tyrosine kinases yielded significant success in treating certain cancer types[189]. The results achieved in treating such cancers arising due to mutated kinases with kinase inhibitors is significant[190]. The target specificity of these drugs is affected by the sequence/structural homology shared by most kinases[189]. Thus, several small molecule inhibitors have more than one target kinase[191]. For example, the Abl inhibitor imatinib that targets oncogenic BCR-ABL, also targets c-KIT and PDGFR kinases[189]. This resulted in testing of imatinib in c-KIT and PDGFR mutated cancers also with significant success[192]. On the other hand, ERBB inhibitors like gefitinib and lapatinib are highly selective with few or no known additional targets[193]. Most of the small molecule targeted drugs are ATP competitive reversible inhibitors although selective irreversible inhibitors were also reported[189].

1.5.1. Small molecule kinase inhibitors – types and mechanism of action

Most of the kinase inhibitors form one to three hydrogen bonds with the hinge region mimicking the binding of the adenine ring of ATP (structure-activity relationships, SARs)[27]. Based on their mechanism of action kinase inhibitors are classified as 1) type 1 inhibitors, 2) type 2 inhibitors, 3) allosteric inhibitors and 4) covalent inhibitors.

Type 1 inhibitors mimic ATP and thus bind to the active (DFG-in) conformation of the kinase[27]. The heterocyclic ring of the type 1 inhibitors occupy the adenine binding site of the kinase[27]. EGFR inhibitors like gefitinib and erlotinib fall into this category.

Type 2 inhibitors bind to the inactive (DFG-out) conformation of the kinase; typically they bind to the hydrophobic binding site created by the movement of the activation loop[27]. Examples of this category include imatinib and sorafenib. Upon inhibitor binding the kinase can undergo several conformations. Crystal structures of imatinib bound to the kinase domain revealed that the ABL kinase adopts an autoinhibited conformation which is seen with many kinases in their inactive state.

Allosteric inhibitors bind outside the active (ATP-binding site) site of the kinase and allosterically inhibits it's activity[27]. For example, the rapamycin-FKBP complex binds to the N-terminal FRB domain of mTOR and inhibits it's activitiy allosterically. Covalent inhibitors are irreversible inhibitors that form covalent bonds with the reactive amino acid residues in the kinase domain[27].

1.5.2. Factors underlying kinase inhibitor sensitivity

Factors that influence the effectiveness of small-molecule kinase inhibitors include oncogene addiction (dependence of the tumor on a particular oncogene or associated pathway) and activation status of downstream signaling molecules. For example, targeting downstream RSK2 kinase is effective in myeloma cells that express oncogenic FGFR3[28]. Similarly, the PTEN status influences the outcome of EGFR inhibitor treatment in glioma and NSCLC[28]. Apart from the above tumor-specific factors, germline polymorphisms, host pharmacogenomics and tumor microenvironment influence the efficacy of inhibitor treatment. For example, gefitinib bioavailability is higher in patients who are heterozygous for *AGCG2*-Q141K compared to the patients with wildtype *ABCG2*[28]. Similarly, the shorter dinucleotide CA repeats in the intron 1 of *EGFR* is associated with increased gefitinib

sensitivity in cell lines with wild-type EGFR[28]. Inhibition of EGFR signaling by gefitinib in endothelial cells inhibited the growth of A375SM melanoma xenograft which lacks EGFR expression demonstrating the role of the tumor microenvironment for inhibitor sensitivity. Thus, the above factors serve as biomarkers to predict *De novo* sensitivity or resistance towards particular inhibitor treatment[28].

1.5.3. RTK inhibitors used in this study

PKC412: PKC412 (Midostaurin) is a staurosporine analog (N-benzoyl-staurosporine) with high specificity towards both serine/threonine kinases (eg. Protein Kinase C) and tyrosine kinases (eg. FLT3, c-KIT and VEGFR2)[194] . It belongs to indolocarbazole class of chemical compounds (Figure 9). PKC412 is currently in phase III trial (Randomized AML Trial In FLT3 in <60 Year Olds, RATIFY) for the treatment of newly diagnosed FLT3-positive AML patients. Being able to inhibit multiple kinases, it is also being investigated in additional cancers. The exact mechanism of PKC412 action is still unclear due to the lack of experimentally verified structural studies.

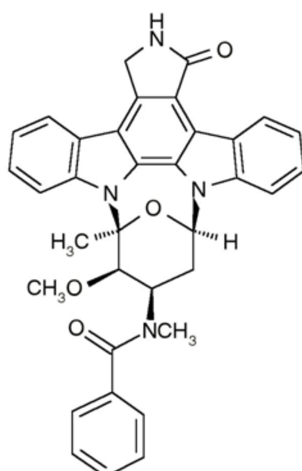


Figure 9. PKC412: N-[(9S,10R,11R,13R)-10-Methoxy-9-methyl-1-oxo-9,13-epoxy-2,3,10,11,12,13-hexahydro-1H,9H-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiazonin-11-yl]-N-methylbenzamide.

Sunitinib: Sunitinib is an indolinone-based compound, and inhibits multiple kinases including FLT3, c-KIT, PDGFR, VEGFR1 and VEGFR2 (Figure 10). Owing to it's effect on tumor angiogenesis, sunitinib has shown significant efficacy in both preclinical models and early trials (phase I and phase II) of renal cell carcinoma (RCC), gastrointestinal stromal tumors (GIST), non-small cell lung cancer (NSCLC), thyroid cancer and

melanoma[195]. Sunitinib is now approved for the treatment of advanced GIST and RCC. In a phase I study, sunitinib showed significant inhibition of mutated FLT3 kinase in AML patients[196]. Co-crystal structures of sunitinib in complex with c-KIT demonstrated that the inhibitor binds to the autoinhibited form of c-KIT similar to the binding of imatinib to ABL[197].

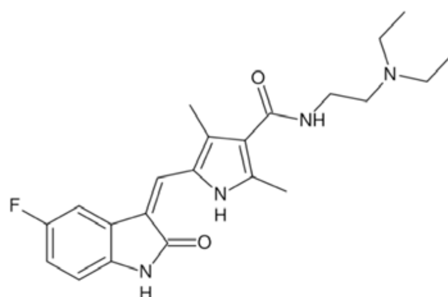


Figure 10. Sunitinib: *N*-[2-(diethylamino)ethyl]-5-[(*Z*)-(5-fluoro-1,2- dihydro-2-oxo-3*H*-indol-3-ylidine) methyl]-2,4- dimethyl-1*H*-pyrrole-3-carboxamide.

<u>Sorafenib</u>: Sorafenib is a multikinase inhibitor targeting both the serine/threonine (Raf1) and tyrosine kinases (FLT3, c-KIT, VEGFR, PDGFR and FGFR1) (Figure 11). Like sunitinib, sorafenib has superior efficacy due to it's tumor-specific antiproliferative as well as antivascular effects. Sorafenib is currently investigated in advance clinical trials for the treatment of RCC, head and neck cancer, SCLC and mesothelioma[26]. A phase I/II study of sorafenib in combination with idarubicin and cytarabine showed significant benefit in FLT3-mutated AML patients (93% achieved complete remission)[198]. Structural studies of sorafenib in complex with B-RAF kinase showed that sorafenib binds to the inactive conformation of the kinase[199].

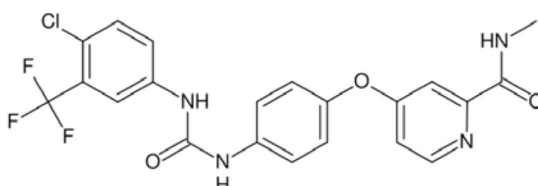
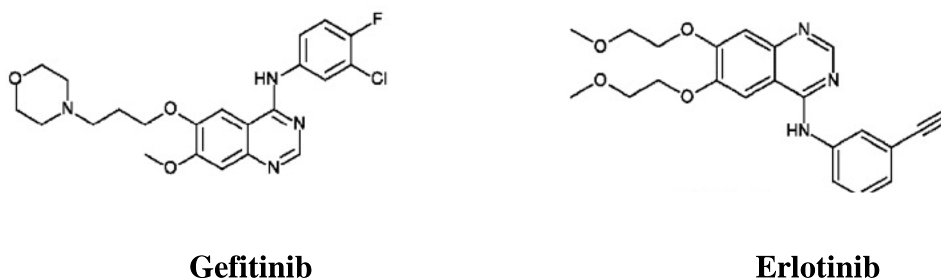


Figure 11. Sorafenib: 4-[4-[[4-chloro-3- (trifluoromethyl)phenyl]carbamoylamino] phenoxy]-*N*-methyl-pyridine-2-carboxamide.

<u>Gefitinib and erlotinib</u>: Gefitinib and erlotinib are ATP-competitive inhibitors sharing a common 4-anilinoquinazoline structure but differ in the substituents attached to the

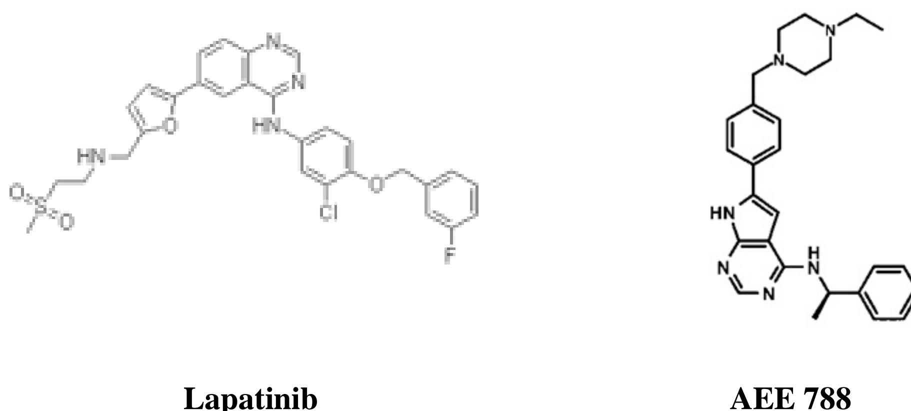
quinazoline and anilino rings (Figure 12)[200]. Crystal structures revealed that the binding modes of gefitinib and erlotinib to EGFR kinase are similar[201, 202]. Both gefitinib and erlotinib are approved for the treatment of NSCLC patients. In a retrospective analysis, gefitinib and erlotinib were shown to have similar antitumor activity in patients with metastatic or recurrent NSCLC[203].



Gefitinib **Erlotinib**

Figure 12. Gefitinib: *N*-(3-chloro-4-fluoro-phenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine; **Erlotinib:** *N*-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy) quinazolin-4-amine.

<u>Lapatinib and AEE788</u>: Lapatinib is a synthetic quinazoline with significant activity against both EGFR and ERBB2 kinases (Figure 13). Unlike gefitinib and erlotinib, lapatinib binds to the inactive conformation of the EGFR kinase[204].



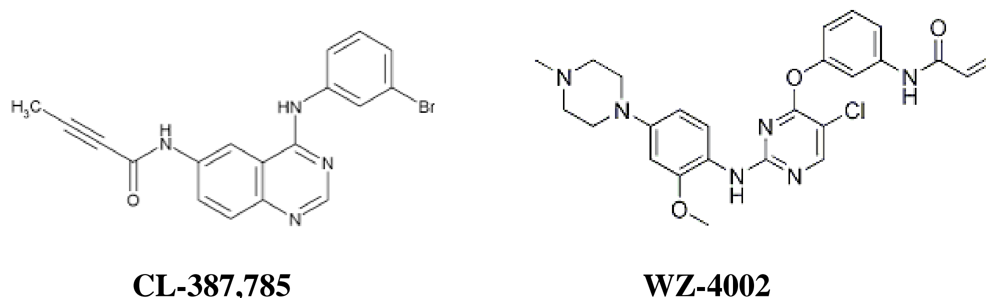
Lapatinib **AEE 788**

Figure 13. Lapatinib: *N*-[3-chloro-4-[(3-fluorophenyl)methoxy]phenyl]-6-[5-[(2-methylsulfonylethylamino) methyl]-2-furyl] quinazolin-4-amine; **AEE788:** (R)-6-(4-((4-ethylpiperazin-1-yl)methyl)phenyl)-N-(1-phenylethyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine.

Lapatinib alone or in combination with chemotherapy is approved for the treatment of advance HER2-positive breast cancer patients. AEE788, like lapatinib, is a reversible dual EGFR/ERBB2 pyrrolopyrimidine inhibitor in early clinical trials (Figure 13). Unlike

lapatinib, AEE788 binds to the active conformation of EGFR kinase. In addition, AEE788 also inhibits VEGFR2 kinase.

<u>CL-387,785 and WZ4002</u>: Irreversible inhibitors CL-387,785 and WZ4002 forms a covalent bond with Cys 797 of EGFR kinase (Figure 14). Both these inhibitors are active against ERBB2 kinase. While CL-387,785 is an anilinoquinazoline, WZ4002 is a pyrimidine[205, 206]. CL-387,785 and WZ4002 are investigational compounds that demonstrated significant promise in pre-clinical studies.



CL-387,785 **WZ-4002**

Figure 14. CL-387,785: N-[4-[(3-Bromophenyl)amino]-6-quinazolinyl]-2-butynamide; **WZ-4002:** N-(3-(5-chloro-2-(2-methoxy-4-(4-methylpiperazin-1-yl)phenylamino)pyrimidin-4-yloxy)phenyl) acrylamide.

1.5.4. Secondary drug resistance

Secondary drug resistance is a phenomenon observed in advance stages of the disease due to several mechanisms such as oncoprotein (target) overexpression, kinase domain mutations and drug efflux[207]. Target overexpression results in insufficient inhibitor concentration and could be partly circumvented by increasing drug concentration. In addition, point mutations in the kinase domain abrogate inhibitor binding to the target. Different point mutations confer varied degree of resistance depending on the location and importance of that particular residue in drug binding. Testing of alternate inhibitors showed that the resistance due to point mutations could be overcome by second generation inhibitors[34]. For example, secondary imatinib resistance due to different mutations in the BCR-ABL kinase domain was shown to be overcome using novel ABL kinase inhibitors like nilotinib, dasatinib and bosutinib[33-35]. Additionally, cell based drug resistance *in vitro* screens have enabled to predict drug resistance mechanisms that might occur in patients thus speeding up the process of testing alternate treatment options to overcome inhibitor resistance[208].

1.6. Aims and objectives

1. To test the efficacy of the multikinase inhibitors sunitinib and sorafenib against activating FLT3 mutants reported in AML patients.
2. To test if sunitinib and sorafenib can overcome PKC412 resistance in FLT3-ITD mutants.
3. To study the effect of EGFR kinase domain mutations (reported in NSCLC patients) on autokinase activity and transformation ability.
4. To establish drug sensitivity profiles for EGFR inhibitors using transduced Ba/F3 cell lines stably expressing EGFR mutants.
5. To study the effect of ERBB2 transmembrane domain polymorphisms on kinase activity and drug sensitivity.
6. To identify ERBB2 kinase domain mutants that are resistant to lapatinib treatment.
7. To test the efficacy of ERBB inhibitors against ERBB2 kinase domain mutants associated with cancer.

2. Materials and methods

2.1. Materials

2.1.1. Standard chemicals and reagents

2-Mercaptoethanol	Sigma-Aldrich, Taufkirchen
Beta-Mercaptoethanol	Gibco/Invitrogen, Karlsruhe
Acrylamide/Bisacrylamide Gel 30	Carl Roth, Karlsruhe
Agarose	Carl Roth, Karlsruhe
Ammoniumpersulfate	Sigma-Aldrich, Taufkirchen
Ampicillin	Sigma-Aldrich, Taufkirchen
Aqua ad injectabilia, sterile	Braun, Melsungen
Bacto Agar	BD Biosciences, Heidelberg
Bacto Yeast extract	BD Biosciences, Heidelberg
Bacto Tryptone	BD Biosciences, Heidelberg
Bromophenol blue	Sigma-Aldrich, Taufkirchen
BSA, Fraction V	Carl Roth, Karlsruhe
Chloroform	Sigma-Aldrich, Taufkirchen
Complete Mini Protease inhibitor tablets	Roche Diagnostics, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Taufkirchen
dNTP mix	Fermentas, St. Leon-Rot
Ethidium bromide	Carl Roth, Karlsruhe
EDTA	Fluka, Taufkirchen
Fugene HD reagent	Roche Diagnostics, Germany
GeneRuler 1kb DNA Ladder	Fermentas, St. Leon-Rot
Glycine	Merck, Darmstadt
Glycerol-2-phosphate	Sigma-Aldrich, Taufkirchen
Isopropanol	Merck, Darmstadt
Lipofectamine 2000	Invitrogen GmbH, Karlsruhe
Methanol	Merck, Darmstadt
Penicillin/Streptomycin solution	PAA, Pasching
Phosphate buffered saline (PBS)	Biochrom AG, Berlin
Polybrene	Sigma-Aldrich, Taufkirchen

Propidium iodide	Sigma-Aldrich, Taufkirchen
PVDF membrane (Immobilon P)	Millipore, Schwalbach/Ts
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
QIAGEN Spin Miniprep Kit	Qiagen, Hilden
QIAquick Gel Extraction Kit	Qiagen, Hilden
QIAquick Spin Purification Kit	Qiagen, Hilden
Rapid DNA Ligation Kit	Roche Diagnostics, Penzberg
Sodium azide	Sigma-Aldrich, Taufkirchen
Sodium chloride	Carl Roth, Karlsruhe
Sodium dodecyl sulphate (SDS)	Carl Roth, Karlsruhe
Sodium fluoride	Fluka, Taufkirchen
Sodium hydroxide	Merck, Darmstadt
Sodium orthovanadate	Sigma-Aldrich, Taufkirchen
Sodium pyrophosphate	Fluka, Taufkirchen
Tetramethylethylenediamine (TEMED)	Fluka, Taufkirchen
Tris (hydroxymethyl) aminomethane (TRIS)	Carl Roth, Karlsruhe
Triton X-100	Sigma-Aldrich, Taufkirchen
TRIzol reagent	Invitrogen GmbH, Karlsruhe
Tween 20	Fluka, Taufkirchen

2.1.2. Antibodies

pY20, mouse monoclonal anti-phosphotyrosine	Transduction Laboratories, USA
4G10, mouse monoclonal anti-phosphotyrosine	Upstate Biotechnology, USA
FLT3, rabbit polyclonal	Upstate Biotechnology, USA
p-Akt (Ser473),	Cell Signaling, Germany
AKT1/2, goat polyclonal	Santa Cruz Biotech., Germany
p-EGFR (Tyr1068), mouse monoclonal	Cell Signaling, Germany
EGFR, rabbit polyclonal	Santa Cruz Biotech., Germany
p-Stat5 (Tyr694),	Cell Signaling (NEB)
Stat5 (G-2),	Santa Cruz Biotech., Germany
p44/42 MAPK (ERK1/2), rabbit polyclonal	Cell Signaling, Germany
p-ERK1/2,	Cell Signaling, Germany

p-ERBB2 (Tyr 1248),	Millipore
ERBB2,	Santa Cruz Biotech., Germany
Actin, mouse	Sigma-Aldrich, Taufkirchen

2.1.3. Enzymes

*BamH*I (10U/ul)	Fermentas, St. Leon-Rot
*Bgl*II (10U/ul)	Fermentas, St. Leon-Rot
CIAP 20-30 U/ul, alkaline phosphatase	Invitrogen, Karlsruhe
*Dpn*I (10U/ul)	Fermentas, St. Leon-Rot
*EcoR*V (10U/ul)	Fermentas, St. Leon-Rot
Pfu-DNA polymerase	Fermentas, St. Leon-Rot
SuperScript II Reverse Transcriptase	Invitrogen, Karlsruhe
T4-DNA ligase	Fermentas, St. Leon-Rot
*Xho*I (10U/ul)	Fermentas, St. Leon-Rot

2.1.4. Vectors and cDNA constructs

The vectors used for cloning cDNA constructs: pcDNA/Zeo 3.1(-), MigR1 (MSCV-IRES-eGFP) and MIY (MSCV-IRES-YFP). Following cDNA constructs were cloned for the study:

MSCV-eGFP-FLT3-ITD	Rebekka Grundler
MSCV-eGFP-FLT3-D835Y	Rebekka Grundler
MSCV-eGFP-FLT3-ITD + N676D	This work
MSCV-eGFP-FLT3-ITD + F691I	This work
MSCV-eGFP-FLT3-ITD + G697R	This work
pcDNA3.1/Zeo-EGFR-WT	This work
pcDNA3.1/Zeo-EGFR + L688P	This work
pcDNA3.1/Zeo-EGFR + P694L	This work
pcDNA3.1/Zeo-EGFR + P694S	This work
pcDNA3.1/Zeo-EGFR + G719C	This work
pcDNA3.1/Zeo-EGFR + G719S	This work
pcDNA3.1/Zeo-EGFR + L730F	This work

pcDNA3.1/Zeo-EGFR + P733L	This work
pcDNA3.1/Zeo-EGFR + G735S	This work
pcDNA3.1/Zeo-EGFR + V742A	This work
pcDNA3.1/Zeo-EGFR + E746K	This work
pcDNA3.1/Zeo-EGFR + E749K	This work
pcDNA3.1/Zeo-EGFR + Del 747-753 ins S	This work
pcDNA3.1/Zeo-EGFR + S752Y	This work
pcDNA3.1/Zeo-EGFR + D761N	This work
pcDNA3.1/Zeo-EGFR + A767T	This work
pcDNA3.1/Zeo-EGFR + S768I	This work
pcDNA3.1/Zeo-EGFR + R776C	This work
pcDNA3.1/Zeo-EGFR + S784F	This work
pcDNA3.1/Zeo-EGFR + T790M	This work
pcDNA3.1/Zeo-EGFR + G810S	This work
pcDNA3.1/Zeo-EGFR + N826S	This work
pcDNA3.1/Zeo-EGFR + L838V	This work
pcDNA3.1/Zeo-EGFR + T847I	This work
pcDNA3.1/Zeo-EGFR + V851A	This work
pcDNA3.1/Zeo-EGFR + I853T	This work
pcDNA3.1/Zeo-EGFR + L858R	This work
pcDNA3.1/Zeo-EGFR + L861Q	This work
pcDNA3.1/Zeo-EGFR + A864T	This work
pcDNA3.1/Zeo-EGFR + E866K	This work
pcDNA3.1/Zeo-EGFR + G873A	This work
MSCV-eYFP-EGFR-WT	This work
MSCV-eYFP-EGFR + L858R	This work
MSCV-eYFP-EGFR + Del 747-753 ins S	This work
MSCV-eYFP-EGFR + L688P	This work
MSCV-eYFP-EGFR + G719C	This work
MSCV-eYFP-EGFR + G719S	This work
MSCV-eYFP-EGFR + V742A	This work
MSCV-eYFP-EGFR + D761N	This work

MSCV-eYFP-EGFR + S768I	This work
MSCV-eYFP-EGFR + R776C	This work
MSCV-eYFP-EGFR + S784F	This work
MSCV-eYFP-EGFR + T790M	This work
MSCV-eYFP-EGFR + G810S	This work
MSCV-eYFP-EGFR + N826S	This work
MSCV-eYFP-EGFR + L838V	This work
MSCV-eYFP-EGFR + V851A	This work
MSCV-eYFP-EGFR + I853T	This work
MSCV-eYFP-EGFR + L861Q	This work
MSCV-eYFP-EGFR + A864T	This work
MSCV-eYFP-EGFR + E866K	This work
MSCV-eGFP-EGFRvIII	This work
MSCV-eGFP-EGFRvIII + G719S	This work
MSCV-eGFP-EGFRvIII + L688P	This work
MSCV-eGFP-EGFRvIII + V851A	This work
MSCV-eGFP-EGFRvIII + I853T	This work
MSCV-eGFP-EGFRvIII + L858R	This work
MSCV-eGFP-EGFRvIII + L861Q	This work
MSCV-eGFP-ERBB2-WT	Heinke Conrad
MSCV-eGFP-ERBB2-I655V	This work
MSCV-eGFP-ERBB2-I654V,I655V	This work
MSCV-eGFP-ERBB2 + L755S	This work
MSCV-eGFP-ERBB2 + L755P	This work
MSCV-eGFP-ERBB2 + V773A	This work
MSCV-eGFP-ERBB2 + V777L	This work
MSCV-eGFP-ERBB2 + T798M	This work
MSCV-eGFP-ERBB2 + N857S	This work
MSCV-eGFP-ERBB2 + T862A	This work
MSCV-eGFP-ERBB2 + H878Y	This work

2.1.5. Standard instruments

Agarose gel electrophoresis chamber	Biometra, Göttingen
CO2 incubator	Heraeus Instruments
ELISA Reader Sunrise	Tecan, Crailsheim
Heat block 5436	Eppendorf, Hamburg
Incubator shaker Innova 4000	New Brunswick Scientific, USA
Cooling centrifuge J2-HS, Rotor JA-14	Beckman, USA
Cooling centrifuge 5417R, 5810R	Eppendorf, Hamburg
Light microscope, Axiovert 25	Zeiss, Jena, Germany
LKB Ultraspec III, spectrophotometer	Pharmacia, Uppsala, Sweden
Neubauer chamber	Reichert, USA
PCR-Thermocycler Primus 96	Peqlab, Erlangen
pH meter	Beckman, USA
Transfer electrophoresis unit	Hoefer, USA

2.1.6. Standard media and buffers

Amidoblack stain	0.2% Naphtol Blau Schwarz
	25% Isopropanol
	10% Acetic acid
Amidoblack destainer	25% Isopropanol
	10% Acetic acid
Blocking solution for western blot	5% BSA or Milk powder
	0.1% Tween 20 in PBS
Cell lysis buffer	10 mM Tris/HCl (pH 7.5)
	130 mM NaCl
	5 mM EDTA
	0.5% Triton X-100
	20 mM Na_2HPO_4/NaH_2PO_4 (pH 7.5)

	10 mM Sodiumpyrophosphate (pH 7.0)
	1 mM Sodiumorthovanadate
	20 mM Sodium fluoride
	1 mM Glycerol-2-Phosphate
	1 Protease-Inhibitor Cocktail tablet
DNA loading buffer	60% Glycerol
	0.2% Bromophenol blue
	0.2 M EDTA in A.d.
LB medium (for bacterial cell culture)	1% Bacto-Tryptone
	0.5% Bacto-Yeast extract
	1% Sodium chloride
LB-Agar (for plates)	1% Bacto-Tryptone
	0.5% Bacto-Yeast extract
	1% Sodium chloride
	1.5% Bacto-Agar
Resolving gel buffer for SDS-PAGE (4X)	1.5 M Tris (pH 8.8)
	0.4% SDS in A.d.
SDS-PAGE running buffer	25 mM Tris
	192 mM Glycine
	0.1% SDS in A.d.
SDS-PAGE loading buffer (2X)	1 M Tris/HCl (pH 6.8)
	200 mM DTT
	4% SDS
	0.2% Bromophenol blue
	20% Glycine in A.d.

Stacking gel buffer for SDS-PAGE (4X)	0.5 M Tris (pH 6.8 < 9) 0.4% SDS in A.d.
TAE buffer (10X)	0.4 M Tris 1.1% Acetic acid 10 mM EDTA (pH 8.0) in A.d.
Western transfer buffer	25 mM Tris 192 mM Glycine 20% Methanol 0.1% SDS in A.d.

2.1.7. Cell lines

Ba/F3	Murine Pro-B cell line
NIH/3T3	Mouse embryonic fibroblast cells
HEK293	Human embryonic kidney cells

2.1.8. Media and reagents for mammalian cell culture

DMEM, cell culture medium	PAA, Pasching
FBS Gold	PAA, Pasching
Human EGF	Chemicon
L-Glutamine	Gibco/Invitrogen, Karlsruhe
Mouse interleukin-3	R&D, Wiesbaden
Opti-Mem	Gibco/Invitrogen, Karlsruhe
PBS, 10X	PAA, Pasching
RPMI 1640 medium	PAA, Pasching
Trypan blue	Gibco/Invitrogen, Karlsruhe
Trypsin-EDTA solution, 10X	PAA, Pasching

2.2. Methods

2.2.1. Methods involving nucleic acids

2.2.1.1. Isolation, purification and measurement of DNA

DNA was isolated and purified as minipreps (from 5 ml of bacteria) and maxipreps (from 200 ml of bacteria) using "QIAprep Spin Miniprep Kit" and "QIAGEN Plasmid Maxi Kit" respectively. DNA was extracted and purified from agarose gels using "QIAquick Gel Extraction Kit".

QIAGEN plasmid purification protocols are based on the principle of alkaline lysis, followed by binding of plasmid DNA to anion-exchange resin under low-salt and low pH conditions. All the contaminants are removed by a medium-salt wash. Plasmid DNA was then eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. The QIAquick gel extraction protocol involves a bind-wash-elute procedure in which gel slices are dissolved in a buffer that allows easy determination of the optimal pH for DNA binding, and the mixture is then applied to the QIAquick spin column. Nucleic acids adsorb to the silica membrane in the high-salt buffer. Impurities are washed away and DNA is eluted with a low-salt buffer. Yield (based on the absorbance at 260 nM) and purity (based on the ratio of absorbances at 260 nm and 280 nm) of the isolated DNA was measured by using a "Nanodrop".

2.2.1.2. Agarose gel electrophoresis

TAE buffer: 0.4% Tris, 1.1% Acetic acid, 2% 0.5M EDTA in A.d.
DNA gel loading buffer (6X): 30% Glycerine (v/v), 0.25% Bromophenol blue (w/v), 0.25% Xylolcyanol, 50mM EDTA in A.d.

Unmodified or modified DNA was separated according to their size by agarose gel electrophoresis. Agarose gel for this purpose was prepared by pouring warm 1% agarose solution (in TAE buffer mixed with ethidium bromide) into a clean gel caster and allowed to solidify. DNA samples were mixed with DNA gel loading buffer and loaded onto the gel. Electrophoresis was performed in a gel chamber at constant voltage until the DNA bands were resolved on the gel. Factors that affect the migration of DNA include the size and conformation. To overcome the role of DNA conformation on migration, linear DNA (cut with DNA restriction enzymes) were usually subjected to electrophoresis. Ethidium

bromide is used to stain DNA for visualization. Ethidium bromide intercalates with nucleic acids and fluoresces when illuminated with UV light. DNA bands were visualised using a UV transilluminator and photographs were taken for records. For further analysis target DNA bands were cut with a clean scalpel and frozen at -20^{0}C. Future experiments were perfomed by isolating DNA from gel pieces using standard gel extraction protocol as described (2.2.1.1).

2.2.1.3. Restriction digestion, modification and cloning of DNA

Compatible DNA for cloning was prepared by digesting the vector and the insert with appropriate restriction enzyme(s) for 2 hours at 37°C. The typical reaction mixture consists of 2 μg of plasmid DNA, 3 μl of 10X enzyme buffer, 1 μl of appropriate restriction enzyme and water to make the final volume to 30 ul. To avoid re-ligation of vector DNA, treatment with alkaline phosphate was performed for 1 hour at 37^{0}C. Digested DNA was then separated on agarose gel, analysed for the expected DNA bands on a UV transilluminator and DNA was extracted using standard gel extraction protocol.

Cloning of vector and insert DNA was done using a DNA ligation kit (Fermentas). Vector and insert DNAs were mixed in appropriate ratios as recommended by the manufacturer. To this mixture, 4 μl of 5X ligation buffer was added along with 1 μl of T4 DNA ligase and sterile water to make the final volume to 20 μl. The mixture was gently mixed and left at room temperature for 1 hour before transforming into DH5alpha *E.coli* bacteria.

EGFR-WT, EGFR-L858R and EGFR-Δ747-753insS were subcloned from pRK into the pcDNA3.1 after double digestion of both vectors with *EcoR*V and *Xho*I. Wild-type (WT) and mutant EGFR were cut with *Xho*I and *Eco*RV from pcDNA3.1 vector and subcloned into the MSCV-YFP (MIY) using *Xho*I and *Hpa*I. EGFRvIII (a kind gift from Frank Furnari, Ludwig Institute for Cancer Research, San Diego, CA) was subcloned from pLERNL (digested with *Sal*I) into the MSCV-eGFP (MigRI) that was digested with *Bgl*II.

2.2.1.4. Site directed mutagenesis and DNA sequence analysis

Point mutations were introduced into target cDNA using a site-directed mutagenesis method as shown (Figure 15). Primers were designed such that the mutation is present at the desired nucleotide location and a PCR was performed for 18 cycles. The resulting PCR product contains both the template DNA as well as the mutated product. To deplete

unmutated template from the PCR product, *Dpn*I restriction digestion was performed. *Dpn*I is a restriction enzyme which digests only methylated substrate DNA only when methylated[209]. Since template DNA is derived from a bacterial culture, it is methylated making it selectively susceptible to *Dpn*I restriction digestion while the mutated PCR product is intact. The nicks left in the product DNA are sealed upon transformation into appropriate bacteria strain. Bacteria colonies were then picked, minipreps were made and confirmation of the presence of mutation was done by restriction analysis and DNA sequence analysis.

All point mutations were introduced into pcDNA3.1/EGFR-WT, MigR1/EGFRvIII and MigR1/ERBB2-WT (a kind gift from Dr. Heinke Conrad) using the QuikChange Site-Directed Mutagenesis kit (Fermentas) according to the manufacturer's instructions. All constructs were confirmed by sequencing.

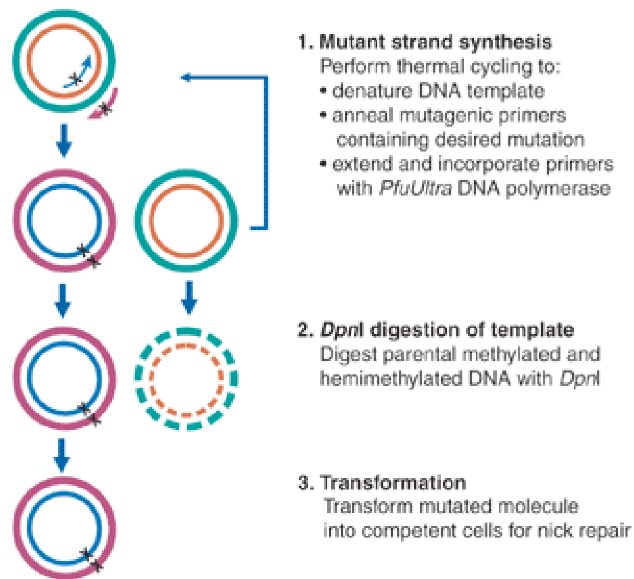


Figure 15. Schematic representation of site directed mutagenesis (Adapted from Smith C *et al.*[209]). Steps involving site directed mutagenesis were depicted in the diagram. Template DNA was shown as green and orange strands while mutated product was shown in blue and violet.

Following primers were used to clone point mutations by site-directed mutagenesis:

MSCV-eGFP-FLT3-ITD + N676D

Forward: 5’-CCACGAGAATATTGTGGACCTGCTGGGGGCGTG-3’

Reverse: 5’-CACGCCCCCAGCAGGTCCACAATATTCTCGTGG-3’

MSCV-eGFP-FLT3-ITD + F691I

Forward: 5’-GGACCAATTTACTTGATTATTGAATATTGTTGCTATG-3’

Reverse: 5’-CATAGCAACAATATTCAATAATCAAGTAAATTGGTCC-3’

MSCV-eGFP-FLT3-ITD + G697R

Forward: 5’-GAATATTGTTGCTATCGTGACCTCCTCAACTAC-3’

Reverse: 5’- GTAGTTGAGGAGGTCACGATAGCAACAATATTC-3’

pcDNA3.1/Zeo-EGFR + L688P and MSCV-eGFP-EGFRvIII + L688P

Forward: 5’-GCTGCAGGAGAGGGAGCCTGTGGAGCCTCTTACAC-3’

Reverse: 5’-GTGTAAGAGGCTCCACAGGCTCCCTCTCCTGCAGC-3’

pcDNA3.1/Zeo-EGFR + P694L

Forward: 5’-GGAGCCTCTTACACTCAGTGGAGAAGCTC-3’

Reverse: 5’-GAGCTTCTCCACTGAGTGTAAGAGGCTCC-3’

pcDNA3.1/Zeo-EGFR + P694S

Forward: 5’-GTGGAGCCTCTTACATCCAGTGGAGAAGCTC-3’

Reverse: 5’-GAGCTTCTCCACTGGATGTAAGAGGCTCCAC-3’

pcDNA3.1/Zeo-EGFR + G719C

Forward: 5’-CAAAAAGATCAAAGTGCTGTGCTCCGGTGCGTTCGGCAC-3’

Reverse: 5’-GTGCCGAACGCACCGGAGCACAGCACTTTGATCTTTTTG-3’

pcDNA3.1/Zeo-EGFR + G719S and MSCV-eGFP-EGFRvIII + G719S

Forward: 5’- CAAAAAGATCAAAGTGCTGAGCTCCGGTGCGTTCGGCAC-3’

Reverse: 5’- GTGCCGAACGCACCGGAGCTCAGCACTTTGATCTTTTTG-3’

pcDNA3.1/Zeo-EGFR + L730F

Forward: 5'-CACGGTGTATAAGGGATTCTGGATCCCAGAAGG-3'

Reverse: 5'-CCTTCTGGGATCCAGAATCCCTTATACACCGTG-3'

pcDNA3.1/Zeo-EGFR + P733L

Forward: 5'-GGGACTCTGGATCCTAGAAGGTGAGAAAG-3'

Reverse: 5'-CTTTCTCACCTTCTAGGATCCAGAGTCCC-3'

pcDNA3.1/Zeo-EGFR + G735S

Forward: 5'-GGACTCTGGATCCCAGAAAGTGAGAAAGTTAAAATTC-3'

Reverse: 5'-GAATTTTAACTTTCTCACTTTCTGGGATCCAGAGTCC-3'

pcDNA3.1/Zeo-EGFR + V742A

Forward: 5'-GTTAAAATTCCCATCGCTATCAAGG-3'

Reverse: 5'-CCTTGATAGCGATGGGAATTTTAAC-3'

pcDNA3.1/Zeo-EGFR + E746K

Forward: 5'-CGTCGCTATCAAGAAATTAAGAGAAGC-3'

Reverse: 5'-GCTTCTCTTAATTTCTTGATAGCGACG-3'

pcDNA3.1/Zeo-EGFR + E749K

Forward: 5'-CGCTATCAAGGAATTAAGAAAAGCAACATCTCCGAAAGC-3'

Reverse: 5'-GCTTTCGGAGATGTTGCTTTTCTTAATTCCTTGATAGCG-3'

pcDNA3.1/Zeo-EGFR + S752Y

Forward: 5'-GAAGCAACATATCCGAAAGCC-3'

Reverse: 5'-GGCTTTCGGATATGTTGCTTC-3'

pcDNA3.1/Zeo-EGFR + D761N

Forward: 5'-GCCAACAAGGAAATCCTCAATGAAGCCTACGTGATGG-3'

Reverse: 5'-CCATCACGTAGGCTTCATTGAGGATTTCCTTGTTGGC-3'

pcDNA3.1/Zeo-EGFR + A767T

Forward: 5'-GAAGCCTACGTGAGGACCAGCGTGGACAACC-3'

Reverse: 5'-GGTTGTCCACGCTGGTCATCACGTAGGCTTC-3'

pcDNA3.1/Zeo-EGFR + S768I

Forward: 5'-CTACGTGATGGCCATCGTGGACAACCCCC-3'

Reverse: 5'-GGGGGTTGTCCACGATGGCCATCACGTAG-3'

pcDNA3.1/Zeo-EGFR + R776C

Forward: 5'-CCCCACGTGTGCTGCCTGCTGGGCA-3'

Reverse: 5'-TGCCCAGCAGGCAGCACACGTGGGG-3'

pcDNA3.1/Zeo-EGFR + S784F

Forward: 5'-GGCATCTGCCTCACCTTCACCGTGCAGCTCATC-3'

Reverse: 5'-GATGAGCTGCACGGTGAAGGTGAGGCAGATGCC-3'

pcDNA3.1/Zeo-EGFR + T790M

Forward: 5'-GCAGCTCATCATGCAGCTCATGC-3'

Reverse: 5'-GCATGAGCTGCATGATGAGCTGC-3'

pcDNA3.1/Zeo-EGFR + G810S

Forward: 5'-CACAAAGACAATATTAGCTCCCAGTACCTGC-3'

Reverse: 5'-GCAGGTACTGGGAGCTAATATTGTCTTTGTG-3'

pcDNA3.1/Zeo-EGFR + N826S

Forward: 5'-GCAAAGGGCATGAGCTACTTGGAGGAC-3'

Reverse: 5'-GTCCTCCAAGTAGCTCATGCCCTTTGC-3'

pcDNA3.1/Zeo-EGFR + L838V

Forward: 5'-GGTGCACCGCGACGTGGCAGCCAGGAACG-3'

Reverse: 5'-CGTTCCTGGCTGCCACGTCGCGGTGCACC-3'

pcDNA3.1/Zeo-EGFR + T847I

Forward: 5’-GTACTGGTGAAAATACCGCAGCATGTC-3’

Reverse: 5’-GACATGCTGCGGTATTTTCACCAGTAC-3’

pcDNA3.1/Zeo-EGFR + V851A and MSCV-eGFP-EGFRvIII + V851A

Forward: 5’-GAAAACACCGCAGCATGCCAAGATCACAGATTTTG-3’

Reverse: 5’-CAAAATCTGTGATCTTGGCATGCTGCGGTGTTTTC-3’

pcDNA3.1/Zeo-EGFR + I853T and MSCV-eGFP-EGFRvIII + I853T

Forward: 5’-CCGCAGCATGTCAAGACCACAGATTTTGGGCTG-3’

Reverse: 5’-CAGCCCAAAATCTGTGGTCTTGACATGCTGCGG-3’

pcDNA3.1/Zeo-EGFR + L861Q and MSCV-eGFP-EGFRvIII + L861Q

Forward: 5’-GCTGGCCAAACAGCTGGGTGCGG-3’

Reverse: 5’-CCGCACCCAGCTGTTTGGCCAGC-3’

MSCV-eGFP-EGFRvIII + L858R

Forward: 5’-GATCACAGATTTTGGGCGGGCCAAACTGCTGGGTG-3’

Reverse: 5’-CACCCAGCAGTTTGGCCCGCCCAAAATCTGTGATC-3’

pcDNA3.1/Zeo-EGFR + A864T

Forward: 5’-CTGGCCAAACTGCTGGGTACGGAAGAGAAAGAATACC-3’

Reverse: 5’-GGTATTCTTTCTCTTCCGTACCCAGCAGTTTGGCCAG-3’

pcDNA3.1/Zeo-EGFR + E866K

Forward: 5’-CTGCTGGGTGCGGAAAAGAAAGAATACCATG-3’

Reverse: 5’-CATGGTATTCTTTCTTTTCCGCACCCAGCAG-3’

pcDNA3.1/Zeo-EGFR + G873A

Forward: 5’-GAATACCATGCAGAAGAAGGCAAAGTGCCTATC-3’

Reverse: 5’-GATAGGCACTTTGCCTTCTTCTGCATGGTATTC-3’

<u>MSCV-eGFP-ERBB2-I655V</u>

Forward: 5'-CAGCCCTCTGACGTCCATCGTCTCTGCGGTGG-3'

Reverse: 5'-CCACCGCAGAGACGATGGACGTCAGAGGGCTG-3'

<u>MSCV-eGFP-ERBB2-I654V</u>

Forward: 5'-CAGCCCTCTGACGTCCGTCGTCTCTGCGGTGGTTG-3'

Reverse: 5'-CAACCACCGCAGAGACGACGGACGTCAGAGGGCTG-3'

<u>MSCV-eGFP-ERBB2 + L755S</u>

Forward: 5'-CAGTGGCCATCAAAGTGCCGAGGGAAAACACATCCCC-3'

Reverse: 5'-GGGGATGTGTTTTCCCTCGGCACTTTGATGGCCACTG-3'

<u>MSCV-eGFP-ERBB2 + L755P</u>

Forward: 5'-CCAGTGGCCATCAAAGTGCCGAGGGAAAACACATCCCC-3'

Reverse: 5'-GGGGATGTGTTTTCCCTCGGCACTTTGATGGCCACTGG-3'

<u>MSCV-eGFP-ERBB2 + V773A</u>

Forward: 5'-GACGAAGCATACGCGATGGCTGGTGTG-3'

Reverse: 5'-CACACCAGCCATCGCGTATGCTTCGTC-3'

<u>MSCV-eGFP-ERBB2 + V777L</u>

Forward: 5'-CATACGTGATGGCTGGTCTGGGCTCCCCATATGTC-3'

Reverse: 5'-GACATATGGGGAGCCCAGACCAGCCATCACGTATG-3'

<u>MSCV-eGFP-ERBB2 + T798M</u>

Forward: 5'-ACGGTGCAGCTGGTGATGCAGCTTATGCCCTATG-3'

Reverse: 5'-CATAGGGCATAAGCTGCATCACCAGCTGCACCGT-3'

<u>MSCV-eGFP-ERBB2 + N857S</u>

Forward: 5'-GCTGGTCAAGAGTCCCAGCCATGTCAAAATTACAG-3'

Reverse: 5'-CTGTAATTTTGACATGGCTGGGACTCTTGACCAGC-3'

<u>MSCV-eGFP-ERBB2 + T862A</u>

Forward: 5'-CCCAACCATGTCAAAATTGCAGACTTCGGGCTGGCTC-3'

Reverse: 5'-GAGCCAGCCCGAAGTCTGCAATTTTGACATGGTTGGG-3'

<u>MSCV-eGFP-ERBB2 + H878Y</u>

Forward: 5'-CGAGACAGAGTACTATGCAGATGGGGG-3'

Reverse: 5'-CCCCCATCTGCATAGTACTCTGTCTCG-3'

Following primers were used to amplify or sequence the kinase domain:

<u>FLT3-KD</u>

Forward: 5'-GCAACAATTGGTGTTTGTCTCCTC -3'

Reverse: 5'-GGTCTCTGTGGACACACGACTTGAAC -3'

<u>EGFR-KD</u>

Forward: 5'-CGGCCTCTTCATGCGAAGGCGCC-3'

Reverse: 5'-CCAGACATCACTCTGGTGGGTATAG-3'

<u>ERBB2-KD</u>

Forward: 5'-GAAAACGGAGCTGGTGGAGCCGC -3'

Reverse: 5'-GCCACTCCTGGTAGATGAGCTGCGGTGCCTGTGGT -3'

2.2.1.5. Transformation of *E.Coli* and inoculation of culture for DNA isolation

LB-Medium: 1% Bacto-Tryptone, 1% NaCl, 0.5% Bacto-Yeast extract in A.d.; autoclaved; pH 7.0
LB-Agar plates: 1.5% Bactoagar in LB-Medium; autoclaved
Ampicillin: 50 mg/ml

DNA was transformed in to the competent *DH5 alpha* strain of *E.Coli*. Competent cells were thawed on ice before mixing with appropriate amount of DNA or reaction mixture that contains DNA. Cells were incubated with DNA on ice for 30 minutes followed by a brief heat shock at 37^0C for 42 seconds. Cells were then kept on ice for 2 minutes and then incubated in LB medium for 45 minutes for recovery. Part of the culture was then plated on

to LB plates that contains ampicillin as antibiotic. Plates were incubated overnight at 37^0C and single clones were picked for inoculation on the following day.
For analysis of transformed DNA, single clones were inoculated into 5 ml of LB medium (with added ampicillin) for minipreps. For maxiprep, single clones were inoculated into 200 ml culture. Cells were then cultured overnight at 37^0C incubator with rotating platform. Following day, cells were centrifuged to make bacterial pellet that was used for DNA isolation after discarding residual medium.

2.2.1.6. RNA isolation, measurement and cDNA synthesis

RNA isolation was performed in highly sterile conditions using TRIzol based method. 5 x 10^6 Ba/F3 cells were pelleted at 1300 rpm for 5 minutes. Cell pellet was then resuspended in 1 ml of TRIzol reagent at room temperature and vortexed briefly. 0.2 ml of chloroform was then added to the lysate and samples were vortexed vigorously for 15 seconds. Centrifugation of samples was then performed at 14000 rpm at 4^0C for 15 minutes. Colourless upper aqueous layer was then collected in a sterile tube without disturbing the lower organic phase. RNA from the aqueous phase was separated by adding 0.5 ml of isopropanol, incubated for 15 minutes at room temperature and centrifuged at 14000 rpm (at 4^0C) for 10 minutes. RNA pellet was then washed with 1 ml of 70% ethanol and air dried for 10 minutes. RNA was dissolved in sterile water and measured for purity and concentration using “Nanodrop” machine.
cDNA synthesis was performed using one step reverse-transcription PCR according to manufacturer instructions. Primers were designed to amplify kinase domain of the ERBB2. A one-step PCR was performed according to the manufacturer’s (Promega) instructions and the cDNA was analyzed on the gel for correct size of the DNA bands. The correct DNA band corresponding ERBB2 kinase was then cut out of the gel, purified using QIAquick gel extraction kit and sequenced. Resulting DNA sequence was then analyzed for the presence of kinase domain mutations in ERBB2 kinase using BLAST program.

2.2.2. Methods involving proteins

2.2.2.1. Isolation of proteins

Cell lysis buffer: 10 mM Tris/HCl (pH 7.5), 130 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 20 mM Na_2HPO_4/NaH_2PO_4 (pH 7.5), 10 mM Sodiumpyrophosphate (pH 7.0), 1 mM Sodiumorthovanadate, 20 mM Sodium fluoride, 1 mM Glycerol-2-Phosphate, 1 Protease-Inhibitor Cocktail tablet

Cells were first pelleted for protein isolation. $5x10^6$ Ba/F3 cells in suspension were subjected to centrifugation at 1300 rpm for 3 minutes. NIH/3T3 cells and HEK293 cells were dislodged from the plates by trypsinization and spun at 1300 rpm for 3 minutes. Medium was drained and cell pellets were frozen in liquid nitrogen before lysis. Cell lysis was performed using standard cell lysis buffer on ice for 30 minutes. Cell lysates were centrifuged at 13000 rpm for 20 minutes and supernatant was separated into fresh tubes. Protein concentration was determined using Bradford method. SDS loading buffer was then added to protein sample and heated at 95^0C for 5 minutes. Samples were briefly spun before performing SDS-PAGE. Remaining protein samples were frozen at -20^0C for future use.

2.2.2.2. SDS gel electrophoresis and gel staining

Stacking gel: 5% Polyacrylamide solution, 12.5mM Tris/HCl (pH 6.8), 0.1%SDS, 0.3% APS, 0.1% TEMED
Resolving gel: 8% Polyacrylamide solution, 375mM Tris/HCl (pH 8.8), 0.1%SDS, 0.3% APS, 0.1% TEMED
SDS-PAGE running buffer: 25 mM Tris, 192 mM Glycine, 0.1% SDS in A.d.
SDS-PAGE loading buffer (2X): 1 M Tris/HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% Glycine in A.d.
Coomassie stain: 0.25% Brilliant Blue, 45% Methanol, 10% Acetic acid in A.d.
Comassie destaining solution: 45% Methanol, 10% acetic acid in A.d.

Polyacrylamide gels were used to separate proteins according to their size. Gel casting apparatus were used to cast gels of appropriate size and percentage of acrylamide/bisacrylamide. Protein samples in loading buffer were then loaded in defined order and proteins were fractionated according to their size under applied electric field. Following SDS gel electrophoresis, gels were either stained with Coomassie stain or used

for western transfer on to the membrane for blotting. For staining, gels were kept in coomassie stain for 30 minutes followed by repeated washes with coomassie destaining solution.

2.2.2.3. Western blotting and assay for activated proteins

Transfer buffer: 25mM Tris, 192mM Glycine, 0.1% SDS, 20% Methanol in A.d.
Amidoblack solution: 0.2% Naphtol Blue Black, 25% Isopropanol, 10% Acetic acid in A.d.
Amidoblack destainer: 25% Isopropanol, 10% Acetic acid A.d.
PBS-Tween buffer: 0.1% Tween 20 in PBS
Blocking solution: 5% skimmed milk powder or BSA in PBS-Tween buffer

Transfer of proteins from gels onto a PVDF membrane was performed in western transfer apparatus that utilized transfer buffer. After western transfer, PVDF membranes were then incubated with 5% of BSA or milk to block non specific binding of antibody to the membrane. PVDF membranes were then incubated in diluted primary antibody at 4^0C overnight. Primary antibodies were diluted in 5% milk or BSA according to manufacturer's recommendation. Following day, PVDF membrane was washed thrice with PBS (+Tween) solution for 10 minutes before incubating with secondary antibody (conjugated to HRP enzyme) for 30 minutes. The membrane was then washed thoroughly and a chemiluminiscence substrate was added to the membrane. Target protein bands were visualized by capturing the signals on a photographic film. Quantification of the bands was done using ImageJ software.
Autokinase activity of receptor tyrosine kinases was measured using the antibodies that specifically recognize phosphorylated proteins. To test the effect of kinase inhibitors, cells were treated with different concentrations of inhibitors for 2 hours and then lysed for analysis. Reduction of phospho protein levels compared to total protein levels was analyzed on western blots. Activation of signaling pathways was tested using antibodies that recognize key activated downstream targets of the kinase.

2.2.2.4. Analysis of EGFR cell surface expression

HEK293 cells were transfected with wildtype and kinase dead EGFR mutants (in pcDNA 3.1 vector) using Lipofectamine 2000 reagent. 48 hours after transfection, cells were

washed twice with PBS and 10^5 cells were resuspended in FACS buffer (0.1% BSA in PBS). 1 μg of cetuximab was added to each sample for 30 minutes at 4^0C. Cells were then washed twice with FACS buffer before staining with a Alexa Fluor 488 goat anti-human IgG (H+L) for 30 minutes at 4^0C. Cells were washed and analyzed by FACS. Empty vector (pcDNA 3.1) transfected cells were taken as a negative control. Wildtype EGFR expressing cells were taken as a positive control.

2.2.3. Mammalian cell culture and transfection

2.2.3.1. Cell culture

Ba/F3 cells (murine pro-B cell line) were cultured as suspension in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1% penicillin/streptomycin solution and 0.2 ng/mL of interleukin-3 (IL-3). Ba/F3 cells that were transformed by stable expression of oncogenes were cultured in the medium devoid of IL-3.

NIH/3T3 (mouse fibroblast cell line) and HEK293 (human embryonic kidney cell line) cells were cultured in DMEM medium supplemented with 10% FCS and glutamine.

2.2.3.2. Transfection

FugeneHD reagent was used for the transient expression of human cDNA constructs in NIH/3T3 cells was achieved by mixing DNA (2 μg) with 6 μl of Fugene HD reagent for 30 minutes in 100 μl serum-free OPTI-MEM medium. The mixture was then added to the cell culture in a 6-well plate. Transfection medium was replaced with fresh medium after 24 hours. HEK293 cells were transfected with Lipofectamine 2000 reagent. 10 μg of DNA and 20 μl of Lipofectamine 2000 reagent were separately mixed with 0.5 ml of serum-free OPTI-MEM medium for 5 minutes. DNA and lipofectamine mixtures were mixed, incubated for 20 minutes and then added to HEK293 cell culture on a 60 mm plate. Transfection medium was replaced by fresh medium after 24 hours of transfection.

2.2.4. Retroviral infection and establishment of stable cell lines

Retrovirus was produced using retroviral vectors and then infected mammalian cells for stable expression of desired proteins. The procedure involved the transfection of PhoenixE

cells (a packaging cell line) with appropriate retroviral cDNA construct, collection of virus and the infection of target cells by spin infection. PhoenixE is a 293T-based cell line designed to produce gag-pol and env proteins for replication-incompetent ecotropic retrovirus (Moloney Murine Leukemia Virus, MMULV) production. Gag-pol (along with the hygromycin resistance marker) is expressed under CMV promoter while env (along with the diptheria resistance marker) is expressed from RSV promoter thus avoiding recombination between the two constructs.

2.2.4.1. Infection of cell lines with retrovirus

Retroviral vector (MiGR1 or MIY) with target cDNA (FLT3 or EGFR or ERBB2) was transfected into the PhoenixE cells (retroviral packaging cell line). For transfection, 10 μg of MSCV-based vector was mixed with 20 μl of Lipofectamine 2000 in 1 ml of serum-free OPTI-MEM for 20 minutes and then added to the PhoenixE cells. 24 hours post transfection, fresh medium was added to the transfected cells (refer to 2.2.3.2 detailed protocol). Cell culture medium which contains retrovirus was collected at 36h and 48h after transfection. This was then purified using a 0.45 μM filter.

For retroviral infection, 1 x 10^5 Ba/F3 cells per well were then taken in a 12 well plate and incubated with 2 ml of collected retrovirus. To increase the efficacy of infection, polybrene (4 μg/ml) was added to the medium along with IL-3 for Ba/F3 cell survival. 12-well plates were then subjected to centrifugation at 2400 rpm for 90 minutes at 32°C. The entire procedure of spin infection was repeated at 12 hours after first infection.

2.2.4.2. Assay to test transformation ability of oncogenic mutants

48h after spin infection, cells were subjected to IL-3 withdrawal. Retroviral MiGR1 vector coexpresses eGFP along with target protein. Cells expressing eGFP can be tracked as green cells in flow cytometer. Since the fraction of infected cell population expressing oncoprotein are green, the outgrowth of such green cells compared to parental Ba/F3 cells (uninfected cells which are not green) can be measured as a percentage in the mixed population by FACS analysis. Ba/F3 cells stably expressing oncogene were said to be transformed if the infected cell population becomes 100% GFP- or YFP-positive (Figure 16). This happens because parental (uninfected) Ba/F3 cells die quickly due to lack of IL-3. On the contrary, oncogenes provide the required survival and proliferative signals in the

infected cells. If the infected cDNA lacks oncogenic properties, Ba/F3 cells won't be transformed. The stronger the oncogene, the faster the Ba/F3 cells become cytokine independent.

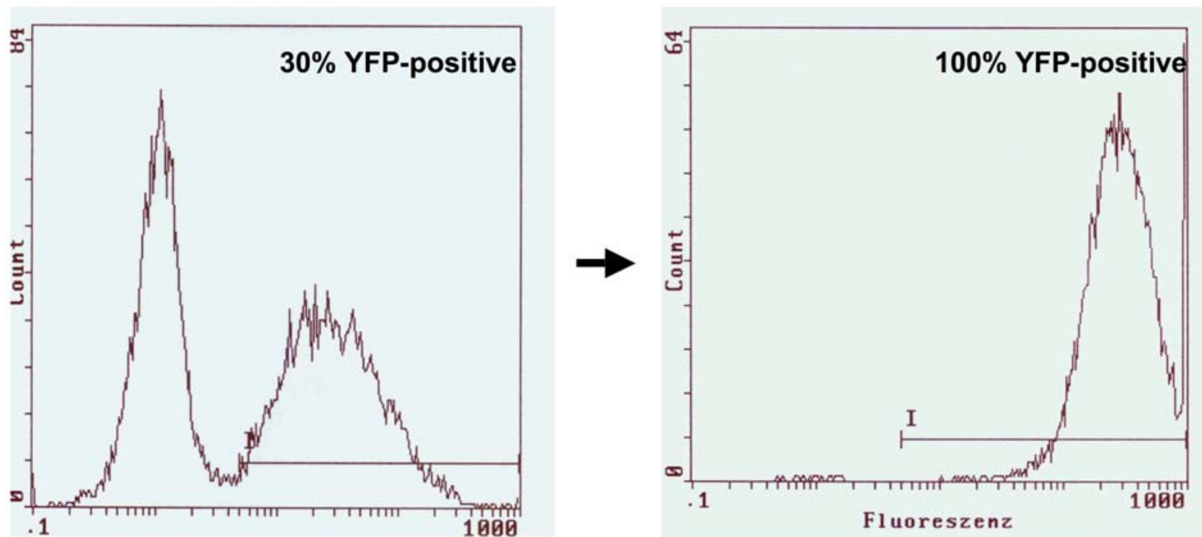


Figure 16. Transformation of Ba/F3 cells by MSCV-YFP-EGFR.
Ba/F3 cells were infected with retrovirus expressing MSCV-YFP-EGFR mutant construct and subjected to IL-3 withdrawal. Before cytokine withdrawal, freshly transduced Ba/F3 cells were 30% positive (left) for YFP. FACS analysis of transformed Ba/F3 cells expressing oncogenic EGFR mutant showed 100% YFP positivity (right).

2.2.5. Drug treatment and identification of drug resistant mutations

The effect of various inhibitors on target cells was measured in a cell proliferation assay using 96-well plates. All inhibitor stock solutions were prepared in DMSO and diluted in RPMI 1640 medium to make appropriate concentrations of inhibitor solutions.

2.2.5.1. Cell proliferation and cell death assay

Cell proliferation analysis was performed using exponentially growing fresh cell cultures. Ba/F3 cells transformed by oncogenic mutant receptor tyrosine kinases were used for the purpose. 1 x 10^4 Ba/F3 cells were plated in 100 μl in each well of a 96-well plate along with the required concentration of the drug. Medium and DMSO controls were taken for all the experiments. Parental Ba/F3 cells in IL-3 were used as control to measure non specific toxicity. Cell proliferation was measured after 48 hours using the CellTiter96 Proliferation Assay (Promega) according to the manufacturer's instructions.

To measure cell death induction by inhibitor treatment, 1 x 10^6 Ba/F3 cells were taken in 4ml of RPMI1640 medium in each well of a 12-well plate. 48 hours after inhibitor treatment, propidium iodide (5 μg/mL) was added to 300 μl of cells and analysed for cell death by FACS. Propidium iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference. PI is membrane impermeant and generally excluded from viable cells. Thus, only the DNA of dead cells are stained which is the basis for identifying the fraction of dead cells in a population.

2.2.5.2. Cell based screen to identify inhibitor resistant mutations

Ba/F3 cells stably expressing wild type ErbB2 were treated twice with 100 μg/mL of a chemical mutagen, N-ethyl-N-nitrosourea (ENU) for 12 hours. Cells were thoroughly washed to remove residual ENU. Mutated cells were then cultured in 96-well plates at a density of 4 x 10^5 cells per well in the presence of 2 μM lapatinib, an EGFR/ERBB2 dual inhibitor. Lapatinib resistant cell colonies were isolated later. Total RNA was extracted using TRIzol reagent (Invitrogen). ErbB2 kinase domain cDNA was amplified by one step reverse-transcription PCR (Promega) and sequenced using the primers described.

3. Results

3.1 Differential sensitivity of FLT3 receptor mutants towards kinase inhibitors

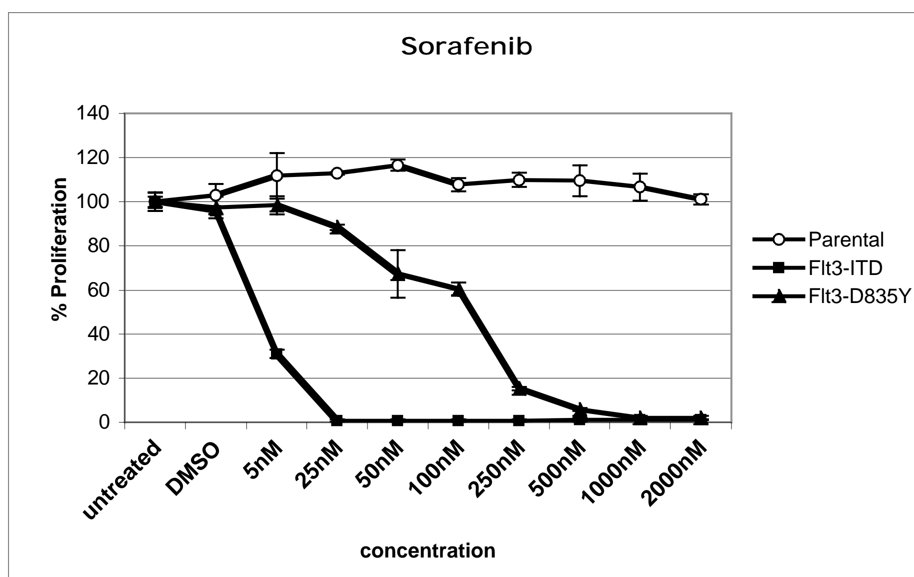
Most common mutations in AML were reported in the FLT3 receptor[89, 90]. Approximately one third of AML patients have an internal tandem duplication (ITD) in the juxtamembrane region of FLT3 receptor. The FLT3-ITD mutation is associated with a decreased survival[89, 90, 210]. Additionally 8-12% of AML patients have a point mutation (D835Y) in the tyrosine kinase domain and FLT3-D835Y is not associated with bad prognosis[89, 90]. Both mutations cause constitutive kinase activity due to loss of autoinhibition leading to the upregulation of promitogenic and prosurvival pathways [86]. The oncogenic potential of both FLT3-ITD and FLT3-D835Y were well studied in hematopoietic cell lines and in murine bone marrow transplantation models[95, 97]. The goal of the present study is to establish sensitivity profiles of FLT3 activating mutants against novel inhibitors that will be useful to select patients based on mutation for specific drug treatment.

3.1.1 Activating FLT3 receptor mutants vary in sensitivity against different inhibitors

FLT3-ITD and FLT3-TKD mutants were previously shown to be in principle sensitive to kinase inhibitors[95]. For example, PKC412 (Midostaurin/Benzoylstaurosporine/CGP41251) is a staurosporine analog and its efficacy against FLT3 mutants was demonstrated previously[194, 211]. In this study, we tested two novel inhibitors sunitinib and sorafenib for their efficacy against activating FLT3 mutants. Sunitinib and sorafenib are multikinase inhibitors with multiple targets: PDGFR alpha, PDGFR beta, VEGFR1, VEGFR2, VEGFR3, FLT3, c-KIT and CSFR for sunitinib, and Raf, VEGFR1, VEGFR2, VEGFR3, PDGFR beta, FLT3, c-KIT and RET for sorafenib[26, 212-214]. Both sunitinib and sorafenib are approved for their use in clinic for the treatment of solid cancers[25, 215].

We first examined the cellular IC_{50} values of sorafenib (Nexavar/Bay 43-9006) against FLT3-ITD and D835Y. Cell proliferation–based assays showed that the sensitivity toward sorafenib differed significantly between FLT3 D835Y (IC_{50} 100 nM) and ITD (IC_{50} < 5 nM) (Figure 17, upper panel). This is in clear contrast to the sensitivity profiles of PKC412

and sunitinib (Sutent/SU11248) against FLT3-ITD and D835Y, which showed similar IC_{50} values for both mutants (Figure 18). Next, we wanted to determine whether the observed differences in growth inhibition by sorafenib correlated with the phosphorylation status of FLT3 and its downstream target AKT. For this purpose, mutant FLT3-expressing Ba/F3 cells were incubated with increasing concentrations of sorafenib prior to cell lysis. The level of FLT3 and AKT activation was determined by Western blot analysis.



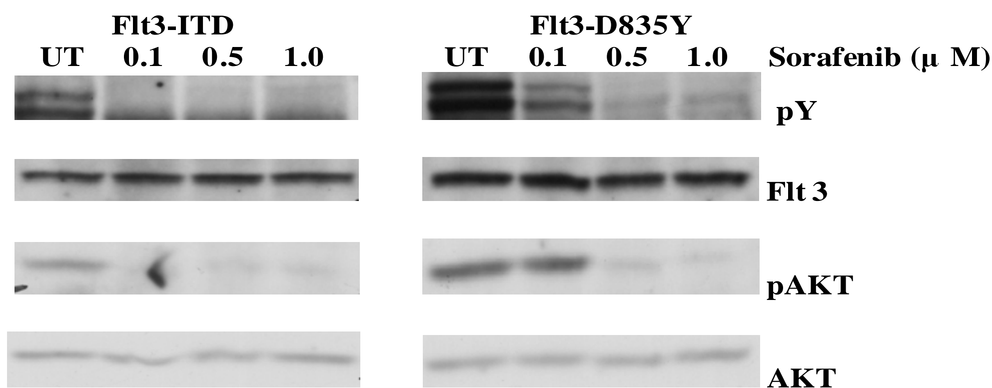


Figure 17. FLT3-ITD is more sensitive to sorafenib than FLT3-D835Y.
Cell proliferation of FLT3-ITD and FLT3-D835Y expressing Ba/F3 cells in the presence of sorafenib was measured after 48 hours and plotted. Parental Ba/F3 cells in the presence of IL3 were totally resistant to sorafenib induced toxicity at concentrations up to 2 μM. Cell lysates of Ba/F3-FLT3-ITD and Ba/F3-FLT3-D835Y treated with indicated concentrations of sorafenib along with untreated control were probed with phospho tyrosine (pY), Flt3, pAkt and Akt antibodies.

Consistent with proliferation data, inhibition of the phosphorylation of FLT3-ITD was more pronounced compared to D835Y mutation (Figure 17, lower panel). Inhibition of AKT phosphorylation correlated with drug response, indicating that inhibitory effects of sorafenib regarding the proliferation were due to specific inhibition of FLT3. Together, these results indicate that patients with FLT3-ITD may be more responsive to sorafenib than patients with the FLT3-D835Y mutation. PKC412 inhibited both FLT3-ITD and FLT3-D835Y at low nanomolar concentrations (Figure 18).

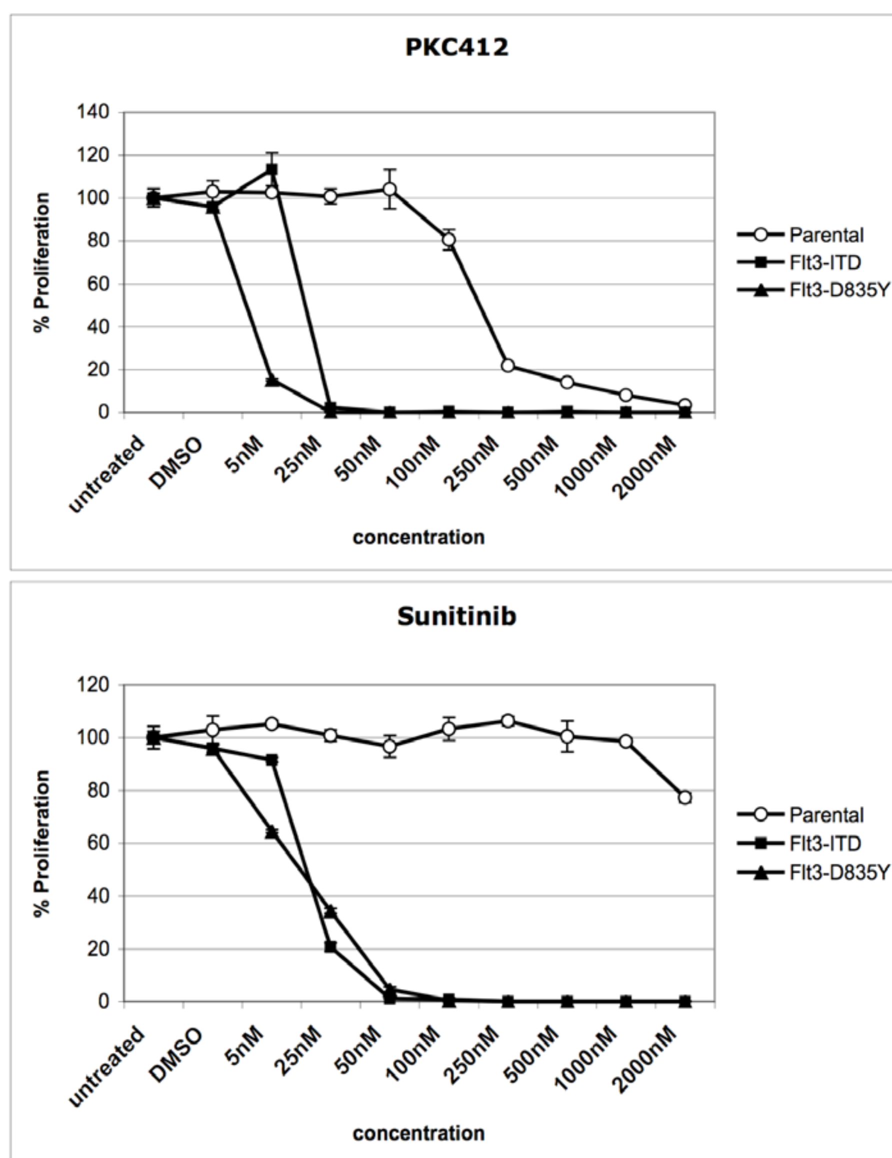


Figure 18. FLT3-ITD and FLT3-D835Y displayed similar sensitivity towards PKC412 and sunitinib. Ba/F3 cells stably expressing FLT3 mutants were treated with PKC412 and sunitinib at indicated concentrations for 48 hours. Parental Ba/F3 cells stimulated with IL3 were taken as negative control to measure non specific toxicity of the drugs tested.

The IC50 values observed were consistent with a previous report[95]. Parental Ba/F3 cells were taken as control to test non specific toxicity. In the presence of IL-3, PKC412 was toxic to parental Ba/F3 cells at concentrations above 100 nM (Figure 18). Sunitinib displayed similar inhibitory activity against the two FLT3 mutated forms without any toxicity in patental Ba/F3 cells up to 2 μM (Figure 18).

3.1.2 Sunitinib and sorafenib are effective against PKC412 resistant FLT3 mutants

Drug resistance due to secondary point mutations in the kinase domain was reported in several kinases. For example, mutations in the kinase domain of BCR-ABL, c-KIT and PDGFR alpha were shown to confer imatinib resistance in CML and GIST patients at the time of relapse[30, 37]. Similarly, the point mutation N676K within the kinase domain in the background of FLT3-ITD was identified in a patient at relapse upon PKC412 treatment. Additionally, several screening strategies were established to predict drug resistance mutations *in vitro*[208]. One such cell based screen has reported FLT3 kinase domain mutations (FLT3-ITD/N676D, FLT3-ITD/F691I and FLT3-ITD/G697R) that confer resistance (IC_{50} > 100 nM) to PKC412 treatment[104]. Thus, we tested whether sorafenib and sunitinib are able to overcome PKC412 resistance induced by secondary mutations in the FLT3-ITD backbone. Upon treatment, FLT3-ITD + N676D was inhibited by both sunitinib and PKC412, with an approximate IC_{50} value of 100 nM (Figure 19). Because PKC412 inhibits the unmutated FLT3-ITD with an IC_{50} value of approximately 5 nM, this represents a large shift in the IC_{50} value. Taking into account the maximal plasma level of PKC412 achievable in patients, this shift seems to be sufficient to explain the PKC412 resistance observed in patients. Also, *in vitro* PKC412 is toxic above the concentration of 100 nM in parental Ba/F3 cells, whereas sunitinib shows no unspecific toxicity at this concentration. Sorafenib was able to inhibit FLT3-ITD + N676D with an IC_{50} value of approximately 50 nM (Figure 19).

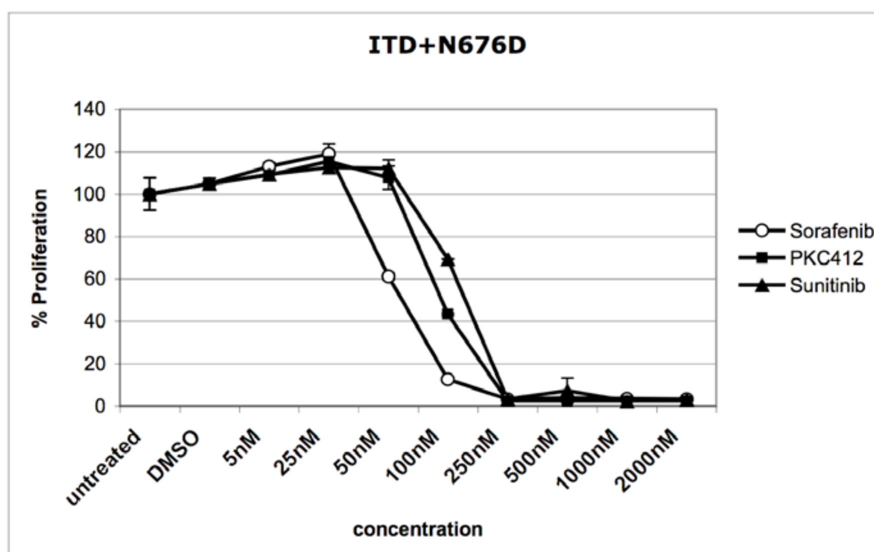


Figure 19. PKC412 resistant FLT3-ITD/N676D is sensitive to sunitinib and sorafenib.
Ba/F3-FLT3-ITD+N676D cells were treated with increasing concentrations of indicated drugs for 48 hours and inhibitory effects on cell proliferation was measured by MTT assay.

F691 is a gatekeeper residue and its mutation to isoleucine is comparable to imatinib-resistant T315I mutation in the Bcr-Abl kinase. Inhibition of T315I of Bcr-Abl or similar mutations in other kinases is a challenge met with less success. Interestingly, FLT3-ITD + F691I can be inhibited by both sunitinib and sorafenib at concentrations not affecting parental cells (Figure 20). Here sunitinib was more effective in inhibiting FLT3-ITD + F691I (IC_{50} 0.5 µM) than sorafenib (IC_{50} 1.0 µM) (Figure 20).

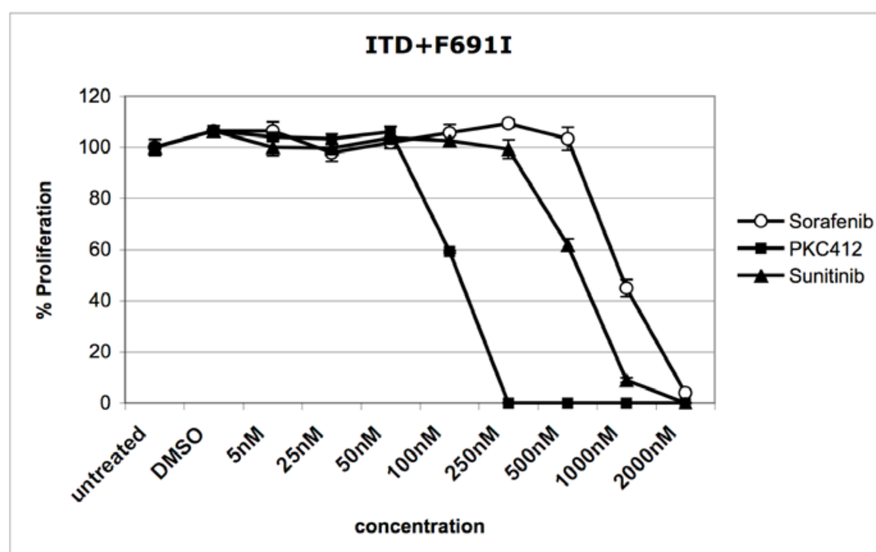


Figure 20. PKC412 resistant FLT3-ITD/F691I is more sensitive to sunitinib than sorafenib.
Stable Ba/F3 cell lines transformed by FLT3-ITD+F691I were tested with 48 hour treatment with indicated concentrations of PKC412, sunitinib and sorafenib.

Finally, the effect of sunitinib and sorafenib on the PKC412-resistant (IC_{50} > 100 nM) FLT3-ITD + G697R was studied. Interestingly, sunitinib had little effect even at concentrations up to 2 µM (Figure 21). In contrast, sorafenib very effectively inhibited FLT3-ITD + G697R with an IC_{50} value of approximately 100 nM, similar or even below the IC_{50} values determined for the other FLT3-ITD–resistant mutants (Figure 21).

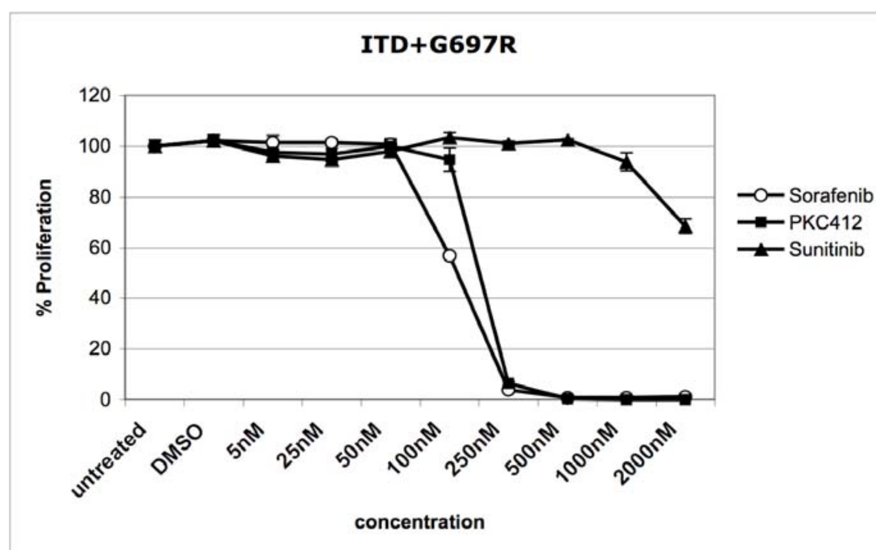


Figure 21. FLT3-ITD/G697R is resistant to both PKC412 and sunitinib but sensitive to sorafenib. Ba/F3-FLT3-ITD+G697R cells were tested for their sensitivity against increasing concentrations of PKC412, sunitinib and sorafenib in a cell proliferation inhibition assay.

Thus, sorafenib inhibited all the PKC412-resistant FLT3-ITD mutations with IC_{50} values (Table 1) within clinically achievable concentrations while sunitinib is ineffective against FLT3-ITD/G697R mutant.

Mutation	PKC 412	Sunitinib	Sorafenib
FLT3-ITD	~ 5 nM	~ 5 nM	< 5nM
FLT3-D835Y	~ 10 nM	~ 5 nM	~ 100nM
FLT3-ITD/ N676D	~ 100 nM	~ 100 nM	~ 50 nM
FLT3-ITD/ F691I	~ 100 nM	~ 0.5 µM	~ 1.0 µM
FLT3-ITD/G697R	~ 100 nM	> 2 µM	~ 100 nM

Table 1 . Approximate IC_{50} values of FLT3 mutants against kinase inhibitors.
Approximate IC_{50} values of FLT3 mutants against indicated kinase inhibitors were calculated from the figures 17 to 21.

3.1.3 Sorafenib potently induces cell death in Ba/F3 cells expressing FLT3 mutations

We then studied the potency of sorafenib to induce cell death in cells expressing the different FLT3 mutations. Sorafenib efficiently induced cell death in a dose-dependent manner in all the mutations tested (Figure 22). Consistent with cell-proliferation inhibition data, D835Y and FLT3-ITD + F691I mutations were relatively less sensitive to cell death upon sorafenib treatment than FLT3-ITD, FLT3-ITD + N676D, and FLT3-ITD + G697R (Figure 22). No cell death was observed in parental Ba/F3 cells upon sorafenib treatment ruling out non-specific toxicity (Figure 22). Thus, sorafenib exhibited both cytostatic as well as cytotoxic activities against activating and PKC412 resistant FLT3 mutants offering a significant promise for the treatment of FLT3 mutant AML patients.

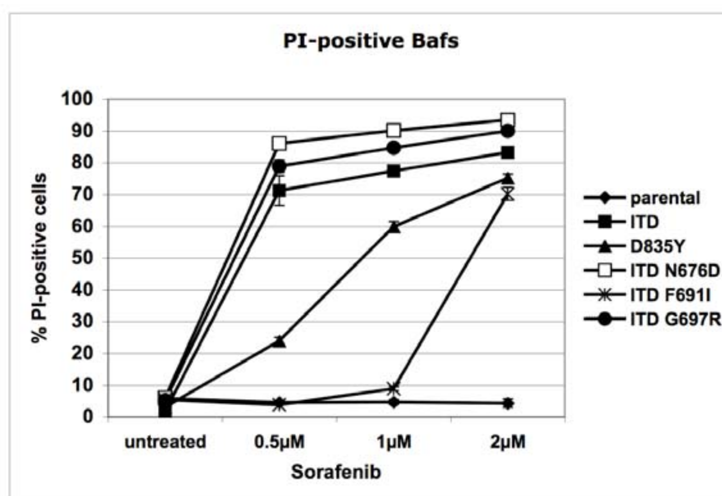


Figure 22. Sorafenib induced cell death in Ba/F3 cells expressing FLT3 mutations.
Parental Ba/F3 and Ba/F3 cells stably expressing FLT3 activating and PKC412-resistant mutations were treated with indicated concentrations of sorafenib for 48 hours and tested for cell death as measured by propidium iodide positivity. Cells were analyzed by FACS.

Since different mutants respond differently against inhibitor treatment, establishing drug sensitivity profiles will give insights in to the role of genetic factors on treatment outcome. In this study, the drug sensitivity profiles of various primary activating and secondary PKC412 resistant mutations were established. Furthermore, induction of cell death in Ba/F3 cells expressing both activating and drug resistant FLT3 mutations was demonstrated with sorafenib at concentrations achievable in patients.

3.2. Functional analysis and drug sensitivity profiles of EGFR kinase domain mutations reported in NSCLC patients

EGFR overexpression or mutation is a frequent genetic abnormality leading to the activation of pro mitogenic and pro survival signaling in several cancers[127, 216]. Recently, activating mutations in the kinase domain of EGFR were shown to sensitize a subset of NSCLC patients to EGFR kinase inhibitor treatment[16]. Several reports followed reporting additional mutations in the EGFR kinase domain[127, 141]. However most mutations were not characterized and their role in cancer progression and drug sensitivity is not known. Correlation between treatment response and EGFR mutation status also varied between clinical studies[141, 142, 217]. Thus we aimed to biochemically characterize individual EGFR kinase domain mutations that were reported in NSCLC patients. We further examined the effect of EGFR inhibitors and established drug sensitivity profiles for individual mutants.

3.2.1. Biochemical characterization of clinically-relevant EGFR mutants

A panel of 30 EGFR kinase domain mutations that were recently reported in NSCLC patients was cloned and expressed for analysis of kinase activity, transforming potential, and drug sensitivity. These mutations affect the N-lobe (exons 18-20) and the C-lobe (exon 21) of the EGFR kinase domain as depicted in Figure 23.

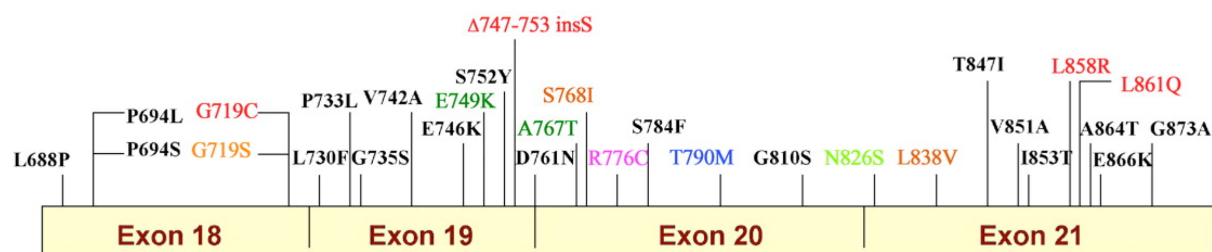


Figure 23. Schematic representation of EGFR kinase domain mutations selected for the study.
EGFR kinase domain mutations reported in NSCLC patients that were selected for the analysis. Mutations in specific exons of EGFR kinase domain were indicated (not drawn to scale). Mutations selected from each study are indicated in different colours: red[16], black[141], orange[134], green[218], pink[135], brown[143], light green[142] and blue[145].

NIH/3T3 cells are devoid of endogenous EGFR and were therefore used for the analysis of autokinase activity of over expressed mutant EGFR proteins. Autophosphorylation of over expressed WT EGFR upon EGF stimulation was taken as a positive control. Most of the

EGFR mutations analyzed showed little or no autophosphorylation without EGF ligand after serum starvation (Figure 24). Addition of EGF resulted in autophosphorylation in the majority of EGFR mutants, except EGFR-L688P, EGFR-V851A, EGFR-I853T, and EGFR-E866K (Figure 24).

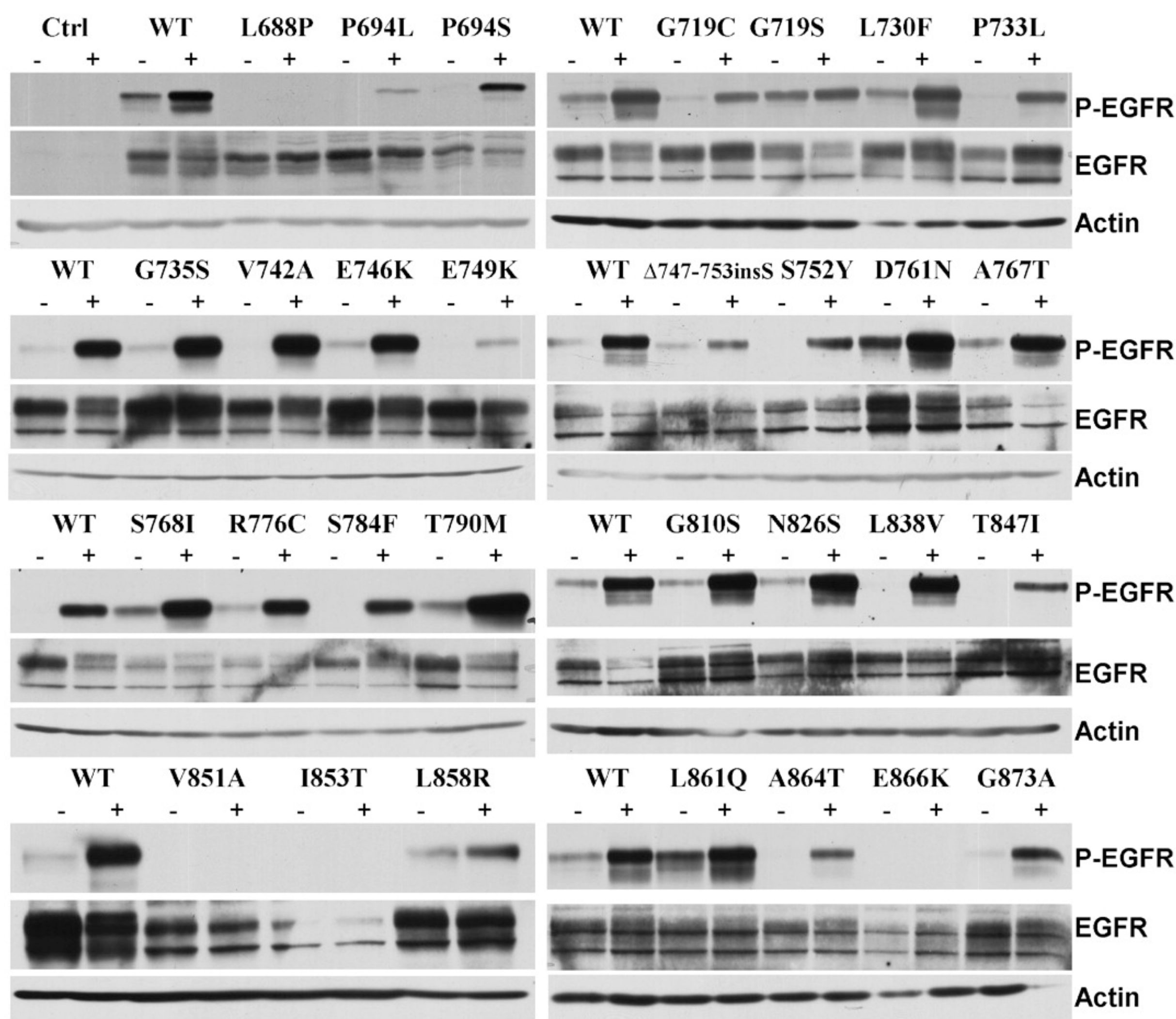


Figure 24. Autophosphorlyation analysis identifies kinase dead EGFR mutants.
Wild type and mutant EGFR in pcDNA3.1 were transfected into NIH/3T3 cells. Cells were serum starved for 12 hours and then stimulated with human EGF for five minutes before lysed for analysis. Autokinase activity was measured using a pEGFR (Tyr 1068) antibody. Untransfected NIH/3T3 cells were taken as a negative control to demonstrated the absence of endogenous EGFR.

Normal cell surface expression of these mutants was detectable and comparable with that of WT EGFR (Figure 25). This indicated that the amino acid changes in these mutations

abrogated the kinase activity of EGFR. These data suggested that some EGFR mutants reported in NSCLC patients lack kinase activity and thus may neither contribute to tumor growth nor serve as a rational target for EGFR kinase inhibitors.

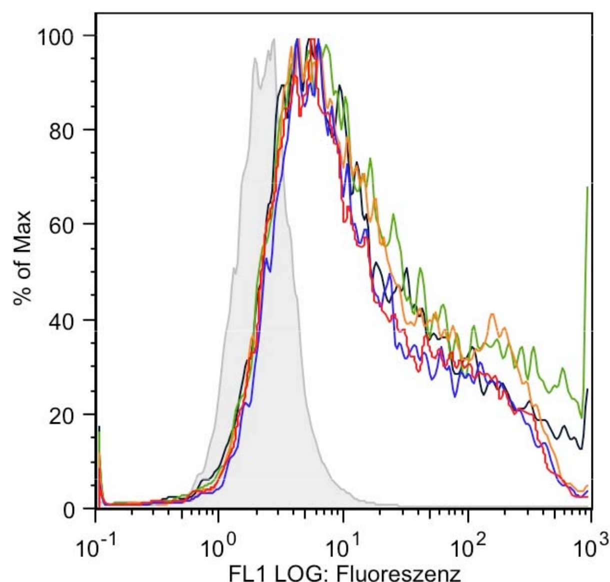


Figure 25. Surface expression of EGFR kinase dead mutations.
Wild type and kinase defective EGFR mutants (in pcDNA 3.1 vector) were transiently overexpressed in HEK293 cells using Lipofectamine 2000 transfection reagent. 48 hours after transfection cells were washed twice with PBS and 10^5 cells were resuspended in FACS buffer (0.1% BSA inn PBS). Cells were then stained with 1 µg of cetuximab and surface expression of EGFR was measured by FACS analysis. Untransfected HEK293 cells were taken as negative control and indicated as shaded peak. Wild type EGFR (black), EGFR-L688P (green), EGFR-V851A (orange), EGFR-I853T (red) and EGFR-E866K (blue) were shown.

3.2.2. Functional characterization of kinase defective EGFR mutations

To test more directly whether the mutations EGFR-L688P, EGFR-V851A, and EGFR-I853T interfere with the kinase activity of EGFR, we cloned these mutations into the background of a constitutively active EGFR mutation (EGFRvIII) and expressed the resulting constructs in HEK293 cells that do not express endogenous EGFR[219]. As a positive control, we introduced the well-characterized activating mutation G719S in the EGFRvIII backbone[201]. Phosphorylation of WT EGFR and its downstream target Stat5 was seen on EGF ligand stimulation (Figure 26). EGFRvIII and EGFRvIII-G719S showed constitutive autophosphorylation and phosphorylation of Stat5 (Figure 26). In contrast, mutations at L688P, V851A, and I853T largely reduced (L688P) or abrogated (V851A and

I853T) the kinase activity of constitutively activated EGFRvIII as evidenced by phosphorylated EGFR and phosphorylated Stat5 levels (Figure 26).

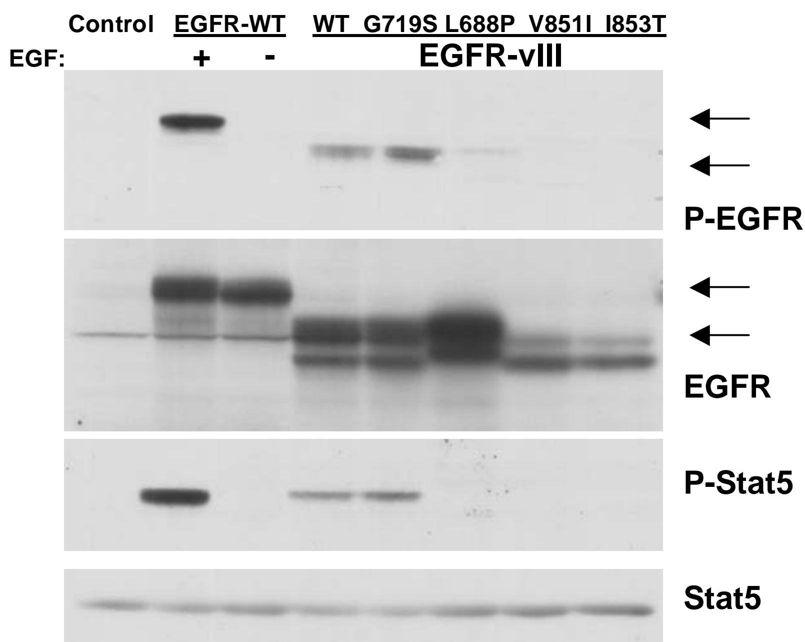


Figure 26. Kinase dead mutations abrogate autokinase activitiy and Stat5 phosphorylation of EGFRvIII.
Kinase defective mutations were cloned into EGFRvIII backbone and transiently expressed in HEK293 cells. Wild type EGFRvIII was taken as a control. EGFRvIII-G719S was taken as positive control. HEK293 cells were transiently transfected with the full length EGFR (unstimulated and stimulated with human EGF) were taken as additional controls. Untransfected cells were taken as a negative control.

We then tested if the observed kinase-dead mutations would have any effect on the oncogenic potency of the constitutively active EGFRvIII. EGFRvIII and EGFRvIII-G719S induced IL-3–independent growth of Ba/F3 cells. In contrast, EGFRvIII-L688P, EGFRvIII-V851A, and EGFRvIII-I853T failed to induce growth factor–independent proliferation of Ba/F3 cells, suggesting that these point mutations indeed result in the loss of the oncogenic potential of EGFRvIII (Table 2).

Mutation	Transforms Ba/F3
EGFR vIII	Yes
EGFR vIII+G719S	Yes
EGFR vIII+L688P	No
EGFR vIII+V851A	No
EGFR vIII+I853T	No

Table 2. Transforming potential of EGFRvIII mutants.
Ba/F3 cells were transduced with wild type and mutated EGFRvIII. Transformation of transduced cells (positive for both EGFRvIII and eGFP) to cytokine independence was measured by FACS analysis.

V851 and I853 are conserved among several receptor tyrosine kinases as shown in Figure 27 and located in the near vicinity of the DFG motif, which is indispensable for kinase activity. It is interesting to note that these residues are conserved in all kinase-active receptors such as EGFR, Her2, and Her4 but absent in the kinase-defective receptor Her3 (Figure 27). Thus, mutations in these conserved residues probably destroy the catalytic core of the EGFR. Identification of EGFR mutations in NSCLC patients that abrogate kinase activity indicates that EGFR mutants in these cases do not serve as a molecular target for EGFR kinase inhibitors. The importance of EGFR-L688 and the aminoacids surrounding the EGFR-V851 and EGFR-I853 in kinase activity was recently reported[124, 220].

EGFR LAARNVLVKTPQHVKITDFGLAKLL
ErbB2 LAARNVLVKSPNHVKITDFGLARLL
ErbB3 LAARNVLLKSPSQVQVADFGVADL
ErbB4 LAARNVLVKSPNHVKITDFGLARLL
PDGFRA LAARNVLLAQGKIVKICDFGLARD
PDGFRB LAARNVLICEGKLVKICDFGLARD
Flt3 LAARNVLVTHGKVVKICDFGLARD

Figure 27. Alignment of receptor tyrosine kinases.
Amino acid sequuences of several kinases surrounding DFG motif were aligned using clustalw program. DFG motif was colored blue and conserved amino acids that were mutated in EGFR kinase were represented in red.

Only a few activating mutations in the kinase domain of EGFR were tested for their potential to confer a growth advantage *in vitro* thus far[221]. Therefore, a panel of kinase domain mutations of EGFR was introduced into Ba/F3 cells and tested whether they are able to induce IL-3–independent growth (Table 3).

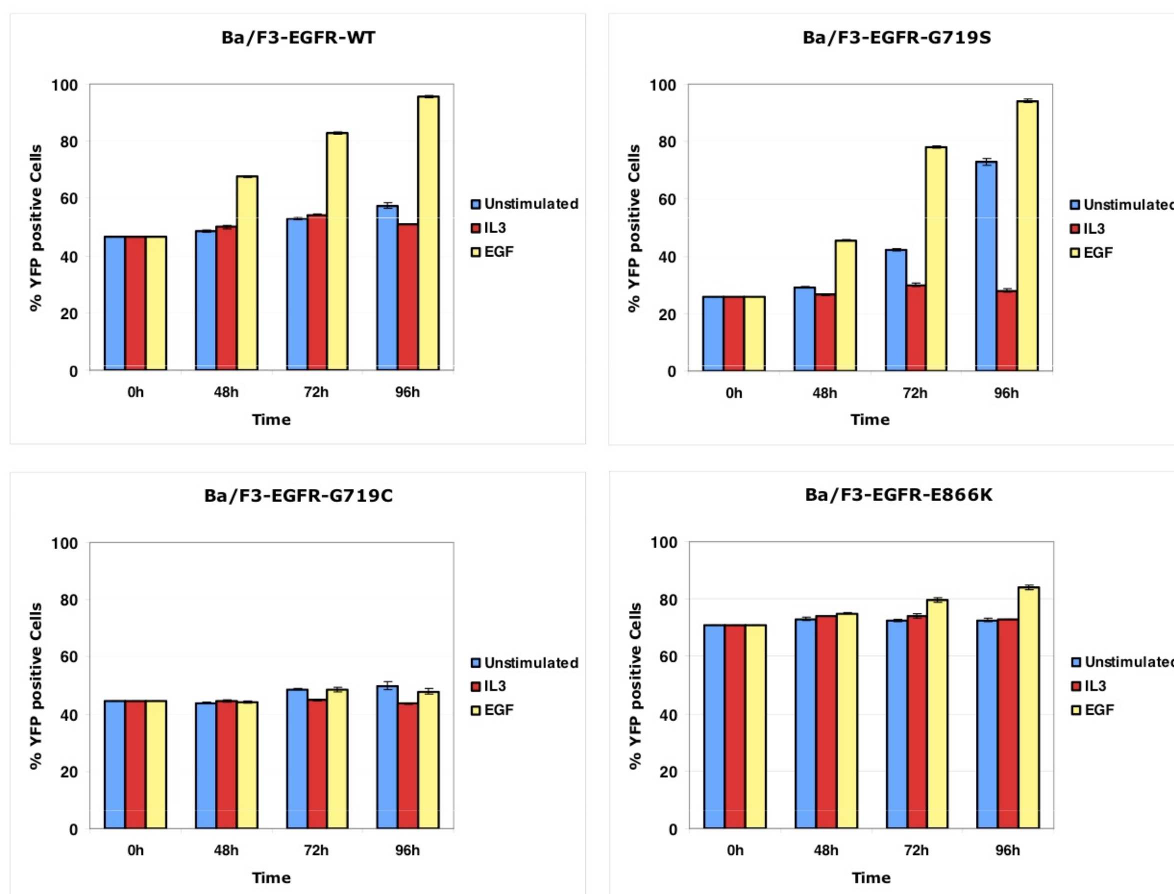


Figure 28. EGFR-G719C and EGFR-E866K didn't confer growth advantage upon EGF stimulation. Fraction of Ba/F3 cells transduced with wild type or mutatnt EGFR (YFP-positive cells) were analyzed for their growth upon stimulation with murine IL3 or human EGF. Outgrowth of YFP-positive cells was measured by FACS analysis for every 24 hours.

As previously reported, EGFR-L858R and EGFR-G719S induced IL-3–independent growth in Ba/F3 cells[221]. This was also observed for the majority of other EGFR mutations tested, indicating that these additional EGFR mutations lead to a growth advantage *in vitro*. In contrast, EGFR-L688P, EGFR-V851A, EGFR-I853T, and EGFR-E866K again failed to induce IL-3–independent growth, presumably due to the absent catalytic activity (Table 3).

In addition, EGFR-G719C also failed to induce IL-3–independent growth (Table 3). We then tested if selected EGFR mutations confer growth in the presence of EGF ligand. As expected, cells expressing EGFR-WT and the constitutive activated EGFR-G719S grew in the presence of EGF, whereas cells expressing kinase-dead EGFR-E866K failed to grow (Figure 28). Interestingly, also cells expressing EGFR-G719C did not grow in the presence of EGF (Figure 28). This mutant is kinase active as shown before and the reason for its lacking growth potential is unclear at the moment.

Biochemical analysis of the transformed cell lines showed constitutive autophosphorylation of the EGFR mutants and revealed activation of key prosurvival and proliferation pathways, such as ERK, Stat5, and AKT, in all cell lines tested (Figure 29). Ba/F3-EGFR-WT showed activation of EGFR and downstream key signaling molecules only on stimulation with EGF ligand (Figure 29).

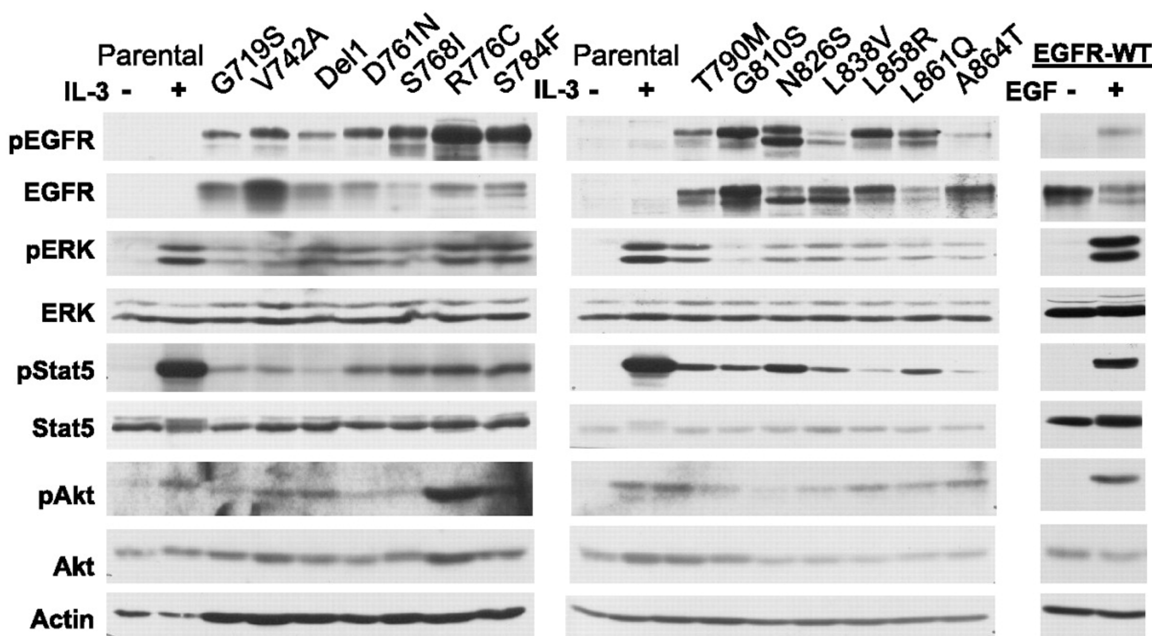


Figure 29. Differential activation of signaling pathways by EGFR mutants.
Ba/F3 cell lines stably expressing EGFR mutants were analysed for the activation of various downstream proteins in EGFR signaling pathway. Unstimulated or IL3 stimulated parental Ba/F3 cells were taken as control for EGFR expression as well as activation of signaling pathways. As a positive control, Ba/F3 cells expressing wild type EGFR were serum starved and stimulated with human EGF.

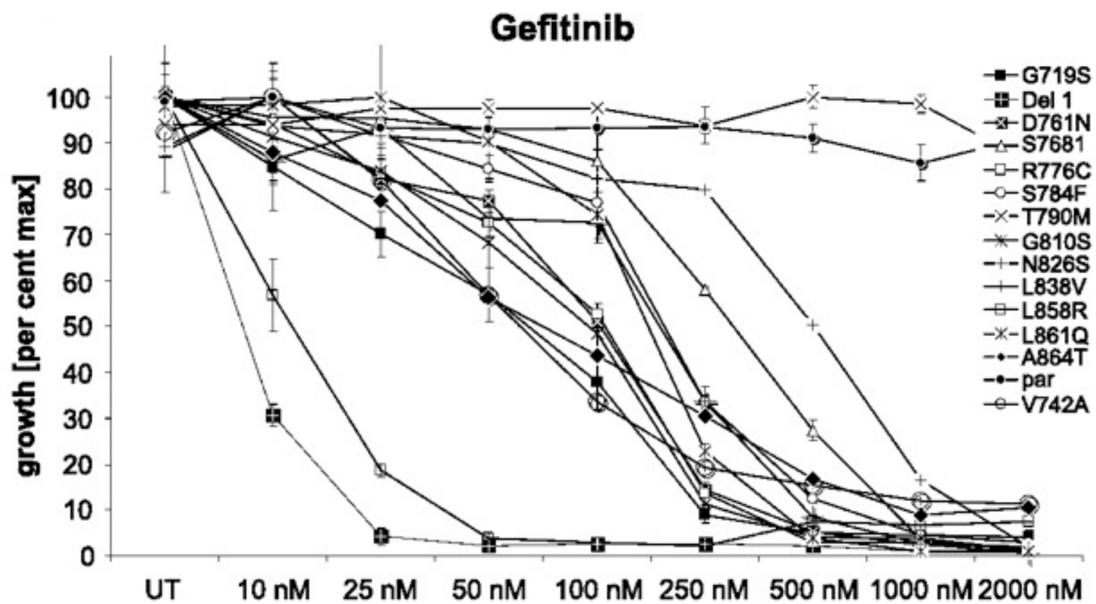
3.2.3. Analysis of drug sensitivity of EGFR mutants against EGFR inhibitors

The small-molecule EGFR kinase inhibitors gefitinib and erlotinib were shown to be effective in inhibiting the most frequent activating mutations EGFR-L858R and EGFR-Δ747-753insS[16]. However, there is growing experimental and clinical evidence that erlotinib and gefitinib may show differential activity toward specific EGFR activating and resistance mutations[222]. We therefore tested the sensitivity of a comprehensive panel of transforming EGFR mutations toward the EGFR inhibitors gefitinib, erlotinib, and AEE788 (Figure 30). Again, we used Ba/F3 cells as readout because these cells do not express endogenous EGFR and none of the drugs showed toxicity against these cells at concentrations of up to 2 µmol/L (Figure 30).

EGFR-L858R and EGFR-Del 747-753insS were extremely sensitive to all three kinase inhibitors with IC_{50} values in the low nanomolar range with no significant differences in IC_{50} values between gefitinib (Iressa, ZD1839), erlotinib (Tarceva, OSI-774), and AEE788 (Figure 30 A-C). In contrast, EGFR-T790M was completely resistant to all drugs tested with IC_{50} values >2 µmol/L (Figure 30 A-C). This mutation is already known to be associated with kinase inhibitor resistance[145]. In addition, EGFR-N826S required high concentrations of all three EGFR kinase inhibitors of ~500 nmol/L for complete inhibition (Figure 30 A-C). Interestingly, EGFR-N826S was detected in a NSCLC patient who did not respond to gefitinib treatment and this lack of response might be explained by the high IC_{50} value[142]. All other EGFR mutations showed IC_{50} values for the kinase inhibitors tested in the range of 10 to 300 nmol/L (Table 3). Several EGFR mutations displayed varying IC_{50} values depending on the kinase inhibitor. EGFR-G719S was relatively more resistant to gefitinib (IC_{50} = 68 nmol/L) than EGFR-L858R (IC_{50} = 12 nmol/L) and this finding is in line with a recent report[201]. However, EGFR-G719S was very sensitive to erlotinib (IC_{50} = 16 nmol/L) and AEE788 (IC_{50} = 13 nmol/L), comparable with EGFR-L858R (IC_{50} for erlotinib and AEE788 = 6 nmol/L) (Table 3). Another frequent mutation EGFR-L861Q was quite insensitive to both gefitinib and erlotinib with IC_{50} values above 100 nmol/L but with a lower IC_{50} value for AEE788 (IC_{50} = 51 nmol/L) (Table 3). Similarly, mutations EGFR-V742A, EGFR-R776C, and EGFR-S784F were more sensitive to erlotinib than gefitinib, with at least two times lower IC_{50} values (Table 3). EGFR mutations EGFR-D761N,

EGFR-S768I, EGFR-S784F, and EGFR-L838V were more sensitive to AEE788 compared with both gefitinib and erlotinib (Table 4).

A



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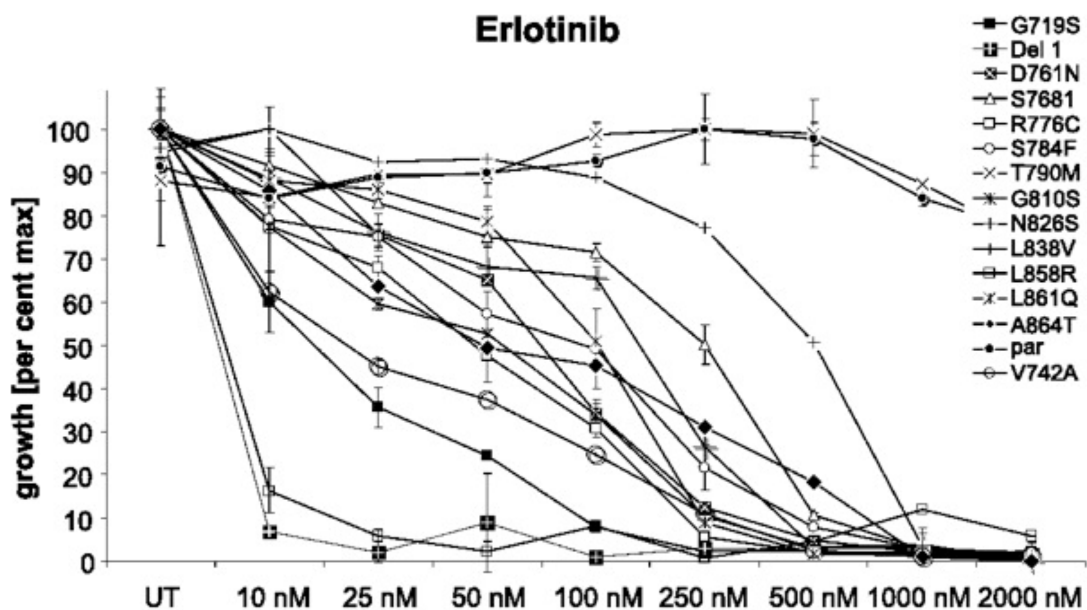

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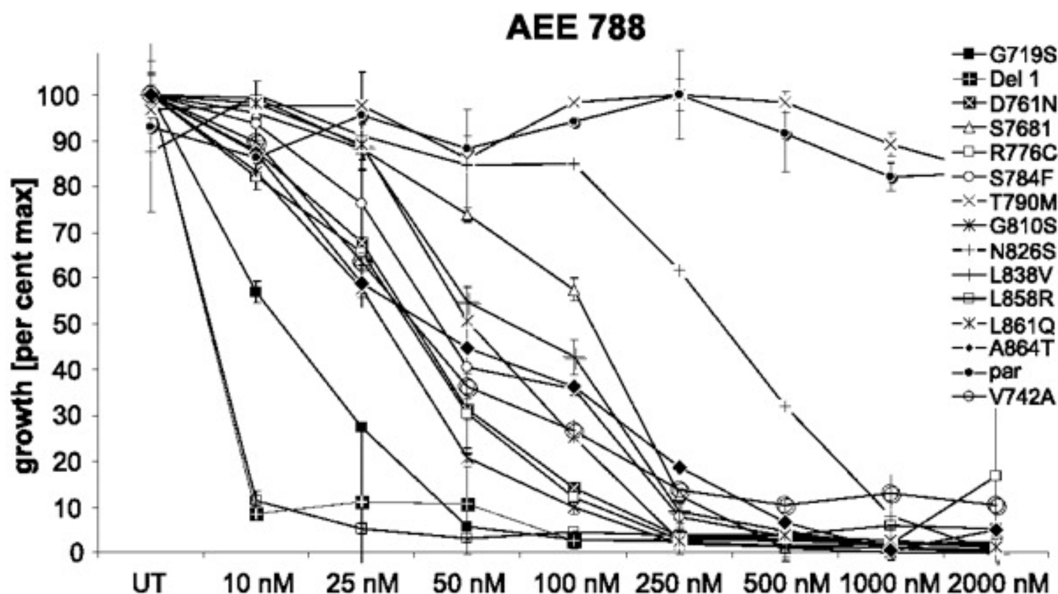


Figure 30. Varied response of EGFR kinase domain mutants towards inhibitor treatment.
Ba/F3 cells transformed by activating EGFR kinase domain mutants were treated with indicated concentrations of gefitinib (A), erlotinib (B) or AEE788 (C) for 48 hours and analysed for cell proliferation inhibition. Experiment was done in triplicates and standard deviation calculated for each point. Parental Ba/F3 cells in the presence of IL3 was taken as a control.

Based on the drug sensitivity profiles, EGFR kinase domain mutations were classified into four sets:

1) Mutations that were highly sensitive to gefitinib, erlotinib and AEE788 with very low nanomolar IC_{50} values : EGFR-Del 747-753insS and EGFR-L858R.
2) Mutations that were relatively less sensitive to gefitinib with IC_{50} greater than 100 nM but sensitive to erlotinib and AEE788 (IC_{50} < 100 nM): EGFR-G719S, EGFR-V742A and EGFR-R776C.
3) Mutations that were less sensitive to both gefitinib and erlotinib but sensitive to AEE788: EGFR-D761N, EGFR-S768I, EGFR-S784F, EGFR-L838V and EGFR-L861Q.
4) Mutations that were resistant to gefitinib, erlotinib and AEE788: EGFR-T790M and EGFR-N826S.

Mutation	Exon	Transforms Ba/F3	IC_{50} value (nM)		
			Gefitinib	Erlotinib	AEE 788
L688P	18	No	x	x	x
G719C	18	No	x	x	x
G719S	18	Yes	68	16	13
V742A	19	Yes	65	21	37
Deletion	19	Yes	7	5	5
D761N	19	Yes	104	75	37
S768I	20	Yes	315	250	125
R776C	20	Yes	110	47	36
S784F	20	Yes	193	95	43
T790M	20	Yes	NA	NA	NA
G810S	20	Yes	96	57	30
N826S	21	Yes	505	505	348
L838V	21	Yes	187	160	70
V851A	21	No	x	x	x
I853T	21	No	x	x	x
L858R	21	Yes	12	6	6
L861Q	21	Yes	170	103	51
A864T	21	Yes	75	49	40
E866K	21	No	x	x	x

Table 3. Summary of IC_{50} values of EGFR kinase mutants against gefitinib, erlotinib and AEE788. Cell proliferation inhibition IC_{50} values for Ba/F3-EGFR mutants cell lines against gefitinib, erlotinib and AEE788 were calculated from the figure 30. EGFR mutants that did not transform Ba/F3 cell lines were indicated in blue. Drug resistant EGFR mutants were indicated in red. EGFR mutants that were relatively more sensitive to erlotinib compared to gefitinib were indicated in green. EGFR mutants that were sensitive to AEE788 compared to both gefitinib and erlotinib were shown in brown. NA = Not Applicable; x = Not tested for drug sensitivity

Gefitinib and erlotinib are anilinoquinazolines, whereas AEE788 is a pyrrolopyrimidine compound. These differences in the chemical structures may account for the distinct responses observed with all three drugs. Thus, the sensitivity of activating EGFR mutations toward different EGFR kinase inhibitors varies significantly and this may have implications

for the sequential and potential combinational use of this compound in EGFR-mutated NSCLC patients.

3.2.4. Hyperactivation of EGFR kinase and transformation ability by L861Q mutation

We have shown that mutations L858R and G719S are very sensitive to all the drugs tested while another common mutation L861Q, is relatively insensitive towards inhibitor treatment. This is in agreement with a previous report[71]. So far, it was not possible to compare the drug sensitivity of ligand independent mutant EGFR to that of EGF-dependent wild type EGFR in a ligand independent cellular assay[221]. Thus, we aimed to establish a cell-based system to compare the drug sensitivities of wild type and mutant receptor kinase domains. The most common point mutations reported in NSCLC patients, EGFR-L858R, EGFR-G719S and EGFR-L861Q were selected for this study.
Our aim was to determine kinase activity and transforming potential of these mutants compared to the wt-EGFR kinase domain. Using the wt-EGFR as reference has several limitations: Ligand stimulation and the required serum starvation of cells may alter cellular responses. In addition, the wt-receptor cannot be used as comparison for the transforming potential of certain EGFR mutants. The use of EGFRvIII as reference abrogates several of these limitations. EGFRvIII contains a wt-kinase domain and thus can be used to study the impact of mutations on the kinase activity in an unaltered cellular setting. Therefore, we first cloned all three point mutations into the EGFRvIII backbone. For analysis of kinase activity and signaling we chose HEK293 cells which lack endogenous EGFR [219, 223]. EGFRvIII-G719S and EGFRvIII-L858R both showed a 2-4 fold increased autophosphorylation compared to EGFRvIII containing a wt-kinase domain (Figure 31). EGFRvIII-L861Q showed the strongest autophosphorylation, which was more than 10-fold higher than EGFRvIII (Figure 31). This data is in agreement with studies using the wt-EGFR receptor with ligand stimulation [224]. EGFRvIII-L861Q was also the strongest activator of Stat5 indicating that not only autophosphorylation but also substrate phosphorylation is enhanced by this mutation. None of the EGFRvIII constructs increased Akt activity above levels observed under normal serum conditions (Figure 31).

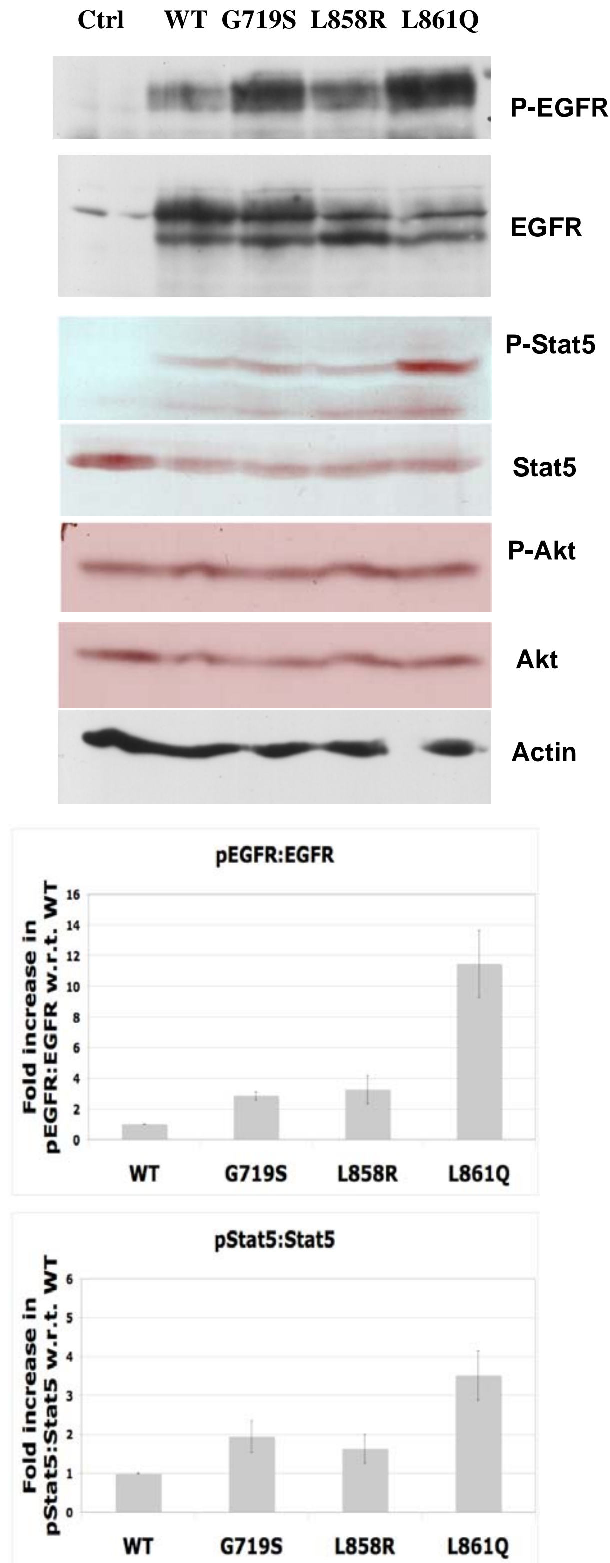


Figure 31. EGFR-L861Q is a hyperactive kinase.
EGFRvIII with wild type or mutant kinase domain were transfected into HEK293 cells and autokinase activity and downstream signaling was measured using phospho specific antibodies. Untransfected cells were

taken as negative control. Quantification of blots was done by ImageJ software and activity of EGFRvIII mutants was compared as a ration of pEGFR:EGFR and pSTAT5:STAT5. Calculated p values were indicated to show the significant differences observed.

To compare the transforming abilities of these mutants we performed a competitive growth assay, in which the outgrowth of oncogene-transduced cells under growth factor withdrawal in a mixed population is measured. To this end newly transduced (transduction efficiency approx. 20%), unselected stable Ba/F3 cell lines expressing EGFRvIII mutants together with eGFP were deprived of IL-3. Outgrowth of eGFP positive cells was measured by FACS analysis over time. EGFRvIII-L861Q expressing cells showed the strongest proliferation advantage in this competition assay (Figure 32). EGFRvIII-G719S and EGFRvIII-L858R expressing cells were also selected more efficiently than cells expressing unmutated EGFRvIII (Figure 32). After prolonged IL-3 deprivation however, all EGFRvIII constructs finally conferred IL-3 independent growth. Thus, mutation at L861Q leads to the strongest gain in kinase activity and most rapid induction of cytokine independent growth compared to the wt-kinase domain.

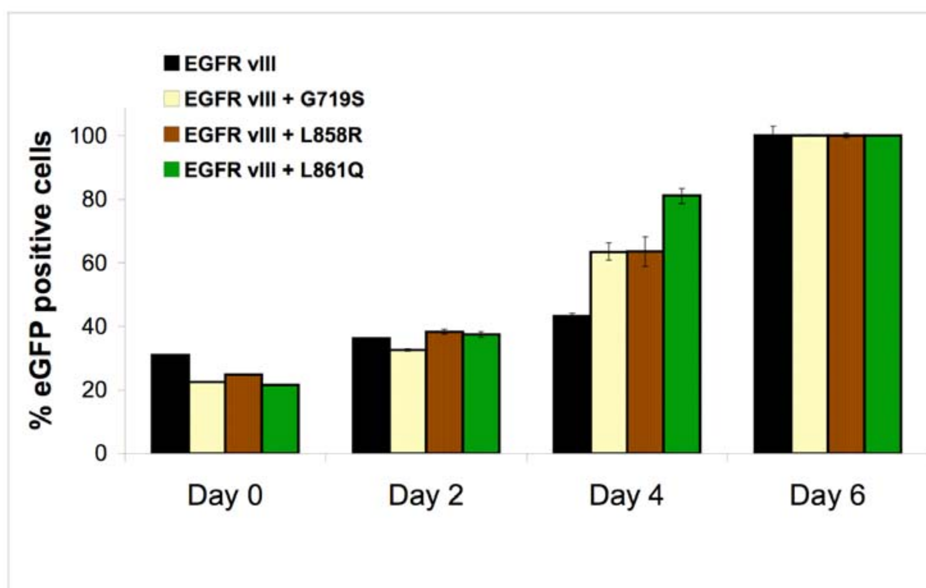
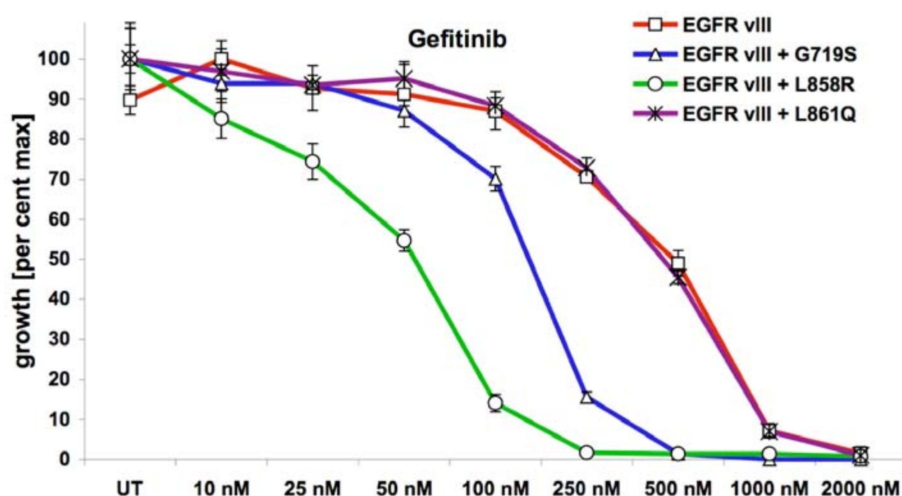


Figure 32. EGFR-L861Q confers strongest transformation potential on Ba/F3 cells.
Ba/F3 cells transduced with either wild type or mutant EGFRvIII were deprived of IL3 and their preferential outgrowth compared to parental Ba/F3 cells was measured by FACS analysis over regular intervals.

3.2.5 EGFR-L861Q is not a drug sensitizing mutation towards EGFR inhibitors

We then wished to determine the effect of oncogenic EGFR kinase domain mutations on drug sensitivity. We used stably transduced Ba/F3 cell lines expressing wild type or mutant EGFRvIII and the EGFR kinase inhibitors gefitinib, erlotinib and AEE788. Gefitinib and erlotinib are selective EGFR inhibitors already approved in the clinic. AEE788 is a compound in development inhibiting both EGFR and Her2. EGFRvIII-L858R expressing cells were very sensitive to all drugs tested with an IC50 value of less than 50nM (Figure 33). EGFRvIII-G719S expressing cells displayed an intermediate sensitivity. Both mutations led to lower IC50 values compared to wild type EGFRvIII thus sensitizing cells to EGFR kinase inhibitors. In contrast, EGFRvIII-L861Q expressing cells displayed IC50 values identical to wt-EGFRvIII (Figure 33). Western blot analysis after treatment of Ba/F3 cells expressing EGFRvIII constructs with gefitinib or erlotinib showed that EGFRvIII-G719S and EGFRvIII-L858R but not EGFRvIII-L861Q displayed enhanced inhibition of EGFR and downstream signaling compared to wild type EGFRvIII (Figure 34). Thus, in this common EGFR mutation activation of the kinase domain seems to be uncoupled from sensitizing effects towards kinase inhibitors. These results suggest, that NSCLC patients with the EGFR-L861Q mutation may not benefit as much from EGFR inhibitor treatment with gefitinib or erlotinib as patients with the EGFR-L858R, EGFR-G719S or EGFR exon 19 deletions.



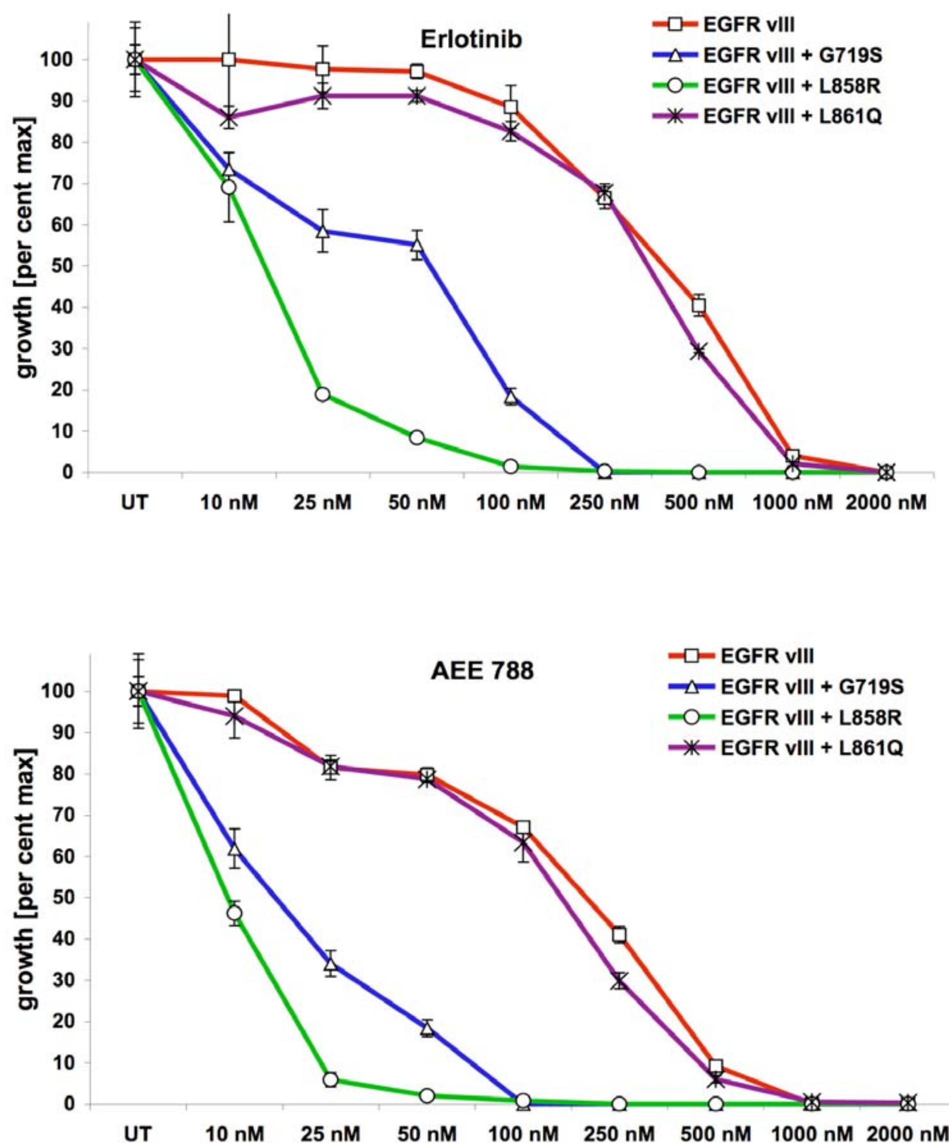


Figure 33. Effect of gefitinib, erlotinib and AEE788 on wild type and mutant EGFRvIII.
Ba/F3 cells transformed by EGFRvIII mutants (WT, G719S, L858R and L861Q) were treated with gefitinib, erlotinib and AEE788 for 48 hours and cell proliferation inhibition was measured by MTT assay.

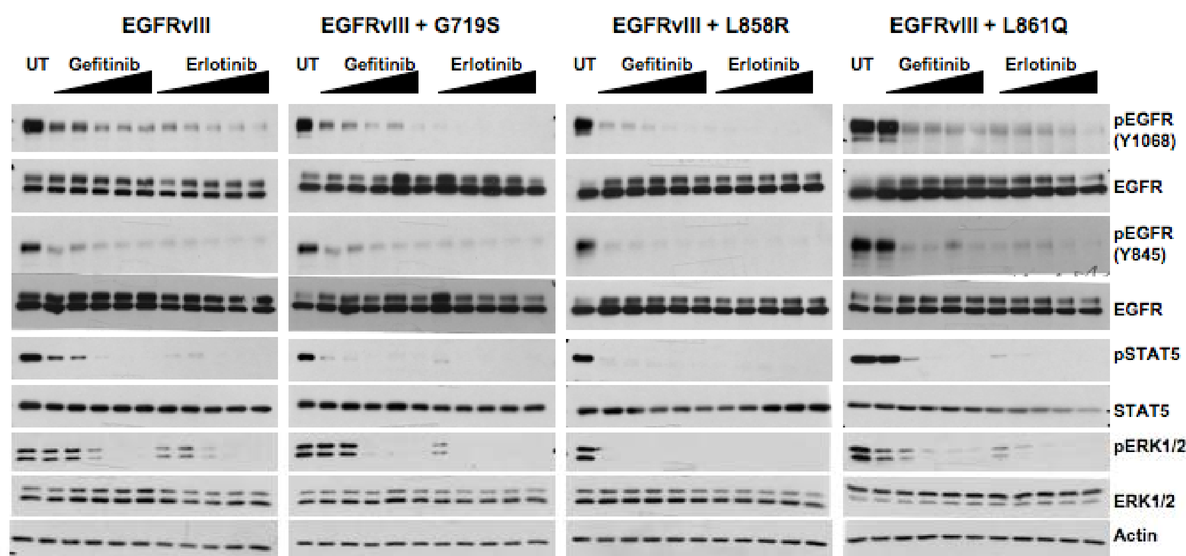


Figure 34. Hyperactivity of EGFRvIII-L861Q is uncoupled from drug sensitizing effect.
Ba/F3 cells expressing wild type or mutant EGFRvIII were treated with increasing concentrations (50nm, 100 nM, 250 nM, 500 nM or 1000 nM) of gefitinib or erlotinib for 30 minutes and analyzed for the inhibition of EGFR autophosphorylation as well as Stat5 and ERK1/2 phosphorylation.

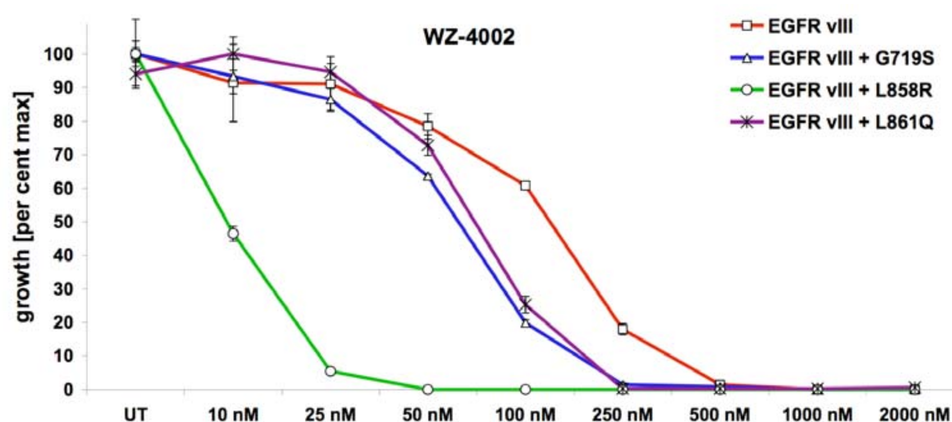
Although L861Q mutation induced the strongest proliferation advantage and highest increase in kinase activity among all mutations tested it did not lead to enhanced kinase inhibitor sensitivity (Table 4).

	EGFR vIII	EGFR vIII + G719S	EGFR vIII + L858R	EGFR vIII + L861Q
Autokinase activity	+	++	++	+++
Transforming ability	+	++	++	+++
Drug sensitivity	+	++	+++	+

Table 4. Hyperactivity of EGFRvIII-L861Q is uncoupled from drug sensitizing effect.
Effect of kinase domain mutations on the properties of EGFRvIII were summarized based on the figures 32, 33 and 34.

Variation in drug response towards different activating mutations in oncogenic tyrosine kinases have been reported in various cancers and accumulating evidence indicates that this may have impact on the clinical outcome upon inhibitor treatment[34, 225, 226]. Therefore it may be beneficial to test alternative EGFR inhibitors towards less sensitive activating EGFR mutations such as EGFR-L861Q. Irreversible EGFR inhibitor was previously shown

to overcome gefitinib resistance due to kinase domain mutation[227]. Thus, we tested if a novel irreversible inhibitor WZ-4002 shows sensitizing effect on EGFR-L861Q. Both EGFRvIII-L858R and EGFRvIII-G719S showed lower IC_{50} values towards WZ-4002 treatment compared to wild type EGFRvIII (Figure 35, upper panel). Interestingly, EGFRvIII-L861Q also showed significantly more sensitivity against WZ-4002 compared to the wild type EGFRvIII (Figure 35, upper panel).



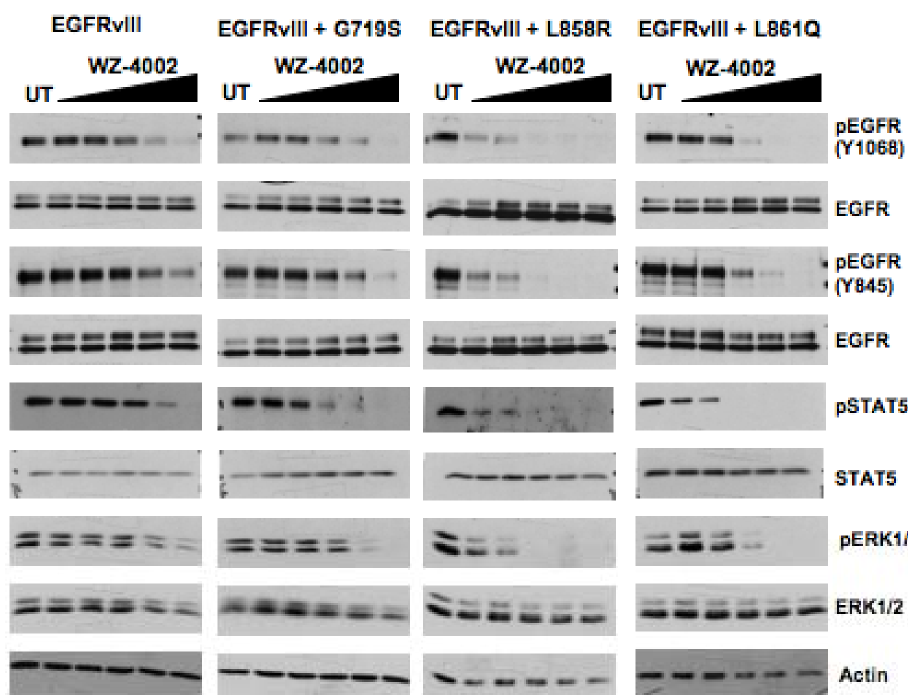


Figure 35. Hyperactivity of EGFRvIII-L861Q is uncoupled from drug sensitizing effect.
Upper panel: EGFRvIII mutant Ba/F3 cells were treated with WZ4002 at indicated concentrations and analyzed for cell proliferation inhibition. **Lower panel**: Ba/F3 cells expressing wild type or mutant EGFRvIII

were treated with increasing concentrations (50nm, 100 nM, 250 nM, 500 nM or 1000 nM) of WZ4002 for 30 minutes and analyzed for the inhibition of EGFR autophosphorylation as well as Stat5 and ERK1/2 phosphorylation.

Biochemical analysis upon inhibitor treatment correlated with the observed cell proliferation data (Figure 35, lower panel). Therefore, WZ-4002 offers potent alternative to selectively inhibit EGFR-L861Q thus avoiding toxicity due to inhibition of wild type EGFR. Another promising EGFR inhibitor in this regard was reported recently and was shown to efficiently inhibit EGFR-L861Q compared to wt-EGFR[228].Thus, the Ba/F3-EGFRvIII-based system described in this study will be a valuable tool to test novel compounds and strategies.

3.3. Irreversible EGFR/ERBB2 inhibitors overcome lapatinib resistance due to ERBB2 kinase domain mutations

ERBB2 belongs to the EGFR family of receptor tyrosine kinases[153]. Activation of ERBB2 kinase due to overexpression was reported in breast cancers thus making it an attractive target for treatment[229]. Polymorphisms in the juxtamembrane region of ERBB2 kinase were reported to cause increased susceptibility to breast cancer[177]. However the role of ERBB2 polymorphisms on biochemical properties as well as drug sensitivity of the receptor is not known. Additionally, mutations in the ERBB2 kinase domain were also reported in solid cancers but the functional significance of these ERBB2 mutants remains unknown.

Tyrosine kinase inhibitors that target both EGFR and ERBB2 are already approved or are already tested within clinical trials. For example, lapatinib (Tykerb/Tyverb, GW572016) is a dual inhibitor of both EGFR and ERBB2 kinases and is approved for the treatment of ERBB2 positive breast cancer[230, 231]. In addition, a monoclonal antibody, trastuzumab (Herceptin) that targets the extracellular region of ERBB2 was approved for the treatment of ERBB2 positive breast cancer[232]. Experimental *in vitro* systems to study the biochemical properties of ERBB2 mutants and to study the effect of ERBB2 specific drugs on these mutants were not described so far. Thus, we aimed to establish a cell-based system to study biochemical properties, transformation abilities and drug sensitivities of these ERBB2 variants *in vitro*.

3.3.1. ERBB2 polymorphisms have no effect on functional properties

A frequent polymorphism ERBB2-I654V was reported in one study to increase the risk of breast cancer incidence and progression[177]. However, several studies found ‘no correlation’ and even ‘inverse correlation’ between breast cancer risk and ERBB2-I654V[179, 180, 233]. Another study showed that ERBB2-I655V in tandem with the ERBB2-I654V polymorphism was shown to increase the risk of familial breast cancer risk[178]. The role of different polymorphisms on the biochemical properties of the ERBB2 kinase is not clear. To test the role of ERBB2 polymorphisms on functional properties, wild type and polymorphic MiGR1-ERBB2 were transfected into HEK293 cells. Autokinase activity was measured using an antibody specific for ERBB2 that is phosphorylated upon activation. pERBB2:ERBB2 levels were calculated and compared between the wild type and

polymorphic ERBB2 variants (Figure 36). Both the wild type and polymorphic ERBB2 variants exhibited similar levels of autokinase activity (Figure 36A). To test if the polymorphisms affect the transformation potential of ERBB2, Ba/F3 cells were transduced with MiGR1-ERBB2 constructs. Oncogenic potential was measured as the increase of eGFP positive cell fraction upon IL-3 withdrawal. Analysis of preferential outgrowth of ERBB2 expressing cells (GFP-positive) showed that both the wild type and polymorphic variants have similar oncogenic potential (Figure 36B).

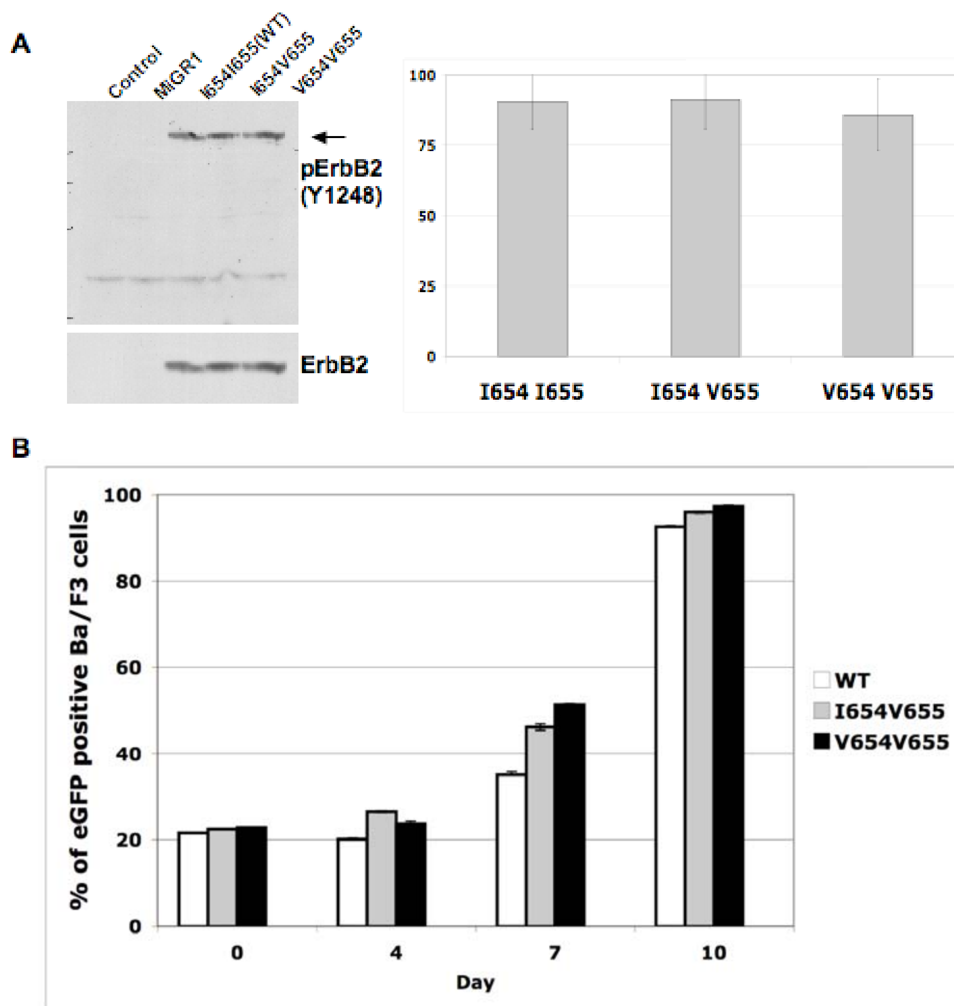
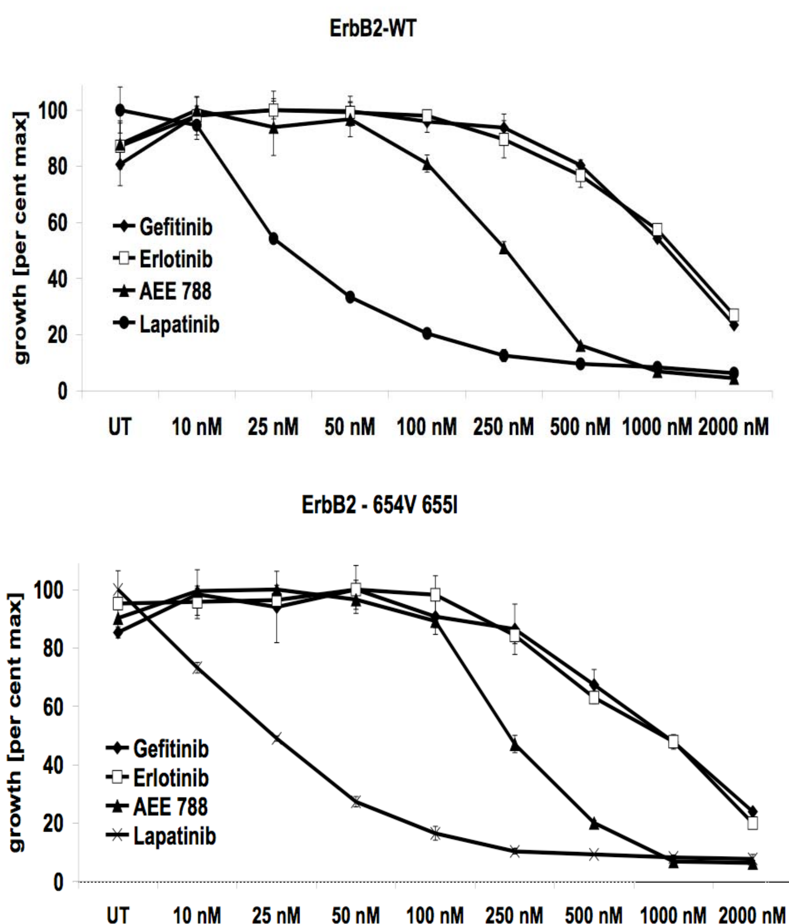


Figure 36. ERBB2 polymorphisms don't alter ERBB2 kinase activity and transforming poteintial. HEK293 cells were transiently transfected with either wild type or polymorphic ERBB2 and tested for autokinase activity using ERBB2-Tyr1248 specific antibody. Relative activation of ERBB2 kinase variants was plotted as a ratio of pERBB2:ERBB2. Ba/F3 cells were infected with wild type and polymorphic ERBB2 retrovirus and preferential outgrowth of transduced cells was measured by FACS analysis.

We then tested the effect of the EGFR/ERBB2 dual inhibitors lapatinib and AEE788 on Ba/F3 cells that are transformed by ERBB2 variants. Lapatinib is already approved for the treatment of ERBB2 positive breast cancer and we intended to test if the ERBB2 polymorphisms affect lapatinib sensitivity (Figure 37). Gefitinib and erlotinib were used as negative controls.

Cell proliferation inhibition analysis upon drug treatment of Ba/F3 cells stably expressing wild type and polymorphic variants showed similar drug sensitivity profiles against gefitinib, erlotinib, lapatinib and AEE788 (Figure 37). The IC_{50} values of all the variants against tested drugs were comparable to that of wild type ERBB2 (Figure 37). Thus, ERBB2 polymorphisms have no effect of kinase activity, transformation ability and drug sensitivity towards EGFR/ERBB2 inhibitors.



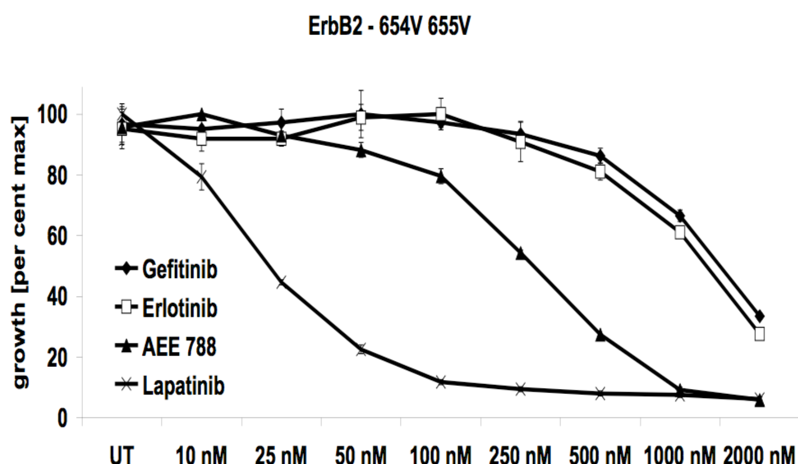


Figure 37. Drug sensitivitiy profiles of wild type and polymorphic ERBB2 variants.
Stable Ba/F3 cells lines expressing wild type and polymorphic ERBB2 kinases were treated with either EGFR inhibitors (gefitinib and erlotinib) or EGFR/ERBB2 dual inhibitors (lapatinib and AEE788) at indicated concentrations for 48 hours and analysed for cell proliferation inhibition by MTT assay.

3.3.2. A cell-based screen identifies lapatinib resistant ERBB2 mutations

Tyrosine kinase inhibitors have revolutionized the treatment of cancers with oncogenic mutations. However secondary drug resistance due to the kinase domain mutations represents a big challenge. This prompted the development of new classes of inhibitors that may overcome the drug resistance. Identification of possible drug resistant mutations *in vitro* is useful to predict possible drug resistant mutations in patients. An *in vitro* cell-based screening method was previously described to identify inhibitor-resistant kinase domain mutations[208]. In this method, cells expressing wild type kinase were treated with a chemical mutagen to induce random mutations. Mutated cells are then cultured in high inhibitor concentrations to select for kinase domain mutations that confer drug resistance. To identify lapatinib resistant mutations, Ba/F3-ERBB2-WT cells were treated with the chemical mutagen ENU and then selected for clones that grow in the presence of lapatinib (Figure 38).

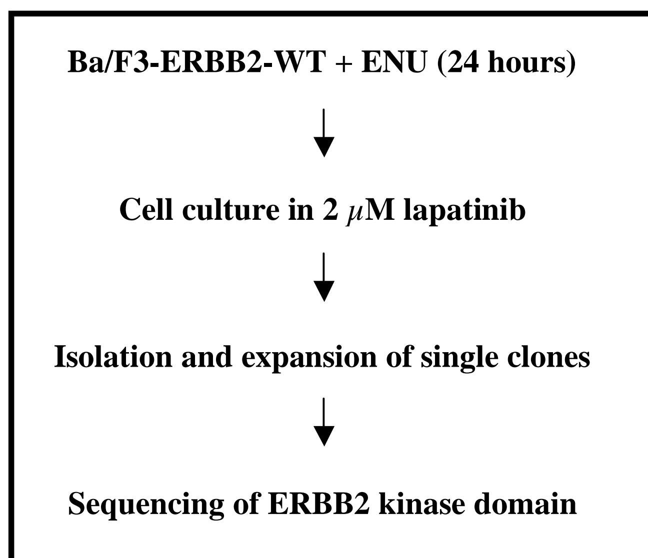


Figure 38. Schematic representation of screen to identify lapatinib resistant ERBB2 mutations. Ba/F3-ERBB2-WT cells were treated twice with ethyl nitroso urea for 12 hours and then cultured for 5 days for the cells to recover. Cells were then cultured in 96-well plates in the presence of 2 μM of lapatinib and drug resistant cell clones were isolated. cDNA for ERBB2 kinase domain was sequenced for the identification of lapatinib resistant mutations.

Sequencing of lapatinib-resistant clones showed that about 50% of the clones contained mutated ERBB2 kinase domain (Figure 39). Interestingly, only two mutations ERBB2-L755S and ERBB2-T862A could be identified in the lapatinib resistance screen indicating that the spectrum of lapatinib resistance mutations maybe limited (Figure 39).

ERBB2-T862A was the predominant mutation identified in nearly 80% of the clones (Figure 39). Interestingly, an analogous mutation was identified in the EGFR (T854A) in NSCLC patients and was shown to cause resistance to gefitinib treatment[234]. Thus, ERBB2-T862 and EGFR-T854 are critical residues for inhibitor binding in both EGFR and ERBB2 kinases.

ERBB2-L755S that was identified in the screen was previously reported in breast cancer patients and analogous EGFR-L747S was reported in NSCLC patients indicating the activating nature of the mutation[183, 235].

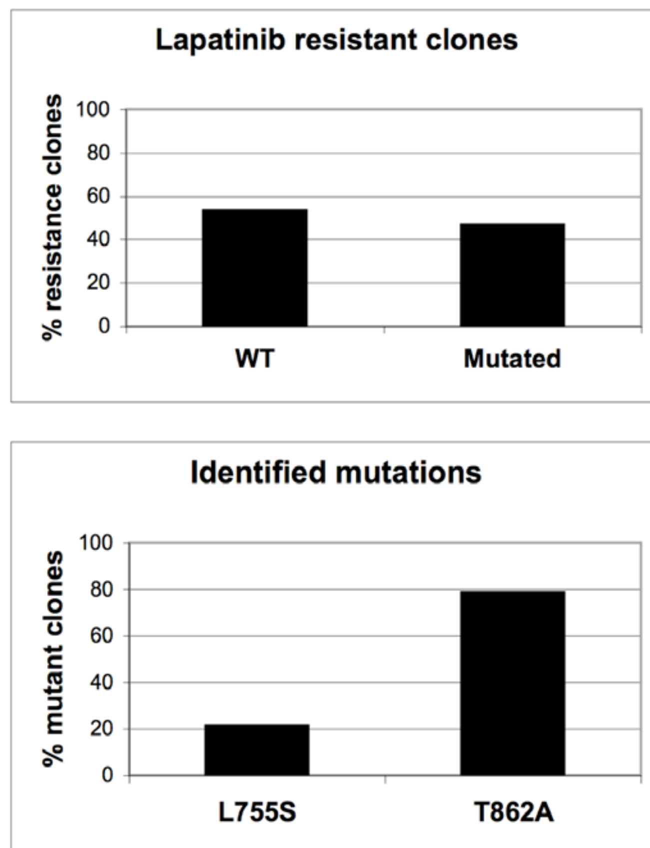


Figure 39. Frequency of identified mutations in lapatinib resistance screen.
Graphical representation of relative percentages of wild type and mutated ERBB2 clones. Number of clones with ERBB2-L755S versus ERBB2-T862A were also shown.

In addition to the mutations identified in the screen, we cloned ERBB2-T798M to test the activity of lapatinib. T798 is a gate keeper residue in ERBB2 kinase and analogous EGFR-T790M was reported in gefitinib resistant NSCLC patients[145]. All these ERBB2 mutants readily transformed Ba/F3 cells to cytokine independence. Stable Ba/F3 cell lines expressing ERBB2-T862A showed a four-fold increase in IC_{50} value compared to wild type ERBB2 but was completely inhibited by lapatinib at concentrations that can be achieved in patients indicating that this mutation causes moderate resistance to lapatinib treatment (Figure 40). ERBB2-L755S was very resistant to lapatinib treatment with IC_{50} value greater than 1 μM (Figure 40). ERBB2-T798M was totally resistant to lapatinib treatment (Figure 40).

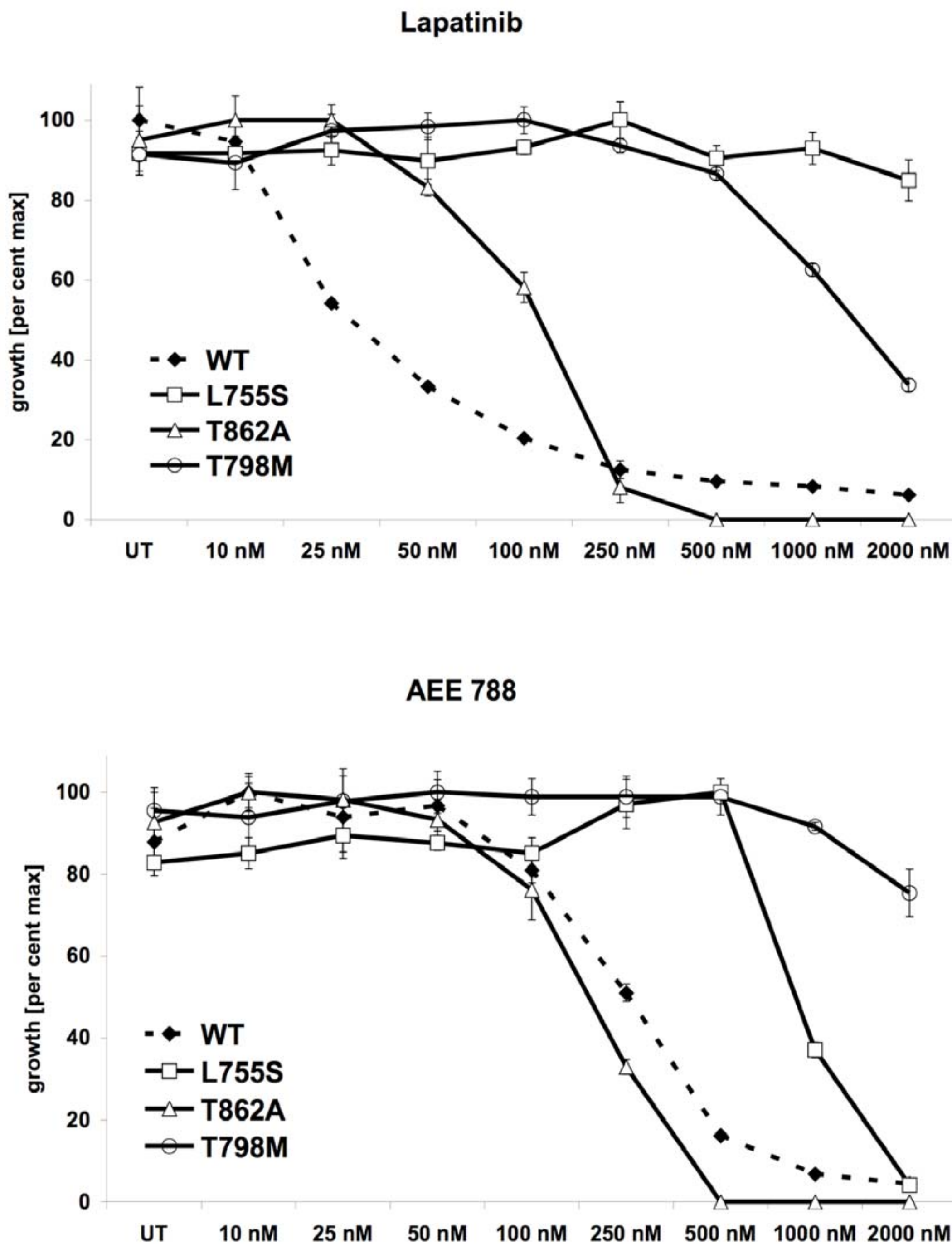


Figure 40. Effect of lapatinib on ERBB2-T862A, ERBB2-L755S and ERBB2-T798M.
Ba/F3 cells stably expressing ERBB2 mutants identified in lapatinib resistance screen were treated with increasing concentrations of lapatinib and AEE 788 for 48 hours and tested for inhibition of cell proliferation. Ba/F3-ERBB2-WT cells were taken as a control for comparison (dotted line).

AEE788 is a dual reversible EGFR/ERBB2 inhibitor and we tested if it can overcome lapatinib resistance due to kinase domain mutations. Wild type ERBB2 kinase showed higher IC_{50} value with AEE788 compared to lapatinib (Figure 40). Interestingly, lapatinib resistant ERBB2-T862A remained sensitive to AEE788 with IC_{50} value comparable to that of wild type ERBB2 (Figure 40). However, ERBB2-L755S and ERBB2-T798M remained resistant to AEE788 treatment (Figure 40).

3.3.3. Drug sensitivity of ERBB2 kinase mutants reported in other solid cancers

Mutations in the ERBB2 kinase were reported in some solid tumors and the role of these mutations in lapatinib sensitivity is not known[181, 182, 186]. To test this, mutations were introduced into MiGR1-ERBB2 construct and stable cell lines expressing mutated ERBB2 mutants were generated. Ba/F3 cells stably expressing ERBB2 mutants were then tested for the efficacy of lapatinib. All the ERBB2 mutants were totally inhibited by lapatinib except ERBB2-L755P which has an IC_{50} value greater than 1 μM (Figure 41). Interestingly, another substitution at the same amino acid (ERBB2-L755S) was detected in the lapatinib resistant screen (Figure 38 and Figure 39). This points to the fact that L755 is a critical residue for lapatinib binding and mutation at this position causes drug resistance. Thus, patients with ERBB2-L755P may not respond to lapatinib treatment suggesting that alternative ERBB2 inhibitors need to be tested that overcome lapatinib resistance.

Importantly, ERBB2-H878Y which was reported in 11% of hepatoma patients, showed enhanced sensitivity to lapatinib treatment with an IC_{50} value nearly half that of the wild type ERBB2 (Figure 41). Thus this mutation can be considered as lapatinib sensitizing mutation similar to EGFR-L858R which is a gefitinib sensitizing mutation reported in NSCLC[16]. This may be significant since hepatoma patients with ERBB2-H878Y may benefit from lapatinib treatment. ERBB2-V777L showed similar IC_{50} value to that of wild type ERBB2 towards lapatinib treatment (Figure 41). ERBB2-V773A and ERBB2-N857S showed higher IC_{50} values compared to wild type ERBB2 but their IC_{90} values were well within 0.5 μM (Figure 41).

We then tested the role of ERBB2 kinase domain mutations on AEE788 sensitivity. All mutants except ERBB2-L755P were sensitive to AEE788 with IC50 values similar to that

of wild type ERBB2 (Figure 41). ERBB2-L755P showed cross resistance to both lapatinib and AEE788 (Figure 41). Structural position of ERBB2 mutants analysed and their IC_{50} values against lapatinib and AEE788 were summarized in the Table 5. Thus, ERBB2-L755S, ERBB2-L755P and ERBB2-T798M were shown to be resistant to both the reversible inhinitors (lapatinib and AEE788) tested.

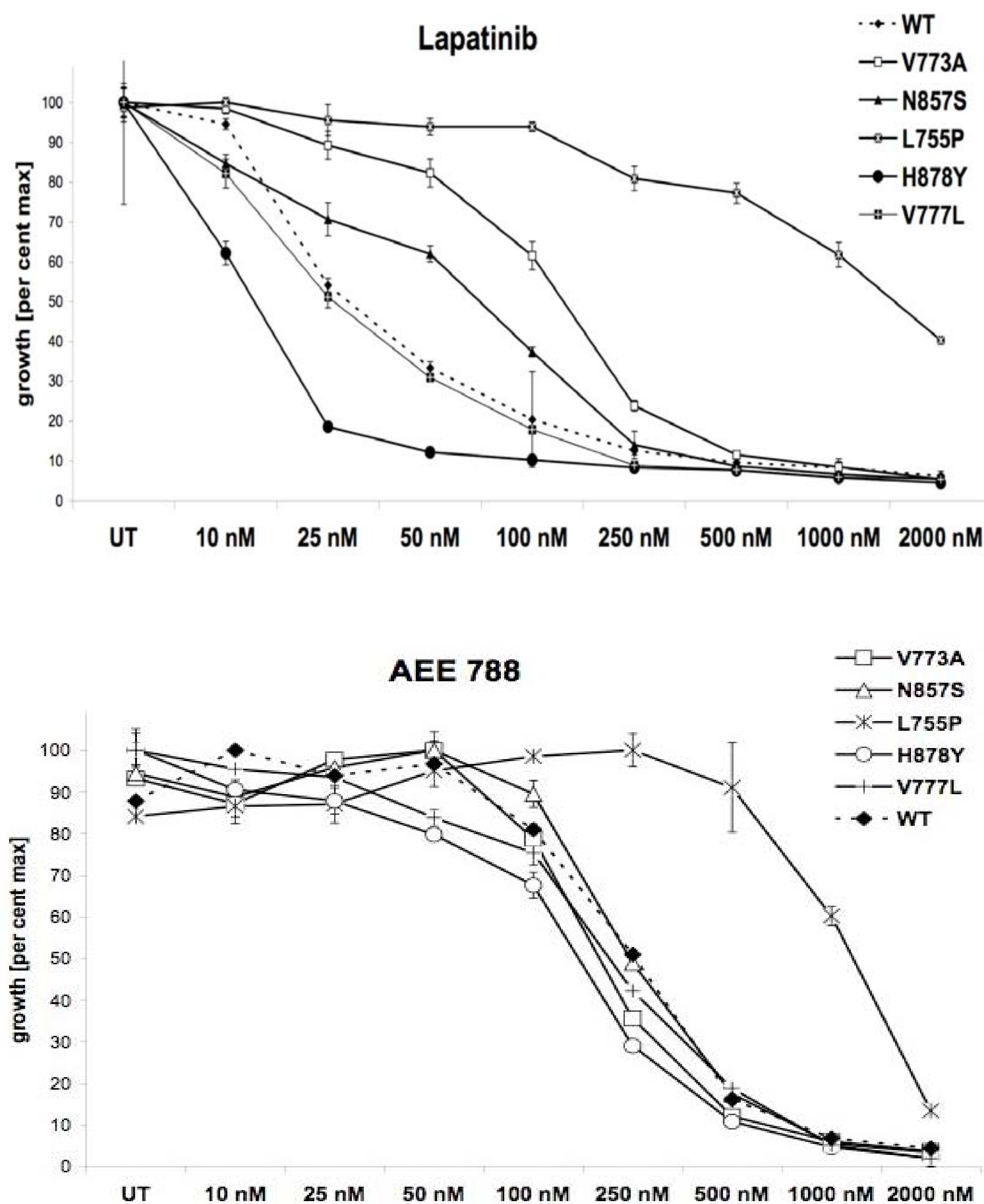


Figure 41. ERBB2-L755P is a lapatinib resistant mutation.
Ba/F3 cells stably expressing ERBB2 mutations that were reported in various cancers were treated with indicated concentrations of lapatinib and AEE 788 and cell proliferation inhibition was measured after 48 hours by MTT assay.

ERBB2 mutation	Exon	Functional region	Cancer type	Lapatinib	AEE788
WT	NA	NA	NA	**30**	**257**
L755S	19	ATP binding region	Breast cancer and gastric cancer	**>2000**	**897**
L755P	19	ATP binding region	NSCLC	**1545**	**1216**
V773A	20	ATP binding region	SCCHN	**146**	**200**
V777L	20	ATP binding region	Gastric cancer, colon cancer and lung adenocarcinoma	**27**	**215**
T798M	20	Gate keeper residue	NA	**1433**	**>2000**
N857S	21	Activation loop	Ovarian cancer	**75**	**246**
T862A	21	Activation loop	Primary gastric cancer	**125**	**191**
H878Y	21	Activation loop	Hepatocellular carcinoma	**14**	**168**

Table 5. Summary of drug sensitivity profiles and transformation ability of ERBB2 mutants.
IC50 values of wild type and ERBB2 mutants were calculated from the figures 40 and 41 were shown along with the position of mutation in ERBB2 gene. The cancer types in which the respective mutations are identified were shown.

3.3.4. Lapatinib-resistant ERBB2 mutants are sensitive towards irreversible inhibitors

It was previously shown for EGFR mutants that irreversible inhibitors can overcome gefitinib/erlotinib resistance[227]. To test if irreversible EGFR/ERBB2 inhibitors overcome lapatinib resistance, CL-387,785 and WZ-4002 were chosen for the study. Upon treatment, Ba/F3 cells lines stably expressing lapatinib resistant ERBB2 mutants (L755S, L755P and T798M) were shown to be sensitive to both the irreversible inhibitors CL-387,785 and WZ-4002 (Figure 42). When compared to wildtype ERBB2 kinase, all lapatinib-resistant ERBB2 mutants showed only 2-4 fold increase in IC_{50} values towards CL-387,785 and WZ4002 (Figure 42). This increase in IC_{50} values towards the irreversible inhibitors is significantly lower compared to lapatinib where the IC_{50} values for ERBB2 mutants were more than 50 fold higher than that of wildtype ERBB2.

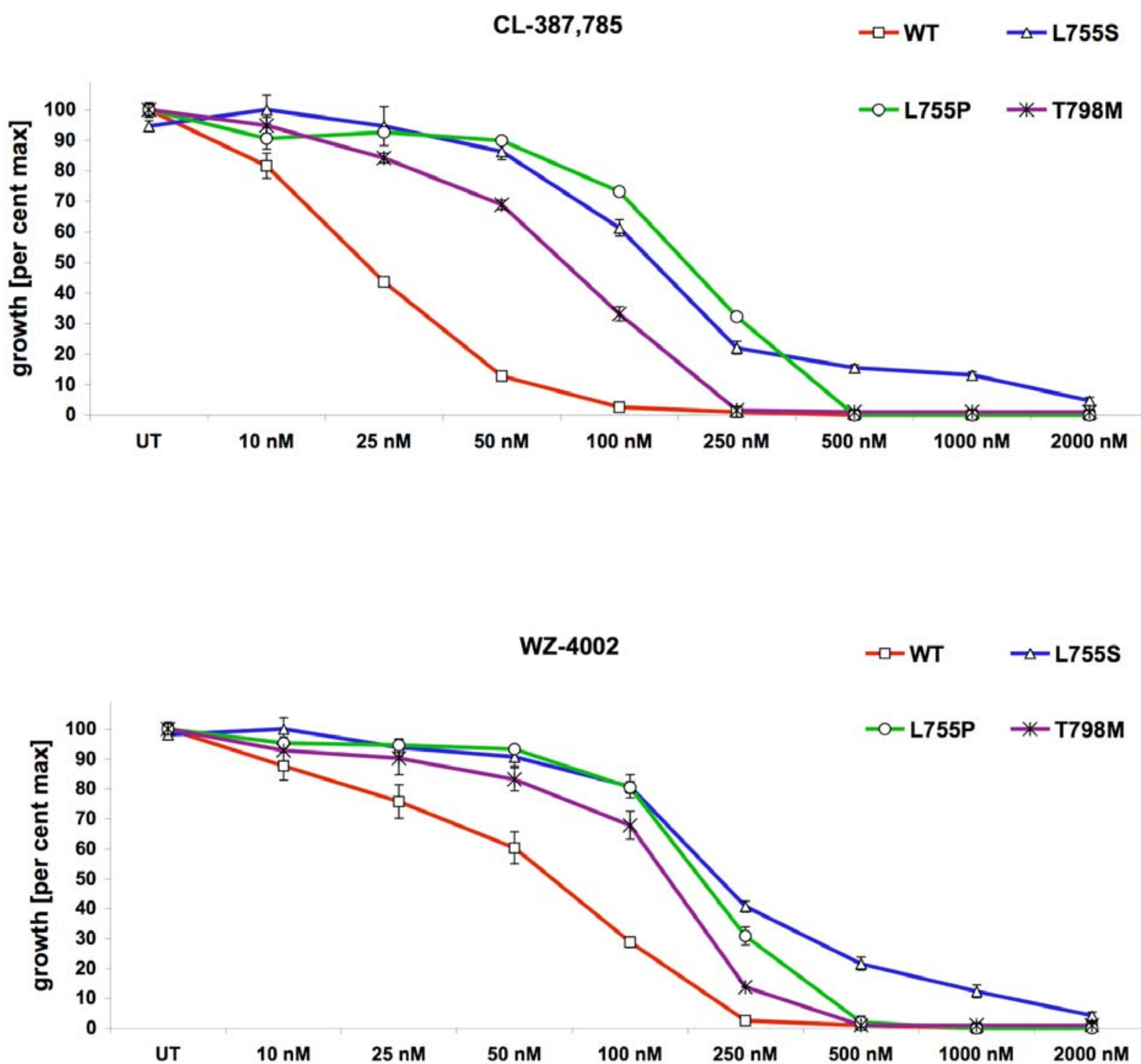


Figure 42. Irreversible inhibitors overcome lapatinib resitance due to ERBB2 kinase domain mutations. Ba/F3-ERBB2 mutant cell lines (L755S, L755P and T798M) were tested for their sensitivity towards CL-387,785 and WZ-4002 at the indicated concentrations. Ba/F3-ERBB2-WT is shown for comparison (red line).

To test if the cell proliferation inhibition is reflected at the biochemical level, mutant ERBB2 Ba/F3 cell lines were treated with CL-387,785 and WZ-4002 and analysed. Inhibition of autophosphorylation (demonstrated by two different phopho-ERBB2 antibodies) as well as activation of downstream signaling molecules (STAT5 and ERK1/2) correlated well with the cell proliferation inhibiton data (Figure 43).

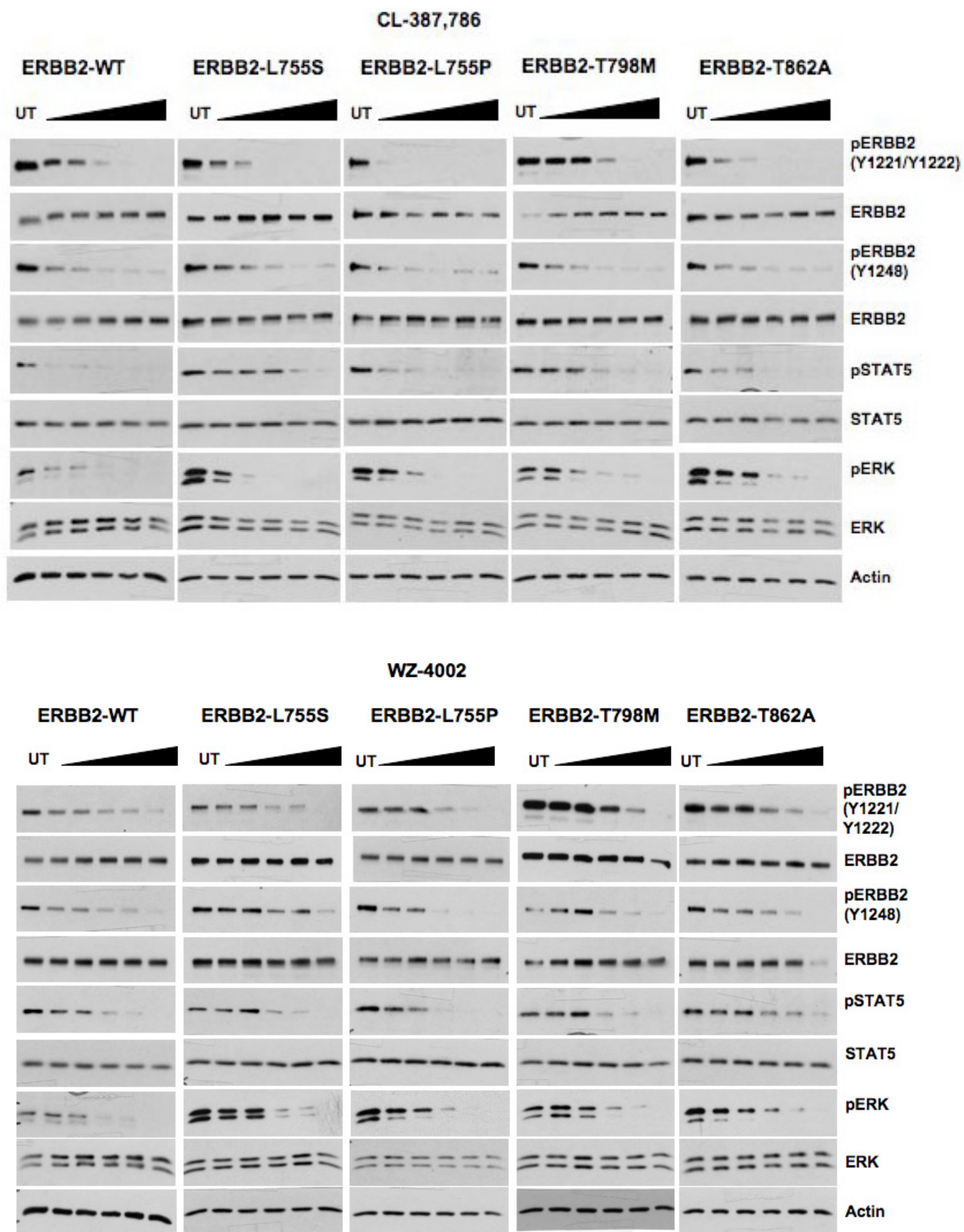


Figure 43. Inhibition of autokinase activity and downstream signaling by CL-387,785 and WZ-4002. Wild type and mutant Ba/F3 cell lines (L755S, L755P, T798M and T862A) were treated with increasing concentrations (50 nM, 100 nM, 250 nM, 500 nM and 1000 nM) of indicated drugs for 30 minutes and analysed for the inhibition of ERBB2 activation as well as the activation of downstream signaling molecules.

Thus, lapatinib resistance due to certain ERBB2 kinase domain mutations can be overcome by irreversible inhibitors. This may offer potent treatment alternatives in the future for lapatinib-resistant ERBB2-positive breast cancer.

4. Discussion

4.1. Drug sensitivity profiles of activating and drug resistant FLT3 mutants

4.1.1. FLT3-D835Y is less sensitive than FLT3-ITD towards sorafenib treatment

Internal tandem duplication (ITD) in the juxtamembrane domain and a point mutation in the activation loop of the kinase domain are the most common activating mutations reported in the FLT3 receptor. Our aim was to test the effect of multikinase inhibitors sunitinib and sorafenib against activating FLT3 mutants. To this end we first established Ba/F3 cell lines that stably express and were transformed by the FLT3 kinase mutants. Analysis of Ba/F3 cells transformed by the FLT3 mutants revealed that FLT3-D835Y was relatively resistant towards sorafenib treatment (cellular IC_{50} approximately 100 nM). Ba/F3-FLT3-ITD remained sensitive to sorafenib treatment with a cellular IC_{50} value of 5 nM. Recently, Auclair et al. showed similar biochemical IC50 values for these activating FLT3 mutants against sorafenib[236]. On the contrary, no significant differences in cellular IC_{50} values was observed between activating mutants against PKC412 and sunitinib treatment. These results indicate that the patients with FLT3-D835Y may not respond well to sorafenib treatment as compared to the FLT3-ITD positive patients. Thus, AML patients with FLT3-D835Y may respond better to sunitinib treatment than sorafenib treatment. However, there are both advantages and drawbacks with this model to test the efficacy of FLT3 kinase inhibitors.

The primary advantage of using the Ba/F3 cell line-based assay is that it is relatively rapid to establish stable cell lines and testing of inhibitors can be done using simple assays. On the other hand, Ba/F3 cells expressing mutant FLT3 receptors is an artificial system and the results should be carefully extrapolated to predict the clinical outcome. However, the added advantage is that the Ba/F3 cells lack any other known oncogenic mutations as evidenced by it's cytokine dependence of cell proliferation and survival. This is important to evaluate the effects of inhibitors on the mutated FLT3 kinase alone in the background of non-transformed cell line. Thus, studies using both the Ba/F3 cells and AML cell lines need to be complemented with each other in order to test the feasibility of findings for clinical application as well as to understand the oncogenic signaling leading to AML pathogenesis.

4.1.2. Sorafenib overcomes PKC412 resistance due to FLT3 kinase domain mutations

Recently, a cell based resistance screen identified that certain kinase domain mutations in the FLT3-ITD background cause PCK412 resistance[104]. Thus, we tested if the multikinase inhibitors sunitinib and sorafenib can overcome PKC412 resistance due to these mutations (N676D, F691I and G697R) in the FLT3-ITD background. All these PKC412-resistant FLT3-ITD mutants (N676D, F691I and G697R) transformed Ba/F3 cells. As shown before[104], these mutants were resistant to PKC412 treatment. Importantly, both sunitinib and sorafenib inhibited FLT3-ITD + N676D with a cellular IC_{50} value within the concentrations that do not have nonspecific toxicity against parental Ba/F3 cells.

Gatekeeper residue mutations were reported to confer very strong inhibitor resistance in several kinases (BCR-ABL-T315I, EGFR-T790M, c-KIT-T670I and PDGFRalpha-T674I)[30, 37, 145, 237]. Similarly, FLT3-ITD + F691I (gatekeeper mutation) is also resistant to all the drugs tested so far[104]. Analysis of cell proliferation inhibition showed that the IC_{50} value for FLT3-ITD + F691I was beyond the PKC412 concentrations that can be achieved in patients. Interestingly, sorafenib inhibited FLT3-ITD + F691I with and IC_{50} value of approximately 1 μM. Eventhough the IC_{50} value for FLT3-ITD + F691I against sorafenib is nearly 200 times higher than that of FLT3-ITD alone, this concentration can be achieved in patients[238]. Importantly, sunitinib was more potent against FLT3-ITD + F691I with an IC_{50} value half to that of sorafenib. We then tested the efficacy of sunitinib and sorafenib on the PKC412 resistant G697R mutation. FLT3-ITD + G697R was totally resistant to sunitinib even at higher concentrations upto 2 μM. On the contrary, sorafenib was very effective against FLT3-ITD + G697R making it an alternate treatment option for FLT3-ITD positive patients who may develop this mutation at the time of relapse. Thus, sorafenib effectively inhibited all the three PKC-412 resistant FLT3-ITD mutations tested. We then examined if sorafenib also induces cell death in these cell lines. Sorafenib potently induced cell death in both the activating and PKC412 resistant FLT3 mutants in a dose dependent manner. The extent of sorafenib induced cell death is in agreement with cell proliferation data observed for all the mutants; FLT3-D835Y and FLT3-ITD + F691I were relatively less sensitive to cell death compared to other FLT3 mutants tested.

PKC412, sunitinib and sorafenib are multikinase inhibitors that are currently in use for AML treatment. Since the effective inhibitor concentrations of sunitinib and sorafenib against FLT3 mutants were well within the range that can be achieved in patients[238, 239],

these drugs offer potent alternatives for treating AML patients who are positive for activating or PKC412 resistant mutations in the FLT3 kinase. Using a mouse model, it was demonstrated that treatment with sorafenib reduced the leukemia burden and prolonged survival[240]. Importantly, in a phase I trial, sorafenib treatment resulted in the decrease of leukemia blasts in FLT3-ITD positive patients but not in patients without this mutation[240]. Moreover, in a recent study, compassionate use of sorafenib before or after allogenic stem cell transplantation showed significant activity in AML patients with FLT3-ITD mutation[241]. Thus, these results suggest that sorafenib monotherapy might offer superior clinical outcome in FLT3-ITD positive AML patients. Another important feature of sorafenib is it's ability to inhibit Raf kinase whose role has been shown previously in AML[236]. Thus, the differential drug responses of different FLT3 mutants observed against tested multikinase inhibitors is an important step towards selecting AML patients for the most suitable drug treatment.

4.2. Functional properties and drug sensitivity profiles of EGFR mutants

4.2.1. Identification of kinase defective EGFR mutations reported in NSCLC patients

Identification of activating mutations in the kinase domain of EGFR in NSCLC patients that sensitize the receptor to small-molecule kinase inhibitors led to a retrospective analysis of several clinical trials to confirm a correlation between EGFR mutational status and treatment response to kinase inhibitors such as erlotinib and gefitinib. One of the largest studies conducted identified several novel EGFR mutations but failed to find a correlation of mutational status and treatment response and thus concluded that the mutational status of EGFR in NSCLC patients is not a predictive factor for erlotinib response[141]. Hence, molecular analysis to predict treatment response was not recommended. Numerous additional studies conducted with both gefitinib and erlotinib produced conflicting results about the mutational status as a predictive factor of drug responsiveness[141, 142, 217, 242]. It is important to note that most of the less frequent mutations reported were not functionally characterized thus far, making it difficult to draw meaningful conclusions from correlative studies comprising only the mutational status but not the type of mutation. Moreover, additional EGFR mutations were reported that do not sensitize but cause resistance toward EGFR inhibitors, making correlative studies even more complicated[36, 222, 227, 243, 244]. Differences in sequencing techniques, interpretation of the results, and probably potential differences in the sensitivity of EGFR mutations toward different EGFR inhibitors sparked a controversy about whether EGFR sequencing analysis has an important role in guiding clinical use of EGFR inhibitors in NSCLC patients[245, 246].

Therefore, we aimed to perform comprehensive analysis of a large panel of published EGFR mutations with respect to kinase activity, transforming potential, and sensitivity toward different EGFR kinase inhibitors. Surprisingly, 4 of 30 EGFR mutations studied were defective in kinase activity even after EGF stimulation. Because EGFR kinase activity is indispensable for the activation of oncogenic signaling pathways, it seems unlikely that these kinase-dead mutations contribute to tumor development. However, a recent study has reported that inhibition of EGFR kinase activity alone does not result in cytotoxicity in tumor cells[247]. Kinase-defective EGFR expression was sufficient to maintain basal glucose levels and tumor cell survival. Thus, kinase-dead mutations identified in patient samples may have a role in tumor maintenance. In any case, however, usage of EGFR inhibitors will

have no effect. The kinase-dead mutation EGFR-V851A was identified in a large retrospective study, which concluded that there is no correlation between EGFR mutation status and response to erlotinib treatment[141]. Interestingly, an EGFR mutation with a different exchange at the same position (EGFR-V851I) has been reported in two patients who were not responsive to gefitinib[139, 248]. Because V851 is critical for the catalytic activity of EGFR, this mutation may not contribute to tumor growth and survival in these cases. It was suggested that the identification of novel EGFR mutations in NSCLC patients may result from PCR artifacts due to the use of formalin-embedded tissue in some cases[245]. Such artifacts include C→T/G→A and A→G/T→C transitions[246], which are present in the kinase-dead mutations identified in this study[141]. On the other hand, EGFR-V851A was independently reported in patients by different investigators[139, 248]. The reason for the detection of kinase-dead EGFR mutation in NSCLC is unclear at the moment and these mutations may also present so-called passenger or bystander mutations as reported previously in lung cancer[249, 250]. Recently, crystal studies of the kinase domain in tandem with juxtamembrane domain showed that the regions around the observed kinase defective mutants were important for the intact intrinsic kinase activity of EGFR[124, 220, 251]. Thus, the residues that are mutated to kinase defective EGFR may be involved in juxtamembrane regulation of EGFR kinase activity. Nevertheless, the variability of kinase activity and sensitivity to EGFR kinase inhibitors may in part be responsible for the discrepancies between clinical studies aiming to correlate mutational status and drug response.

4.2.2. Drug sensitivity profiles of EGFR kinase domain mutants

Sensitivity toward different kinase inhibitors can vary significantly between individual activating and resistance mutations, as it has been shown for Bcr-Abl, c-Kit, or Flt-3[207, 225]. This prompted us to establish drug sensitivity profiles for a comprehensive panel of EGFR mutations toward three EGFR kinase inhibitors. From these studies, we have identified four sets of mutations based on their drug sensitivity profiles: (*a*) mutations that are very sensitive to all three drugs tested with IC_{50} values in the low nanomolar range (L858R and Del 747-753insS), (*b*) mutations that are less sensitive to gefitinib (IC_{50} > 100 nmol/L) but sensitive to both erlotinib and AEE788 (G719S, V742A, and R776C; IC_{50} < 100 nmol/L), (*c*) mutations that are less sensitive to both gefitinib and erlotinib but sensitive to AEE788 (D761N, S768I, S784F, L838V, and L861Q), and (*d*) mutations that are resistant to all

three drugs tested (N826S and T790M). Interestingly, EGFR-V742A was also reported in the stroma of two breast cancer patients[252]. Thus in these patients with gefitinib resistant EGFR-V742A, treatment with erlotinib may result in positive outcome. Does such a dose-response profile for EGFR mutations have any effect on the clinical management of NSCLC and do differences of IC_{50} values below or above 100 nmol/L constitute a clinically significant difference? With both gefitinib and erlotinib, mean plasma concentrations well above 1 μmol/L can be achieved, and this is well above the *in vitro* concentrations at which most of the EGFR mutants can effectively be inhibited. However, mean plasma concentrations do not provide information about drug concentration within a tumor cell and whether the EGFR target is efficiently inhibited. Two recent articles describe secondary EGFR mutations in gefitinib- and erlotinib-resistant patients. In one article, it was shown that erlotinib treatment could overcome gefitinib resistance in a NSCLC patient caused by a EGFR-L858R+L747S mutation[243]. *In vitro* IC_{50} values for this mutant were 200 and 80 nmol/L for gefitinib and erlotinib, respectively. Similarly, in a second article, erlotinib resistance caused due to an EGFR-L858R+E884K mutation could be overcome by gefitinib treatment. Again, *in vitro* data suggested IC_{50} differences in the 100 nmol/L range[253]. This indicates that in a clinical setting, IC_{50} values for a particular EGFR mutant above and below 100 nmol/L might well be important for whether a patient responds to EGFR kinase inhibitor treatment and that *in vitro* sensitivity profiles could be used to improve treatment strategies. In summary, our results suggest that not all EGFR mutations reported to date are of pathophysiologic relevance for NSCLC development and maintenance and underscore the need of functional characterization of every new EGFR mutation discovered in NSCLC patients as it has been done in other malignancies such as chronic myelogenous leukemia.

Identification of comprehensive drug resistance profiles opens the opportunity to test alternative EGFR inhibitors *in vitro* such as AEE788. Other recent studies have shown that irreversible inhibitors of EGFR kinase were effective to overcome the resistance caused by reversible inhibitors such as gefitinib and erlotinib[227]. Such preclinical investigations will undoubtedly accelerate the development of second-generation EGFR kinase inhibitors. However, as it is the case for chronic myelogenous leukemia, several resistance

mechanisms, including the switch to alternate oncogenic pathways, will add complexity to the resistance issue[254].

4.2.3. EGFR-L861Q is a hyperactive kinase but not drug sensitizing mutation

EGFR-L861Q is one of the frequent mutations reported in NSCLC patients but cell proliferation analysis showed that this mutation has a higher IC_{50} value compared to other common EGFR mutants such as L858R and G719S against the EGFR inhibitors gefitinib and erlotinib. The higher cellular IC_{50} value for EGFR-L861Q against erlotinib was also reported previously[71]. In the absence of EGF ligand, all these mutants can transform cell lines while the wild type EGFR cannot[221]. Thus, it is not possible to compare drug responses of ligand-independent EGFR mutants to that of the ligand dependent wild type EGFR[221]. To overcome this obstacle, EGFRvIII was used as a test backbone to compare biochemical properties and drug sensitivities of EGFR mutants to that of the wildtype kinase domain. EGFRvIII is an oncogenic receptor reported in glioblastoma patients and contains a large deletion in extra cellular ligand binding domain but contains an intact kinase domain[255]. Because EGFRvIII retains the wildtype kinase and can transform cell lines in the absence of EGF ligand, it was chosen for the study of the role of kinase domain mutations on intrinsic properties of the EGFR. Using this strategy, EGFR-L861Q showed enhanced autokinase activity as well as transformation ability compared to both wild type (EGFRvIII) as well as the most common mutants EGFRvIII-L858R and EGFRvIII-G719S. Upon cell proliferation inhibition analysis, the most common EGFR mutants reported in NSCLC patients, L858R and G719S in the EGFRvIII background showed significant decrease in cellular IC50 values compared to EGFRvIII. Interestingly, EGFRvIII-L861Q did not confer enhanced drug sensitivity towards EGFR inhibitor (gefitinib, erlotinib and AEE788) treatment compared to EGFRvIII. These results demonstrated that unlike mutations L858R and G719S, EGFR-L861Q is not a drug sensitizing mutation. A mutation can be considered as drug sensitizing if its IC_{50} value against a particular drug is less than that of the wild type kinase. Thus, NSCLC patients with EGFR-L861Q may not have significant benefit upon EGFR inhibitor treatment compared to those with either EGFR-L858R or EGFR-G719S. Thus, alternate EGFR inhibitors need to be tested that might confer enhanced sensitivity on EGFR-L861Q compared to the EGFR-WT kinase.

WZ-4002 is a novel irreversible inhibitor of EGFR kinase that was recently shown to have significant activity both *in vitro* and *in vivo*[205]. Both EGFRvIII-L858R and EGFRvIII-G719S showed lower IC_{50} values towards WZ-4002 treatment compared to wild type EGFRvIII. Interestingly, EGFRvIII-L861Q also showed significantly more sensitivity against WZ-4002 compared to the wild type EGFRvIII. Therefore, second-generation kinase inhibitors such as WZ-4002 may offer more potent alternative to treat patients with EGFR-L861Q mutation[228]. The Ba/F3-EGFRvIII-based system described in this report will be a valuable tool to test novel compounds and strategies.

4.3. Effect of cancer associated ERBB2 variants on kinase activity and drug sensitivity

4.3.1. Genetic polymorphisms in ERBB2 kinase do not effect drug sensitivity

The importance of the role of genetic factors in determining drug sensitivity towards inhibitor treatment is evident in CML, AML and NSCLC[127, 256]. Polymorphisms and mutations in the ERBB2 kinase were reported mostly in breast cancer and less frequently in other cancers. ERBB2 polymorphism I655V either alone or in tandem with another polymorphism I654V has been postulated to increase the risk of breast cancer incidence[177, 178]. Methods to test ERBB2 polymorphisms were also described[179, 257]. But, contradicting reports exist questioning the role of ERBB2 polymorphisms in breast cancer risk[179, 180, 233]. However, the functional role of ERBB2 polymorphisms is not known making it difficult to assess their role in breast cancer pathogenesis. Functional analysis revealed that these ERBB2 polymorphisms do not enhance kinase activity, transformation potential and drug sensitivity suggesting that there is no need to test for ERBB2 polymorphism for lapatinib treatment. Thus, ERBB2 polymorphisms has no role in predicting outcome upon lapatinib treatment.

4.3.2. Identification of lapatinib resistant ERBB2 kinase domain mutations

Development of secondary drug resistance upon inhibitor treatment is a major problem in advanced diseases[30, 145]. In vitro screens which identify kinase domain mutations that abrogate inhibitor binding were shown to be useful in predicting the mechanisms of secondary drug resistance[208]. Lapatinib is approved for the treatment of ERBB2 positive breast cancer and it is important to identify lapatinib resistant ERBB2 kinase domain mutations. Thus, we performed an in vitro screen to identify ERBB2 kinase domain mutations that cause resistance to lapatinib treatment. Using ENU mutagenesis screen, ERBB2-L755S, ERBB2-T862A and ERBB2-T798M were identified as lapatinib resistant kinase domain mutations. Importantly, ERBB2-L755S was also reported previously in an independent lapatinib resistance screen[258]. Moreover, ERBB2-L755S was previously identified in breast cancer as well as in gastric cancer patients[183]. Thus patients with ERBB2-L755S may not respond to lapatinib treatment (primary resistance). Interestingly, similar mutation at the homologous position in EGFR (L747S) was identified in a lapatinib

resistance screen indicating a common mechanism of lapatinib activity against both EGFR and ERBB2[259]. Recently, EGFR-L747S was reported in lung cancer and as a gefitinib resistant mutation[235, 260].

Lapatinib resistant ERBB2-T862A (identified in the screen) is an activation loop mutant and was recently reported in primary gastric cancer[261]. Moreover, EGFR-T854A is homologous to ERBB2-T862A and was reported as a gefitinib-resistant mutation in a lung adenocarcinoma patient[234]. EGFR-T854A was also identified as an erlotinib resistant kinase domain mutant in an in vitro screen indicating that this mutation is cross-resistant to different inhibitors[259]. Eventhough identified in lapatinib resistance screen, ERBB2-T862A was totally inhibited at higher lapatinib concentrations suggesting that dose escalation may overcome the intermediate resistance caused by this mutation.

Gatekeeper residue in the kinase domain is critical for inhibitor binding and its mutation in several kinases was shown to cause secondary resistance against various kinase inhibitors[30, 145]. T798M is the gatekeeper residue mutation in ERBB2 kinase and conferred lapatinib resistance. Interestingly, similar mutation in EGFR kinase (T790M) was shown to cause lapatinib resistance in *in vitro* resistance screen suggesting a common role of this residue in lapatinib activity against both EGFR and ERBB2[259].

4.3.3. Drug sensitivity profiles of ERBB2 mutations reported in cancer patients

Kinase domain mutations in ERBB2 were reported in various cancers and these mutants are not characterized functionally. A panel of selected ERBB2 mutants transformed Ba/F3 cells to cytokine independence which were then used to test inhibitor sensitivity. The locations of the kinase domain mutants considered in this study are depicted in Figure 44 (A and B). Of the four mutations in the N-lobe of the kinase, L755S/P emerges from a loop adjacent to helix C, V773 and V777 are at or near the C-terminal portion of helix C, and T798 is at the gatekeeper position in the ATP binding site (Figure 44A and B). Of the remainder, N857 emerges from helix D, T862A forms the base of the ATP binding site, and H878 is in the activation loop.

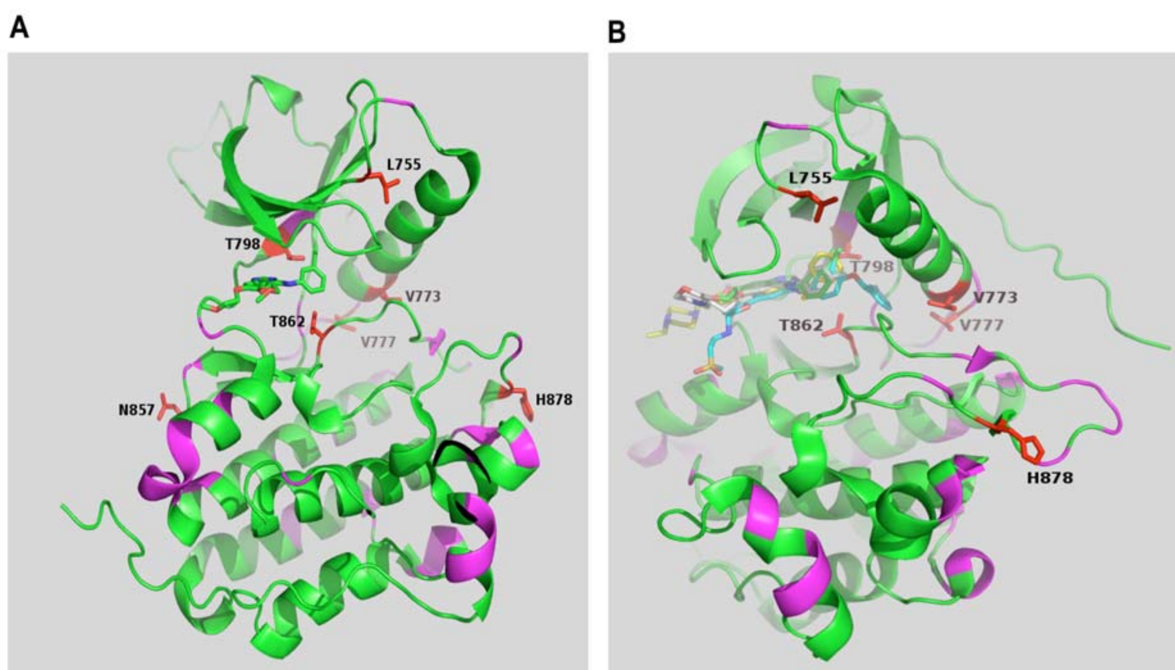


Figure 44. Structural analysis of lapatinib resistant ERBB2 kinase domain mutants.
The side chains of mutants considered in this study are plotted (red sticks) together with a schematic representation of the protein fold using the crystal structure of EGFR kinase in complex with erlotinib (green sticks). B) is a view roughly orthogonal to A) and shows additional inhibitors gefitinib (yellow sticks) and lapatinib (blue sticks) superimposed at the ATP binding site (performed in collaboration with Prof. Richard A. Engh (NORSTRUCT, Department of Chemistry, University of Tromsø, Norway).

ERBB2-H878Y was identified as the most sensitive mutant towards lapatinib treatment with IC_{50} value less than that of the wild type ERBB2 receptor. Similar sensitizing effect of ERBB2-H878Y was reported recently in an autophosphorylation assay[262]. ERBB2-H878Y was reported in 11% of hepatoma patients and thus these patients may benefit from lapatinib treatment[185].

ERBB2-V777L was reported in gastric and colon cancer and showed significant sensitivity to lapatinib treatment[183]. Mutations ERBB2-V773A and ERBB2-N857S showed higher IC_{50} values compared to wild type ERBB2 kinase, but were totally inhibited within clinically achievable concentrations of lapatinib. Interestingly, ERBB2-L755P was very resistant to lapatinib treatment. Another mutation affecting the same residue (ERBB2-L755S) also caused lapatinib resistance. Thus, the position L755 is critical for lapatinib activity and its mutation may confer drug resistance in patients treated with lapatinib.

Previously, treatment with alternative inhibitors was shown to overcome inhibitor resistance due to kinase domain mutatins in BCR-ABL, FLT3 and EGFR[34, 225, 227, 263]. Thus,

we tested another reversible EGFR/ERBB2 inhibitor AEE788 against Ba/F3-ERBB2 mutant cell lines. Upon analysis, AEE788 retained activity against lapatinib resistant ERBB2-L755S but not towards ERBB2-L755P and ERBB2-T798M prompting the need to test new inhibitors. Previously, lack of preclinical models was postulated to hamper the understanding mechanisms leading to lapatinib resistance[264, 265]. Thus, the models presented in this study would be useful to study drug sensitivity profiles of ERBB mutants as well as lapatinib resistance. Furthermore, these cell lines are useful tools to test novel inhibitors that might overcome lapatinib resistance.

4.3.4. Structural basis of lapatinib resistance

To elucidate the mechanism underlying, structural studies were performed in collaboration with Prof. Richard A. Engh (NORSTRUCT, Department of Chemistry, University of Tromsø, Norway).

To date, no structural data is available for ERBB2 kinase. Thus, the high degree of identity between EGFR and ERBB2 was exploited to perform homology modeling by simple replacement of side chains. The ligand binding surfaces at and near the ATP-binding site for EGFR and ERBB2 are nearly identical. The following lapatinib-resistant ERBB2 kinase domain mutations are analysed:

L755S/P: Figure 45A shows contacts between L755 and helix C that are seen in the active EGFR structures. While mutations at L755 will not affect inhibitor binding directly, they do affect the packing interactions with helix C, and thus will influence the structure of the active state and the transition between active and inactive forms. In the active form (Figure 45A), L755 packs against the helix with hydrophobic interactions. In inactive forms (Figure 45B), the C-helix is translated away from the active site, the activation loop may adopt a helical turn, and L755 does not make ordered contact with helix C. Because the mutations are transforming in the absence of inhibitor, the L755 mutations either stabilize the active state relative to the inactive state or lower a barrier to activation. L755P may do this by reducing disorder of the inactive state and stabilizing the loop favorable for an active conformation. L755S likely destabilizes the interactions in the inactive state, observed to be hydrophobic. It is also possible that L755S introduces stabilizing polar interactions of a structurally altered active form.

<u>T798M</u>: Threonine 798 is the ERBB2 "gatekeeper", the ATP site residue long known as a primary selectivity determinant among protein kinases. The gatekeeper is also known as the most prominent site of drug resistant mutations of Abl kinase against imatinib and other CML drugs[30]. The mutation of the gatekeeper to methionine is the principle mechanism for drug resistance in EGFR kinase (T790M)[145]. It is known to enhance the affinity of oncogenic forms of EGFR kinase to ATP, explaining its drug resistant properties despite retention of tight binding to inhibitors. Figure 45C shows how the binding mode of AEE788 remains unaffected by the mutation. Unlike AEE788, lapatinib binds the inactive conformation preferentially. Figure 45D shows different binding modes for lapatinib in EGFR kinase and ERBB4, which share high identity with ERBB2.

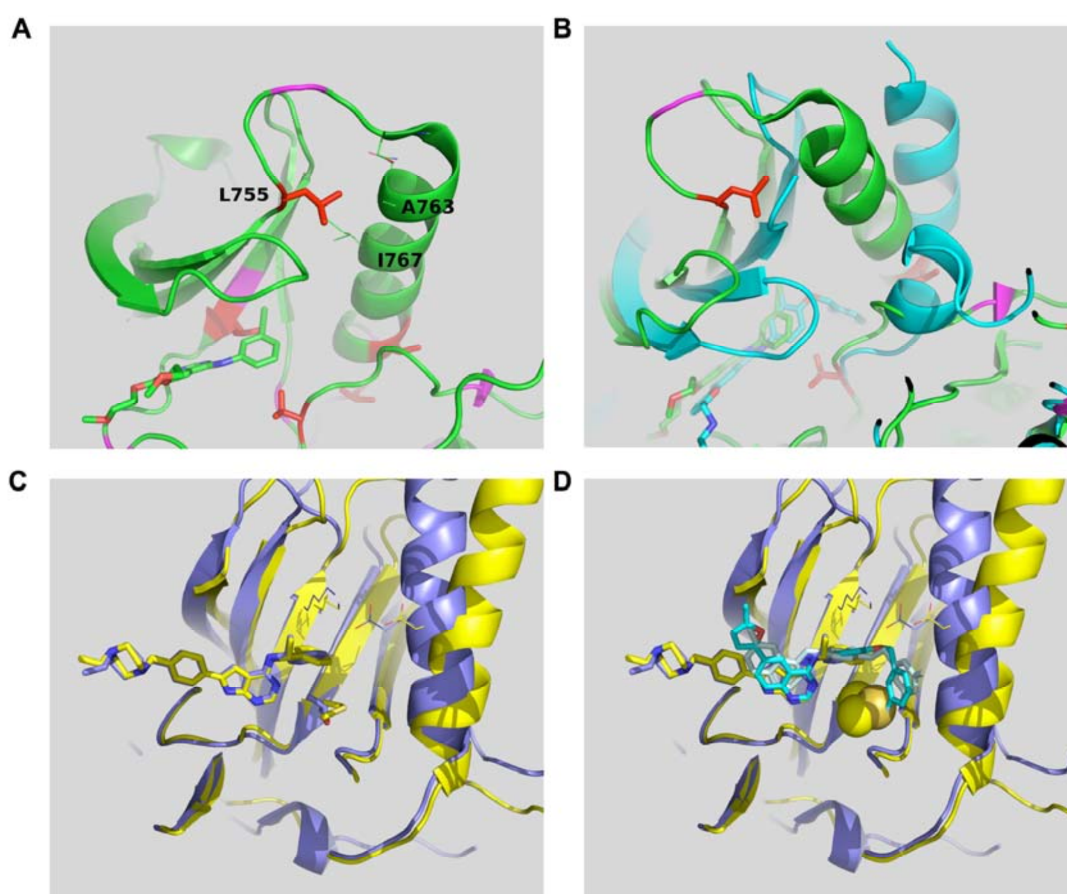


Figure 45. Structural analysis of lapatinib resistant ERBB2 kinase domain mutants.
(A) L755 packs against helix C, closest to residues Ala763 and Ile767, and makes no contacts with the inhibitors (structure 1M17 with inhibitor erlotinib is depicted lower left). **(B)** Comparing the active structure of 1M17 (green) to an inactive representative 1XKK bound to lapatinib shows the loss of L755 interactions (cyan). **(C)** Overlay of AEE788 bound structures of EGFR (2J6M, active, blue) and EGFR T790M (2JIU, inactive, yellow). The existence of the salt bridge linking the active site lysine K753 with the helix C E770 is a marker for the active state. The T798M (ERBB2 numbering) mutation does not significantly alter binding,

although a rotation of the inhibitor aromat is apparent. **(D)** Superposition of two binding modes of lapatinib onto the overlay of figure C) and display of the T798M atoms as Van der Waals spheres shows how the binding mode seen in 1XKK (cyan) obviously clashes with the mutation, but the binding mode of 3BBT (pale blue, ERBB4, which also has threonine as gatekeeper) does not (performed in collaboration with Prof. Richard A. Engh (NORSTRUCT, Department of Chemistry, University of Tromsø, Norway).

4.3.4. Irreversible EGFR/ERBB2 inhibitors overcome lapatinib resistance

Treatment with an irreversible inhibitor was previously shown to overcome gefitinib reistance due to EGFR kinase domain mutation[227]. We then tested if irreversible dual EGFR/ERBB2 can overcome lapatinib resistance due to ERBB2 kinase domain mutations. Cell proliferation analysis showed that both the irreversible inhibitors (CL-387,785 and WZ-4002) tested inhibited lapatinib resistant ERBB2 mutant Ba/F3 cell lines. Western blot analysis showed that autokinase activity of ERBB2 as well as phosphorylation of downstream signaling molecules was inhibited in a dose dependent manner. Thus, irreversible inhibitors offer a potent alternative to treat lapatinib resistant cancer due to ERBB2 kinase domain mutations.

In conclusion, our findings suggest that only a subset of patients with select ERBB2 kinase mutations may benefit from lapatinib treatment. Thus, a careful molecular diagnosis is needed to treat patients with ERBB2 kinase inhibitors.

5. Summary

The identification of mutations in druggable kinases as oncogenic events is a major advancement in molecular medicine. These mutations include gene fusions, point mutations, insertions and deletions that result in constitutive kinase activity and can confer a transformed phenotype. Prime example is the BCR-ABL oncogene in chronic myeloid leukemia patients, which can be efficiently targeted by small molecule kinase inhibitors such as imatinib. Recently several oncogenic mutations were reported in tyrosine kinases in hematological malignancies as well as in solid tumors that can be targeted by kinase inhibitors. Even though treatment with kinase inhibitors shows promising success in several neoplasias, development of secondary drug resistance due to mutations that abrogate inhibitor binding has emerged as a major problem. The aim of this study is to establish cellular systems for studying biochemical and signaling aspects of oncogenic tyrosine kinases, and to use these systems to test the effectiveness of small molecule inhibitors that target them. Further objectives include the establishment of cell based screening system to identify drug resistant mutations that will be useful in the future to predict resistance towards kinase inhibitors in patients.

We have successfully established cell models to study the biochemical properties and drug sensitivity of oncogenic mutations in EGFR, HER2 and FLT3. Interesting findings include 1) overcoming PKC412 resistant FLT3 mutants using sunitinib and sorafenib 2) the identification of EGFR kinase dead mutations that were reported in NSCLC patients, 3) differential sensitivity of EGFR mutations towards reversible inhibitors gefitinib, erlotinib and AEE788, 4) differential sensitivity of ERBB2 mutants towards AEE788 and lapatinib, 5) the identification of drug resistant mutations in EGFR and ERBB2 kinase domains and 6) overcoming lapatinib resistance due to ERBB2 kinase domain mutations by WZ-4002 treatment.

6. Zusammenfassung (Summary in German)

Ein großer Fortschritt der Molekularmedizin war die Entdeckung von Kinasemutationen als Ursache zahlreicher Neoplasien. Diese durch Genfusionen, Punktmutationen, Insertionen oder Deletionen konstitutiv aktivierten Kinasen führen zu zellulärer Transformation und tragen so zur Entstehung von Neoplasien bei. Eine medikamentöse Inhibition der deregulierten Kinasen kann somit zur Behandlung dieser Neoplasien eingesetzt werden.

Als bestes Beispiel dient die chronische myeloische Leukämie, die auf molekularer Ebene durch das Fusionsgen BCR-ABL definiert ist und effektiv mit Kinaseinhibitoren wie Imatinib behandelt wird.

Heutzutage sind zahlreiche Tyrosinkinasemutationen in hämatologischen Erkrankungen sowie soliden Tumoren beschrieben, die gezielt mit Kinaseinhibitoren behandelt werden können. Obwohl die Behandlung verschiedener Neoplasien mit Kinaseinhibitoren Erfolge zeigt, stellen Resistenzmechanismen aufgrund von zusätzlichen Mutationen ein Problem dar.

Ziel dieser Arbeit war es, in Zellsystemen die Aktivität von Tyrosinkinasen auf biochemischer sowie signaltransduktionaler Ebene und ihre Sensitivität gegenüber niedermolekularen Inhibitoren zu untersuchen. Darüber hinaus wurde ein in-vitro screening System entwickelt, mit dem Resistenzmutationen identifiziert werden können. Mit den dabei gewonnenen Erkenntnissen lassen sich Vorhersagen bei der Behandlung von Patienten mit auftretenden Sekundärmutationen treffen. Im Rahmen dieser Arbeit wurde mit den oben beschriebenen Modellsystemen die Sensitivität von onkogenen Mutationen in EGFR, HER2 und FLT3 gegenüber verschiedenen Inhibitoren bestimmt. Die Ergebnisse beinhalten den Nachweis 1) der Inhibition PKC412-resistenter FLT3-Mutanten durch Sunitinib und Sorafenib; 2) kinasedefekter EGFR Mutationen, welche in NSCLC-Patienten beschrieben wurden; 3) der unterschiedlichen Sensitivität von Mutationen des EGFR gegenüber Gefitinib, Erlotinib und AEE-788 und 4) der verschiedenen Sensitivitäten von ERBB2 Mutanten gegenüber AEE-788 und Lapatinib. Des Weiteren 5) die Identifikation von Resistenzmutationen in den Kinasedomänen von EGFR und ERBB2 und 6) die erfolgreiche Behandlung Lapatinib resistenter ERBB2 Klone mit dem WZ-4002 Inhibitor.

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9. Publications

1) von Bubnoff N, Gorantla SH, **Kancha RK**, Lordick F, Peschel C, Duyster J.
The systemic mastocytosis-specific activating cKit mutation D816V can be inhibited by the tyrosine kinase inhibitor AMN107.
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2) **Kancha RK**, Grundler R, Peschel C, Duyster J.
Sensitivity toward sorafenib and sunitinib varies between different activating and drug-resistant FLT3-ITD mutations.
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Imatinib and leptomycin B are effective in overcoming imatinib-resistance due to Bcr-Abl amplification and clonal evolution but not due to Bcr-Abl kinase domain mutation.
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4) **Kancha RK**, von Bubnoff N, Peschel C, Duyster J.
Functional analysis of epidermal growth factor receptor (EGFR) mutations and potential implications for EGFR targeted therapy.
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British Journal of Haematology. 2009 Mar; 144(6):865-74.

6) **Kancha RK**, Peschel C, Duyster J.
The EGFR-L861Q mutation increases intrinsic kinase activity without leading to enhanced sensitivity towards EGFR kinase inhibitors.
(**In Press**)

7) **Kancha RK**, von Bubnoff N, Peschel C, Duyster J.
Study of transforming potential and drug sensitivity of ErbB2 variants identifies lapatinib-resistant kinase domain mutations
(**Manuscript in preparation**)

10. Conference presentations

Oral Presentations:

1) "Study of transforming potential and drug sensitivity of ErbB2 variants identifies lapatinib-resistant kinase domain mutations" at the German Society for Hematology and Oncology (DGHO) Annual Meeting, Vienna, Austria, October 10-14, 2008.

2) "The EGFR-L861Q mutation increases intrinsic kinase activity without leading to enhanced sensitivity towards EGFR kinase inhibitors" at the DGHO Annual Meeting, Mannheim, Germany, October 2-6, 2009.

Poster presentations:

1) **Rama K. Kancha**, Cornelius Miething, Nikolas V. Bubnoff, Katharina Gotze, Christian Peschel, and Justus Duyster.
Title: Imatinib and Leptomycin B Are Effective in Overcoming Imatinib-Resistance Due to Bcr-Abl Amplification or Clonal Evolution but Not Due to Bcr-Abl Kinase Domain Mutation.
American Society of Hematology (ASH), 47th Annual Meeting, Atlanta, USA, December 10-13, 2005.

2) **Rama K. Kancha**, Nikolas V. Bubnoff, Christian Peschel and Justus Duyster.
Title: Differential effect of Gefitinib and Erlotinib on Epidermal growth factor receptor mutations observed in NSCLC. DGHO Annual Meeting, Leipzig, Germany, November 04-08, 2006.

3) **Rama K. Kancha**, Nikolas von Bubnoff, Christian Peschel, and Justus Duyster.
Title: Differential effect of gefitinib and erlotinib on epidermal growth factor receptor mutations observed in NSCLC. American Association for Cancer Research (AACR), 98th Annual Meeting (Centennial), Los Angeles, USA, April 14-18, 2007.

4) **Rama K. Kancha**, Nikolas von Bubnoff, Christian Peschel and Justus Duyster.
Title: Sorafenib (Nexavar) in vitro is a potent inhibitor of imatinib resistant c-Kit mutations observed in gastrointestinal stromal tumors (GIST). DGHO Annual Meeting, Basel, Switzerland, October 05-09, 2007.

5) **Rama K. Kancha**, Rebekka Grundler, Christian Peschel and Justus Duyster.
Title: Sensitivity towards sorafenib and sunitinib varies between different activating and drug resistant FLT3-ITD mutations. DGHO Annual Meeting, Basel, Switzerland, October 05-09, 2007.

6) **Rama K. Kancha**, Nikolas von Bubnoff, Christian Peschel and Justus Duyster.
Title: Molecular basis for differential clinical outcome in NSCLC patients with kinase domain mutations in epidermal growth factor receptor upon targeted therapy. DGHO Annual Meeting, Basel, Switzerland, October 05-09, 2007.

7) F. Heidel, F.K. Mirea, D.B. Lipka, S. Dove, R. Grundler, **Rama K. Kancha**, J. Duyster, Ch. Huber, F.D. Böhmer, T. Fischer.
Title: Bis(1*H*-indol-2yl)methanones Are Effective Inhibitors of Mutated FLT3 Tyrosine Kinase, Partially Overcome Resistance to PKC412A *In Vitro* And Show Synergy With Chemotherapy. DGHO Annual Meeting, Basel, Switzerland, October 05-09, 2007.

8) **Rama K. Kancha**, Nikolas von Bubnoff, Christian Peschel, and Justus Duyster.
Title: Study of transforming potential and drug sensitivity of ErbB2 variants identifies lapatinib-resistant kinase domain mutations. AACR 100th Annual Meeting, Denver, USA, April 18-22, 2009.

9) **Rama K. Kancha**, Nikolas von Bubnoff, Christian Peschel, and Justus Duyster.
Title: Study of transforming potential and drug sensitivity of ErbB2 variants identifies lapatinib-resistant kinase domain mutations. British Association for Cancer Research (BACR)/European Association for Cancer Research (EACR) Symposium "**Cancer Drug Discovery, Development and Evaluation**", Nottingham, UK, 3rd July 2009.

10) **Rama K. Kancha**, Nikolas von Bubnoff, Christian Peschel, and Justus Duyster.
Title: Study of transforming potential and drug sensitivity of ErbB2 variants identifies lapatinib-resistant kinase domain mutations. <interact 2010> PhD Symposium, Munich, Germany, 23rd March 2010.

11) **Rama K. Kancha**, Nikolas von Bubnoff, Richard A. Engh, Rebekka Grundler, Christian Peschel, and Justus Duyster.
Title: Functional analysis and inhibitor sensitivity profiling of clinically relevant oncogenic mutations in druggable tyrosine kinases. "EMBO Global Exchange & Wellcome Trust/DBT India Alliance meeting: Life Science Research in India", Barcelona, Spain, 4th September, 2010.