EGFR AS TARGETTED THERAPY

2.1 Epidermal Growth Factor Receptor (EGFR)

EGFR is family of four structurally related receptor tyrosine kinases, viz: EGFR (Epidermal Growth Factor Receptor), ErbB-1 (The gene symbol, ErbB, is derived from the fact that these receptors are homologous with a viral oncogene named erythroblastic leukemia viral oncogene), HER 2 (Human epidermal growth factor Receptor 2)/neu (ErbB-2), HER 3 (ErbB-3) and HER 4 (ErbB-4). Kinase receptors contain an extracellular-domain having ligand-binding site and an intracellular domain for enzymatic function, linked by a single transmembrane alpha helix. There are various ligands which regulate and stimulate ErbB receptors that include EGF agonists (bind to EGFR) and Neuregulins (bind to ErbB3 and ErbB4) [19].

Epidermal Growth Factor (EGF) and its receptors were discovered by Stanley Cohen working at Vanderbilt University, USA. For discovery of growth factors, Stanley Cohen and Rita Levi-Montalcini were given Nobel Prize in Medicine in 1986. Epidermal Growth Factor Receptor (EGFR) is a cell surface kinase receptor, which phosphorylates tyrosine. Ligand binding to EGFR results in activation of tyrosine kinase in the intracellular region, which in turn, phosphorylates multiple intracellular substrates leading to cell growth. EGFR, if amplified, has been linked to formation of many solid tumors including lung, head and neck, ovary, prostate, breast, kidney, colon, brain, and bladder cancers [20].

2.2 Structure and downstream signaling of EGFR

EGFR is a glycoprotein of 170 kDa, encoded by a gene located on chromosome 7p11.2. It has cysteine rich extracellular region, intracellular domain having tyrosine kinase site and C-terminal tail having autophosporylation sites. Extracellular portion is subdivided into I-IV regions, where I and III are cysteine poor regions that contain the sites for EGF and transforming growing factor (TGF-α) binding, whereas II and IV are cysteine rich domains and contain N-linked glycosylation sites. Using X-Ray crystallography, EGFR structure has been resolved and helps in understanding the ligand induced activation of EGFR tyrosine kinase family. Crystal structure revealed the basics of ligand binding, ectodomain dimerization and the conformation changes of the apo-kinase domain [21].

EGFR ligands can be specific such as epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), epigen, amphiregulin, β-cellulin, heparin-binding EGF and epiregulin or

nonspecific, which bind to more related receptors (Figure 2.1). Neuregulins binds to ErbB3 and ErbB4 whereas HB-EGF, epiregulin, and β-cellulin activate ErbB1 and ErbB4. For activation, first step involves the ligand induced dimerization of EGFR leading to stimulation of intracellular kinase domain and results in autophosphorylation of EGFR at multiple residues (TYR1016, TYR 1092, TYR1110, TYR1172 and TYR1197). This activation of EGFR further recruits downstream signaling molecules which regulate the EGFR functions. These proteins include many Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain containing proteins. For further signaling these proteins involve adaptor proteins, such as Src homology domain-containing adaptor protein C (Shc), Crk, growth factor receptor bound protein 2 (Grb2), Grb7, Grb2-associated binding protein, Gab1, phospholipase Cγ (PLCγ), Cbl and Esp15 [22]. The kinases involved are Src, Chk and phosphatidylinositol-3-kinase (PI3K) and the protein tyrosine phosphatases such as PTP1B, SHP1 and SHP2 [23] [24]. It activates downstream signaling pathways, including the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3'-kinase (PI3K)-Akt pathway. Ras activation initiates multistep phosphorylation events that lead to the activation of MAPKs. The MAPKs, extracellular-related kinase, (ERK1, and ERK2) regulate gene transcription.

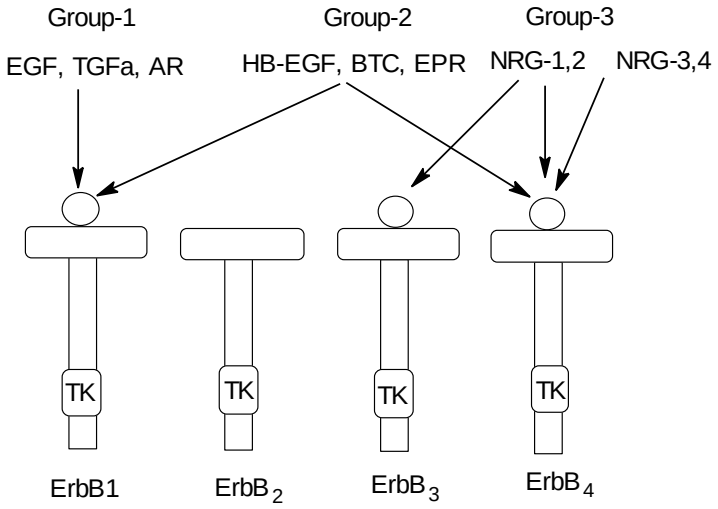

Figure 2.1: EGFR family members and their ligands

One ligand may be a weak activator of one pathway but it activates strongly the other one. The degree of pathway activation by specific growth factors depends on the amount of growth

factor present and the degree of expression of growth factors. Cell surface localization of their cognate receptor tyrosine kinases (RTKs) and the co-expression also affect the pathway activation. Positive and negative feedback loops function in both the Ras-ERK and PI3K-mTORC1 pathways.

2.2.1 The MAPK/ERK pathway

The MAPK/ERK pathway (also known as the Ras-Raf-MEK-ERK pathway) is shown in (Figure 2.2). Upon ligand binding induced receptor dimerization, EGFR intrinsic kinase activation and auto-phosphorylation results. ERK pathway is triggered by Grb2 binding to EGFR at Y1068 and Y1086 and indirectly through Shc binding at Y1148 and Y1173 [25].

Grb2 contains an SH2 domain which binds to phosphotyrosine residues of activated EGFR receptor. Grb2 also recruits SOS (son of sevenless), a set of guanine nucleotide exchange factors, to EGFR receptor complex with the help of two SH3 domains and results in SOS activation. SOS mediates activation of Ras proteins (H-Ras, K-Ras, and N-Ras) at the plasma membrane. Ras activation induces the activation of Raf family kinases including A-Raf, B-Raf, and C-Raf. Activated Raf further activates MEK1 and MEK2 by phosphorylating serines 218 and 222 in the activation loop, and as a result ERK1 and ERK2 are activated [26]. Activation of MAPK/ERK affects translation of mRNA to proteins i.e. it phosphorylate 40s ribosomal protein S6 kinase (RSK) which further phosphorylates ribosomal protein S6. Further, MAPK can also phosphorylate and activate MNK (MAPK interacting kinase) and result in phosphorylation of CREB (cAMP response element binding). CREB is a key Regulator of gene expression, is activated by phosphorylation and results in multiple intracellular signaling cascades [27].

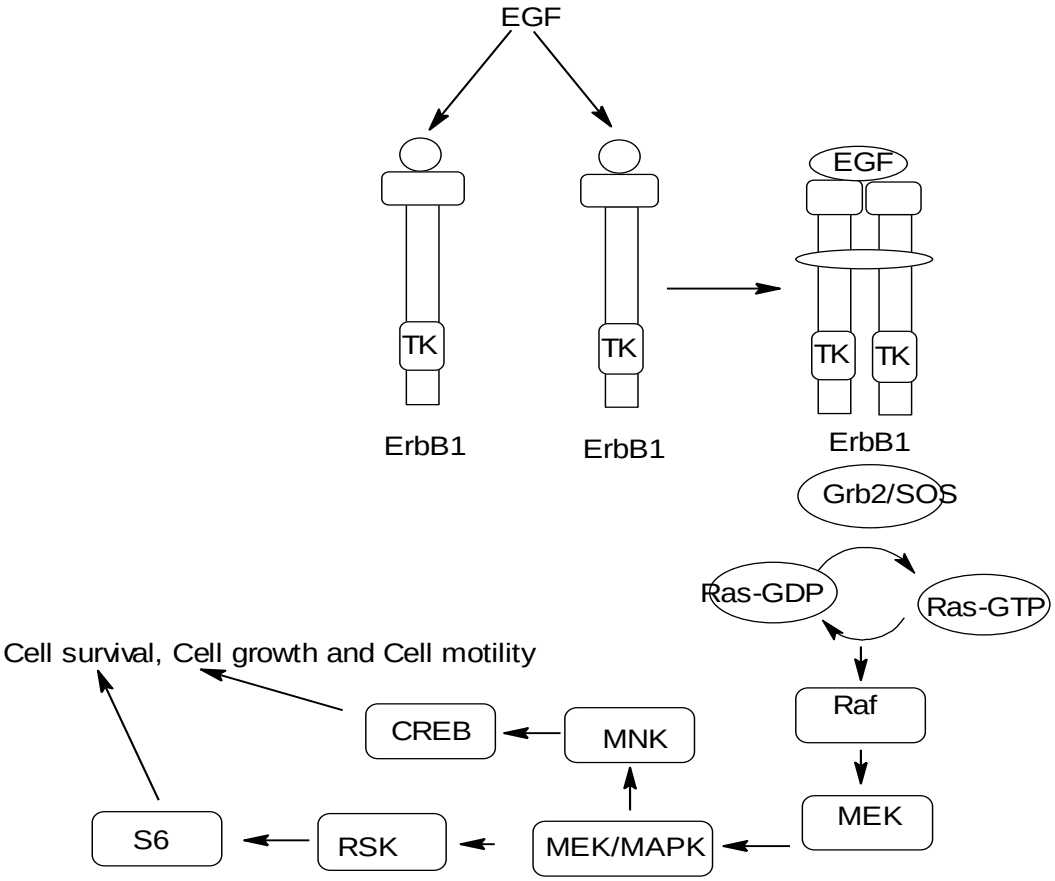

Figure 2.2: The Ras-ERK-MAPK Pathway

Inactive Ras-GDP associates with the plasma membrane whereas inactive MEK, Raf and ERK are largely cytoplasmic. Growth factor (GF) binds and activates RTK autophosphorylation, which further generates binding sites for the Shc and Grb2 adaptor molecules. These adaptor molecules recruit SOS, the Ras GEF (GTPase exchange factor) to membrane. Further SOS catalyzes Ras GTP exchange and Ras-GTP then recruits Raf to the membrane, where it gets activated. Raf activates MEK, which further activates ERK via activation loop phosphorylation. ERK also feeds back to negatively regulate the pathway.

2.2.2 The PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR pathway is an important intracellular signaling pathway (Figure 2.3) which regulates the cell cycle and has been directly linked to cellular quiescence, proliferation and cancer.

In humans, there are three highly homologous isoforms of Akt (Akt1, Akt2, and Akt3). Akt is a serine/threonine kinase, called protein kinase B (PKB) because its catalytic domain is similar to PKA and PKC family members. In quiescent cells, the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) maintains minimum levels of PIP3 thus maintaining AKT inactivation state. PTEN is the primary negative regulator of Akt [28]. Loss of PTEN or PTEN mutation is the most common cause of hyperactivation of the PI3K/Akt pathway in many human cancers [29]. Insulin and growth factor IGF1 binds to RTKs and results in receptor autophosphorylation creating binding sites for recruitment of insulin receptor substrate (IRS), an adaptor protein for PI3K. Once PI3K is activated it phosphorylates phosphatidylinositol-4,5-diphosphate (PIP2), leading to accumulation of phosphatidylinositol-3,4,5-triphosphate and activates AKT in the plasma membrane. Pleckstrin homology (PH) domains of AKT and phosphinositide dependent kinase 1 (PDK1) identifies PIP3 and further phosphorylates the activation loop.

Tuberous sclerosis complexes (TSC2 and TSC1) maintain GTPase Ras homolog enriched in brain (RHEB). AKT phosphorylation of TSC2 releases TSC inhibition of the GTPase RHEB. mTORC2 phosphorylates the hydrophobic motif of AKT, thus resulting in AKT activation and phosphorylation of TSC2. TSC2 phosphorylation inhibits TSC2 GTPase activating protein (GAP) activity. RHEB-GTP activates mammalian target of rapamycin1 (mTORC1) followed by S6K which results in cellular activities [30].

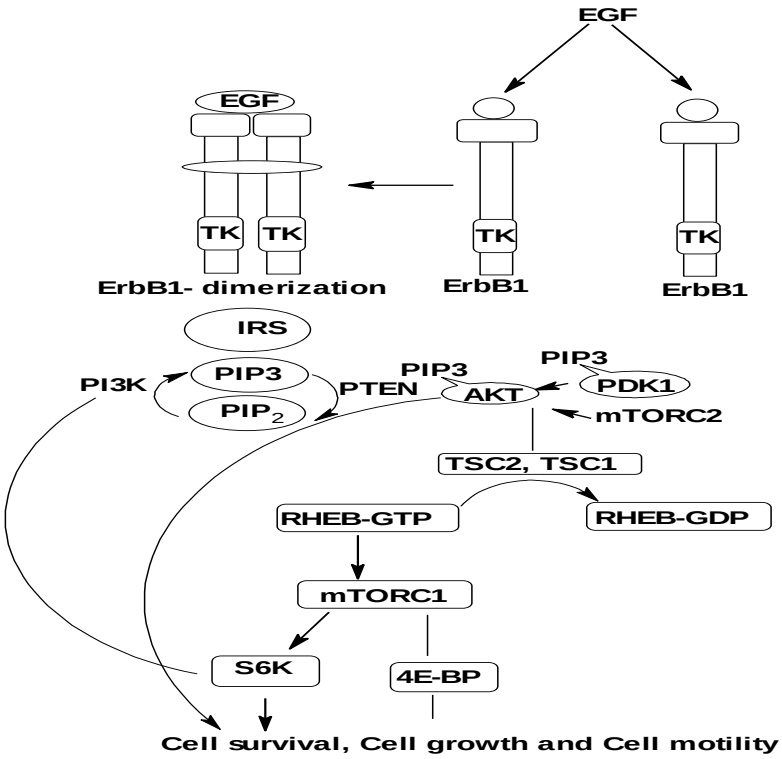

Figure 2.3: The PI3K-mTOR Pathway

The lipid phosphatase PTEN maintains low levels of PIP3 and keeps AKT in inactivation stage. TSC2 and TSC1, maintains RHEB in the GDP-bound state. Insulin and IGF1 binding to RTKs and receptor autophosphorylation creates binding sites that then recruit IRS, an adaptor protein for PI3K. Activated PI3K phosphorylates PIP2 and generates membrane-bound PIP3. mTORC2 phosphorylates the hydrophobic motif of AKT, thus promoting AKT activation and phosphorylation of TSC2 which results in inhibition of TSC2 GAP activity. RHEB-GTP complex activates mTORC1 following its recruitment by the Rag GTPase [31].

2.3 EGFR over expression in various carcinomas

EGFR is expressed in tissues of epithelial, messenchymal and neuronal origin. It helps in normal cellular processes such as proliferation, differentiation and development [32]. EGFR and EGF like peptides are usually over expressed in various types of human carcinomas. Either amplification or mutations in EGFR tyrosine kinase domain have been reported. Most often EGFR are over expressed in lung, colon, breast, ovarian and gastric carcinomas [33]. Over

expression of EGFR has also been found in Non-Small Cell Lung Carcinomas (NSCLC), metastatic colorectal cancers (mCRC), head and neck squamous cell cancer (HNSCC) [34] [35].

2.3.1 EGFR and Colorectal Cancer (CRC): EGFR is not considered as a prognostic factor in CRC but it plays an important role in tumor cell proliferation. Consequently, it's over expression in stage II disease (a situation in which chemotherapy is not a standard) opens opportunity to test the efficacy of EGFR inhibitors in CRC. It has been shown that, EGFR mRNA in colorectal carcinoma cells plays a significant role during tumor progression. EGF and EGFR levels have been shown to be higher in malignant zones of colorectal cancer specimens than in the surrounding mucosa. Nevertheless, the presence of EGFR in CRC may also justify the application of specific EGFR targeted therapy. Some preclinical and clinical studies have already demonstrated the efficacy of EGFR inhibitors in advanced colorectal carcinomas and their potential synergistic effects with chemotherapy and radiation therapy [36].

2.3.2 EGFR and Pancreatic Cancer: Surgical therapy is presently the only therapeutic approach associated with long-term survival in pancreatic adenocarcinoma. Over expression of EGFR in pancreatic cancer correlates with more advanced disease, poor survival and the presence of metastases. Therefore, inhibition of the EGFR signaling pathway is an attractive therapeutic target. Although, several combinations of EGFR inhibitors with chemotherapy demonstrate inhibition of tumor-induced angiogenesis and tumor cell apoptosis but these benefits remain to be confirmed. Multimodality treatment incorporating EGFR inhibition is emerging as a novel strategy in the treatment of pancreatic cancer [37].

2.3.3 EGFR and Breast Cancer: EGFR over expression in breast cancer is associated with large tumor size, poor differentiation, and poor clinical outcomes. Though EGFR over expression is observed in all subtypes of breast cancer, EGFR is more frequently over expressed in triple-negative breast cancer (TNBC) and inflammatory breast cancer (IBC), which are especially aggressive. Approximately half of cases of triple-negative breast cancer (TNBC) and inflammatory breast cancer (IBC) over express EGFR. EGFR and its downstream pathway regulate epithelial-mesenchymal transition, tumor invasion and high EGFR expression is an independent predictor of poor prognosis in IBC. Thus EGFR targeted therapy might have a promising role in TNBC and IBC [38].

2.3.4 EGFR and Head and Neck Squamous Cell Carcinoma: Treatment for early stage Squamous-cell carcinoma of the head and neck (SCCHN) is usually done by surgery and radiation therapy (RT). Locally-advanced tumors are best treated with concurrent chemotherapy to RT. EGFR is expressed on the surface of healthy cells, and is commonly expressed at high levels in a variety of epithelial tumors, including SCCHN. The abnormal activation of the EGFR leads to enhanced proliferation and other tumour-promoting activities. The clearest benefit of EGFR-inhibitor treatment to date is noted when it is combined with RT for treatment of locally advanced head and neck cancer [39].

2.3.5 EGFR and NSCLC: In NSCLC, malignant cells develop in the lung tissue. There are different kinds of cancer cells and each type grows and spreads in different way. EGFR amplification and over expression is seen in upto 85% of patient and with NSCLC. Treatment options available for NSCLC are surgery, radiation, chemotherapy and biological therapy. Early stage of localized NSCLC can be treated by surgery, and upto 70% patients survive for 5 years if treated at this stage. Patient who cannot be operated are offered radiotherapy along with chemotherapy. Chemotherapy is used when NSCLC is diagnosed at advanced stage and has already spread to other parts of body [40]. Enzyme inhibitors such as EGFR TKIs, mTOR inhibitors, proteosome inhibitors, growth factor inhibitors, signal-transduction inhibitors, multikinase inhibitor etc. are useful in the treatment of NSCLC.

Currently, EGF inhibitors, EGFR inhibitors and EGFRTKIs are used as targeted therapy of NSCLC. All three approaches result in inhibition or reduction of downstream signaling. EGFR inhibitors or Anti-EGFR monoclonal antibodies (MAbs) function as competitive antagonist of binding and prevent intracellular phosphorylation inhibiting further signaling cascade. EGFR TK inhibition is an emerging strategy for NSCLC and many EGFR TKIs are being approved for clinical use for WT as well as mutant types.

Structural requirements for EGFR TK inhibitors

Various crystal structures of EGFR (2ITO, 2ITP, 2ITQ, 2ITT) reveal the necessity of groups required for being a EGFR inhibitor. Crystal structures of EGFR inhibitor complexes have revealed key features regarding the possible protein-ligand interactions, and information

related to the nature of binding sites has been of critical importance in the design and development of inhibitors as potential EGFR TK inhibitor drug candidates.

Structural elements of kinase region are N-lobe (grey, red and cyan), C-lobe (White), hinge region (residues 788-797-Violet), P loop (residues 712–731-Red), C helix (residues 752–767-green) and activation loop (855–877, in blue) as given in Figure 2.4.

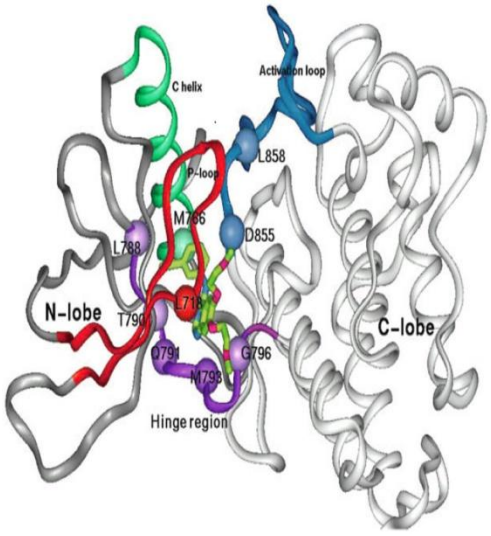

Figure 2.4: Structural domains of EGFR kinase region ([41]

Hydrogen bond donor or acceptor groups are necessary to form hydrogen bonding with MET793, CYS797, GLY796, ASP800, ASP855 etc. Interactions in hinge region involving MET793 and GLY796 are required for better EGFR inhibitory activity. From the same lobe, CYS797 interactions are necessary to result in covalent inhibition and more residence time in the receptor. In the C-helix, MET766 hydrogen bonding and from the activation loop, ASP855 hydrogen bonding imparts inhibitory activity. Hydrophobic interactions with hinge region amino acids PHE795, GLN791, THR790, LEU792, PRO794, LEU788 and LEU718, SER719, VAL726 from P-loop, MET766, GLU762 from C-helix and THR854 are required for the inhibitory activity.

2.4 EGFR TK mutations and resistance

Mutations occur in four exons: 18 to 21, which encode the tyrosine kinase domain of EGFR. Of all the TK activating mutations, in frame deletions in exon 19 mutations account for 44% and include amino-acid residues leucine-747 to glutamic acid-749. The predominant single-point mutation-exon 21, L858R point mutation accounts for 41%. Glycine-719 (G719) change to

serine, alanine or cysteine accounts for 10% of the activating mutations. Duplication and/or insertion in exon 20 accounts for the remaining 5% of EGFR TK activating mutations. Overall, deletions in exon 19 and the point mutation of L858R are termed ‘classical’ activating mutations [42]. These mutations result in enhanced EGFR kinase activity leading to enhanced downstream events. After activating mutations, second point mutation in the TK domain in Codon 790 (T790M) of Exon 20 of EGFR gene has been reported. The threonine-790 to methionine (T790M) point mutation is found in approximately 50% of all patients with acquired resistance to EGFR TKI therapy. Crystal structure reveals that T790, referred as "gatekeeper residue", gets interchanged with a bulkier methionine and enhances interaction of EGFR and ATP, that results in enhanced phosphorylation and cellular events.

MET gene amplification is also involved in resistance to EGFR TKIs. It encodes kinase domain and is involved in metastasis, invasion and angiogenesis of tumors. Cell line with EGFR exon 19 deletion shows MET mutations, when exposed to increased concentration of gefitinib. MET is involved in phosphorylation of ErbB3 and activates phosphatidylinositol 3-kinase (PI3K)/Akt which results in downstream signaling.

Following are the major mutations (Figure 2.5) reported in EGFR TK region:

1. 90% of the mutations are ‘classical’ mutations ie. single-point L858R mutation in exon 21 and deletion of exon 19.
2. Acquired resistance mechanism to EGFR TKIs was first reported in 2005. The main reason for this inhibition was T790M mutation in Codon 790 of Exon 20 of EGFR gene. T790, also referred as "gatekeeper residue", gets replaced by a bulkier methionine and it sterically hinders the binding of first generation EGFR TKIs [43] [44][45].
3. Other mutations reported are D761Y, L747S, and T854A. Most of exon 20 insertion mutations result in decreased EGFR TK inhibitor sensitivity ([46] [47] [48].

Resistance mechanisms to latest EGFR TKIs have been identified in preclinical in vitro models, but clinical evidence of resistance is lacking. Mutations at the EGFR C797 codon, of kinase-binding region, have been confirmed in vitro. There is a loss of the potential for covalent bond formation at position 797 because of missense mutation in C797S and results in reduced cellular potency of EGFR TKIs [49].

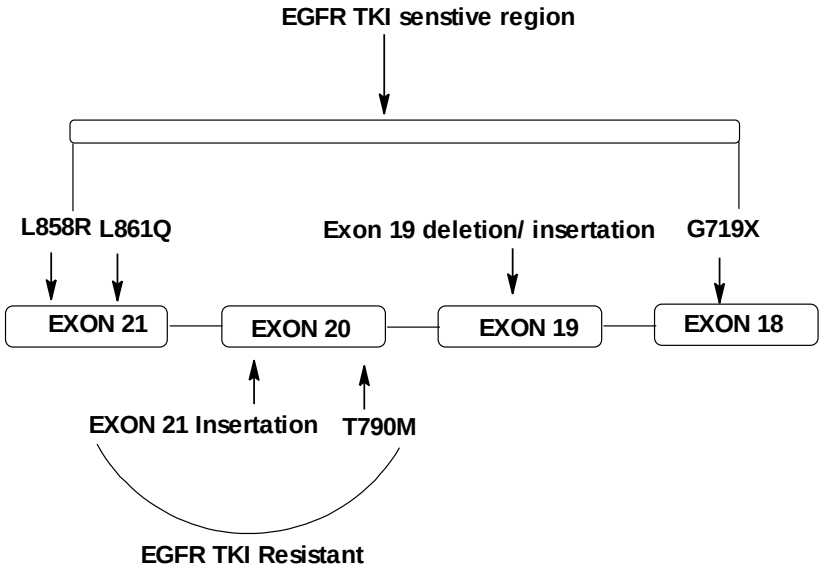

Figure 2.5: EGFR mutations and domains

2.5 EGFR targeted therapies

EGF inhibitors, EGFR inhibitors and EGFR TKIs are most promising strategies used as targeted therapy. All three approaches result in inhibition or reduction of downstream signaling, which ultimately results in inhibition of cellular events. EGFR antibodies bind to extracellular domains whereas TK inhibitors target the intra cellular TK domain. Recent studies have indicated the use of various chemo preventive agents in down regulating EGFR at gene level. Furthermore, several studies have substantiated and conferred significant benefits of anti-EGFR agents in several types of solid tumors including colorectal, head and neck cancer, NSCLC and pancreatic cancer in terms of overall survival, progression free survival and overall response rate.

2.5.1 Monoclonal antibodies

Cetuximab and Panitumumab are FDA approved monoclonal antibodies against EGFR for colorectal cancer. Panitumumab is the first FDA (2006) approved human monoclonal antibody used for the treatment of EGFR expressing metastatic colorectal cancer. Cetuximab is an FDA approved human–murine chimeric anti-EGFR monoclonal antibody. Cetuximab binds to the second (L2) domain of EGFR thereby blocking its downstream signaling by prompting receptor internalization and encumbering ligand-receptor interaction [50]. Bevacizumab (Avastin, Genentech/Roche, CA, USA), is a monoclonal antibody targeted to vascular endothelial growth

factor (VEGF). Bevacizumab is thus defined as an anti-angiogenic drug due to its ability to prevent VEGF from interacting with appropriate receptors in vascular endothelial cells.

2.5.2 EGFR TK inhibitors

Concept of kinase inhibition started in 1960s when details of kinase characterization and signaling came in focus. Some of the important kinase inhibitors along with the year of FDA approval are given in Figure 2.6. Till 2016, more than 28 Small-molecular kinase inhibitors (SMKIs), are approved by the US Food and Drug administration (FDA). More than 30% of approved SMKIs have a molecule weight (MW) exceeding 500 and all have a total ring count between three and five [51]. These compounds have half life of 36-48 h, whereas MAbs have 3-7 days [52].

Many EGFR TKIs are being approved for clinical use. The development can be categorized into first generation, second generation and third generation. EGFR TKIs used in NSCLC are given in Figure 2.7.

First generation EGFR TKIs: Anilino quinazolines have been reported to competitively inhibit EGFR TK. Two compounds of this class Gefitinib and Erlotinib have been approved for NSCLC with oncogenic mutations. Gefitinib was approved by USFDA in May 2003 as monotherapy for patients with metastatic non-small cell lung cancer (NSCLC). Gefitinib was found to cause significant shrinkage in tumors in approximately 10% of patients with NSCLC. Erlotinib was approved by USFDA in 2004 for treatment of locally advanced or metastatic NSCLC. It was also approved by FDA in 2004/2005 in combination with gemcitabine for treatment of locally advanced, metastatic pancreatic cancer [53]. Epithelium based toxicities as a result of inhibition of EGFR are drawback of erlotinib and gefitinib. Resistance to these drugs has emerged due to mutations in kinase domain (particularly T790M). First generation EGFR TKIs are reversible and clinical efficacy remains limited because of dose dependent toxicity due to inhibition of WT EGFR.

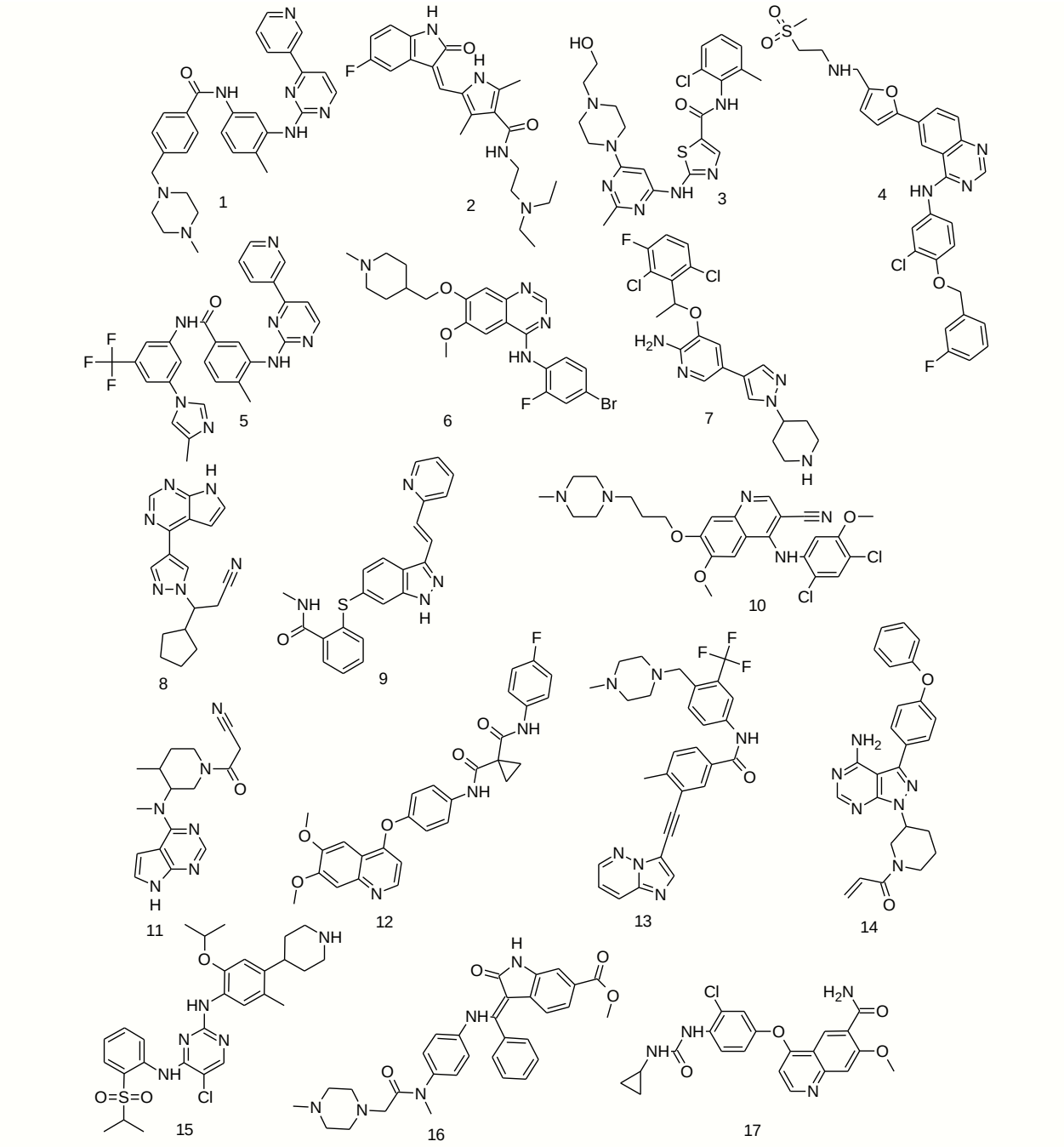

Figure 2.6: Drugs approved as kinase inhibitors during 2001-2015.

1. Imatinib (2001) 2. Suntinib (2006) 3. Dasatinib (2006) 4. Lapatinib (2007) 5. Nilotinib (2007) 6. Vandetanib (2011) 7. Crizotinib (2011) 8. Ruxolitinib (2011) 9. Axitinib (2012) 10. Bosutinib (2012) 11. Tofacitinib (2012) 12. Cabozatinib (2012) 13. Ponatinib (2012) 14. Ibrutinib (2013) 15. Ceritinib (2014) 16. Nintedanib (2014) 17. Lenvatinib (2015).

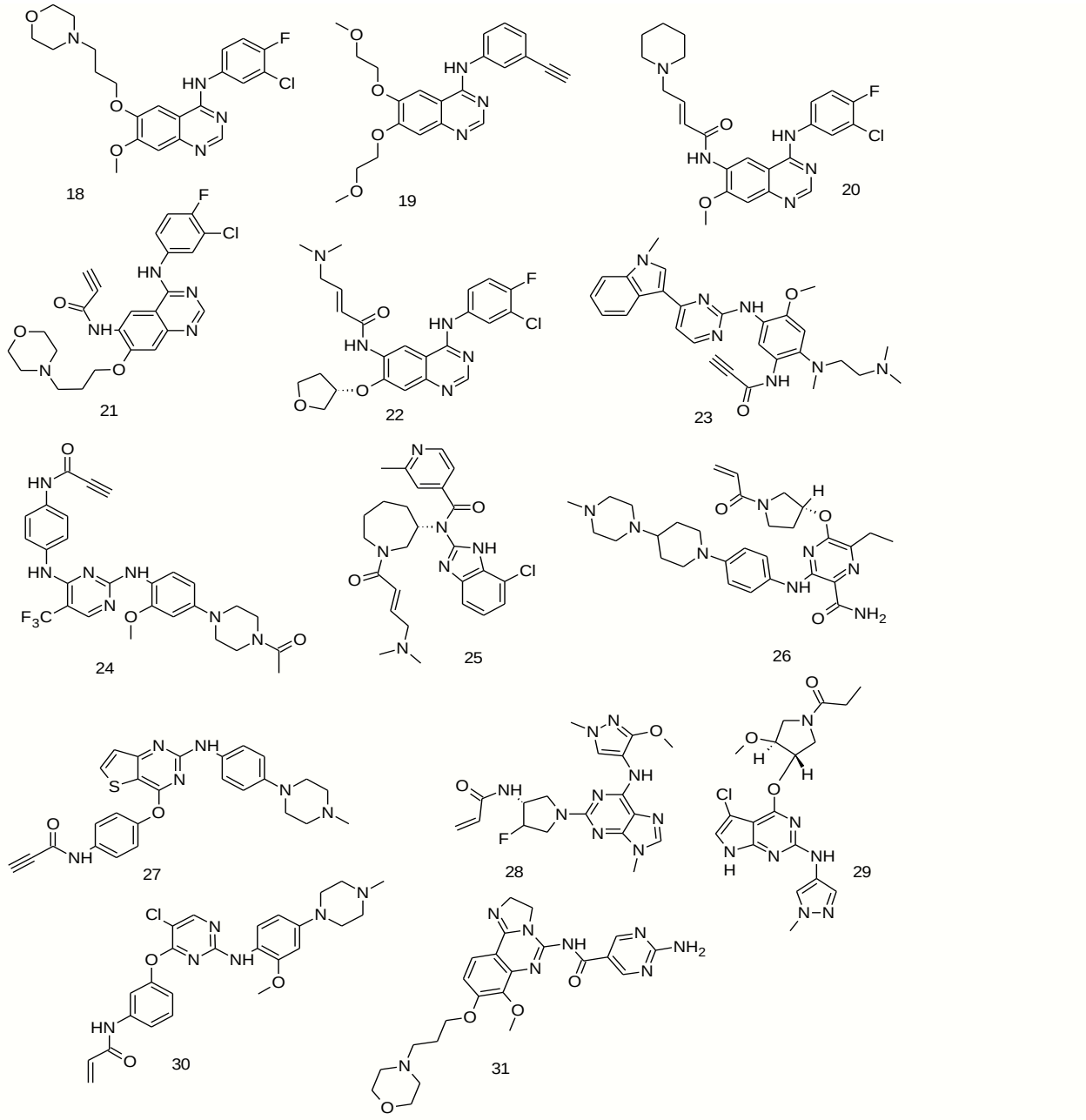

Figure 2.7: EGFRT KIs: All generations used in NSCLC

18. Gefitinib 19. Erlotinib 20. Dacomitinib 21. Caneritinib, 22. Afatinib 23. Osimertinib 24. Rociletinib 25. EGF816 26. ASP8273 27. HM-61713, BI-1482694/ Olmutinib 28. PF-06747775 29. PF-06459988 30. WZ4002 31. Copanlisib

Second generation EGFR TKIs: These again belong to the anilinoquinazoline class and examples are dacomitinib, canritinib and afatinib. These ligands attenuate EGFR-T790M activity but WT EGFR inhibition results in severe epithelium based toxicities (e.g., skin rash and diarrhea). Despite promising *in vitro* inhibitory potency against the gefitinib resistant EGFR L858R/T790M mutants, these inhibitors show insufficient efficacy at clinically achievable concentrations. These drugs covalently modify EGFR CYS797 and demonstrate increased cellular potency against T790M mutants. However, because of aniline moiety in the structure, clashes with MET790 side chain, their T790M activity is less against the primary activating EGFR mutants. As a result, their clinical efficacy against T790M patients is limited due to dose-limiting toxicity associated with inhibition of WT EGFR [54] [55].

Third generation EGFR TKIs: These are ceritinib, crizotinib, nintedanib, osimertinib, rociletinib, EGF 816, ASP8273, HM-61713/BI-1482694/olmutinib, PF-06747775, PF-06459988 and WZ4002. These compounds have good potency against T790M mutants and are free of epithelium toxicities resulting from non selectivity for WT EGFR. Major interactions which contribute to the potency against T790M mutant are: covalent bond formation with CYS797, hydrogen bonding with MET793 and with gatekeeper MET790. A potent irreversible inhibitor, PF-06459988, acts on double mutant L858R/T790M [56].

Some of the third generation EGFRTKIs and their interactions with enzyme are discussed below.

In 2011, the FDA granted accelerated approval to crizotinib for the treatment of patients with locally advanced or metastatic non-small cell lung cancer. In 2014, the USFDA granted accelerated approval to ceritinib (ZYKADIA, Novartis Pharmaceuticals Corporation) for the treatment of patients with metastatic non-small cell lung cancer. In the same year, US FDA approved nintedanib for the treatment of idiopathic pulmonary fibrosis (IPF), a debilitating and fatal lung disease, which has a median survival of 2-3 years after diagnosis.

AZD9291 (Osimertinib/Mereletinib/Tagrisso)

Osimertinib (Tagrisso) is developed by AstraZeneca and has been approved by USFDA in 2015 for metastatic EGFR T790M mutation-positive NSCLC. It is a potent, orally active, structurally different irreversible EGFR TKI which is mutant selective and has very less inhibition of WT EGFR. The activity profile against mutated EGFR is: Exon 19 deletion-IC_{50} 12.92 nM, double

mutant IC_{50} 11.44 nM and WT IC_{50} 493.8 nM [57] [58]. It is a mono anilino pyrimidine compound and its interactions include pyrimidine forming hydrogen bond with MET793, indole group with gatekeeper residue and covalent bonding of CYS797 with acrylamide. It is a safe drug having few adverse effects such as rash, diarrhea, nausea, and poor appetite [59].

Rociletinib (CO-1686)

Orally active small molecule irreversible inhibitor selectively targets EGFR mutant forms (exon 19 deletion, L858R and T790M) and spares wild type. It is approved by USFDA in May 2014 for NSCLC where progression of disease was observed even after EGFR TKIs therapy. The common toxicity was hyperglycemia [60].

EGF816

EGF816 is a third generation potent EGFR TKI which spares the WT EGFR and has potent inhibitory activity against L858R, del19 resistant mutations. In mouse xenograft model EGF816 was found to be better as compared to earlier ligands and is better option against T790M mutations. Diarrhoea, stomatitis, rash and pruritis were the most common AEs reported [61] [62].

ASP8273

ASP8273 is a small molecule, irreversible EGFR TKI which inhibits the kinase activity of EGFR including T790M mutation and shows limited activity against EGFR wild-type (WT) in NSCLC. In the *in vitro* assays (enzymatic and cell-based), ASP8273 has been evaluated against EGFR mutants (L858R, exon 19 deletion, L858R/T790M and del19/T790M) and found to retard progression of cell growth. In animal models, ASP8273 induces complete regression in 15 days therapy. It efficiently suppresses ERK/Akt pathway and is also active against cell lines which show resistance to AZD9291and rocelitinib [63].

HM-61713/ BI-1482694/ Olmutinib

It is a novel third-generation, orally active, irreversible EGFR mutant-specific EGFRTKI. It has been approved in South Korea for the treatment of patients with locally advanced or metastatic EGFR T790M mutation. In cell lines, olmutinib has shown potent EGFR inhibition against L858R and T790M mutants and spares WT EGFR. Olmutinib demonstrated promising clinical activity and favorable safety profile in clinical trials [64] [65].

WZ4002

WZ4002 is a new anilinopyrimidine mutant-selective EGFR TK inhibitor. It irreversibly suppresses the ATP-dependent auto-phosphorylation of WT EGFR and EGFR mutants, which includes EGFR L858R/T790M, EGFR T790M and EGFR L858R. At lower concentrations, it is more effective against EGFR mutants rather than wild-type EGFR resulting in less toxicity in normal tissues. WT EGFR, however is susceptible to higher concentrations of WZ4002 as well as prolonged administration of WZ4002, especially in tissues where it accumulates.[66] [67]

PF-06459988

Pyrrolopyrimidine derivative PF-06459988 is another inhibitor with additional CYS797 covalent interaction. It is a potent third generation irreversible inhibitor of EGFR L858R/T790M double mutant, which offers high potency and specificity to the T790M-containing double mutant EGFRs and has reduced proteome reactivity relative to earlier irreversible EGFR inhibitors. IC_{50} value of PF-06459988 for double mutant L858R/T790M was found to be 13nM [68].

PF-06747775

It is a small molecule pyrrolidine derivative inhibitor with high potency against EGFR (del 19 or L858R/T790M) mutants. Compared to some other EGFR inhibitors, PF-06747775 offers several advantages in treatment of tumors with T790M-mediated drug resistance: it binds significantly and inhibits T790M mutation, prevents cell death and thus results in antineoplastic activity. It also spares wild type EGFR and produces less toxicity as it has greatly reduced proteome reactivity relative to earlier EGFRTKIs [69].

To overcome all types of mutations, an ideal inhibitor should have sustained target engagement in the presence of even high intracellular concentrations of the competitive ligand (ATP). It should be selective to T790M mutants and should spare wild type EGFR. CYS797 interactions are critical in irreversible binding of inhibitor, whereas MET793 also helps through hydrogen bonding. All new inhibitors have shown exciting clinical results in NSCLC for T790M mutants but acquired resistance to these inhibitors have also come in picture [70]. In osimertinib treated patients, resistance mechanism includes C797S mutation with T790M mutations. Rocelitinib resistance developed because of loss of T790M mutation, EGFR over expression and transformation to small cell lung cancer. It is believed that, C797S mutation impairs the

covalent binding of thiol side chain in cysteine. Larger patient population and deeper analysis is required to confirm the mechanism of resistance to third generation inhibitors [71]. Number of EGFRT KIs are in clinical trials and their status is given below in table 2.1.

Table 2.1: Clinical trial status of various EGFR agents

Condition	Phases/ NCT NO	Sponsor/ Collaborator	Drug candidate(s)	Status
To see the effects of erlotinib hydrochloride and how well it works in reducing duodenal polyp burden in patients with familial adenomatous polyposis at risk of developing colon cancer.	NCT02961374	National Cancer Institute (NCI)	Erlotinib Hydrochloride	November 10, 2016
To access Intracranial response of AZD9291 With a dose of 80 mg once daily by brain MRI scan and PET-CT scan.	II/ NCT02736513	Rabin Medical Center	AZD9291	First received: March 2016 Last updated: April 2016
To see the effect of combination therapy in colorectal cancer, triple negative breast cancer and NSCLC.	NCT02900664	Novartis Pharmaceuticals	PDR001 ACZ885 CJM112 TMT212 EGF816	First received: May 2016 Last updated September 2016
To investigate the absorption, metabolism and excretion of [14C] labeled ASP8273 in patients with solid tumors harboring EGFR mutations	I/ NCT02674555	Astellas Pharma Global Development, Inc.	Radio-labeled ASP8273	First received: January 2016 Last updated: June 2016

To conduct a clinical trial of rociletinib in patients with EGFR-mutant NSCLC with activating EGFR mutations (including exon 19 deletion or L858R mutation), or without EGFR T790M mutation.	II/ NCT02705339	Washington University School of Medicine/ Clovis Oncology, Inc.	Rociletinib	First received: January 2016 Last updated: May 2016
For management pancreatic neuroendocrine tumors (pNET) are the control of symptoms and tumor growth.	NCT02713763	Grupo Espanol de Tumores Neuroendocrinos	Sunitinib	First received: March 2016 Last updated: March 2016
To compare the blood levels of oral and IV dose of AZD 9291.	I/ NCT02491944	AstraZeneca	AZD9291	First received: July 2015 Last updated: September 2015
To see the effect of rociletinib in patients with advanced or metastatic EGFR-mutant NSCLC who have been treated previously with EGFR directed therapy and have evidence of a T790M mutation.	NA/ NCT02547675	Clovis Oncology, Inc.	Rocelitinib	First received: September 2015 Last updated: March 2016
To evaluate the progression free survival (PFS), of ASP8273 compared to erlotinib or gefitinib in NSCLC.	III/ NCT02588261	Astellas Pharma Global Development, Inc.	ASP8273, Erlotinib, Gefitinib	First received: October 2015 Last updated: March 2016

To observe the safety of the combination of rociletinib and MPDL3280A in EGFR-mutant NSCLC patients.	I/ NCT02630186	Clovis Oncology, Inc./ Genentech Inc.	Rociletinib, MPDL3280A	First received: December 2015 Last updated: May 2016
To evaluate the safety and anti-tumor effect of rociletinib when administered in combination with trametinib in NSCLC.	I,II/ NCT02580708	Clovis Oncology, Inc./ Novartis	Rociletinib, Trametinib	First received: October 2015 Last updated: January 2016
To determine safety, antitumor activity and the pharmaco kinetics of ASP8273 EGFR-TKI-native Patients with NSCLC harboring mutations.	II/ NCT02500927	Astellas Pharma Inc.	ASP8273 capsules	First received: June 2015 Last updated: December 2015
To evaluate the efficacy, safety and pharmacokinetic parameters of HM61713 (BI 1482694) in patients with T790M-positive NSCLC.	II/ NCT02485652	Hanmi Pharmaceutical Company Limited/ Boehringer Ingelheim	HM61713/ BI1482694	First received: June 2015 Last updated: February 2016
To evaluate the efficacy and safety of HM61713 (BI 1482694) in NSCLC.	II/ NCT02444819	Hanmi Pharmaceutical Company Limited/ Boehringer Ingelheim	HM61713/ BI1482694	First received: May 2015 Last updated: June 2016

To evaluate pharmacokinetic, and pharmacodynamic dose escalation study of PF 06747775 as a single agent in patients with advanced EGFR mutated NSCLC patents (del19 or L858R, +/ -T790M).	I,II/ PF-06747775	Pfizer		First received: January 2015 Last updated: June 2016
To evaluate the safety and anti-tumor effect of rociletinib in Non-small Cell Lung Cancer	II/ NCT02147990	Clovis Oncology, Inc.	Rociletinib	First received: May 2014 Last updated: October 2015
To compare the safety and anti-tumor effect of rociletinib with erlotinib in patients whose tumors have specific EGFR mutations.	I,II/ Clovis Oncology, Inc.	Clovis Oncology, Inc.	Rociletinib, Erlotinib	First received: June 2014 Last updated: February 2016
To assess the safety and tolerability of ASP8273 and to determine the maximum tolerated dose (MTD) in patient of NSCLC	I/ NCT02113813	Astellas Pharma Global Development, Inc.	ASP8273, Midazolam	First received: April 2014 Last updated: February 2016
To compare the anti-tumor efficacy of oral single-agent rociletinib with that of single-agent cytotoxic chemotherapy in advanced/metastatic NSCLC.	III/ NCT02322281	Clovis Oncology, Inc.	Rociletinib Pemetrexed or gemcitabine or paclitaxel or docetaxel	First received: December 14 Last updated: June 2016

The study determines the maximum tolerated dose (MTD) or recommended phase 2 dose of EGF816 in combination with INC280 in patients with NSCLC with EGFR mutation.	I, II/ NCT02335944	Novartis Pharmaceuticals	INC280, EGF816	First received: October 2014 Last updated: December 2015
To determine the safety and tolerability, pharmacokinetics (PK) of and antitumor activity of ASP8273.	I,II/ NCT02192697	Astellas Pharma Inc.	ASP8273	First received: May 2014 Last updated: August2015
To estimate the maximum tolerated dose (MTD) of EGF816 and to investigate the anti cancer activity of EGF816.	I,II/ NCT02108964	Novartis Pharmaceuticals	EGF816	First received: April 2014 Last updated: June 2016
To determine the safety, tolerability and preliminary anti-tumour activity of AZD9291 with one of either MEDI4736, AZD6094 or selumetinib in patients with EGFR mutation positive advanced lung cancer.	I/ NCT02143466	AstraZeneca	MEDI4736, AZD6094, selumetinib	First received: May 2014 Last updated: June 2016
To determine the efficacy and safety of Nivolumab with EGF816 and Nivolumab in combination with INC280 in previously treated NSCLC patients.	II/ NCT02323126	Novartis Pharmaceuticals	EGF816, INC280, Nivolumab	First received: December 2014 Last updated: June 2016

To investigate the anti-tumor activity of single agent BYL719, INC280, LDK378 and MEK162 in Adenocarcinoma Lung Cancer; Squamous Cell Lung Carcinoma.	II /NCT02276027	Novartis Pharmaceuticals	BYL719,INC280, LDK378, MEK162	First received: October 2014 Last updated: May 2016
To evaluate effect of rifampicin on the pharmacokinetic (PK) parameters of AZD9291 in patient with NSCLC.	I/ NCT02197247	AstraZeneca	AZD9291	First received: July 2014 Last updated: April 2016
Effect of food on the pharmacokinetics (PK) of AZD9291 in NSCLC.	I/ NCT02163733	AstraZeneca	AZD9291	First received: May 2014 Last updated: March 2016
To evaluate the interaction of AZD9291 with omeprazole in approximately 50 healthy, adult male volunteers.	I/ NCT02224053	AstraZeneca	AZD9291, Omeperazole	First received: August 2014 Last updated: March 2016
To assess the efficacy and safety of AZD9291 versus a standard in Locally Advanced or Metastatic EGFR Sensitizing Mutation Positive NSCLC.	III/ NCT02296125	AstraZeneca / Parexel	AZD9291,Gefitinib, Erlotinib	First received: October 2014 Last updated: June 2016
To determine the relative bioavailability of oral formulations of AZD9291 and the effect of food in healthy volunteers.	I/ NCT01951599	AstraZeneca	AZD9291	First received: September, 2013 Last updated: April 2016

To study safety and effectiveness of an investigational drug in NSCLC.	II/ NCT01854034	Massachusetts General Hospital	Hsp-90-7/AUY922	First received: May 2013 Last updated: August 2015
To study the side effects and the best dose of Hsp90 inhibitor AUY922 with pemetrexed disodium in treating patients with previously treated stage IV NSCLC.	I/ NCT01784640	Jonsson Comprehensive Cancer Center/ NCI	Hsp-90-7/AUY922 and Premetrexed disodium	First received: February 2013 Last updated: March 2016
To estimate the maximum tolerated dose of the combination of LDK378 and AUY922 in Anaplastic Lymphoma Kinase (ALK) NSCLC.	NCT01772797	Novartis Pharmaceuticals	LDK378 and Hsp-90-7/AUY922	First received: January 2013 Last updated: June 2016
To evaluate the pharmacokinetics of HM61713 tablet in Healthy Volunteers.	I/ NCT01894399	Hanmi Pharmaceutical Company Limited	HM61713/BI1482694	First received: July 2013 Last updated: February 2016
To evaluate the safety and tolerability of HM61713 (BI 1482694).	I,II/ NCT01588145	Hanmi Pharmaceutical Company Limited/ Boehringer Ingelheim	HM61713/ BI1482694	First received: April 2012 Last updated: June 2016

To evaluate the pharmacokinetic (PK) and safety profile of oral rociletinib in Locally Advanced or Metastatic NSCLC.	I,II/ NCT01526928	Clovis Oncology, Inc.	Rocelitinib	First received: January 2012 Last updated: July 2015
To evaluate the safety and efficacy of retaspimycin HCl (IPI-504) and everolimus in patients with KRAS mutant NSCLC.	I,II /NCT01427946	Infinity Pharmaceuticals, Inc.	Hsp-90-4/ IPI-504/Retaspimycin and Everolimus	First received: August 2011 Last updated: November 2014
To compare the impact of IPI-504 in combination with docetaxel in NSCLC.	II /NCT01362400	Infinity Pharmaceuticals, Inc.	Hsp-90-4/ IPI-504/Retaspimycin with Docetaxel	First received: May 2011 Last updated: May 2014
To study the side effects and best dose of Hsp90 inhibitor AUY922 with erlotinib hydrochloride In Adenocarcinoma of the Lung.	I,II/NCT01259089	Northwestern Univrsity/ Robert H. Lurie Cancer Center	Hsp-90-7/AUY922 and Erlotinib Hcl	First received: December 2010 Last updated: June 2015
To check if STA-9090 has a therapeutic effect on small cell lung cancer.	II/ NCT01173523	Dana-Farber Cancer Institute.	Hsp-90-6/STA-9090	First received: July 2010 Last updated: February 2013
This is a phase 2 clinical study of the HSP90 inhibitor, STA-9090 (ganetespib) in papients with stage IIIB or IV NSCLC.	II/ NCT01031225	Synta Pharmaceuticals Corp.	Hsp-90-6/STA-9090	First received: November 2009 Last updated: September 2014

For investigating the effectiveness and safety of the combination of the study drugs cetuximab and erlotinib n patients with advanced (metastatic) refractory CRC.	NCT00784667	Austin Health/ Ballarat Health Services	Cetuximab and Erlotinib	First received: November 2008 Last updated :November 2010
To study the effectiveness of gefitinib in treating patients who have locally advanced or metastatic CRC.	NCT00030524	University of Texas Health Sci. Center, San Antonio /NCI	Gefitinib	First received: Februrry 2002 Last updated: October 2012

Recent reported inhibitors in literature:

Hu et al. have reported new salicylanilide derivatives as potent EGFR TKIs. IC_{50} value of compound 32 was found to be 10.4 ± 2.25 µM and was comparable to that of gefitinib [72]. Zhang et al. have reported 4-anilinoquinazoline-acylamino derivatives as EGFR and VEGFR-2 dual TK inhibitors with good IC_{50} values. Three compounds viz: 33-35 exhibited the inhibitory activity against EGFR (with IC_{50} values of 0.13µM, 0.15µM and 0.02µM, respectively) and VEGFR-2 (with IC_{50} values of 0.56µM, 1.81µM and 1.71µM respectively). Compound 33 interacts in the EGFR ATP binding site with GLU804 and forms hydrogen bond with CYS797 with the nitrogen of the quinazoline ring. Hydrophobic interactions have been observed with amino acids VAL726, LEU718 and LEU844 [73][74].

Bugge et al have reported thienopyrimidine based EGFR inhibitors having IC_{50} values less than 1 nM. Most potent compound (compound 36) was found to have IC_{50} of 0.3nM towards EGFR and its mutants L858R and L861Q. The results were discussed in light of in silico studies, wherein it showed interaction of CHO group with LEU718, methoxy with GLY796, pyrimidine nitrogen with MET793, THR854 and ASP855 with CH_2OH group [75].

Ismail et. al. have reported 4-aminoquinazoline derivatives targeting EGFR tyrosine kinase. In cell line study, compound 37 was found to inhibit autophosphorylation of the receptor in A431 cells at a concentration of 0.1 µM [76].

Yang et. al. have reported thiourea modified 3-chloro-4-fluoroanilino-quinazoline compounds having thiourea directly attached to quinazoline ring or with a ethyl amino chain. Compound 37 was found to have IC_{50} values of 4.2 µM and 1.7 µM for NCI-H460 (WT) and NCI-H1975 (a lung cancer cell line having L858R/T790M mutation in EGFR) respectively [77]. All the reported newer inhibitors are given in Figure 2.8.

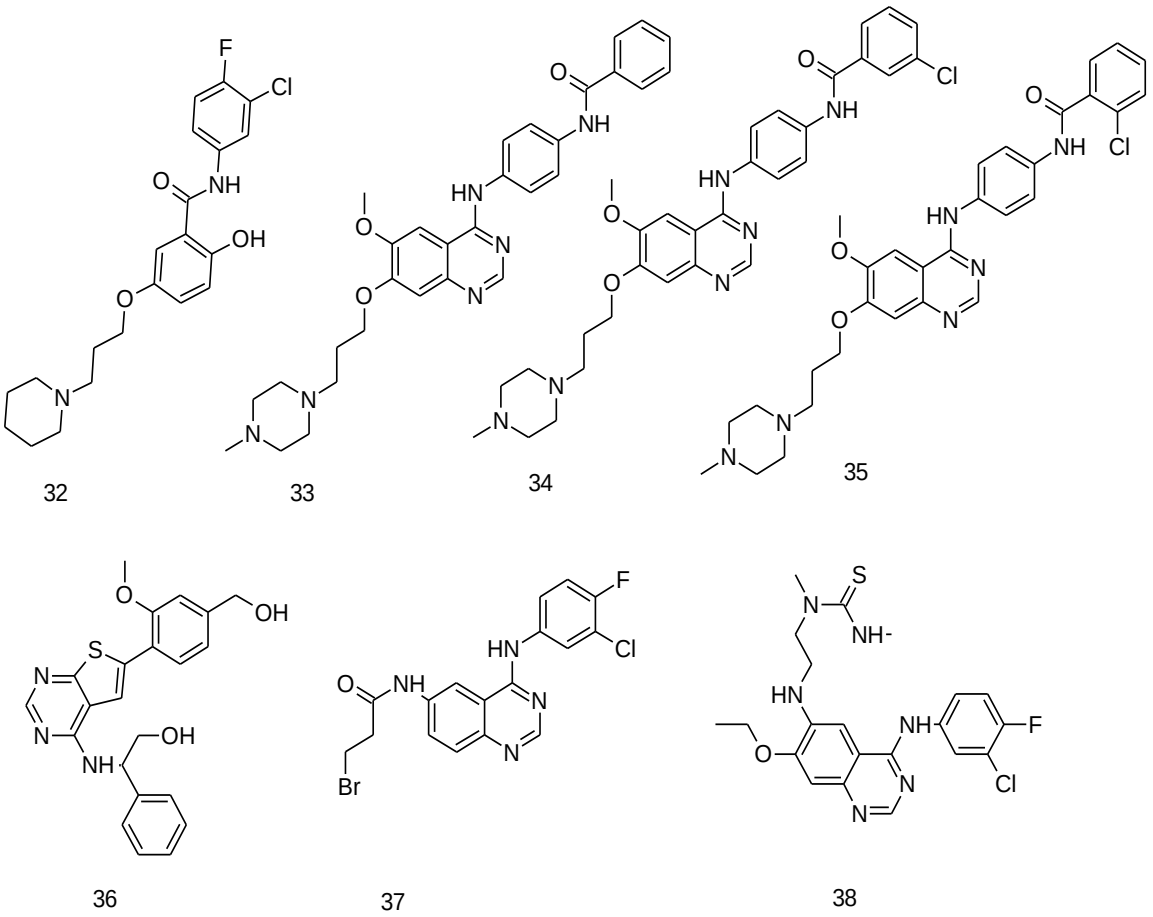

Figure 2.8: New EGFRT KIs

32. Salicylamide derivative 33-35. Anilino quinazoline derivatives 36. Thienopyrimidine derivative 37. Aminoquinazoline derivative 38. Thiourea quinazoline derivative

2.6 Rationale for EGFR as target

The epidermal growth factor receptor-tyrosine kinase (EGFR TK) is an important selective target for various carcinomas, as it is activated in many tumor cells whereas strictly controlled in normal cells. In normal tissues, the availability of EGFR ligands is tightly regulated to control the kinetics of cell proliferation. However, in cancer, EGFR is often perpetually over expressed because of the sustained production of EGFR ligands in the tumor microenvironment. It could result from mutation in EGFR itself. The EGFR and EGF-like peptides are often over-expressed in human cancers such as breast, colon, pancreas, lung, kidney etc. Amplification of the EGFR gene and mutations of the EGFR tyrosine kinase domain have been recently demonstrated to occur in NSCLC.

The EGFR TK initiates diverse signal transduction pathways in tumor cells that have a profound effect on their biology. Activation of the EGFR TK provides signals that drive dysregulated proliferation, invasion and metastasis, angiogenesis, and enhanced cell survival. A direct correlation also exists between growth factors and cellular proto-oncogenes. In fact, several proto-oncogenes code for proteins that are either growth factors, or growth factor receptors, or proteins that are involved in the intracellular signal transduction pathway for growth factors. The expression levels of TGFα, EGF and EGFR have been shown to correlate with progressive tumor growth, development of metastasis, and resistance to current chemotheraputic agents. Measurements of EGFR expressed in human colon cancer cells *in vitro* indicate that metastatic cells may express as much as five-times more EGFR in comparison to non metastatic cells.

2.7 Conclusion

First generation EGFR TKIs gefitinib and erlotinib was approved by FDA in 2002 and 2004. A number of EGFR TKIs have been discovered and evaluated for the treatment of carcinoma and are in clinical trials. Overall, to develop more active, selective, low-molecular-weight EGFR TKIs, different chemical skeletons might give a new hope.