EGFR tyrosine kinase inhibitors and multidrug resistance: perspectives

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1. ABSTRACT

Aim of present review is to provide an evidence-based update of mechanisms responsible for the onset of resistance to drug therapy by EGFR inhibitors, particularly with regards to TKIs. Among ABC transporters involved in MDR, P-glycoprotein and BCRP have been considered the pumps responsible for TKIs treatment failure. Moreover, two subtypes of EGFR mutations have been described: mutations of the exons coding for tyrosine kinase domain (18 to 21) and truncating mutations (exons 2 to 7) that involve downstream effectors such as MAPK, PI3K/Akt, STAT. The first group of mutations can be considered as a hallmark of NSCLC and are responsible for the failure of TKIs while the second group of mutations leads to resistance. The strategies to overcome MDR and to bypass the kinase domain mutations have been addressed. Finally, for some first generation TKIs some perspectives as radiotracers for PET/SPECT diagnosis in tumor displaying P-gp and BCRP overexpression have been suggested.

2. INTRODUCTION

The Epidermal Growth Factor Receptor (EGFR or ErbB-1) is a transmembrane glycoprotein that displays specific tyrosine kinase activity and regulates proliferation and differentiation of epidermal cells (1, 2). It belongs to the ErbB family of Receptor Tyrosine Kinases (RTK) that includes 4 members: ErbB-1/EGFR, ErbB-2/HER-2/neu, ErbB-3/HER-3, ErbB-4/HER-4 (3, 4). EGFR signaling is activated by the binding of growth factors, such as epidermal growth factor (EGF), amphiregulin, TGF-alpha which induce the omodimerization of the receptor or its heterodimerization with other receptors belonging to the ErbB family. EGFR activation can be induced by ligand-interaction or by itself leading to the autophosphorylation or transphosphorylation of the cytoplasmic kinase domains resulting in the recruitment of adapter proteins responsible for several pathways. All the induced pathways result in pro-growth and survival signal transduction and include PI3K (phosphatidylinositol 3-kinase)-AKT, RAS-RAF-
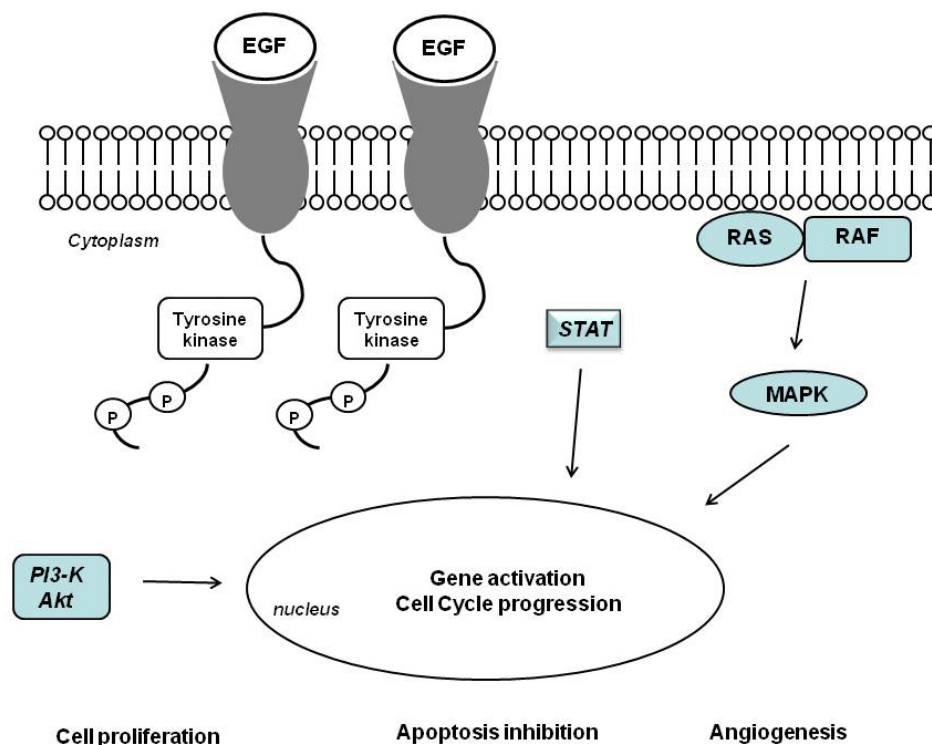
Figure 1. EGFR activation pathway.

MAPK (mitogen-activated protein kinase) and JAK-STAT (signal transducer and activator of transcription) (5, 6) as depicted in Figure 1.

EGFR overexpression has been detected in several human epithelial malignancies and induces the modification of a normal cell in a malignant form by supporting angiogenesis, cell proliferation, survival promotion, apoptosis inhibition and metastasis (7). Therefore, since EGFR signaling is involved in cancer development and progression, EGFR inhibition is an attractive strategy for tumor treatment.

At the present in clinical practice, two strategies have been developed to inhibit EGFR receptor: Monoclonal Antibodies (MAbs) (Cetuximab, Panitumumab, Gentuzumab, etc.) (8, 9) and small molecules, known as Tyrosine Kinase Inhibitors (TKIs), such as Gefitinib (10-12), Erlotinib (13-15), AG1478 and Lapatinib (8). As depicted in Figure 2, MAbs and TKIs differ for their interaction with the target; indeed, MAbs bind EGFR receptor at its extracellular domain by a mechanism independent of the receptor phosphorylation status and exerting EGFR competitive antagonist activity (8).

Conversely, TKIs interact with the intracellular tyrosine kinase domain of EGFR and inhibit its phosphorylation. The difference between the two strategies is due to the specificity towards EGFR; indeed, TKIs, exerting a competitive activity towards ATP binding site of the tyrosine kinases, can interact with other HERB-B family such as HER-2 (16).

The aim of present review is to deepen the pharmacodynamic and pharmacokinetic properties of TKIs focusing, for the mostly studied ligands, all the aspects linked to MDR.

TKIs mostly employed for the treatment of locally advanced or metastatic non-small-cell lung cancer (NSCLC) are Gefitinib, Erlotinib, and Lapatinib (Figure 3) characterized by a common pharmacophoric moiety 4-anilino quinazoline.

Gefitinib and Erlotinib, approved by the FDA for the treatment of locally advanced or metastatic non-small-cell lung cancer (NSCLC), are actually under evaluation in clinical trials for other tumors (17, 18) and several TKIs are considered as a pharmaceutical class, orally available and well-tolerated, for the treatment of several neoplasms, including lung, breast, kidney and pancreatic cancer as well as gastro-intestinal stromal tumors and CML.

Lapatinib, a reversible inhibitor of both EGFR and HER-2 tyrosine kinases, has been recently approved by the FDA for its use in combination with Capecitabine in the treatment of advanced breast cancer overexpressing HER2 (HER2+). Lapatinib monotherapy was well tolerated, even if the response was low in patients with advanced breast cancer. The combination of Lapatinib with Capecitabine significantly improved the response of the antineoplastic drug. This evidence suggests that the clinical effectiveness of Lapatinib in breast cancer is limited to HER2+ disease (19).
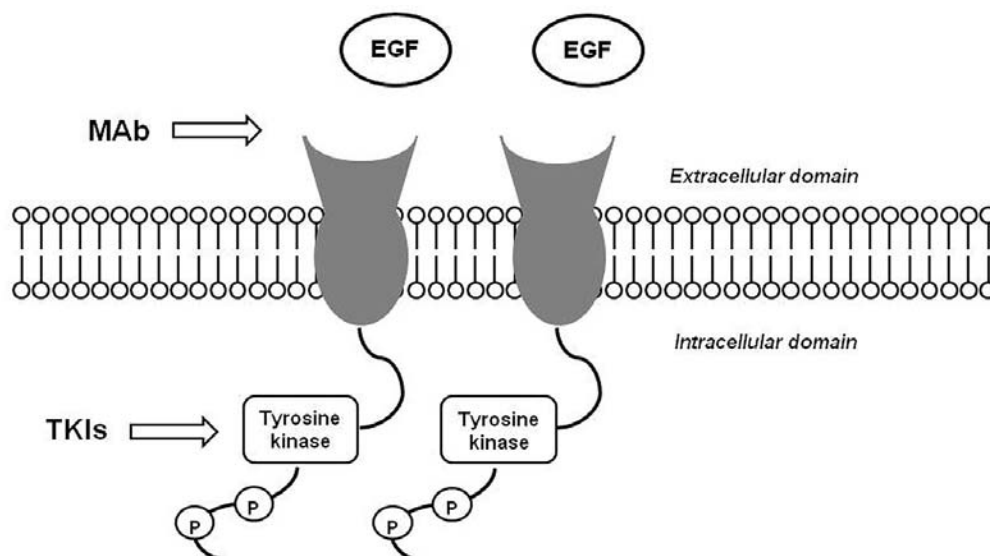
Figure 2. Sites of interaction of Tyrosine Kinase Inhibitors (TKIs) and Monoclonal Antibodies (MAb).

Several patients treated with TKIs displayed an initial response to TKIs therapy with a subsequent resistance to these agents, putting in evidence the urgent need to develop new and more potent EGFR inhibitors able to overcome MultiDrug Resistance (MDR).

Several literature reports evidenced that one of the mechanisms involved in the establishment of tumor resistance to TKIs is the overexpression and/or the activation of some ABC transporters. The overexpression of P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP) and MultiDrug-associated Proteins (MRPs) (20) is reported as the most important factor that leads to Multi Drug resistance (MDR) onset in cancer treatment. Another hypothesis involves the presence or the insurgence of a mutation in specific codons of the receptor (20).

Several strategies have been performed to overcome the limitations of the TKIs treatment such as: i) to develop TKIs second generation; ii) to co-administrate a MDR inhibitor with a TKI; iii) to plan innovative ligands acting at various steps of EGFR pathway.

2. MULTI DRUG RESISTANCE (MDR) TOWARDS TKIS

2.1. Overexpression of some ABC transporters

MDR is the development of resistance to a variety of anticancer drugs that are structurally and mechanistically unrelated, representing a major obstacle to successful chemotherapy treatment (21). A significant effort to elucidate the mechanism of MDR has been focused on the study of ATP-Binding Cassette (ABC) transporters, and their abilities to extrude drugs from the cells (22, 23). The ABC transporters are a superfamily of transmembrane proteins that transport a wide variety of substrates across extracellular and intracellular membranes, such as ions, sugars, aminoacids, vitamins, lipids, oligosaccharides, oligopeptides, and drugs (24). These ABC transporters are

coupled to an ATP hydrolysis process, thereby using energy to transport drugs outside of cells. In the human genome, 49 different ABC transporters have been identified and divided into seven subfamilies (A-G) based on sequence similarities (24). The major members of the ABC transporters leading to MDR in cancer cells include ABCB1 (Pgp), ABCCs (MRPs) and ABCG2 (BCRP) (Figure 4). Drugs transported by P-gp include vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes (25). MRPs transport drugs such as vinca alkaloids, anthracyclines, epipodophyllotoxins and some heavy metal anions (26) while drugs transported by BCRP include anthracyclines, mitoxantrone, antifolates, and flavopiridol (27).

Recently, it has been reported that several TKIs interact with P-gp and/or BCRP as substrate or inhibitor. Indeed, resistance to Imatinib is related to P-gp overexpression (28) while Gefitinib has been reported to interact with BCRP and P-gp (29). Recent data suggest that AG1478 interacts with P-gp and BCRP (30), and that Lapatinib and Erlotinib were P-gp and BCRP substrates (31, 32). In Table 1 the transporters involved in the efflux of TKIs, actually in clinical practice in the treatment of cancer diseases, are reported.

2.2. MDR due to TK receptor mutation

Two subtypes of EGFR mutations have been described: mutations of the exons coding for tyrosine kinase domain (18 to 21) and truncating mutations (exons 2 to 7) (21). The first group of mutations can be considered as a hallmark of NSCLC and are responsible for the failure of TKIs while the second group of mutations leads to resistance.

The most common point mutation in the EGFR tyrosine kinase domain is T790M. In this mutation a methionine at 790 position (a key position in the ATP binding cleft) is substituted by a threonine. T790M seems
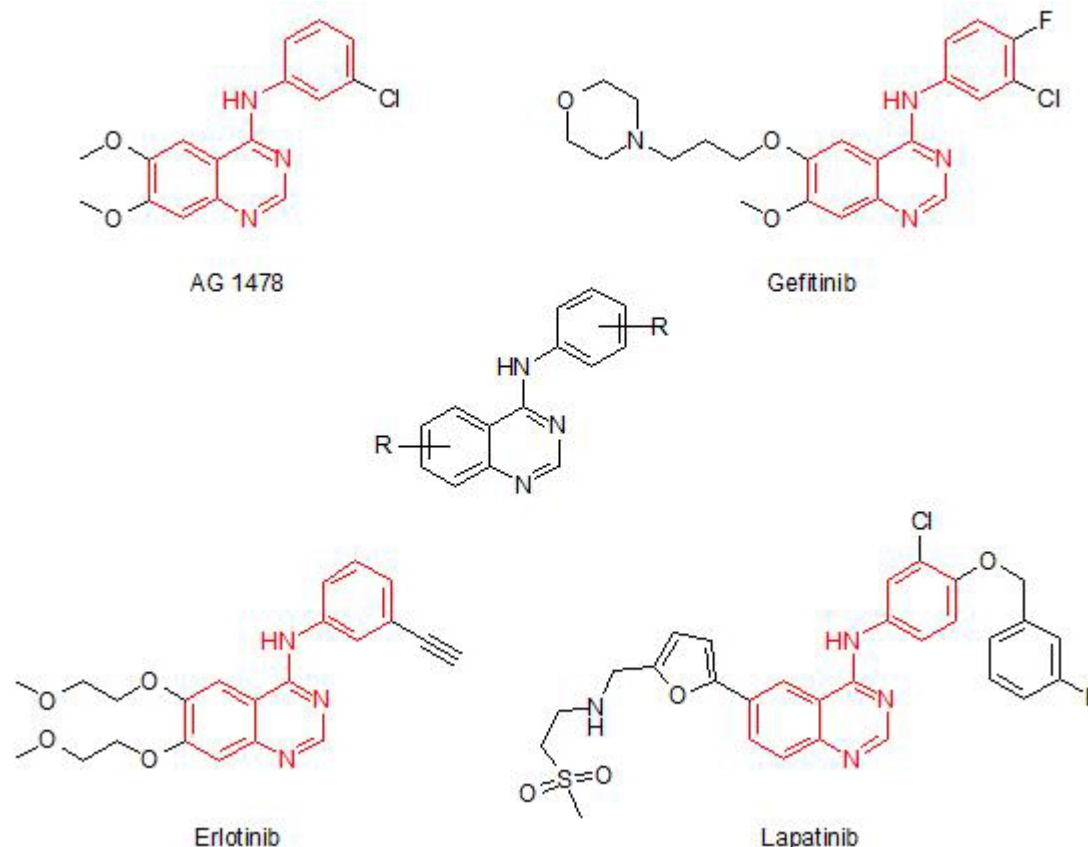
Figure 3. TKIs structures and their pharmacophoric moiety 4-anilino quinazoline.

Table 1. ABC transporters involved in TKIs effluxes

TKIs	ABCB1/Pgp	ABCG2/BCRP	ABCC10/MRP7
Gefitinib	++	++	NR
AG1478	+	+	NR
Erlotinib	++	++	+
Lapatinib	++	++	+

NR =Not Reported

to be involved in the acquired resistance to TKIs (33, 34). Another known mutation, known as L858R, occurs at codon 858 and is a missense mutation at exon 21 (35). T790M mutant cells exhibits tyrosine phosphorylation levels comparable to wild-type EGFR, while the combination of both mutations, T790M and L858R, leads to a substantial increase in phosphorylation, compared with the L858R mutant alone. Therefore, T790M resistance mutation, combined with EGFR kinase domain mutations, enhance catalytic phosphorylating activity and this could explain the additional role of the T790M mutation in predisposing to tumorigenesis (36). Animal models with T790M mutation, alone or together with L858R mutation, developed lung adenocarcinomas displaying a longer latency when express T790M mutation alone (37). These findings demonstrated that T790M mutation is not only responsible for the resistance to TKIs (especially to Gefitinib and Erlotinib) but is also an oncogenic mutation that is maximized in combination with other common EGFR activating mutations.

Moreover, EGFR mutations are constitutively activated without ligand binding and mutated cancer cells, as dependent on the EGFR pathway, are in a state defined as "oncogene addiction" (38, 39). In EGFR-mutant cells the pathway mainly activated is the PI3K-Akt signaling and its down-regulation is responsible for Gefitinib-induced apoptosis (40). Moreover, since mutant EGFR kinases displayed high affinity towards EGFR-TKIs (41), patients with lung cancer with EGFR mutations often displayed an initial dramatic response to EGFR-TKIs but acquired resistance after a period of approximately 10 months (42).

3. STRATEGIES TO REVERT MDR

The classical pharmacological strategy for reverting MDR consists in the co-administration of a transporter inhibitors together with the chemotherapeutic agent transported by the same pump of drug efflux (43). However, although several P-gp modulators have been tested in clinical trials, no satisfactory results have been obtained (44).Alternative approaches to revert P-gp-mediated MDR is the encapsulation of P-gp substrate drugs in liposomes or other nanoparticles, targeting P-gp function by specific monoclonal antibodies and the use of antisense strategies or transcriptional regulators to target *MDR1* gene expression (45).

Drug delivery approaches using liposomes or nanocarriers can target drugs to tumor tissue, tumor cells,
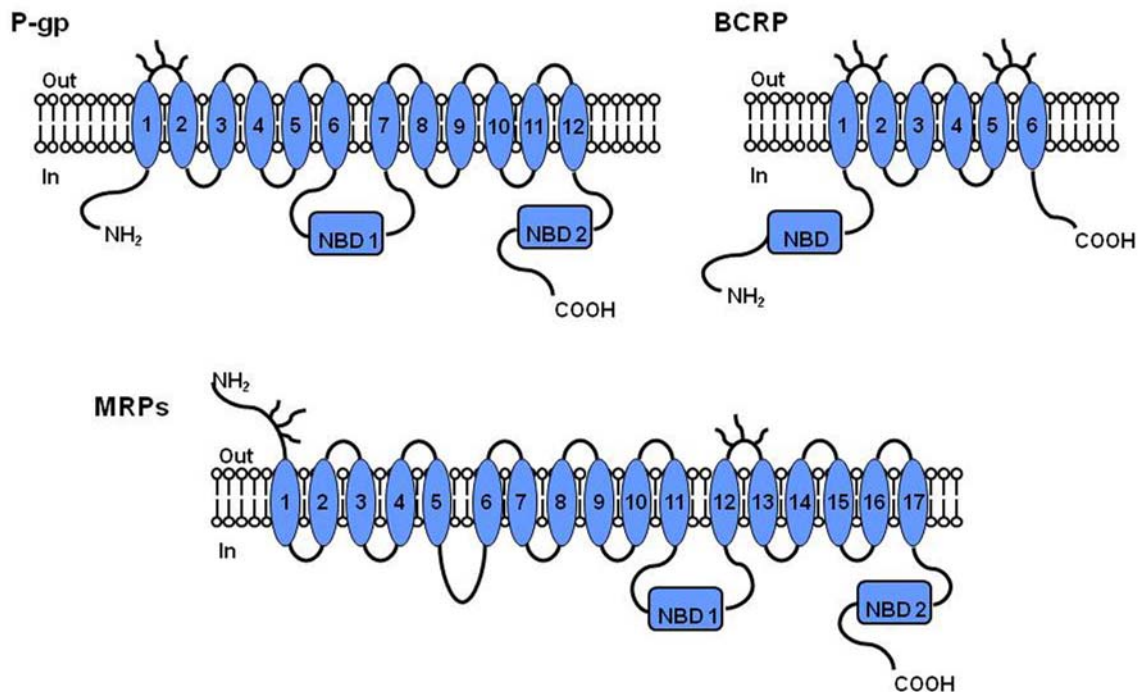
Figure 4. ABC Transporters.

subcellular compartments within tumor cells. Certain liposomal formulations passively accumulate in solid tumors due to the increased permeability of tumor blood vessels compared to the vascular lining of healthy tissues (46). The main objective of liposomal anticancer drug delivery is to reduce the side effects of conventional chemotherapeutic agents without reducing their efficacy. Some liposomal encapsulated drug formulations have been approved for clinical cancer chemotherapy including liposomal carriers of P-gp substrate anticancer agents (e.g. anthracyclines, taxanes, vinca alkaloids, platinum compounds). Interestingly, circumvention of drug resistance in MDR tumors leading at least in part to enhanced antitumor activity has been observed (47).

Several monoclonal antibodies that specifically recognize extracellular or intracellular P-gp epitopes have been generated. These antibodies may be used either as diagnostic tools to detect MDR transporters and as therapeutic tools to reverse MDR. Indeed, results from an *in vivo* study show that, when monoclonal antibody is administered prior to chemotherapeutic drug in nude mice xenotransplanted with colorectal cancer cells, it reverses resistance to this drug (48).

The down-regulation of *MDR1* gene expression at the mRNA level has been widely investigated in an effort to decrease the amount of protein expressed. The most common strategies designed to decrease the expression of P-gp are represented by the administration of antisense molecules, ribozymes or small interfering RNA which help degrade P-gp RNA. Antisense oligonucleotides, widely used to downregulate gene expression, have been used to inhibit the biosynthesis of P-gp and consequently to chemosensitize drug-resistant cells.

Other strategies to revert MDR have been undertaken such as the development of a second generation of TKIs (49) or the design of novel irreversible TKIs (50-52). This topic is detailed in the section 3.1. Moreover, few experimental data have been reported for reversing MDR by co-administration of a TKI with an inhibitor of ABC transporter involved in MDR (53). By contrast, several papers have been published reporting the co-administration of a TKI with a chemotherapeutic agent. In this strategy TKI acted as pump modulator restoring cell accumulation of antineoplastic agents (28-32).

Although several papers speculated on the possibility that TKIs, known as P-gp or BCRP substrates, could be considered as potential agents for reversing MDR, it is evident that this possibility displayed several limitations. In particular, high concentrations of TKIs are needed to saturate the efflux sites of transporters involved in MDR with respect to other specific inhibitors such as Elacridar and Tariquidar that blocked the efflux activity of transporters at concentration 100-fold lower than TKIs.

3.1. Strategies to revert MDR: Second generation TKIs
The resistance induced by T790M mutation, especially towards Gefitinib and Erlotinib, is due to the increased affinity of EGFR to ATP with respect to TKIs (54). Indeed, T790M mutation led to the acquisition of a ''second site'' point mutation in the EGFR kinase domain resulting in a substitution of a methionine for a conserved threonine at residue 790 (T790M). This residue is a ''gatekeeper'' threonine in EGFR and is localized within
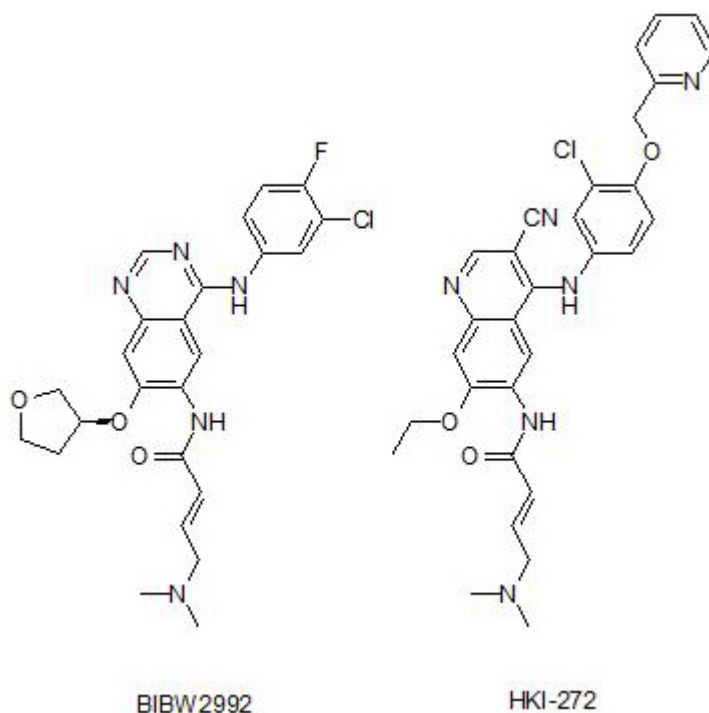
Figure 5. Structures of some "second generation" TKIs.

the ATP-binding pocket (34). This change probably inhibits the binding of Gefitinib or Erlotinib by hydrogen bond within the ATP-binding pocket of the catalytic region (55).

Therefore, one strategy to overcome this kind of resistance is the development of a novel class of TKIs showing an affinity towards T790M kinase higher than that of ATP for the mutant kinase. Currently, "second generation" TKIs are in various stages of development: BIBW2992 (50) and HKI-272 (52) reported in Figure 5 and PF00299804 (51). These "second generation" covalently bind the sulfhydryl group of cysteine 797 at the catalytic pocket of EGFR.

This irreversible interaction is sufficient to allow a reversible binding of the inhibitor towards EGFR-T790M with an affinity sufficient to compete with ATP such as for XL647 (55) reported in Figure 6.However, it has been reported an acquired resistance, due to T790M mutation, also for the irreversible EGFR-TKI, HKI- 272 so that HKI-272 are able to overcome T790M only at doses (1 μM) higher than that clinically achievable (0.2 μM) (56).

3.2. Multisteps inhibitors on activation pathway

Starting from the Structure-Activity Relationships (SAR) and Quantitative Structure-Activity Relationships (QSAR) carried out on the 4-anilinoquinazoline derivatives (57-61), two series of analogues have been developed using Gefitinib as lead compound (62) as depicted in Figure 7.

The first series (I) bears a benzothiophene nucleus instead of the benzene ring, a secondary amino-substituted propoxy side chain at position 6 and methoxy group at

position 7 of the quinazoline nucleus. The second series (II) bears the benzothiophene nucleus, a methoxy group at position 6 and secondary amino-substituted propoxy side chain at position 7 of the quinazoline nucleus. Both series displayed a decreased activity with respect to the lead compound as EGFR inhibitors while, tested for their ability to inhibit two other EGFR-related receptor tyrosine kinases, HER-2 and MET, displayed a different interaction potency. Indeed, the second series showed a comparable HER-2 inhibitory activity and an increased MET inhibitory activity compared to Gefitinib while the first series displayed less inhibitory potency towards both kinases. These findings demonstrated that Gefitinib analogues bearing a secondary amino-substituted propoxy side chain at position 7 were more potent RTK inhibitors than the ligands substituted at position 6. Since resistance to Gefitinib was also due to the crosstalk between EGFR and HER-2/MET (60, 61), these molecules, acting as "pan-RTK inhibitors", could overcome the limitations of TKIs due to resistance suggesting a potential alternatives to Gefitinib. Therefore, the novel "pan-RTK inhibitors" is an alternative strategy to Gefitinib therapy in the treatment of Gefitinib-resistant tumors by blocking simultaneously multiple RTK signaling pathways.

3.3. TKI and ABC inhibitor co-administration

Many drugs have been tested for modulating the P-gp activity and among them the calcium channel blocker Verapamil (63) and the antisteroid Tamoxifen (64) (first generation P-gp inhibitors). Unfortunately, these compounds displayed side effects such as interferences with several enzyme systems. Second generation of P-gp inhibitors, such as Valspodar (65) and Biricodar (66), have been developed. These compounds, more potent and less
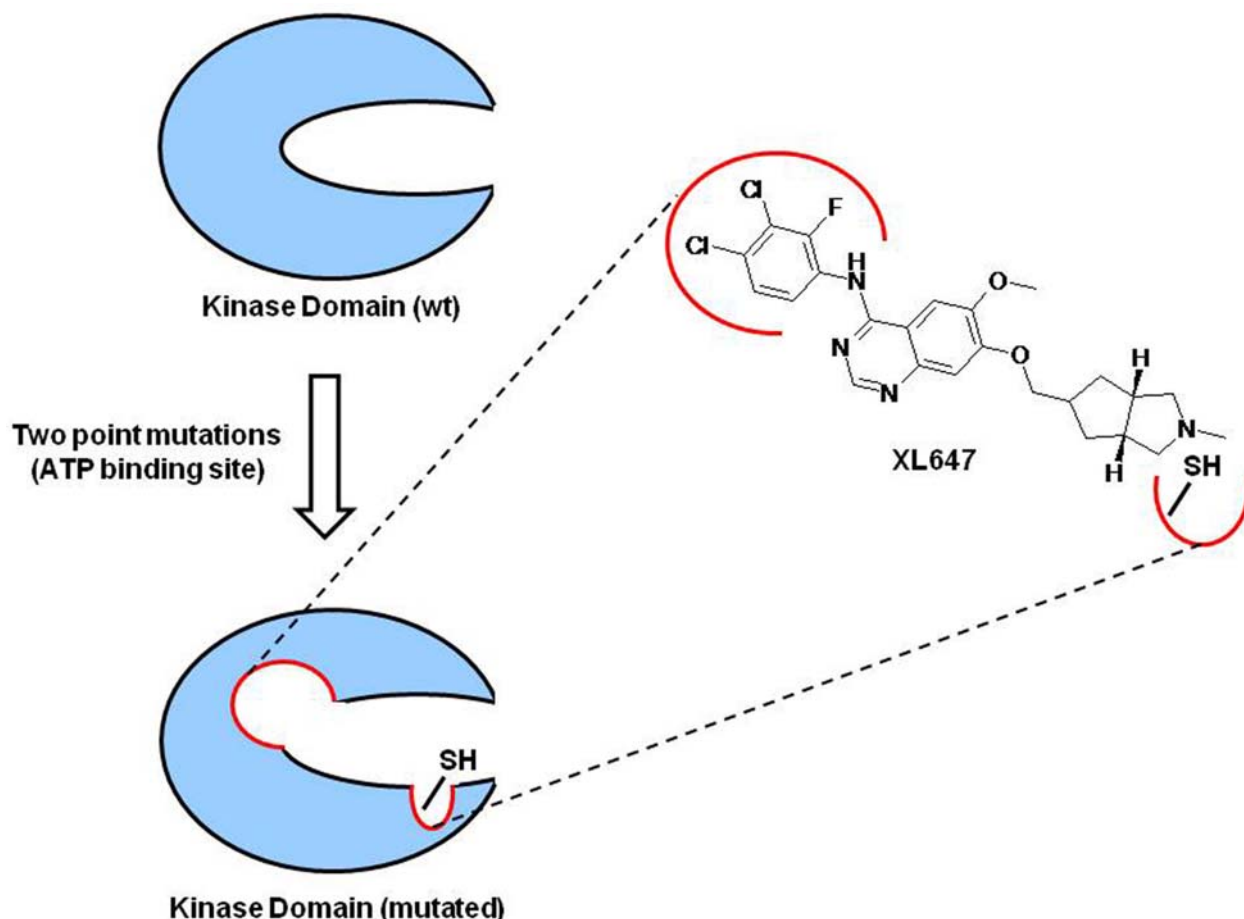
Figure 6. XL647 binding towards kinase domain mutated in two different points.

toxic than first generation ligands, evidenced some limits because significantly inhibited the metabolism and excretion of cytotoxic agents (67). The most studied third generation P-gp modulators are Elacridar (68), Tariquidar (69) and Laniquidar (Figure 8) (70). These inhibitors displayed no pharmacokinetic interaction with chemotherapeutic drugs and showed high potency and specificity for P-gp and at the present these compounds are in different phases of clinical trials.

Currently, three generations of P-gp inhibitors and a number of MRP1 and BCRP inhibitors have been developed to enhance the effect of chemotherapeutic drugs on MDR cancer cells *in vitro* and *in vivo* (71-73). Recently, it has been reported that several TKIs are dual substrates of P-gp and BCRP (28-32).

A potential application of these dual P-gp/BCRP ligands could be as ^{11}C or ^{18}F radiotracers in resistant tumor diagnosis by PET analysis because several unresponsive tumors (breast, colon) are characterized by overexpression of these ABC transporters (74, 75). Therefore, since to date radiotracers binding with similar kinetic profile both pumps (P-gp and BCRP) are not available, for this diagnostic investigation these dual ligands could improve the characterization of refractory tumors by PET imaging.

Even if the co-administration of TKIs with antineoplastic agents has been routinely performed in cancer therapy, some parameters should be pointed out because of the high TKIs concentration requested for restoring antineoplastic agents activity in unresponsive tumors (76).

The perspective of TKIs belonging to the first generation inducing MDR could be the co-administration with P-gp/BCRP inhibitors such as Elacridar, Tariquidar for restoring their pharmacological activity although the kinetics related to the transporters binding of TKIs and third P-gp inhibitors should be better assessed.

4. PERSPECTIVES AND CONCLUSIONS

Small molecules, acting as TKIs, were developed against EGFR and tested in lung cancer such as Gefitinib, Erlotinib and Lapatinib, this last compound exerted dual inhibition activity towards EGFR and HER2. In fact, it has been demonstrated that it caused antitumor activity in cancer cell lines overexpressing HER2 by apoptosis induction (77). However, clinical trials evidenced the onset of resistance after the first response to the treatment with
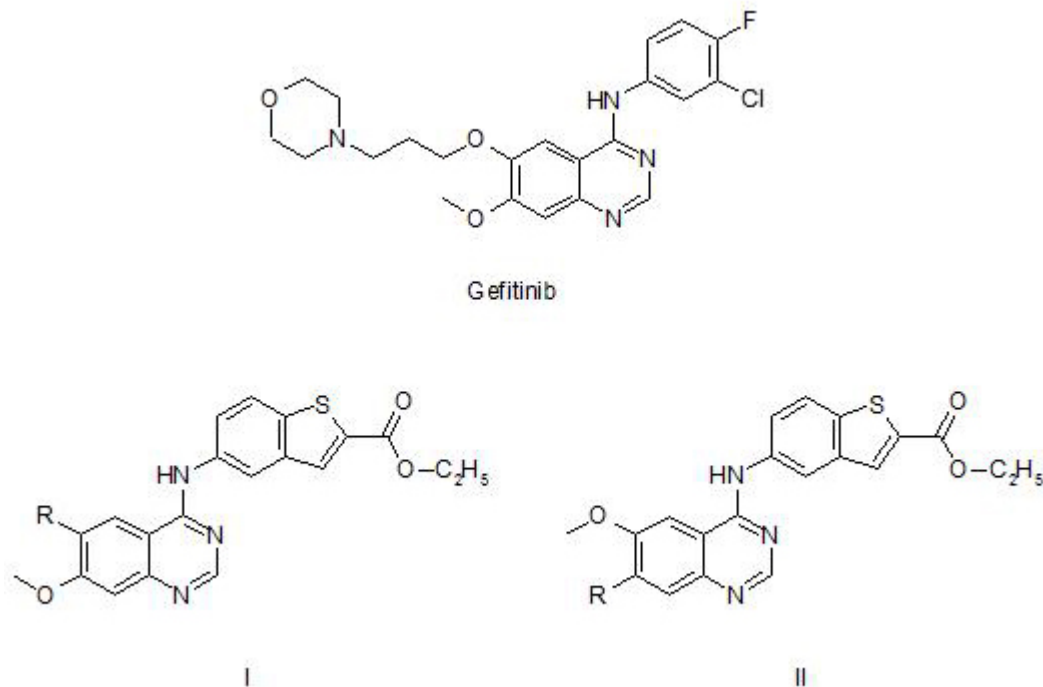
Gefitinib

I

II

Figure 7. Multisteps inhibitors structures.
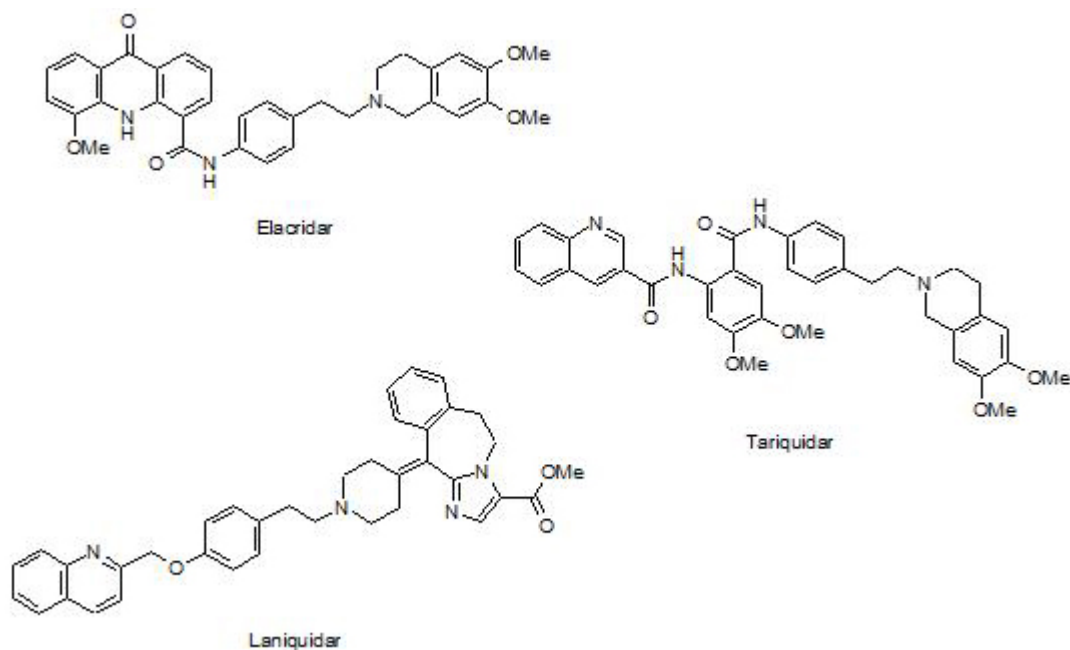

Elacridar

Tariquidar

Laniquidar

Figure 8. Third generation P-gp inhibitors.

TKIs. Since, as already reported, resistance can be due to some known mutations, to overcome this problem, irreversible TKIs were developed (50-52, 78).

4.1. Perspectives of irreversible TKIs
TKIs competing for ATP binding are defined competitive inhibitors because they recognize the kinase active conformation. By contrast, irreversible inhibitors induced the inactivation of kinase receptor binding in a covalent and irreversible manner usually interacting with a nucleophilic cysteine residue (79). Among these irreversible TKIs, HKI-272 and BIBW-2992 were developed in the last years and to date are under evaluation in clinical trials in different tumor types. Actually, the

irreversible TKIs could be considered the perspective in medicinal chemistry field to overcome the resistance limitations associated with the reversible agents. Irreversible TKIs are promising molecules in cancer treatment especially for their potential to overcome the possible mechanism of resistance displayed by TKIs first generation. These TKIs, evaluated in phase II studies in tumors overexpressing HER2, led to successful results displaying an increased response rates and patients survival (80). These drugs can be used also in combination with either chemotherapy or endocrine therapy in order to establish the optimal administration route, their long-term toxicity, their efficacy and their role in combination with other anti-HER2 therapy.

4.2. Perspectives of TKIs first generation

As reported for several anticancer drugs such as doxorubicin, vinca alkaloids, mitoxantrone, TKIs belonging to the first generation, such as Erlotinib, Gefitinib and Lapatinib interacted with some ABC transporters (P-gp, BCRP) involved in MDR. It has been ascertained that they are substrates of these efflux pumps so that their therapeutic employement has been limited. Starting from this finding, they could be employed to recognize refractory tumors by imaging tecniques such PET and SPECT. In fact, dual ligands, binding P-gp and BCRP pumps, are needed as radiotracers for PET/SPECT investigation because, to date, only unslective (towards others ABC transporters not involed in MDR) P-gp or BCRP ligands are available. In particular, breast and colon tumors displaying P-gp and BCRP overexpression could be monitored by using simultaneously specific biomarkers and not invasive tecniques such as PET/SPECT investigation.

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