

Tyrosine Kinase Inhibitors

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Abstract: Over the last ten years, several new and therapeutically relevant cancer drugs targeting tyrosine kinase signaling pathways have been developed. Tyrosine kinase inhibitors (TKIs) are a pharmaceutical class of small molecules, orally available, well-tolerated, worldwide approved drugs for the treatment of several neoplasms, including lung, breast, kidney and pancreatic cancer as well as gastro-intestinal stromal tumors and chronic myeloid leukemia. This comprehensive review focuses on the most relevant members of the first and the second generation TKIs designed to interact with receptor and nonreceptor TKs. Attention is mainly focused on molecular mechanisms *in vitro* and *in vivo* models related to the clinical activity of the drugs and to the development of resistance to treatment, still the major challenge in cancer research and care.

Keywords: Angiogenesis, HER family, multitarget, nonreceptors, receptors, resistance, tyrosine kinase inhibitors.

INTRODUCTION

Protein tyrosine kinases (PTKs) are a family of proteins that play significant roles in cancer development [1]. They can be broadly subdivided into two classes based on their structure, function and localization: receptor tyrosine kinases (RTKs) and nonreceptor tyrosine kinases (NRTKs). Both of them have been involved in multiple cancer types including, but not limited to, leukemia, lung cancer, breast cancer, pancreatic cancer and gastrointestinal stromal tumors (GISTs) [2].

RECEPTOR TYROSINE KINASES

Receptor tyrosine kinases have crucial functions in signaling pathways involved in many cellular processes such as proliferation, differentiation and metabolism, migration, angiogenesis and survival. Aberrant RTK activity has been linked with tumor development and abnormal cell growth. Because of this high oncogenic potential, RTKs have become a preferred target in cancer therapy.

In the human genome 58 RTKs have been identified, distributed into 20 subfamilies as defined by receptor and/or ligand, including receptors for insulin and growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and nerve growth factor [1]. The RTKs catalyze the transfer of the γ -phosphate of adenosine triphosphate (ATP) to hydroxyl groups of tyrosines on target proteins. All RTKs contain an extracellular highly glycosylated ligand binding domain (ECD) connected to the carboxy-terminal tail cytoplasmic catalytic domain with the kinase activity by a single transmembrane domain. Ligand binding to the extracellular domain induces homo- or hetero-dimerization of receptors [3] and subsequent activa-

tion of the kinase. Dimerized receptors induce phosphorylation of tyrosine residues on the receptors themselves and on downstream proteins, thus initiating signaling cascades [4]. Multiple cytoplasmic signaling pathways [Ras/mitogen activated protein kinase (MAPK), phospholipase C, PI3-kinase, signal transducer and activator of transcription-3 (STAT3), SRC/FAK, protein kinase C and JAK/STAT] may be activated [5] leading to altered DNA synthesis and cell division as well as effects on a variety of biological processes, including cell growth, migration, differentiation and death [6].

RECEPTOR TYROSINE KINASE INHIBITORS

Due to their key role in signaling pathways that affect every aspect of cell growth, differentiation and metabolism, PTKs have become prime targets for cancer-directed therapy. A great deal of interest has been devoted to the development of small molecules, the TKIs, a new class of drugs with a unique mechanism of action. Unlike monoclonal antibodies (mAbs), TKIs can translocate through plasma membranes and interact with the cytoplasmic domain of cell-surface receptors and can be orally administered.

First Generation Inhibitors of the HER Family

The HER family and the RTKs encoded by these genes function in a wide range of cellular processes including cell proliferation and apoptosis. Four HER family members are known: the EGF Receptor 1 (EGFR, HER1 or ErbB1) [7]; the EGF Receptor 2 (HER2, HER2/neu or ErbB2) [8]; the EGF Receptor 3 (HER3 or ErbB3) [9] and the EGF Receptor 4 (HER4 or ErbB4) [10]. All, except HER3 [11], contain intracellular catalytically active TK domain and all, except HER2, bind to known extracellular ligands [12]. The HER family ligands include EGF, amphiregulin and transforming growth factor- α (TGF- α), betacellulin, heparin-binding EGF-like growth factor and several other molecules [13]. HER2 seems to be the preferred dimerization partner for all the other HER receptors [14]. HER2 heterodimers are more stable [15] and their signaling is more potent than other receptor combinations without HER2 [16].

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Aberrant expression and signaling of the HER family has been associated with bladder, brain, colon, head and neck, lung, ovarian, and pancreatic as well as breast cancer [17]. Amplification of the HER2 gene, resulting in HER2 protein overexpression [18] is present in about 25% of human breast cancers and is associated with a more aggressive form of the disease with significantly shortened disease-free survival (DFS) and overall survival (OS) [19]. As a result, this RTK family has been extensively studied as therapeutic target of many types of human carcinomas. Three EGFR TKIs are currently in clinical use: gefitinib [20], erlotinib [21] and lapatinib [22]. All these compounds share a common 4-anilinoquinazoline core even if they have distinct HER inhibitory profiles and mechanisms of action.

Overexpression of EGFR has been linked to the pathogenesis of many human solid tumor types, playing a key role in promoting tumor invasion and metastasis [23]. The first signaling cascade downstream of the EGFR is the Ras/MAPK pathway [24]. Following dimerization and autophosphorylation of specific tyrosine residues, the SOS (Son of Sevenless) guanine nucleotide exchange factor is recruited to the plasma membrane *via* the Grb2/Drk/Sem5 adapter protein. SOS stimulates the exchange of GTP for GDP on Ras which becomes activated and, in turn, binds to and activates Raf. Raf then binds to, phosphorylates and activates MEK, a dual kinase that can phosphorylate tyrosine and serine residues. MEK phosphorylates and activates MAPK pathway to promote cell proliferation [25]. EGFR also mediates cell functions through the phospholipase C gamma (PLC γ) and phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR pathways. The complexity of signaling is further increased by cross-talk among individual pathways. Ras is able to regulate PI3K activity and PLC γ can indirectly regulate Ras [26]. Another signaling cascade initiated by activation of EGFR is the Janus kinase (JAK)/signal transducer and the activator of transcription (STAT) pathway, which is also implicated in cell survival responses [27]. JAK phosphorylates STAT proteins localized at the plasma membrane leading to the translocation of STAT proteins to the nucleus where they activate the transcription of genes associated with cell survival.

Non small cell lung cancer (NSCLC) still remains the leading cause of death due to cancer despite progresses of the last years in diagnosis and treatment [28]. NSCLC is associated with a high rate of EGFR overexpression [23] and somatic mutations of the EGFR have been detected in approximately 10% of European and North American NSCLC patients and in 30-40% of East Asian patients [29]. These mutations have been related to a higher sensitivity to EGFR TKIs [30].

Erlotinib and gefitinib are orally active compounds belonging to the first generation of inhibitors of the intracellular TK domain of the EGFR [21].

Gefitinib

Gefitinib (Iressa[®]; ZD1839) is a low molecular weight synthetic anilinoquinazoline compound with a high, selective and reversible capacity to inhibit the TK activity the EGFR in a dose-dependent and complete manner. Gefitinib targets the ATP-binding cleft of the EGFR TK domain thus inhibiting ATP binding, completely blocking EGFR autophos-

phorylation and signal transduction. Among several quinazoline derived TKIs, gefitinib has been selected for clinical evaluation for its high EGFR TK inhibitory activity and long half-life compatible with once-daily oral administration [20]. As a single agent and in combination with various chemotherapy agents, gefitinib inhibits tumor cell proliferation, apoptosis and angiogenesis in a variety of EGFR-positive human cancer cell lines and xenografts [31], but its activity does not depend on the levels of expression of the EGFR [32]. In NSCLC, responses to TKIs were noted in subsets of patients, notably of East Asian ethnicity, female gender, light- or never-smoking status and adenocarcinoma histology [33, 34]. In the same subset of patients, EGFR mutations predicting for response to TKIs were identified in the TK domain [30]. In addition, more than 70% of NSCLCs with EGFR mutations responded to TKIs, whereas only 10% of wild type tumors responded [35]. Mutations are limited to the first 4 exons (exons 18–21), in the ATP-binding region of the EGFR gene TK domain leading to constitutive autophosphorylation and aberrant activation of EGFR [36]. The TK domain normally exists in on (active) or off (inactive) conformational states. It consists of two roughly globular-shaped lobes, the N-terminal (N-lobe) and C-terminal (C-lobe) lobes [1] and the ATP-binding catalytic cleft lies between them. The smaller N-lobe consists largely of a β -sheet and the prominent highly conserved α C helix, which has a role in the conformational changes of the binding pocket and for activation. The C lobe is larger and mostly helical. The α C helix, the activation loop and the phosphate-binding loop represent key structures required for function and activation and surround the cleft. The most common mutations target these key structures and can be classified as either class (i), class (ii) or class (iii). Class (i) mutations are in-frame deletions in exon 19, that almost always include amino acid residues leucine-747 to glutamic acid-749 and are located at the N-terminus of the kinase domain C-helix. Deletions in exon 19 shorten a loop at the N-terminus of the α C helix (which borders the catalytic cleft in the N-lobe and participates in substrate binding), restrain the α C helix and shift the axis of the helix downward. These deletions account for approximately 44% of the activating EGFR TK domain mutations. Class (ii) mutations are dominated by a single point mutation in exon 21 in which the replacement of hydrophobic leucines at residue 858 with an arginine (L858R) may thermodynamically favor the stabilization of the activation loop in the activated position. This point mutation accounts for approximately 41% of EGFR TK activating mutations. An additional 4% of EGFR TK activating mutations result in glycine-719 mutation to either serine, alanine, or cysteine, and a further 6% are represented by other missense mutations. Class (iii) mutations account for the remaining 5% of EGFR TK activating mutations [37]. These mutations appear to selectively activate Akt and STAT signaling, which promotes cell survival by inducing resistance to apoptosis [38].

Clinical data have shown that lung cancers showing EGFR mutations are sensitive to EGFR TKIs [30]. High response rates ($\sim$ 70%) to gefitinib in patients with EGFR mutations have been confirmed in recently published prospective phase II studies [39]. In addition, patients with EGFR mutations have a significantly longer survival when treated with EGFR TKIs [40]. An increase in EGFR gene copy

number has also been associated with significant survival benefit from EGFR-TKIs treatment [41]. Further studies indicated that other factors, like interactions with other members of the EGFR family i.e. HER2 and HER3 might play a role in determining response and survival after TKIs [42]. On the other hand, transformation by the D770_N771 ins NPG (exon 20) EGFR insertion confer resistance to gefitinib or erlotinib treatment [36, 43]. A further complexity is the clinical observation that somatic mutations of *kRAS* confer intrinsic resistance to TKIs as it has been demonstrated in patients with *kRAS*-mutated colorectal cancer [44, 45]. The actual role of *kRAS* mutations will be discussed below in the erlotinib paragraph. In addition, mesenchymal-epithelial transition factor (MET) amplification and over-expression of its ligand, the hepatocyte growth factor (HGF), have also been reported as causes of acquired resistance of EGFR-TKI [46, 47], and it is hypothesized that other mechanisms of resistance may exist [48].

Based on the data from two phase II trials [33, 49], gefitinib initially received accelerated approval by the U.S. Food and Drug Administration (FDA) in May 2003 for patients with locally advanced or metastatic NSCLC who had progressed after platinum-based and docetaxel chemotherapy. Later, a phase III trial comparing gefitinib with Best Supportive Care (BSC) did not show any statistically significant difference in OS [50]. After the results of this trial, in June 2005 the FDA restricted the use of gefitinib to patients enrolled in clinical trials or deriving clinical benefit from ongoing treatment. This effectively removed gefitinib from routine clinical practice within the U.S. However, gefitinib was approved in other countries and clinical trials investigating the role of gefitinib are still in progress. Of particular relevance is the phase III study INTEREST comparing gefitinib with docetaxel in pretreated advanced NSCLC patients from 24 countries worldwide [51]. The trial established non-inferior survival benefit of gefitinib vs docetaxel in almost all subgroups with added advantages of improved tolerability and quality-of-life, suggesting that gefitinib is a valid treatment for pretreated patients with advanced NSCLC [52]. The European Medicines Agency (EMA) granted marketing authorisation to gefitinib in 2009 for treatment of locally advanced or metastatic NSCLC bearing activating mutations of EGFR, across all lines of therapy.

Erlotinib

Erlotinib (Tarceva[®]; OSI-774) is an orally active quinazoline derivative that selectively and reversibly inhibits the TK activity of the EGFR by competing with ATP for the ATP-binding site within the intracellular TK domain of the EGFR. Binding modality of erlotinib to the EGFR is essentially the same as gefitinib [53]. Nanomolar concentrations of erlotinib inhibit the growth of various cancer cell lines that express the EGFR and this effect is accompanied by a significant reduction of intratumoral EGFR phosphorylation [21]. Erlotinib has additive growth inhibitory effects when used in combinations with cytotoxic drugs, including paclitaxel and cisplatin [54]. The main circulating metabolites of erlotinib are products of the O-demethylation of the side chains (such as OSI-420 and OSI-413) and their activity against EGFR is similar to that of erlotinib [55]. The pharmacokinetics of erlotinib and its metabolite OSI-420 are

dose-independent and not appear to be altered by the concomitant administration of paclitaxel and carboplatin [56, 57]. The recommended dose for the orally continuous schedule is 150 mg/day. The primary route of metabolism is oxidation by the cytochromes CYP3A4, CYP3A5, CYP1A1 and CYP1A2 isoforms of the P450 enzymes [58]. Polymorphisms of these genes have been linked to interindividual variability in erlotinib toxicity, i.e. acneiform skin rash and diarrhea [33, 41].

Similarly to gefitinib, early experience with erlotinib revealed that some patients experienced a rapid and tremendous response to therapy, and that these responses were associated with a history of never-smoking, female sex, Asian ethnicity, adenocarcinoma histology [44]. These clinical subgroups have been found to have a higher prevalence of activating EGFR mutations in the TK domain [59].

Despite the high response rates to gefitinib and erlotinib in EGFR-mutant NSCLC, all patients will ultimately develop acquired resistance [60]. Mechanism of resistance, in more than 50% of patients, is secondary to EGFR mutations that affect drug efficacy. There are currently two documented point mutations linked to acquired resistance to erlotinib, the threonine-790 to methionine (T790M) [44, 61] and aspartic acid-761 to tyrosine [62]. EGFR T790M mutation has been found in about 50% of tumors from patients that have developed acquired resistance to therapy [63]. Crystal structure modelling has revealed that position T790 is located in the ATP-binding pocket of the catalytic region and it is critical for the binding of erlotinib. Substitution of the threonine at this codon with a methionine, is thought to sterically hinder the binding of these two drugs.

Other mechanisms of resistance have been related to MET deregulation and to *kRAS* mutations. Pitfalls in the MET/HGF signaling pathway can cause resistance via different mechanisms, including HGF and/or MET overexpression, MET gene amplification, mutations or rearrangements [64]. Moreover MET interacts with the HER3-dependent activation of PI3K thus allowing the cells to maintain activity of downstream signaling pathways even in the presence of EGFR inhibitors [59]. Somatic mutations of the *kRAS* oncogene have been assessed as a mechanism of *de novo* resistance to TKIs in about 20% of NSCLC patients [65, 66]. The Ras/Raf/ERK pathway activation plays a key role in tumor growth and progression of various tumor types [67]. *kRAS* mutations, most frequently found in codons 12, 13 and 61 [68], lead to impaired GTPase activity resulting into constitutive activation of RAS signaling. This permits the cell to evade apoptosis and acquire a growth advantage independent of EGFR signaling and thereby resistant to EGFR TKIs therapy [69]. However, an intact *kRAS* is necessary but not sufficient to derive benefit from EGFR inhibition [70]. *kRAS* and EGFR mutations seems mutually exclusive thus suggesting that they might have functionally equivalent roles in lung tumorigenesis. About 15-30% of lung adenocarcinomas contain activating mutations of *kRAS* while they are quite uncommon in lung squamous cell carcinomas [68, 71]. Recent reports indicate that alterations in other members of the EGFR signaling cascade could also drive resistance to anti-EGFR therapy, including BRAF mutations [72] and deregulation of the PIK3CA/PTEN pathway [73].

Erlotinib has been approved by the FDA on November 2004 and by the EMEA in October 2005 for the treatment of locally advanced or metastatic NSCLC after failure of at least one prior chemotherapy regimen on the basis of the BR.21 trial, which showed a significant improvement in progression free survival (PFS) and OS with erlotinib in comparison to BSC in previously treated patients [34]. On the other hand, two randomized, double-blind, placebo-controlled phase III trials assessing the efficacy of erlotinib in combination with chemotherapy in the first-line setting, the TRIBUTE and the TALENT trial, failed to demonstrate any survival advantage for patients receiving the addition of erlotinib to a standard platinum-based doublet chemotherapy [74, 75]. According to the last update of the American Society of Clinical Oncology Clinical Practice Guidelines, erlotinib is now recommended as third-line therapy for the treatment of patients with stage IV NSCLC who have not received prior erlotinib or gefitinib [76]. Erlotinib has also been approved by the FDA and EMEA in combination with gemcitabine for the treatment of patients with locally advanced, unresectable or metastatic pancreatic carcinoma, although clinical data showed a modest benefits in survival rates [77].

Lapatinib

The HER family receptors, EGFR and HER2, are both overexpressed in approximately 25% of primary breast cancers [18]. This overexpression is associated with poor prognosis, suggesting HER targeting therapies as an attractive modality for breast cancer treatment [19]. Nowadays the standard of care for early and advanced stage HER2 overexpressing breast cancers is the anti-HER2 mAb trastuzumab [78]. Unfortunately, most HER2-positive breast cancers do not respond to trastuzumab and the majority of responders progress within 12 months after initiating therapy [79]. Consequently, other approaches to block HER2 signaling have been developed including small molecules that compete with ATP for binding the catalytic kinase domain of both EGFR and HER2. On the other hand, EGFR is not a major therapeutic target in breast cancer [80] but it has been shown to be relevant in other tumor types [23] or breast tumors that develop insensitivity to HER2 targeted therapy [81]. The knowledge of the mechanisms of trastuzumab resistance is of paramount relevance to understand the role of lapatinib in breast cancer treatment. The PI3K pathway can be activated by a loss or low levels of the tumor suppressor phosphatase and tensin homolog (PTEN) [82]. PTEN negatively regulates cell proliferation and survival by inhibiting downstream effectors of PI3K, such as Akt [83]. Downregulation, mutations and/or deletions of PTEN, reported in up to 50% of breast cancer cases [84], may lead to trastuzumab resistance [82]. The insulin-like growth factor (IGF) signaling system regulates multiple levels of tumor progression, including proliferation, evasion from apoptosis, metastasis and invasion [85]. Overexpression of the IGF-1 receptor (IGF-1R) or increased levels of IGF-1R/HER2 heterodimers may hyperactivate PI3K and its downstream effector Akt, thus causing trastuzumab resistance [86]. Amino terminally truncated carboxyl terminal fragments of HER2 are termed p95^{ErbB2}; they are HER2 receptors that have a truncated ECD, but retain their kinase activity. p95^{ErbB2} may originate through the proteolytic shedding of the extracellular domain of full-

length HER2 [87] or alternative initiation of translation from methionines located near the transmembrane domain of the full-length molecule [88]. They are found in up to 30% of HER2-positive breast cancer cell lines and tumors [89] and high levels of these molecules are correlated with reduced DFS and OS [87, 89]. Expression of p95^{ErbB2} may disrupt trastuzumab binding at the cell surface, hence signaling pathways remain active even if trastuzumab is present.

Other unconfirmed mechanisms of resistance have been reported [90] such as high levels of EGFR and HER3 ligands [81, 89], CD44 tumor cell overexpression [91], Fc receptor status and lack of ADCC response [92], microRNA signatures [93], phosphorylated HER2 receptor [89] and c-MYC amplification [94].

Lapatinib (Tykerb®/Tyverb®; GW572016) is a synthetic, low molecular weight, orally-active compound, developed from the quinazoline core found in other HER RTKIs. It shows a reversible, non-covalent inhibition of the TK domain of both EGFR and HER2 [22], but it has a very slow off-rate compared with gefitinib or erlotinib. This allows for prolonged effects after drug concentrations have dropped, even if the clinical relevance of this phenomenon is yet to be established [95]. Lapatinib has been shown to inhibit the growth of HER2-overexpressing breast cancer cells and to have a synergistic effect with trastuzumab, the two compounds being apparently non cross-resistant [96, 97]. Lapatinib also sensitizes HER2-overexpressing breast cancer cells to radiation [98] and restores tamoxifen sensitivity in tamoxifen-resistant breast cancer models [96]. A recent meta-analysis of randomized phase III trials in metastatic breast cancer has shown that the clinical benefit from treatment with lapatinib is limited to patients with HER2-positive breast cancer, showing no activity in HER2-negative disease [99].

The activity of lapatinib in trastuzumab resistant cells has been related to different possibilities. Lapatinib seems to have activity in tumors expressing the truncated p95^{ErbB2} directly inhibiting phosphorylation of the constitutively activated TK activity within the cell [93]. Lapatinib is also able to reduce the IGF-I signaling pathway in trastuzumab-resistant cells and to inhibit signaling in tumor cells even in the presence of elevated IGF-I [100]. These results are further reinforced by data showing that co-administration of lapatinib with an antibody that blocks IGF-1R, alpha IR3, has an additive effect on decreasing breast cancer cell proliferation [100]. Lapatinib is effective also in cells in which PTEN has been downregulated [67]. It has also been shown to inhibit HER2 phosphorylation and to prevent its ubiquitination thus causing a marked accumulation HER2 at the cell surface. This increase in receptor number at the cell surface may enhance trastuzumab-mediated ADCC [101].

Lapatinib and trastuzumab have recently been shown to interact synergistically in the inhibition of the proliferative activity of a panel of HER2-amplified human upper gastrointestinal cancer cell lines and xenografts, supporting a role for these drugs in metastatic gastric cancer treatment [102, 103].

Lapatinib has been approved by the FDA in 2007 and by EMEA in 2008 for use in combination with capecitabine for the treatment of patients with advanced or metastatic HER2-

positive breast cancer who have received prior therapy including an anthracycline, a taxane and trastuzumab [104]. On January 2010, the FDA approved lapatinib in combination with letrozole to treat hormone receptor positive and HER2-positive advanced breast cancer in postmenopausal women for whom hormonal therapy is indicated [105].

Second Generation Inhibitors of the HER Family

BIBW 2992

BIBW 2992 (Tovok[®]) is a still investigational, second generation, orally available, irreversible *in vitro* and *in vivo* inhibitor of EGFR and HER2 receptors [106]. BIBW-2992 inhibits proliferation of EGFR and HER2 positive tumors [107], including NSCLC cell lines and animal models over-expressing EGFR with the T790M point mutation, responsible for acquired resistance to gefitinib and erlotinib [108]. From phase I trials, the recommended dose is 70 mg once-daily in a 14-day on, 14-day off schedule or 50 mg continuously; it is generally well tolerated with only diarrhea and skin rash as limiting toxicities [106, 109]. BIBW 2992 is currently under clinical evaluation in NSCLC, breast and prostate cancer, head and neck carcinoma as well as glioma. The clinical efficacy and safety of BIBW 2992 versus standard chemotherapy (cisplatin/pemetrexed) as first-line treatment in NSCLC patients with EGFR mutations is currently being investigated in a Phase III study. BIBW 2992 has received a Fast Track Designation status by the FDA for NSCLC patients resistant to prior treatment with reversible EGFR inhibitors.

Canertinib

Canertinib dihydrochloride (CI-1033; PD183805) is a still investigational, second generation, orally available, irreversible pan-HER TKI. It irreversibly blocks ATP binding site of all four members of the HER receptor family and induces receptor ubiquitination and degradation [110]. Canertinib has been shown to have anticancer activity against a wide variety of tumor models, including NSCLC, glioblastoma, breast and ovarian cancer [111, 112]. It has also been shown to act as a radiosensitizing agent and to synergize with chemotherapeutic agents [113-115]. Canertinib has been evaluated in phase II clinical studies at orally administered daily doses ranging between 50 and 450 mg, using an intermittent or a continuous-dosing schedule, however only the 50 mg dose was well tolerated [116, 117]. Higher doses of canertinib have been associated with dose-limiting toxicities such as diarrhea, edema, rash, anorexia, and thrombocytopenia [116, 117]. Single-agent canertinib did not show clinically significant activity in pretreated patients with metastatic breast cancer expressing one or more HER receptors. The drug is currently under evaluation in several clinical trials.

Neratinib

Neratinib (HKI-272) is a still investigational, second generation, orally available, potent irreversible pan-HER TKI selected among a series of 6,7-disubstituted 4-anilinoquinoline-3-carbonitriles, all targeting of EGFR, HER2, HER3 or HER4 kinases [118, 119]. It binds to a conserved cysteine residue (Cys 797) within the ATP-binding pocket of the HER receptors, forming a covalent complex

[120]. Neratinib inhibits cell proliferation of most HER2-positive breast cancer cell and EGFR-dependent cells [120]. It also blocks the growth of HER2 overexpressing tumor xenografts in a way compatible with a once-daily oral dosing [120]. Neratinib has also been shown to overcome multiple mechanisms of gefitinib resistance in EGFR-T790M point mutant NSCLC cells [121]. Neratinib is currently being tested in the clinical setting at the once-daily dosing of 320 mg orally administered [122] mainly in patients with gefitinib and erlotinib resistant NSCLC and HER2 overexpressing breast cancer, with promising preliminary results [119]. A randomized phase III trial comparing neratinib versus lapatinib and capecitabine in HER2-positive trastuzumab-resistant metastatic or locally advanced breast cancer is currently recruiting participants (NCT00777101).

Pelitinib

Pelitinib (EKB-569) is a still investigational, second generation, orally available, irreversible EGFR TKI [118]. It binds covalently to cysteine residue in EGFR and HER2 thereby inhibiting receptor phosphorylation and downstream signaling pathways [123, 124]. Pelitinib inhibits tumor cell proliferation in *in vitro* [124] and *in vivo* animal models [123]. It has good tolerability profile and its prolonged inhibitory effect on EGFR phosphorylation allows once-daily oral administration with either intermittent or continuous schedule, either in monotherapy or in combination with chemotherapy [125-127]. Development of pelitinib for the breast cancer treatment has been suspended for lack of activity in this setting.

Multitarget Tyrosine Kinase Inhibitors (MTKIs)

Multikinase inhibitors are small molecules capable to inhibit multiple RTKs and related signaling pathways [128]. Among them, multikinase angiogenesis inhibitors have shown relevant clinical activity in several tumor types, including kidney and liver cancer. In healthy individuals, the formation and maintenance of new vasculature is a complex multi-step process and its dysregulation has significant impact on many disease states including inflammation, cardiovascular diseases and cancer [129]. Angiogenesis is physiologically controlled by the balance between of pro- and anti-angiogenic factors, while pathological angiogenesis is shifted to a sustained production of pro-angiogenic factors [130]. Pathological angiogenesis is necessary for tumors to grow beyond the limits of passive diffusion and it is essential for tumor cell survival, local invasion and metastatic spreading [131]. Many extracellular, cell surface and intracellular molecules able to induce angiogenesis have so far been identified, including VEGF, FGF, PDGF, TGF- α 1, adhesion molecules, matrix metalloproteinases, urokinase plasminogen activator, fibronectins and collagens, transcription factors such as hypoxia inducible factor (HIF), nuclear factor- κ B (NF- κ B) and signaling molecules such as mTOR, Akt, cyclooxygenase-2 and others [132]. The role of angiogenesis inhibitors such as thrombospondin-1, metalloproteinase inhibitor-1, interleukin-10, angiostatin, endostatin and a number of other molecules, is to physiologically counteract stimulatory effects on blood vessel formation [133]. The net outcome between of pro- and antiangiogenic factors depends mainly on hypoxia, oncogenes or growth factor signaling.

VEGF (or VEGF-A) is a tumor-secreted cytokine with a key role in promoting angiogenesis [134]. VEGF is a dimeric glycoprotein, member of the PDGF superfamily of growth factors that includes VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PlGF). The VEGF gene, located in the 6p21.3 region [135], is composed of eight exons and alternative exon splicing results in generation of four major VEGF isoforms (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF_{206is}) [136, 137]. VEGF expression is mainly induced by hypoxia mediated by HIF-1 overexpression [138] but also other factors including cytokines [139], growth factors [140], tumor suppressor genes [141] and hormones [142] contribute to its regulation.

The VEGF-Receptor (VEGFR) family consists of three membrane RTKs predominantly, but not exclusively, expressed in endothelial cells: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (flt-4) [143]. VEGF binding to VEGFRs induces receptor homo- or hetero-dimerization leading to autophosphorylation and activation of the intracellular TK domain of the receptor [143]. Intracellular signaling activated by VEGFRs include PLC- γ , MAPK and PI3K-Akt pathways [143, 144]. Members of the VEGF family have different affinities for one of the three VEGFRs: VEGF-A binds to VEGFR-1 and VEGFR-2; VEGF-B and PlGF bind selectively to VEGFR-1 while VEGFR-3 is bound only by VEGF-C and VEGF-D [143]. VEGFR-1 and VEGFR-2 are prominently expressed by vascular endothelial cells in addition to selected other cell types; VEGFR-3 is prominently expressed in lymphatic endothelial cells, although it can also be expressed on activated blood endothelial cells [145]. It is now agreed that VEGFR-2 is the major mediator of mitogenic, angiogenic and permeability-enhancing effects of VEGF [146].

Interference with VEGF pathways is the most validated approach to block tumor angiogenesis. Currently approved antiangiogenic drugs are represented by mAbs against circulating VEGF (i. e. bevacizumab) and small MTKIs of VEGFRs and related signaling molecules [147, 148].

First Generation MTKIs

Sunitinib

Sunitinib malate (Sutent[®]; SU11248) is an oral MTKI with antitumor and antiangiogenic activities. Sunitinib selectively inhibits members of the split-kinase domain RTKs, including all three isoforms of VEGFR, PDGF receptor (PDGFR) α , PDGFR β , FLT3, KIT, colony-stimulating factor 1 receptor, RET and RET/PTC subtypes 1 and 3 [149, 150]. The VEGF and PDGF signaling pathways are critical in transduction of extracellular signals and induction of angiogenesis [151]. Simultaneous inhibition of both targets is expected to suppress tumor neoangiogenesis to a greater extent than inhibition of either of the targets alone [152]. Moreover, use of such a multitargeted strategy might delay or prevent the emergence of resistance to therapy [153]. *In vitro*, sunitinib inhibits the VEGF-induced proliferation of human endothelial cells, abrogates migration and blocks their ability to form capillary-like tubes [149, 151]. *In vivo*, sunitinib treatment prevents new blood vessel formation in the tumor vascular window model, and slows progression of tumor growth [154]. On the other hand, receptors such as c-

KIT, FLT3, and RET are also relevant in signal transduction and growth of solid tumors, including GISTs, small-cell lung cancer, thyroid carcinoma and hematologic malignancies such as lymphomas and leukemias [151, 155]. Preclinical data have shown that sunitinib antitumor activity may result also from a direct antiproliferative effect on tumor cells [149].

Sunitinib is primarily metabolized by cytochrome P450 3A4 to SU12662, an active N-desethyl metabolite. SU12662 is also metabolized by cytochrome P450 3A4. The combination of sunitinib and SU12662 represents the total active drug in plasma [156].

Sunitinib has been evaluated in the clinic as an antiangiogenic agent in several types of cancer [157, 158] but its higher activity has been detected in patients with metastatic clear-cell renal carcinoma (RCC) [159, 160] and with imatinib resistant or intolerant GIST patient [161, 162].

The pathogenesis of clear-cell RCC is associated with the loss of function of the von Hippel-Lindau (VHL) gene [163]. Inactivation of both VHL alleles *via* mutation or promoter hypermethylation is present in more than 60% of sporadic, non-inherited clear-cell RCC and in RCC secondary to the VHL syndrome, an inherited multi-system disorder characterized by vascular tumors including RCC [164]. The VHL gene product forms stable complexes with other proteins, such as elongin C/B, Cullin2 and ring box1 and targets the α -subunit of HIF-1 α and HIF-2 α for ubiquitin- and proteasome-mediated degradation [5, 165]. Under hypoxic status or when the VHL gene is mutated, HIF overaccumulates in the cell, thus promoting increased transcription of several pro-angiogenic factors such as VEGF, PDGF, TGF α , Glucose transporter-1 and EGFR. Understanding the VHL gene pathways has allowed development of targeted approaches in the treatment of patients with advanced RCC, i. e. with the use of drugs such as sunitinib and sorafenib targeting the VEGF and PDGF receptors.

A multicenter randomized phase III study comparing sunitinib with IFN- α in front-line therapy of patients with untreated metastatic clear-cell RCC defined the clinical activity of the drug in this setting [160]. Sunitinib treatment resulted in a response rate of 31% vs. 6% ($p < 0.001$) and a median PFS of 11 months (95% CI: 10–12) against the 5 months (95% CI: 4–6) in the IFN- α group (HR: 0.42; 95% CI: 0.32–0.54; $p < 0.001$). These data allowed to recommend sunitinib as the new reference standard for the first-line treatment of metastatic RCC. Treatment with sunitinib has recently been associated with a significant three-fold increase in the risk of arterial thromboembolic events (ATEs) with no differences between RCC and non-RCC patients, warranting a careful, continued surveillance and prompt reporting specially in high risk patients [166]. The role of sunitinib in the adjuvant setting is currently under evaluation in two large phase III studies (S-TRAC and ASSURE) in high-risk patients.

The majority of adult GIST cases result from activating mutations of the KIT exon 11 gene, which encodes for the KIT RTK (> 80%) or of PDGFR α (5-7%) [167, 168]. The remaining 10-15% of wild type GISTs have no known molecular alterations even if it has been recently suggested

other molecular alterations may be responsible, such as mutations in the neurofibromatosis type 1 gene, mutations in BRAF or amplification of the IGF-1R [169, 170]. GISTs harbouring KIT exon 11 mutations achieve the best responses with TKI imatinib [171], a drug that targets the ATP binding site of KIT and PDGFRA thus blocking signaling pathway and cell growth (see below) while the KIT exon 9-activating mutations predict resistance to imatinib therapy [172]. However, about 12-14% of patients have primary resistance and about 2-3 years after commencing treatment, half of the patients show secondary resistance [173]. Following progression during treatment with the imatinib at the standard dose of 400 mg/day, it is advised to increase the imatinib dose to 800 mg/day. The higher dose of 800 mg/day is also recommended for patients with KIT exon 9-activating mutations. In case of further progression or intolerance to imatinib, second line therapy with sunitinib is now the treatment of choice [161, 174]. In this setting, patients treated with sunitinib have a response rate of 7%, with a median PFS of about 6.3 months versus 1.5 months for patients receiving placebo [161]. Of interest, sunitinib was not as effective in patients with secondary KIT exon 17 or 18 mutations (which encode the activation loop) [162]. Genotype testing, when available, could be an aid to select the most appropriate first-line therapy for individual patients [162].

Sunitinib has received regulatory approval in 2006 by the FDA and by EMEA for the treatment of advanced renal cell carcinoma and imatinib-resistant or imatinib-intolerant GISTs and it is actually in phase III development across multiple tumor types.

Sorafenib

Sorafenib tosylate (Nexavar[®]; BAY 43-9006) is a biaryl urea, orally available, multikinase inhibitor. Sorafenib was originally developed as an inhibitor of the mutant B-RAF and C-RAF kinases [175], members of the MAPK signaling pathway [176] and later, after the hypertensive effect observed in clinical trials, sorafenib has been shown to block VEGFR-2 and VEGFR-3, PDGFR- β , FLT3, RET, c-KIT, the serine/threonine kinases c-RAF and both wild-type and mutant^{V599E} BRAF [175, 177, 178].

Sorafenib inhibits *in vitro* tumor cell proliferation at a concentration within plasma levels shown to be active in clinical trials [179] and blocks *in vivo* the growth of human tumor xenografts including colon, lung, breast, ovarian, pancreatic, thyroid and melanoma cancers in a dose-dependent manner [175]. The growth inhibiting activity of sorafenib has been related to a direct antitumor effect and to its antiangiogenic properties depending on the tumor type, even if it is difficult to determine the relative contribution to the sorafenib clinical efficacy [175].

In the multicenter, double-blind, randomized, phase III TARGET trial, sorafenib extended PFS from 2.8 to 5.5 months (HR: 0.44; 95% CI: 0.35–0.55; $p < 0.01$) in patients who failed on cytokine therapy as compared with placebo [180]. However, partial responses were reported in only 10% of patients treated with sorafenib but many patients had stable disease. Even if the trial did not show an OS gain in the sorafenib arm at the first preplanned interim analysis, pa-

tients still on placebo were allowed to cross over to sorafenib. Interestingly, a preplanned secondary analysis censoring patients crossing over from placebo to sorafenib, treatment with sorafenib led to a survival advantage, suggesting an important cross-over effect [181]. Data from this trial allowed FDA approval in 2005 of the drug as second line treatment, in cytokine-refractory advanced RCC patients. A recently published randomized phase II trial failed to show a PFS advantage of sorafenib vs IFN- α -2a in patients with untreated RCC [182].

Liver cancer is highly resistant to cytotoxic chemotherapy and death rates for are increasing worldwide [28]. Before sorafenib, there was no standard treatment for patients with non-resectable hepatocellular carcinoma (HCC). Hepatocarcinogenesis is a complex event, which has been mainly related to angiogenesis [183] and to the activation of the MAPK signaling pathway [184]. Upregulation of the MAPK pathway and overexpression of RAS has been reported in preneoplastic lesions and HCC cells [185]. HCC is a hypervascularized tumor and VEGF increases both HCC development and metastases in preclinical models [186]. Moreover, in animal models, overexpression of PDGFR- β , a pro-angiogenic factor [187], on endothelial cells of HCC tumors is associated with a highly metastatic phenotype [188]. In HCC models, sorafenib has been reported to inhibit cell proliferation, to induce apoptosis and tumor regression by reducing tumor angiogenesis and blocking the MAPK cascade [189].

The international, multicenter, randomized, placebo-controlled, phase III, SHARP trial confirmed these preclinical data suggestions [190]. Previously untreated patients with advanced HCC were randomized to receive either sorafenib or placebo. The median OS was 10.7 months in sorafenib-treated patients vs 7.9 months in placebo patients (HR: 0.69; $p = 0.00058$); time to progression was also significantly improved (median 5.5 vs 2.8 months; HR: 0.58; $p = 0.000007$). These data are confirmed by a recent meta-analysis showing that sorafenib-based therapy is superior to other agent-based therapy in terms of time to disease progression and OS. However, treatment with sorafenib, as shown for sunitinib, has been associated with a significant increase in the risk of ATEs [166].

Sorafenib has been approved by FDA and EMEA for treatment of advanced unresectable HCC and it is the only approved targeted therapy for these patients, thus becoming the new reference standard [191]. The possible efficacy of sorafenib as adjuvant treatment in order to prevent recurrences after resection or local ablation is currently being evaluated in clinical studies [192].

Second Generation MTKIs

Axitinib

Axitinib (AG013736) is a still investigational, second generation, orally available, angiogenesis inhibitor of multiple RTKs including VEGFR-1, VEGFR-2, VEGFR-3, PDGFR and cKIT. It has a relatively high potency against VEGFR-2 with respect to other inhibitors [193, 194]. It has been shown to reduce tumor angiogenesis, vascular permeability, blood flow [195] and tumor growth of a murine

Lewis lung carcinoma model in a dose-dependent manner [193].

In phase I and II trials single-agent axitinib demonstrated anti-tumor activity in multiple cancers, including advanced RCC, NSCLC, melanoma, colorectal and thyroid cancer [183-186]. The drug is administered at a daily dose of 10 mg and the safety profile is acceptable, with secondary side-effects such as diarrhoea, nausea, hypertension and voice modification [196-199].

Cediranib

Cediranib maleate (Recentin[®]; AZD2171) is a second generation, orally available, highly potent inhibitor of VEGFR-2 and, to a lesser degree, of VEGFR-1, VEGFR-3, PDGFR- β , and cKIT [200]. Cediranib has been shown to inhibit VEGF signaling and angiogenesis dependent processes *in vitro* and *in vivo* [200, 201]. Interestingly, cediranib is able to prolong survival in glioblastoma bearing mice by reducing tumor-associated edema with no effect on tumor growth [202]. A similar effect on vascular normalization has been shown in recurrent glioblastoma patients as early as after 1 day after cediranib treatment (as evaluated by magnetic resonance imaging) and this reduction has been reported to be predictive of increased PFS and OS [203].

Cediranib has been tested in phase I and phase II clinical trials at 30-45 mg/day, either as a single agent or in combination with other agents in solid tumors and leukemias, showing a significant antitumor activity and a good tolerability profile, hypertension being the most commonly reported adverse effect [204-209]. Cediranib is currently being evaluated in phase III trials (HORIZON II and HORIZON III) in combination with FOLFOX for the first-line treatment of metastatic colorectal cancer and in a phase II study as second-line treatment (HORIZON I) [210].

Pazopanib

Pazopanib hydrochloride (Armala[®]; GW-786034) is a still investigational, second-generation, oral angiogenesis inhibitor of multiple RTKs such as VEGFR-1, -2 and -3, PDGFR α , PDGFR β and cKIT [211] with interesting antitumor and antiangiogenic activity against a broad range of cancer cell lines and human tumor xenografts [211]. Pazopanib has been selected among a large collection of similar molecules because of its high biochemical potency and cell-based selectivity together with a pharmacokinetic profile supporting a once-daily oral therapy [212]. Preclinical studies have shown that a steady-state plasma concentration of ≥ 40 $\mu\text{mol/L}$ is required for optimal *in vivo* activity, levels required for the inhibition of *in vivo* VEGFR-2 phosphorylation [213]. Pazopanib is generally well tolerated, the most frequent adverse events being nausea, anorexia, and vomiting, hair color change, diarrhea, hypertension and a low incidence of hand-foot syndrome [214].

A recently published, randomized, phase III clinical trial, shows that pazopanib (at the dose of 800 mg once daily) compared with placebo, is able to significantly prolong PFS of patients with advanced RCC (median, PFS 9.2 v 4.2 months; hazard ratio, 0.46; 95% CI, 0.34 to 0.62; $P < .0001$) [214]. Pazopanib is currently under clinical development for other solid tumors, including glioblastoma, gynecologic cancer and sarcomas [215-217].

Pazopanib has been approved by FDA on October 2009 for the treatment of patients with advanced RCC [218].

Vandetanib

Vandetanib (Zactima[®]; ZD6474) is an ATP mimetic small molecule, orally available, with inhibitory activity on different intracellular signaling pathways: VEGFR-2, EGFR and RET [219, 220]. The RET (rearranged during transfection) proto-oncogene encodes a trans-membrane TK for the glial cell line-derived neurotrophic factor family of ligands [221]. RET signaling leads to activation of the Ras/MAPK and the PI3K/Akt pathways, involved in the regulation of cell growth, differentiation, and survival [222]. Activating mutations of RET have been reported in nearly all hereditary cases of medullary thyroid carcinoma and in about one quarter to half of sporadic tumor cases [223]. Recent data show that EGFR might contribute to RET kinase activation, signaling and cell growth stimulation [224], reinforcing the already well known concept that different signal transduction pathways cooperate to promote tumorigenesis [225]. Vandetanib inhibits tumor-induced neovascularization and growth of a variety of cancer cell lines and human tumor models, including lung, colon, liver and medullary thyroid cancer [226].

In the clinical setting, vandetanib is generally well tolerated at 300 mg/day, with a once-daily oral administration schedule [227]. From phase II studies, rash, diarrhea, hypertension and fatigue were the most common and manageable adverse events [226]. Interestingly, vandetanib demonstrates a significant clinical activity in patients with metastatic medullary thyroid carcinoma, a disease lacking effective medical therapy [228, 229]. Clinical activity of vandetanib in NSCLC is currently being tested in two large randomized phase III trials, in combination with docetaxel (ZODIAC trial) and with pemetrexed (ZEAL trial).

Vatalanib

Vatalanib succinate (PTK787/ZK 222584) is an oral angiogenesis inhibitor selected through high-throughput screening, for its ability to block VEGF receptors, PDGFR β and cKIT [230, 231]. It inhibits tumor growth and reduce tumor microvasculature, without impairing wound healing [232]. Vatalanib has also an antiaromatase activity and it is a competitive aromatase inhibitor, of potential interest for estrogen receptor positive breast cancer treatment [233].

From phase I trials, the recommended single agent dose for administration of vatalanib as single agent is 750 mg BID because of its relative short half-life of 3-6 hours [234]. The clinical antitumor activity of vatalanib is currently under evaluation, even if two phase III clinical trials in advanced colorectal carcinoma using vatalanib in combination with chemotherapy showed a non-significant benefit in terms of PFS and OS [235, 236]. The most common toxicities associated with vatalanib administration have been grade 3-4 hypertension, diarrhea, fatigue, nausea and vomiting.

NONRECEPTOR TYROSINE KINASES

NRTKs are a large family of cytosolic proteins that play an important role in regulating cell proliferation, differentiation, metabolism, migration and survival. Nine families of

NRTKs have been identified, which include ABL, Src, Tec, CSK, FAK, SYK, Jak, TnK and FeS for a total of 32 NRTKs [237]. Most NRTKs are localized in the cytoplasm while other NRTKs are anchored to the cell membrane through aminoterminal modification [238]. In addition to a TK domain, NRTKs possess domains that mediate protein-protein, protein-lipid and protein-DNA interactions. NRTKs catalytically transfer a phosphate group from ATP to a tyrosine residue on an effector polypeptide/protein, thus initiating signaling cascades involved in cellular processes such as proliferation and differentiation. As observed for RTKs, the activity of these proteins can be dysregulated due to multiple causes such as genetic mutations or overexpression, leading to abnormal signaling and/or loss of autoregulatory processes. NRTKs have been involved in the pathogenesis of many types of cancer thus becoming an important therapeutic target for the treatment of neoplastic diseases [30, 239].

First Generation Nonreceptor Tyrosine Kinase Inhibitors (NRTKIs)

Imatinib

Imatinib mesilate (Gleevec®/Glivec®; STI571) acts through a competitive inhibition at the ATP-binding site and selectively blocks the BCR-ABL oncoprotein, cKit and PDGFR [240].

The Philadelphia Chromosome (Ph) is the cytogenetic hallmark of chronic myeloid leukemia (CML) [241]. This chromosomal rearrangement, due to the reciprocal translocation of the long arm of chromosomes 9 and 22 [t(9;22)], generates a fusion gene encoding for an oncoprotein, referred to as BCR-ABL, endowed with a constitutively activated TK activity [241]. The BCR-ABL fusion protein may be present in three forms: p190, p210 and p230 depending on the specific breakpoint on the BCR gene fusing with exon a2 of ABL. P190+, p210+, and p230+ leukemias show distinct clinical phenotypes reflecting the different oncogenic potential of the three fusion proteins [242]. In particular, p190 is mostly associated to Ph+ acute lymphoblastic leukemia (ALL) and rarely with CML, while p210 is found in the large majority of CML, and in one third of Ph+ ALL cases [242]. Finally, p230+ leukemias usually show the longest latency period and the less aggressive clinical behavior. Early experimental models allowed to recognize that the presence BCR-ABL alone is sufficient to induce CML and the TK activity of the protein is required for its tumorigenic activity [243]. These data set the stage for the discovery and development of imatinib, the first-in-class BCR-ABL inhibitor, now considered the first-choice treatment option for chronic phase CML [244-246]. In contrast, treatment with imatinib is not so effective for blast crisis patients, suggesting that blastic transformation may occur as a result of a BCR-ABL-independent cell growth.

The BCR-ABL activation of PI3K is essential to leukemic transformation [247] and PI3K inhibiting molecules can block both myeloid and lymphoid leukemic transformation [248]. Activation of PI3K by BCR-ABL is an indirect process *via* the autophosphorylation of the BCR region on the fusion protein [248]. Among the downstream PI3K signaling pathways, Akt plays a key antiapoptotic role, activating a number of proteins that promote cell survival, including

FoxO, Bad, and glycogen synthase kinase 3 β [249]. Another pathway activated by BCR-ABL is the Jak/STAT one. Indeed, BCR-ABL constitutively activates STAT5 and, to a lesser extent, STAT3 and STAT1 thus circumventing cytokine or growth factor dependent dimerization of STAT5 [250]. This pathway results in increased expression of the Bcl-2 family members MCL-1 and BCL-xL, as well as of cyclin D1, thereby facilitating the G1-to-S phase cell cycle progression [251, 252]. It has been experimentally observed that BCR-ABL-transformed 32D cells can proliferate in low serum conditions [253] and the induction of BCR-ABL expression is sufficient to initiate cell cycle progression in cells arrested due to culture in low serum conditions [254]. This phenomenon is associated with BCR-ABL-dependent activation of CDK2, a kinase required for G1/S progression, which can be attributed in part to reduced nuclear accumulation of the CDK inhibitor p27^{kip1} [255]. Growth inhibition by imatinib is selective for BCR-ABL+ hematopoietic cells and does not affect normal cells [244].

The very high response rates and good safety profile in clinical trials made imatinib the gold standard treatment for previously untreated CML patients in chronic phase [245, 246]. Indeed, the six-year follow up of the phase III, randomized, open-label IRIS trial of imatinib vs. interferon plus low-dose cytarabine for newly diagnosed chronic-phase CML patients, showed that nearly two thirds of patients receiving imatinib 400 mg daily were still on treatment, almost all of them being in stable complete cytogenetic remission (CCyR) with an estimated OS of 88% [256].

Criteria to establish failure and suboptimal and optimal responses to imatinib have been recently defined as well [257]. Imatinib dose escalation can be an option for patients who achieve a suboptimal response, whereas those with imatinib treatment failure should be switched to a second-generation TKI, such as nilotinib or dasatinib (see below) [257-259]. However there are cases of true drug resistance. The predominant mechanisms of resistance to imatinib include: BCR-ABL kinase domain mutations, BCR-ABL oncoprotein overexpression and clonal evolution with activation of additional transformation pathways [260]. The most studied resistance mechanisms are point mutations within the kinase domain of the BCR-ABL fusion protein. BCR-ABL mutations are found in 40-90% of imatinib-resistant patients according to the CML phase and method of detection [261]. Depending on the gene fragment involved, mutations can act by disrupting key drug-target contact points or by inducing a protein conformational changes that precludes imatinib binding. Currently, more than 100 different BCR/ABL mutations have been identified, the most "dangerous" of which occur at the ATP binding site of the protein, known as phosphate-binding loop [261]. Moreover, patients with mutations have been reported to have inferior progression-free survival compared to those without mutations [262].

In addition to the inhibitory effect on BCR-ABL, imatinib is also able to block other RTKs including KIT and PDGFR α [263]. This finding has provided rationale to explore the clinical activity of this agent in tumors in which these target molecules are known to play a critical pathogenic role.

GISTs are among the best examples of fruitful application of the TK inhibiting strategy to a solid tumor. The rate of relapse after radical surgery is from 40% to more than 80%. Median survival associated with unresectable or metastatic disease in the pre-imatinib era was 9 to 20 months with any therapeutic approach [264]. For the pathogenetic mechanisms, see above in the sunitinib paragraph. Initial clinical experiences with imatinib in patients with advanced GIST have shown high overall response rates and improvement in quality of life as well as increased PFS and OS [245]. The recommended starting dose of imatinib is 400 mg daily, although phase III dose-randomized trials suggested that a higher dose (600 or 800 mg daily) could be of benefit for patients with metastatic or progressing disease [265]. Mutational status of KIT or PDGFR α correlates with clinical outcome: exon 11 mutated patients have a statistically significant PFS advantage compared to exon 9 mutated or unmutated (wild type allele) patients [168, 266, 267].

Several mechanisms of imatinib sensitivity and resistance have been proposed [268], including acquisition of a new KIT or PDGFR α point mutations, KIT amplification with subsequent protein overexpression and KIT activation outside the juxtamembrane region. One further mechanism of imatinib resistance consists in an unbalanced expression of intra- and extracellular drug transporters (i.e. influx and efflux drug pumps) [269]. Some of these resistant patients may still respond to the imatinib dosage increase, while for “truly” imatinib-refractory patients, novel agents are being considered, including new MTKI such as sunitinib (see above), or molecules targeting downstream of KIT or PDGFR α such as the mammalian target of rapamycin-inhibitor everolimus [270].

Activating mutations of KIT or PDGFR α genes play a central role in the pathogenesis of other neoplastic diseases including myelodysplastic/myeloproliferative neoplasms (MDS/MPN), systemic mastocytosis (SM), hypereosinophilic syndrome (HES) or chronic eosinophilic leukemia and dermatofibrosarcoma protuberans (DFSP).

Molecular pathogenesis of MDS/MPNs has largely remained elusive until it was noticed that some patients displayed reciprocal chromosomal translocations involving regions 4q12, 5q31-33, 8p11, 9p24 and 13q12, where candidate TK-encoding genes [namely PDGFR α , PDGFR β , Fibroblast Growth Factor Receptor 1 (FGFR1), JAK2 and FLT3] are mapped. Such chromosomal aberrations result in fusion genes where partners of PDGFR β encode for proteins able to induce its dimerization or oligomerization, thus mimicking the physiological mechanism of activation [271]. Small series and case reports have reported encouraging results with the use of imatinib in PDGFR β -positive MDS/MPN patients, in some cases showing remarkable CCyR rates and durable remissions with continuous treatment [272].

A clonal rearrangement involving the PDGFR α gene which fuses with FIP1L1 to originate the FIP1L1-PDGFR α chimeric sequence has been reported in about 20% of HES patients [273, 274]. Treatment of FIP1L1-PDGFR α positive HES patients with imatinib has resulted in impressive reduction in fusion transcript levels [275], as well as remission of lung, spleen, liver, soft tissue, but not cardiac abnormalities

[276]. Notably imatinib suppresses but not eliminates the neoplastic clone, as indicated by rapid rise of FIP1L1-PDGFR α levels upon treatment withdrawal. This phenomenon suggests that optimal initial dosing is a major determinant of therapeutic success and should be tailored according to FIP1L1-PDGFR α molecular monitoring. A number of new TKIs, including nilotinib, dasatinib and sorafenib are presently tested in clinical trials and might be useful in the treatment of imatinib-resistant PDGFR α -positive patients [276, 277].

SM, characterized by the accumulation of mature-appearing mast cells (MC) in several tissues, may be associated with aggressive clinical course and shortened patient survival [278]. The recent identification of KIT-D816V mutations in the large majority of these patients prompted research for new molecularly-targeted therapies directed at specifically inhibiting KIT-mediated signaling. It has been shown that imatinib can block wild type-KIT *in vitro* [279] and suppress the growth of certain KIT-mutated cell lines [280]. Imatinib-sensitive mutations include the V560G, the F522C, and the K509I, while D816V-positive cells, due to conformational protein changes that prevent imatinib binding, are resistant to this agent [280]. The Spanish Network on Mastocytosis, REMA, has recommended imatinib treatment for: *i*) aggressive SM or MC leukemia KIT-D816V mutation negative; *ii*) patient carrying the juxtamembrane region mutation and with aggressive disease; *iii*) aggressive SM associated with a clonal hematopoietic non-MC disorder [281, 282].

DFSP is a rare sarcoma involving the dermis and underlying soft tissues. A characteristic cytogenetic aberration, the t(17;22) (22;q13), found in up to 90% of cases [283], determines the fusion of collagen type 1 alpha 1 (COL1A1) and PDGFR β genes with generation of the COL1A1-PDGFR β chimeric sequence. PDGFR β undergoes autocrine stimulation with subsequent increased proliferation of DFSP cells [284]. The discovery of COL1A1-PDGFR β as a constant finding in DFSP has provided rationale for the use of imatinib in these patients. Clinical experience with imatinib in DFSP has demonstrated that 50% of patients with recurrent or metastatic disease harboring t(17;22) can achieve objective responses while lack of FISH and/or RT-PCR-detectable fusion gene is associated resistance to therapy [285, 286]. However, several issues regarding the optimal positioning of the drug, the duration of treatment or the monitoring of response, still need to be addressed.

In light of all above mentioned considerations, the first registered TKI, imatinib, is currently approved by the FDA and EMEA for the treatment of Ph⁺ CML, cKIT-positive GIST and for rare diseases such as MDS/MPN-associated with PDGFR gene rearrangements, SM, HES or chronic eosinophilic leukemia, recurrent and/or metastatic DFSP.

Second Generation NRTKIs

Dasatinib

Dasatinib (Sprycel[®]; BMS-354825) is an orally available, multi-kinase inhibitor showing an *in vitro* potency against unmutated BCR-ABL which is 325 times greater compared to that of imatinib [287]. It also inhibits most known BCR-

ABL mutants with the remarkable exception of the T315I [287]. Additional targets of dasatinib include PDGFR, cKIT, SRC family kinases (SFK), FGFR1, and EGFR [288]. The ability of dasatinib to inhibit other TKs, including SFKs such as Lyn, is of particular clinical relevance since these molecules may play an important role in the development of resistance to imatinib [289, 290]. The SFK family is the largest family of NRTKs including nine members [291] and its aberrant activity has been widely implicated in the development and progression of human cancer, thus representing a new attractive target for potential novel anticancer therapies [292]. Treatment with dasatinib at a dose of 70 mg twice daily results in hematologic, cytogenetic and molecular responses in all CML phases in both imatinib-intolerant and -resistant patients [259, 293, 294]. Dasatinib is generally well tolerated and main toxicities include pleural effusions and myelosuppression [293, 294]. Dasatinib is currently being tested either alone or in combinations with other agents in a variety of solid tumors [295, 296].

Dasatinib has recently been approved by the FDA and EMEA for the treatment of patients with CML (chronic, accelerated, or myeloid or lymphoid blast phases), with resistance or intolerance to prior therapy including imatinib and for Ph⁺ acute lymphoblastic leukemia with resistance or intolerance to prior therapy.

Nilotinib

Nilotinib (Tasigna[®]; AMN-107) is an orally available, second generation TKI characterized by a 20- to 50-fold enhanced selectivity and potency for BCR-ABL inhibition compared to imatinib, the parental analog [297-299]. As observed for dasatinib, nilotinib exhibits inhibitory activity against a wide range of mutant BCR-ABL kinases with the exception of the T315I mutation [297-299]. Nilotinib is also active against c-KIT, PDGFR, and ephrin receptor kinase [300].

In phase I and II clinical trials, nilotinib at a dose of 400 mg BID produced cytogenetic and hematologic as well as molecular responses across all phases of CML [259, 301, 302]. Nilotinib treatment is generally well tolerated and the associated toxic effects may include myelosuppression, skin rashes and biochemical abnormalities such as lipase, transaminase and bilirubin elevations [303]. QT interval prolongation, of initial concern, is usually minimal and clinically irrelevant [301, 304]. Recent data have shown an early and potent therapeutic effects of nilotinib, 400 mg twice daily, in previously untreated, early chronic phase Ph⁺ CML patients supporting a role for nilotinib for the frontline treatment of CML [305]. Nilotinib has been approved in 2007 by the FDA and EMEA for the treatment of adult patients with Ph⁺ CML resistant to or intolerant of prior treatment that included imatinib. Results from the ENESTnd (Evaluating Nilotinib Efficacy and Safety in Clinical Trials-Newly Diagnosed patients) trial, a phase III multicenter study comparing the efficacy and safety of nilotinib at two separate dosages, namely 300 mg BID and 400 mg BID, versus imatinib in patients with newly diagnosed CML in chronic phase, have been presented at the 2009 American Society of Hematology meeting. Nilotinib has shown clear superiority over imatinib in terms of molecular response rates at both the 6- and 12-month timepoint (33 and 30 vs. 12% and 44 and 43 vs. 22%

for nilotinib 300 and 400 mg BID and imatinib, respectively)¹.

CONCLUDING REMARKS

In the early '90s, impressive clinical activity of the first targeted therapies, such as trastuzumab and imatinib, prompted worldwide scientists to claim that the "magic bullet" against cancer was finally available. Today we cannot say that the prediction has come true; many "battles" are won but not yet the "war". A great number of new drugs developed against several specific targets, all of them in some way related to cancer development and progression, are currently available, either registered or in clinical trials. Among them, TKIs have a primary role. As reported in this review, clinical studies conducted in the recent years confirmed that most TKIs are safe and therapeutically active drugs for selected cancer histologies, and many of them are now part of the standard treatment regimen for specific tumor types. However basic and clinical research has clearly assessed that most tumors have complex multifactorial pathogeneses and are frequently linked to defects in more than one signaling cascade, with interferences among receptors within one as well as distinct pathways regulating cell proliferation and survival [306]. From a clinical point of view, this means that almost all cancer patients who initially respond to treatment, will ultimately develop resistance. Overcoming primary and acquired resistance to targeted therapies is the major goal of the 21st Century Oncology.

ABBREVIATIONS

ALL	=	acute lymphoblastic leukemia
ATE	=	arterial thromboembolic event
ATP	=	adenosine triphosphate
CCyR	=	complete cytogenetic remission
C-lobe	=	C-terminal lobes
CML	=	chronic myeloid leukemia
COL1A1	=	collagen type 1 alpha 1
DFS	=	disease-free survival
DFSP	=	dermatofibrosarcoma protuberans
ECD	=	extracellular highly glycosylated ligand binding domain
EGF	=	Epidermal growth factor
EGFR	=	EGF Receptor
EMEA	=	European Medicines Agency
ErbB1	=	EGF Receptor 1
ErbB2	=	EGF Receptor 2
ErbB3	=	EGF Receptor 3

¹Saglio, G.; Kim, D. W.; Issaragrisil, S.; leCoutre, P. D.; Reiffers, J.; Lobo, C.; Pasquini, R.; Clark, R. E.; Hughes, T. P.; Hochhaus, A.; Gallagher, N. J.; Hoenekopp, A.; Dong, M.; Haque, A.; Kantarjian, H. M.; Larson, R. A. Nilotinib Demonstrates Superior Efficacy Compared with Imatinib in Patients with Newly Diagnosed Chronic Myeloid Leukemia in Chronic Phase: Results From the International Randomized Phase III ENESTnd Trial. *Blood*, **2009**, *114*, LBA-1.

ErbB4	=	EGF Receptor 4
FDA	=	U.S. Food and Drug Administration
FGF	=	Fibroblast growth factor
FGFR1	=	Fibroblast Growth Factor Receptor 1
GISTs	=	gastrointestinal stromal tumors
HER1	=	EGF Receptor 1
HER2	=	EGF Receptor 2
HER2/neu	=	EGF Receptor 2
HER3	=	EGF Receptor 3
HER4	=	EGF Receptor 4
HES	=	hypereosinophilic syndrome
HGF	=	hepatocyte growth factor
HIF	=	hypoxia inducible factor
HCC	=	hepatocellular carcinoma
IGF-1R	=	IGF-1 receptor
mAbs	=	monoclonal antibodies
MAPK	=	mitogen activated protein kinase
MDS/MPN	=	myelodysplastic/myeloproliferative plasmas
MET	=	mesenchymal-epithelial transition factor
MTKIs	=	multitarget tyrosine kinase inhibitors
NF- κ B	=	nuclear factor- κ B
N-lobe	=	N-terminal lobes
NRTKs	=	nonreceptor tyrosine kinases
NSCLC	=	non small cell lung cancer
OS	=	overall survival
p95 ^{ErbB2}	=	amino terminally truncated carboxyl terminal fragments of HER2
PDGF	=	platelet derived growth factor
PDGFR	=	PDGF receptor
PFS	=	progression free survival
Ph	=	Philadelphia chromosome
PI3K	=	phosphatidylinositol-3-kinase
PLC γ	=	phospholipase C gamma
PIGF	=	placenta growth factor
PTEN	=	tumor suppressor phosphatase and tensin homolog
PTKs	=	protein tyrosine kinases
RCC	=	clear-cell renal carcinoma
RET	=	rearranged during transfection
RTKs	=	receptor tyrosine kinases
SFK	=	SRC family kinases
SM	=	systemic mastocytosis
SOS	=	Son of Sevenless

STAT	=	Signal transducer and activator of transcription
STAT	=	activator of transcription
T790M	=	threonine-790 to methionine
TGF- α	=	transforming growth factor- α
TKIs	=	tyrosine kinase inhibitors
VEGF	=	Vascular endothelial growth factor
VEGFR	=	VEGF-Receptor
VHL	=	von Hippel-Lindau gene

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