GFR tyrosine kinases inhibitors in cancer treatment: in vitro and in vivo evidence

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1. ABSTRACT

The increasing understanding of the molecular mechanisms of neoplastic transformation and progression has prompted the search for novel drugs that could interfere with the intracellular targets involved in this process. EGFR is implicated in the development and progression of the majority of the common human epithelial cancer; therefore different agents have been developed to block EGFR activation in cancer cells. This review focuses on EGFR-tyrosine kinase inhibitors in clinical practice that interfere with ATP binding, inhibiting tyrosine kinase activity and subsequently blocking signal transduction from EGFR. We report current knowledge on molecular mechanisms underlying the anticancer activity of EGFR-tyrosine kinase inhibitors in preclinical models, with particular attention to EGFR downstream effectors responsible for treatment efficacy or resistance.

2. INTRODUCTION

The EGFR (ErbB1, HER1) is the prototypic member of the ErbB family of receptor tyrosine kinase (RTKs), which further consists of ErbB2-4 (HER2- 4) (1). The ErbB receptors share a similar protein structure, consisting of: i) an extracellular domain involved in recognizing and binding ligands; ii) a membrane spanning sequence involved in the interaction between receptors and iii) an intracellular C-terminal domain with tyrosine kinase activity (2, 3).

Upon autophosphorylation, the EGF receptor undergoes a conformational change to a more extended form which removes an inhibitory constraint and allows the recruitment of SH2-containing signaling proteins involved in signal transduction (4).

Crystallographic studies demonstrated that the EGFR kinase domain adopts the bilobate-fold characteristic
of all protein kinase domains. The NH2-terminal lobe (N-lobe) and the COOH terminal lobe (C-lobe) are separated by a cleft similar to those in which ATP, ATP analogues, and ATP-competitive inhibitors bind to. The two lobes form an opening angle between them, whose width differs depending on the presence or absence of ATP or a close analogue.

An important element of the catalytic machinery, bordering the cleft, is the activation loop (A-loop) that assumes its catalytically competent conformation only after phosphorylation (5). A part of the A-loop is the DFG motif of kinases directly involved in coordinating ATP binding (6).

Protein kinases exhibit similar conformations once activated. However they show several differences when being in the inactive state. In fact, these are characterised by (i) a different orientation of the two lobes that reflects flexibility about the hinge region in the kinase fold; (ii) different orientations of the C-helix, which creates an extra pocket for inhibitor groups in many inactive states; (iii) displacement of the activation segment, which is disordered in many inactive kinases; and (iv) a different conformation of the DFG motif that has been identified as a switch between active and inactive kinase conformations (7).

The inhibitors of EGFR tyrosine kinase activity consist of small molecules (TKIs and monoclonal antibodies (MoAbs), (8).

Nowadays, three small-molecules EGFR tyrosine kinase inhibitors (TKIs) are currently used in clinical practice for anti-tumor treatment. They have been synthesized, based on a 4-anilinoquinazoline scaffold; the quinazoline ring is hydrogen bonded to the hinge region between the NH2- and COOH terminal lobes of the EGFR kinase domain (6). The ATP binding site of the kinase can be bound in its active form by gefitinib (Iressa) and by erlotinib (Tarceva) or in its inactive conformation by lapatinib (Tykerb) (7).

The inhibition of the EGFR tyrosine kinase activity results in the blockade of the activation of signal transduction pathways that mediate EGFR functions (2). This review aims to provide an evidence-based update of in vitro and in vivo characterization of TKIs activity pointing out the molecular pathways that are activated and required for treatment efficacy in several tumor study models.

At least five different cell signaling pathways are known to be responsible for TKIs response, including that of the mitogen-activated protein kinase (MAPK), the phospholipase C, the phosphatidylinositol 3-kinases (PI3K) / Akt, the JAK / STAT, and the SRC / FAK.

The MAPK signaling pathway is mainly involved in the control of cell proliferation, differentiation, survival and migration and the phospholipase C signaling pathway in the regulation of tumor invasion and migration. The PI3K/Akt signaling pathway controls a variety of critical cell functions including glucose metabolism, cell proliferation and survival, the STAT signaling pathway the cell proliferation, cell survival, angiogenesis, and immune system evasion and finally, the SRC / FAK signaling pathway promotes tumor angiogenesis, through the vascular endothelial growth factor (VEGF) (2).

3. GEFITINIB

3.1 Mechanism of action

Gefitinib (‘Iressa’, ZD1839) is an orally-active inhibitor of EGFR tyrosine kinase activity (9). It binds to the active conformation of the EGFR kinase at the ATP binding site in a conformation similar to that observed for quinazolines binding to CDK2 and p38 MAP kinases (7). Gefitinib [4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholino propoxy) quinazoline] has small aniline substituent off the quinazoline ring, and exhibits relatively rapid off-rates, that may reflect the fact that the inhibitor binds and dissociates from the enzyme, in its active form, without requiring changes in protein conformation (6).

3.2. Biological properties

3.2.1. Inhibition of cell cycle progression and induction of apoptosis

As a consequence of receptor inhibition, gefitinib has been shown to mainly affect cell survival and growth signaling pathways such as the extracellular signal-regulated kinase (Erk1/2) and the PI3K/Akt pathway (10–16). However, the downstream EGFR intracellular signal transducers involved have not yet been elucidated (17).

It has been demonstrated that gefitinib treatment resulted in cytotoxic effect in EGFR overexpressing cells such as A431 vulval squamous carcinoma cell line. After treatment with gefitinib, an increase of the portion of A431 cells in the G1-G0 phase of the cell cycle was observed with a corresponding decrease of cells in S and G2-M phases. Moreover, a significant gefitinib-dependent increase in the sub-G1 cell population was detected, which represents apoptotic cells thus, suggesting that apoptosis contributes to the cytotoxic effect of gefitinib in A431 cells. The activation status of the MEK/Erk and PI3K/Akt pathways has been investigated after treatment with gefitinib in A431 cell line. These are the two major intracellular signaling pathways actively blocked in this cell line by gefitinib. Consistently, in addition to decreased Erk and Akt phosphorylations status, in treated cells, also a sub-sequent decrease of p90PK and GSK3-beta phosphorylation level was observed as a consequence that both kinase pathways were blocked by gefitinib (18).

Other studies conducted with quinazoline inhibitors of EGFR tyrosine kinase activity in A431 cells have shown a cell-cycle accumulation in G1 phase, a dose-dependent up-regulation of p27kip1 and a hypophosphorylation of the retinoblastoma protein (19).

Conversely, gefitinib treatment doesn’t change cell cycle distribution in non-small cell lung cancer (NSCLC) cell lines and this could be correlated with the limited antiproliferative effects observed in this cancer models. Moreover, one or both pathways analyzed remain
active upon gefitinib treatment and contribute to the resistance of this cells to anti-EGFR treatment. The induction of apoptosis was observed only when both pathways were blocked and cell sensitivity to gefitinib is closely correlated with Erk1/2 and Akt activation status EGFR-dependent (18,20).

It has been demonstrated that the mechanisms by which gefitinib induces apoptosis in mammary cells involve the activation of the proapoptotic Bcl-2 family member BAD and of the caspases (18).

Engelman et al. reported a link between the Akt pathway and gefitinib responsiveness. The Akt pathway is down-regulated in response to gefitinib only in NSCLC cell lines that are growth-inhibited by this drug (21).

Erk1/2 and Akt are significantly inhibited also in gefitinib-treated human gallbladder adenocarcinoma cells (HAG-1), in which the drug causes growth inhibition by arresting the cells in the G0/G1 phase and increasing the amount of apoptotic cells. This arrest in G0/G1 phase is associated to decreased cyclin D1 mRNA expression as well as to p27 protein accumulation. Apoptosis may be due to the accumulation of Bax, consequent to the simultaneous inhibition of Erk1/2 and Akt activity by gefitinib (17).

Matheny et al. demonstrated that phosphorylated MAPks level was decreased and p27KIP1 level increased after gefitinib treatment in head and neck squamous carcinoma SCC-012 cells. They reported an increase in G1-phase and a decrease in S-phase accumulation of these cells along with a dose-dependent decrease in p63, at protein and messenger RNA levels, induced by gefitinib treatment. This is consistent with growth arrest observed and suggests that p63 is a downstream target of EGFR signaling in this tumor model (22).

Recent studies have shown that gefitinib inhibits the proliferation of pancreatic cancer cells because it induces a delay in cell cycle progression provoking the arrest in G0/G1 and in G2/M phase. These results were associated with a combined double effect: increased expression of p27KIP1 and decreased expression of aurora B, down-regulated by gefitinib (23).

Lee et al. demonstrated that gefitinib induces a delay in cell cycle progression and a G1 arrest also in oral squamous carcinomas (OSCCs) cell line. They found that this effect was associated with increased expression level of the cyclin-dependent kinase inhibitors (CDK1) p27KIP1 and p21CIP1/WAF1 and this up-regulation could be mediated by a p53-independent pathway (24).

When human immortalized keratinocyte HaCaT cells were incubated with EGF and gefitinib, a reduction of EGFR and MAPK phosphorylation in a dose-dependent manner is observed; moreover, this drug up-regulates p15INK4b which results in RB hypophosphorylation and consequent G1 arrest (25,26).

The antitumor activity of gefitinib does not depend on the expression level of EGFR. In fact, expression of the target receptor is not a marker of sensitivity to EGFR-TKIs: either cell lines with high or low levels of EGFR expression can respond to gefitinib treatment or, conversely, can be resistant to this drug (2).

Campiglio et al. analyzed the ability of gefitinib to induce apoptosis or inhibition of proliferation in six human breast cancer cell lines expressing various levels of EGFR and HER2 and to affect the activation status of Akt and MAPKs. They showed that the drug effects were independent of EGFR expression levels, but were influenced by high HER2 expression. Moreover, they didn’t find any correlation between the cytostatic/cytotoxic effects of gefitinib and its ability to down-regulate MAPK and Akt activity in the tumor cell lines; they supposed that the antitumor activity of gefitinib is due to a cytostatic effect, and involves apoptosis only in sensitive cells (27).

Others studies investigated the effects of gefitinib in vivo demonstrating that it caused growth inhibition in human tumour xenografts not depending on high levels of EGFR expression (28).

Gefitinib is a potent inhibitor of proliferation also in ErbB-2 overexpressing cells (29). Anderson et al. investigated the effects of gefitinib, in vitro and in vivo, on the growth of human cancer cell lines expressing various levels of EGFR and ErbB-2. They found that gefitinib in vitro potently inhibits the proliferation of A431 and MDA-MB-231, EGFR overexpressing cells; it blocks autophosphorylation of EGFR and prevents activation of PLC-gamma1, Erk MAP kinases and PKB/Akt by EGF. It also inhibits proliferation in EGFR cancer cell lines overexpressing ErbB-2 (SKBr3, SKOV3, BT474). This effect is correlated with inhibition of EGFR-dependent ErbB-2 phosphorylation and activation of Erk MAP kinase and PKB/Akt in SKOV3 cells (29).

In in vivo experiments, MDA-MB-231 and SKOV3 cells, respectively as models of EGFR and ErbB-2 overexpression, were injected into athymic nude mice and xenografts were treated with oral administration of gefitinib. The drug inhibited growth of tumour, reduced proliferation but didn’t cause any change in apoptotic index (29).

Anido et al. demonstrated that gefitinib inhibited the growth of breast cancer cell lines with high levels of EGFR expression and the drug was equally potent in inhibiting the growth of BT-474 and SK-BR-3 cells, with high levels of HER2 and low EGFR levels, respectively. This is consistent with a possible gefitinib double complementary mechanism of action against ErbB receptors: a direct inhibition of EGFR TK and sequestration of HER2 and HER3 receptors in an inactive heterodimer configuration with the EGFR (EGFR/HER2 and EGFR/HER3 heterodimers), which ultimately causes reduction of HER2/HER3 levels. In fact, gefitinib induces conformational changes in the EGFR that modify its ability to form dimers with other members of ErbB receptors family (2,30).
Finally, a relationship between gefitinib and vandetanib, another VEGFR/EGFR TKI, and multi-drug resistance (MDR) transporters was demonstrated, through a series of direct and indirect experimental evidences which showed that TKIs appeared to act as breast cancer resistance protein (BCRP) substrates (31). These data suggested that the interaction of these agents with MDR proteins may be an important determinant of drug resistance, particularly when these agents are combined with certain chemotherapeutic agents, like camptothecins (31).

3.2.2. Inhibition of angiogenesis, invasion and metastatization

Activation of epidermal growth factor receptors and intracellular growth factor-activated signal transduction pathways control not only cancer cell proliferation but also several processes important for tumor progression such as invasion, angiogenesis, and metastasis formation.

Ciardiello et al. investigated the antiangiogenic activity of gefitinib in human colon (GEO, SW480, and CaCo2), breast (ZR-75-1 and MCF-7 ADR), ovarian (OVCAR-3), and gastric (KATO III and N87) cancer cells that coexpress TGF-alfa and EGFR. They found that this treatment determines a dose- and time-dependent growth inhibition and a decrease in the production of VEGF, bFGF and TGF-alfa in vitro, which are angiogenic growth factors for endothelial cells.

This antiangiogenic activity may contribute to the antiproliferative and antitumor effect of gefitinib treatment in vivo. The same authors extended their studies in nude mice bearing human GEO colon cancer xenografts evidencing that gefitinib treatment produces a dose-dependent cytostatic inhibition of GEO tumor growth, a reduction of TGF-alfa, bFGF, and VEGF expression in cancer cells and of neoangiogenesis, as determined by microvessel count. Moreover, this effect can be potentiated by the combined treatment with other cytotoxic drugs (32).

In in vivo studies showed a complete regressions of A431-derived tumors in mice treated with gefitinib. This strong antitumor activity compared with the limited antiproliferative effects in vitro may be explained with the antiangiogenic activity displayed by this drug (18).

Moreover, it was demonstrated that gefitinib completely blocks EGF-induced neovascularization in an avascular area of the mouse cornea. This effect could be mediated directly by blocking EGF-induced neovascularization and also indirectly by the inhibition of VEGF or interleukin-8 production, that are mediated through PI3K/Akt pathway (20).

Several studies showed that gefitinib blocks EGF-induced cytoskeleton remodeling, cell growth, and in vitro invasiveness of cancer cells. Kedrin et al. demonstrated that this drug blocks lamellipodia extension, chemotaxis and invasion of MTLn3E cells, a highly metastatic mammary adenocarcinoma, at lower concentrations rather than proliferation. In fact low concentrations of gefitinib (1 microM) are able to block EGF-induced phosphorylation of ErbB-1 and ErbB-2 in vitro. Moreover, they found that in vivo motility of tumor cells in the primary tumors is significantly reduced by gefitinib treatment, showing that the endogenous in vivo motility is ErbB-1 dependent. Therefore, they affirmed that gefitinib is able to inhibit motility and invasion and to reduce the efficiency of approach to blood vessels while it doesn’t directly affect invasation (33).

Barnes et al. found that gefitinib treatment alone blocks EGFR phosphorylation, lamellipodia formation and motile cell phenotypes. Moreover, this treatment also induces the formation of thick actin cables, characteristic of nonmotile differentiated cells. They evidenced also that gefitinib treatment inhibits p42/44 mitogen activated protein kinase (MAPK) and p21-activated kinase 1 (Pak1) activation in immortalized keratinocytes (HaCaT cells) and cutaneous squamous cell carcinoma cells. Pak1 is a component of many growth factor receptor-mediated signal transduction pathways which leads to directional cell motility, cell invasiveness, and angiogenesis (25).

Yang et al. evaluated the effect of gefitinib on the stimulation of Pak1 and c-Src, using human head and neck squamous cancer and breast cancer cells. They found that it inhibits Pak1 and c-Src activity only in EGF-stimulated cancer cells thus, gefitinib acts inducing a secondary inhibition affecting cytoskeleton remodeling (34).

EGFR-TK activity increases the expression of matrix metalloproteases (MMP) (35). Lee et al. found that treatment with gefitinib doesn’t induce cytotoxicity conversely, it exerts an inhibitory effect, in a dose- and time-dependent manner, on invasion, migration, and adhesion in human oral squamous carcinoma cells (OSCCs). This effect is due to gefitinib-dependent inhibition of MMP-2 and MMP-9, demonstrating a significant proteolytic role in both invasion and metastasis inhibition (24).

4. ERLOTINIB

4.1. Mechanism of action

Erlotinib (Tarceva; OSI-774) is an orally bioavailable 4-anilinoquinazolines that selectively inhibits the EGFR tyrosine kinase domain through binding to the site occupied by ATP during phosphotransfer (5). It targets the active form of the tyrosine kinase and the interaction with wild-type EGFR kinase is similar to that of the kinase with gefitinib; i.e., it is also characterized by small aniline substituent off the quinazoline ring, and by rapid off-rates like gefitinib (6,7).

4.2. Biological properties

4.2.1. Inhibition of cell cycle progression and induction of apoptosis

Erlotinib blocks the signal transduction pathways implicated in cell proliferation and survival of cancers. In
vivo and in vitro studies have shown that erlotinib is active against a wide variety of tumor types.

Ling et al. found that erlotinib displays potent antitumor effects against human non–small-cell lung cancer (NSCLC) cell growth however, the mechanism of such effect is still not elucidated. They found that the drug inhibits cell growth through G1/S phase arrest accompanied by a decline of activities of cyclin-dependent kinase (CDK) 2 and CDK4. Moreover, retinoblastoma protein phosphorylation decrease was found directly associated with the inhibition of Erk1/2, and induction of CDK inhibitor p27KIP-1 may be caused by the increase of p27KIP-1 transcriptional level and/or by the increase of p27KIP-1 protein stability. In several human NSCLC cell lines, erlotinib inhibits the activation of PKB/Akt, Ras/Raf/mitogen-activated protein kinase, and Stat family of transcription factors (36).

Moyer et al. demonstrated that erlotinib inhibits the proliferation of DiFi human colon tumor cells at submicromolar concentrations and blocks cell cycle progression in G1 phase. Moreover, a hypophosphorylation of the retinoblastoma protein and an accumulation of p27KIP-1 are evident, which may contribute to the cell cycle block. Inhibition of EGFR also triggers apoptosis by inducing DNA fragmentation (37).

Further studies have been conducted in vitro by Lu et al. in human pancreatic cancer cell line BxPC-3. They found that erlotinib treatment can suppress cell growth in a dose-dependent manner, by means of two mechanisms: cell cycle arrest (the drug led to the accumulation cells in G0/G1 phase, thereby decreasing the proportion of cells in the S phase) and apoptosis (erlotinib induces a decrease in the expression of bcl-2 and bcl-x1) (38).

In in vitro studies showed that erlotinib inhibits MAPK and Akt phosphorylation at concentrations higher than that required for inhibition of HER1/EGFR TK phosphorylation. Furthermore, drug concentration inhibiting EGFR is correlated to the cytoplasmic concentrations of the receptor in each tumor cell line (39).

The relationship between erlotinib concentrations and antitumor effect depends on the human tumor models utilised; i.e; the drug showed antitumor effect in HN5 head and neck tumor xenografts at lower doses as respect to that utilised in the A549 non–small-cell lung cancer xenografts (39).

Chen et al. evaluated the antitumor activity of erlotinib in two human colorectal tumor xenograft models (LoVo and HCT116, characterized by similar HER1/EGFR expression levels) in athymic mice. They found that HCT116 xenograft model was not responsive to any dose of erlotinib treatment and they demonstrated that resistance to erlotinib in these tumors may be a result of persistent activation of Erk (40).

The effects of erlotinib have been studied also in human pancreatic cancer xenografts implanted orthotopically in immunodeficient mice. Tumors from two pancreatic cancer patients were analysed: OCIP# 2, with high HER1/EGFR expression level and moderate HER2, and OCIP#7, with moderate HER1/EGFR and HER2. Erlotinib inhibits HER1/EGFR phosphorylation in both in vivo models conversely, Erk phosphorylation decreases only in OCIP#2. These different behaviour may depend from selective activation of alternative pathways or from the incomplete inhibition of HER1/EGFR by the drug (41,42).

EGFR can form heterodimers with HER2 to drive mitogenic signaling pathways. Emlet et al. showed that erlotinib inhibits cell growth in a panel of unrelated human breast cancer cell lines and this effect was not related to HER2 expression levels (43).

Schaefer et al. demonstrated that erlotinib treatment in HER2-overexpressing cells decreased basal EGFR and HER2 receptors phosphorylation and this in turn induced cell growth inhibition. Nevertheless, it is unclear whether this decreased phosphorylation is due to direct inhibition of both HER2 and EGFR receptors or to direct inhibition of EGFR and the subsequent loss of HER2 transactivation. Schaefer et al. proposed an intermediate mechanism (44).

4.2.2. Inhibition of angiogenesis, invasion and metastatization

Erlotinib can also inhibit angiogenesis in vitro and in vivo. Lu et al. investigated the ability of this drug to suppress tumor vessel formation in vitro by using ECV304 cells that rapidly align and form tube-like structures when cultured on ECMatrix. They found that the length of the tubes is markedly reduced in the erlotinib treated cells compared with the untreated ones. Moreover, drug treatment on BxPC-3 cells causes down-regulation in VEGF expression. In vivo, erlotinib-treated mice demonstrate a reduced tumor volume, along with weight and microvessel density (MVD) as compared to the control. The decrease in MVD could be attributable to a decrease in endothelial cell proliferation, in agreement with results from in vitro studies (38).

Cell adhesion, motility, exocytosis and endocytosis, cell morphology and cell division are maintained by actin, an ubiquitous protein. Alteration of actin remodeling is a marker of malignant-transformation. Jin et al. tested erlotinib on actin remodeling in a bladder carcinogenic model consisting of untransformed HUC-PC cells and transformed MC-T11 cells, both derived from the same normal human urothelial clone immortalized by SV40. They showed that the actin remodeling effect was more prominent at lower dosage levels and it was accompanied by an increased cell adhesion and decreased motility. Erlotinib induced a decreased adhesion and anoikis (detachment associated apoptosis) at higher dosage levels. Moreover, it caused a more pronounced effect on actin polymerization in transformed cells compared with untransformed cells.

In fact, erlotinib significantly reduced migration of MC-T11 cells as respect to the control, already in short term. These cells, but not the untransformed ones, showed a
weak constitutive EGFR phosphorylation activity, which was inhibited by erlotinib in a dose-response manner. Nevertheless, Jin et al., using an inhibitor of MAPK (P44/42), found that there is not a specific effect on actin remodelling; suggesting that other pathways than MAPK may be involved (45).

5. LAPATINIB

5.1. Mechanism of action

Lapatinib (‘Tykerb’, GW572016) is a novel tyrosine kinase inhibitor which binds EGFR and ErbB-2. This drug is now approved by the US Food and Drug Administration (FDA) for the treatment of advanced or metastatic breast cancer with overexpression of HER2 receptors, and it is in phase III clinical trials for head and neck squamous cell carcinoma (HNSCC) (46, 47).

Structural and biochemical studies indicate that lapatinib is a potent inhibitor of the tyrosine kinase activity in vitro. The drug interacts with the inactive-like conformation of EGFR with high potency; and it inhibits also ErbB-2 which becomes unable to adopt an active-like conformation.

Lapatinib consists of the quinazoline core and of a bulky aniline substituent. The latter moiety reaches deep into an opened back pocket and makes predominantly hydrophobic interactions with the protein. The dissociation of lapatinib may require a conformational change of EGFR explaining the slow dissociation rate observed. Though the slow dissociation rate might be due to a very tight binding affinity between receptor and drug. In fact, lapatinib has a unique mechanism of action: it exhibits reversible, non-covalent inhibition of EGFR and ErbB-2, and it has a very slow off-rate compared with other reversible 4-anilinoquinazoline compounds (6).

5.2. Biological properties

5.2.1. Inhibition of cell cycle progression and induction of apoptosis

Several studies conducted in various cancer models demonstrated that cancer cells co-expressing EGFR and HER2 are sensitive to lapatinib with IC50 values ranging from nanomolar to low micromolar concentrations (48). However, the dependency of the antitumor activity from EGFR expression has not yet been established (47, 49).

Konecny et al. tested lapatinib activity against a panel of 22 breast cancer cell lines having varying degrees of EGFR and HER2 expression. They found that lapatinib is more potent to inhibit cell growth in cell lines overexpressing HER2 compared to those with high levels of EGFR or low levels of either receptor. They analyzed cell cycle of lapatinib-treated cells overexpressing HER2 and observed an increased percentage of cells with sub-2N DNA, generally considered a hallmark of apoptosis, and a reduced percentage of cells undergoing G1 phase. Moreover, they investigated the effects of this drug on HER2 and EGFR signaling and they found a dose- and time-dependent phosphorylation reduction of EGFR, HER2, Akt, and Erk. Furthermore, they showed that lapatinib completely inhibits the growth of HER2-overexpressing human breast cancer xenografts (50). Similar results have been shown by Rusnak et al. who evaluated lapatinib efficacy in cell growth assays on EGFR-overexpressing human tumor cell lines (HN5, a head and neck cancer cell line) or ErbB-2 overexpressing (BT474, a breast cancer cell line). They found that in HN5A cells, lapatinib treatment results in the accumulation of cells in G1 phase of cell cycle to a greater extent than in BT474 cells which were, after drug treatment, strongly accumulated in sub-G1 (51).

Thus, they investigated the ability of the compound to inhibit the phosphorylation of PKB/Akt, known to be involved in the inhibition of apoptotic pathways. They found that, although EGFR and ErbB-2 are inhibited similarly in BT474 and HN5 cells, there are dramatic differences in the amount of Akt phosphorylation inhibition; It was inhibited to a greater extent in the BT474 cells than in the HN5 cells, consistently with the ability of lapatinib to initiate cell death or growth arrest in BT474 and HN54, respectively.

Thus, inhibition of EGFR by this compound results preferentially in cell growth arrest, whereas inhibition of ErbB-2 yields growth arrest followed by cell death in vitro. Rusnak et al. confirmed the lapatinib ability to inhibit the growth of human tumor cells in vivo, using HN5 and BT474 xenograft models (51). Xia et al. also demonstrated that lapatinib inhibits activation of Erk1/2 and Akt and they showed a complete inhibition of activated Akt in ErbB-2 overexpressing cells associated with significant induction of apoptosis. Xia et al. extended their studies in vivo and reported that lapatinib treatment inhibits activation of EGFR, ErbB-2, Erk1/2 and Akt in human tumor xenografts.

These results agree with other studies, from the same author, in which the correlation between constitutive activation of Akt and resistance to chemotherapeutics are reported (52).

Zhou et al. found similar effects in human colon adenocarcinoma GEO cells that express EGFR and ErbB-2 receptors at relatively “basal” levels. They demonstrated that lapatinib suppresses the activation of EGFR, ErbB-2, MAPK, and Akt in a concentration-dependent manner and inhibits cell proliferation. Moreover, the drug induces apoptosis at low concentrations as reflected by the induction of poly(ADPribose) polymerase (PARP) cleavage and DNA fragmentation. Additional in vivo studies confirmed that lapatinib treatment blocks GEO xenograft growth in a concentration-dependent manner (53).

D’Alessio et al. investigated the effects of the combined blockade of EGFR and ErbB-2 on signal transduction and regulation of cell cycle in breast cancer cells. In this regard, they found that lapatinib inhibits Akt and MAPK activation and blocks cell cycle progression in G0/G1 phase. Moreover, lapatinib increases the levels of
Recent studies in non-small cell lung cancer cell lines (NSCLC) have been conducted by Diaz et al. They investigated the activity of lapatinib in A549 cells, which harbors genomic amplification of EGFR and HER2. They found that this compound reduces cell proliferation, DNA synthesis, and colony formation capacity in this cell line. Furthermore, lapatinib alters cell cycle rate inducing the arrest in G1 phase with a corresponding decrease of cells in S and G2-M phase. Moreover, a significant increase of cells in the sub-G1 phase was evident with a concomitant reduction of cyclin A/B1 level and increase of cleaved PARP and caspase-3. The same authors also showed that lapatinib treatment reduces the expression levels of p-EGFR, p-HER2, p- Erk1/2, c-Myc and PCNA in A549 cells. Furthermore, lapatinib reduces the levels of two antiapoptotic proteins IAP-2 and Bcl-xL, and increases the levels of the proapoptotic protein Bak-1 (46).

Similar effects have been shown in nasopharyngeal carcinoma cells, in which lapatinib treatment causes survivin, Mcl-1 and cyclin D1 down-regulation, too (48).

Recently, Wainberg et al. have performed studies in human upper gastrointestinal cancer cell lines demonstrating that lapatinib displays antiproliferative activity in a concentration-dependent manner, especially in HER2-amplified cells. Conversely, no association between EGFR protein expression and sensitivity to this compound has been evidenced. Lapatinib induces an increase of cells in the G1 phase in sensitive cell lines while it inhibits Akt and Erk phosphorylation (55). Kondo et al. investigated the antitumor effects of lapatinib in head and neck squamous cell carcinoma (HNSCC) cell lines evidencing that the drug shows antiproliferative effects in vitro and induces apoptosis in nude mice bearing an established xenograft of YCU-H891 cells. Nevertheless, they did not observe any correlation between the effect on cell proliferation and the expression of EGFR or HER2 in this in vitro model (56).

Konecny et al. analyzed the activity of lapatinib in endometrial cancer cells and showed a concentration-dependent antiproliferative effect, which is not associated with PTEN status. Moreover, they evidenced a significant association between HER2/EGFR expression and response to lapatinib in vitro. This is in contrast with all other in vitro models in which this correlation is not evident (57).

Zhang et al. demonstrated that lapatinib sensitivity is independent from EGFR expression level in HER2-positive breast cancer cells. In fact, they showed that EGFR small-interfering RNA (siRNA) knockdown doesn’t affect lapatinib sensitivity in cells which express high levels of HER2 and moderate levels of EGFR. Moreover cells transfected with HER2 siRNA become significantly more resistant to lapatinib. This suggests that the antitumor effect of lapatinib is the result of its anti-HER2 activity (49).

Hedge et al. analyzed the molecular mechanisms underlying the sensitivity of several breast cancer cell lines to lapatinib. In particular BT474 and SKBr3 breast cancer cells, constitutively over-expressing ErbB-2, are responsive to the drug. Conversely, MDA-MB-468 and T47D cells, with basal level of the receptor, are not responsive to lapatinib. Furthermore they found that lapatinib treatment results in differential expression of genes associated with cell cycle regulation and in particular related to the G1-S phase transition. In lapatinib-responsive cells, CDC6, MCM2, and CDK4 RNAs are more strongly down-regulated. AKT1 and BAD1 are also down-regulated conversely, FOXO3A, the proapoptotic gene, is upregulated in sensitive cell lines (58).

Scaltriti et al. demonstrated that lapatinib treatment of HER2-overexpressing breast cancer cells, SKBR-3 and MCF-7HER2, results in inhibition of HER2 and MAPK phosphorylation, in prevention of receptor ubiquitination and in induction of HER2 receptor accumulation on cell surface. Furthermore, lapatinib treatment induced the stabilization of inactive HER2 homo- (HER2/HER2) and hetero- (HER2/EGFR and HER2/HER2) dimers, which act as a ligand trap, being able to bind (and sequester) the ligands, thus preventing receptor phosphorylation (59).

5.2.2. Inhibition of angiogenesis, invasion and metastatization

Diaz et al. demonstrated that lapatinib impairs angiogenesis in an in vivo model of A549 cells and this may be one the most relevant mechanisms elicited by this drug in vivo. They found that in treated-mice this compound dramatically reduces vessel density compared to controls; moreover, it reduces also circulating endothelial progenitor cells (CEPs), which contribute to tumor angiogenesis.

It is possible that inhibition of EGFR downstream signaling reduces the expression of angiogenic factors (VEGF, IL-8, etc.) through an indirect mechanisms. Consistently, it has been recently demonstrated that lapatinib is able to inhibit VEGFR1 phosphorylation in A549 cells (46).

Inhibition of ErbB-1 and ErbB-2 also contributes to inhibit other characteristics of tumor progression such as cell motility and invasation. This latter effect is extremely important because it may affect the ability of tumor cells to spread and metastatize. In particular, Kedrin et al. demonstrated that ErbB-1 is important for local stromal invasion, whereas ErbB-2 is more directly important for invasation i.e., lapatinib treatment also significantly reduces invasation of tumor cells (33).

Lui et al. investigated the effect of lapatinib in nasopharyngeal carcinoma (NPC) cells (HK1-LMP1 and HONE-1 cells) which are intrinsically invasive, anokis-resistant and able to proliferate in suspension. Using the matrigel invasion assay, they demonstrated that lapatinib is very efficacious in inhibiting NPC cell invasion in vitro and it is able to induce anoikis-sensitization, by inhibiting cells growth in suspension (in detached state) (48).
6. PERSPECTIVES

The relevance of EGFR as target for anticancer treatment is extensively supported by several literature evidences. Two main classes of EGFR activity inhibitors has been developed: small-molecules (TKIs) binding to the intracellular TK domain of the receptor and monoclonal antibodies directed to the extracellular receptor domain. Focusing on the first class of agents, in the recent past, the molecules in preclinical and then in clinical practice are gefitinib and erlotinib, both reversible EGFR TKIs and lapatinib, a reversible EGFR/HER2 TKI.

However several clinical trials demonstrated that clinical benefit derived from first-generation EGFR TKIs is modest and translational research is currently focusing on discovery of new predictive factors, which could help to select patients with a high chance of benefitting from this target-oriented therapy, and on development of a second generation of EGFR TKIs. These new agents are able to i) form covalent bonds with the receptor, increasing their effectiveness by prolonging the inhibition of EGFR signaling to the entire lifespan of the drug-bound receptor molecule and/or ii) inhibit several EGFR correlated targets, such as additional members of the ErbB family (HER2) or other downstream or parallel pathways (the vascular endothelial growth factor receptor (VEGFR) pathway).

Several compounds belonging to the second-generation of EGFR inhibitors have been synthesized to prevent the development of acquired resistance and/or to overcome acquired resistance in patients previously treated with gefitinib or erlotinib (60). One of these new molecules, in preclinical and clinical trials, is BIBW 2992, an orally bioavailable aniline - quinazoline derivative, which irreversibly binds to the Cys residues of EGFR and of HER2/neu (61). This drug is used in the treatment of patients with solid tumors, including NSCLC, overexpressing EGFR and with T790M mutation, which confers resistance to the first-generation EGFR inhibitors (62). Another compound used in clinical trials is neratinib (HKI-272), an irreversible pan-ErbB TKI inhibitor that binds covalently to cysteine in the EGFR kinase domain and binds also ErbB-2 and ErbB-3 (60, 63). It was three-fold more effective than gefitinib in suppressing cell proliferation, and it is active also in patients with EGFR mutations. Recent studies have shown that neratinib has substantial activity also in HER2-positive breast cancer (63). PF-00299804 is a quinazoline-based irreversible inhibitor that potently inhibits wild-type ErbB-1, ErbB-2, and ErbB-4 as well as a mutated form of EGFR associated with resistance to gefitinib and erlotinib (64). Finally, XL647 and ZD6474 (vandetanib, Zactima) inhibit reversibly EGFR, like erlotinib and gefitinib, and are able to simultaneously inhibit also vascular endothelial growth factor receptors (VEGFRs) and/or HER2, Flt-4, and EphB4. Their broader spectrum of inhibition allows to overcome acquired resistance to erlotinib or gefitinib (60, 65).

The results from ongoing phase I/III trials of these agents in cancer patients are eagerly awaited because these new drugs could represent a novel strategy in EGFR targeted therapy also valid in tumors resistant to reversible EGFR TKIs.

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