Antitumor effect in medulloblastoma cells by gefitinib: Ectopic HER2 overexpression enhances gefitinib effects in vivo

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The effects of the epidermal growth factor receptor (EGFR) inhibitor gefitinib on cell growth and signaling were evaluated in three medulloblastoma (MB) cell lines (D283, D341, Daoy), one supratentorial primitive neuroectodermal tumor cell line (PFSK), and four MB primary cultures. Cell lines showed diverse expression of EGFR and human epidermal receptor 2 (HER2), with high levels of constitutively activated HER2 in the HER2-overexpressing D341 and D283 cells. Gefitinib sensitivity varied across lines and was not related to expression of HER receptors or receptor baseline activation. Gefitinib induced G0/G1 arrest in all lines, whereas apoptosis was dose-dependently induced only in D283 and D341 cells. The molecular response to gefitinib was investigated in Daoy and D341 lines, which showed a higher (half-maximal inhibitory concentration [IC50], 3.8 μM) and lower (IC50, 6.6 μM) sensitivity to the agent, respectively. Gefitinib inhibited constitutive and EGF-triggered EGFR phosphorylation in both lines but was ineffective in constitutive activation of HER2 in D341 cells. Phosphorylated AKT inhibition paralleled that of phosphorylated EGFR, suggesting the presence of an autocrine gefitinib-sensitive EGFR/AKT pathway. On the whole, EGF-dependent signaling was less responsive to ligand stimulation and gefitinib inhibition in D341 cells, which correlated with the lower sensitivity to gefitinib’s antiproliferative effect of this line. In vivo, the growth of D341 and Daoy xenografts treated with gefitinib at 150 mg/kg per day was inhibited by approximately 50%. Ectopically overexpressed HER2 in Daoy cells significantly increased sensitivity to gefitinib’s antitumor effects in vivo (tumor volume inhibition = 78%). Our data indicate that gefitinib might be a molecularly targeted agent for the treatment of MB. Neuro-Oncology 11, 250–259, 2009 (Posted to Neuro-Oncology [serial online], Doc. D08-00060, November 25, 2008. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2008-095)

Keywords: EGFR, gefitinib, HER2, in vivo, medulloblastoma

Medulloblastomas (MBs) are highly aggressive embryonal tumors of the cerebellum, which represent the most common malignant primary brain tumor of childhood. Despite multimodal aggressive treatment regimens, only 60%–70% of the patients survive for 5 years after diagnosis, and most of them will suffer significant iatrogenic sequelae. Therefore, there is an urgent need to develop more effective and less toxic anticancer agents than conventional cytotoxic drugs. To this end, selective targeting of the molecular alterations that drive MB development may represent alternate means to improve outcome, while reducing treatment-related morbidities.

Epidermal growth factor receptor (EGFR, also known as HER1, ErbB1) is the prototypic member of
the human epidermal receptor (HER) family of tyrosine kinases, which also contains HER2 (Neu, ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Ligand-induced receptor oligomerization triggers downstream signaling cascades, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/AKT pathways, which play fundamental roles in cellular proliferation, survival, and differentiation.\(^4,5\) Overactivity of EGFR signaling leads to unrestrained cellular proliferation and increased survival, resulting in cellular transformation and tumor progression.\(^6\) A number of mechanisms, such as autocrine growth factor loops and heterodimerization within the HER family, appear to modulate the EGFR-mediated signaling. Amplification/overexpression of EGFR and/or HER2 has been found in tumors of different origin, including MB. A causative role for HER2 in MB might be driven from the observation that HER2, which is detected in approximately 80% of MBs and overexpressed in approximately 40%, is not detected at any stage of cerebellar development.\(^7\) Furthermore, HER2 overexpression in MB has consistently been associated with more aggressive disease, poor survival, and resistance to cytotoxic agents.\(^8,9\)

Recently, EGFR has attracted many efforts to develop novel molecularly targeted agents that selectively block its signaling cascades. One such agent is the EGFR tyrosine kinase (TK) inhibitor gefitinib (ZD1839, Iressa; AstraZeneca, Macclesfield, Cheshire, England), which competes with ATP for binding to the EGFR-TK pocket. In preclinical studies, gefitinib has shown antitumor activity in a variety of human cancer cell lines and xenograft models.\(^10,11\) Although gefitinib inhibits EGFR in the nanomolar range and HER2 at approximately 100-fold higher concentrations, endogenous or ectopic overexpression of HER2 increases sensitivity to gefitinib’s antiproliferative effects in lung and breast cancer in vitro and in vivo.\(^12-14\) Gefitinib has been approved for previously treated advanced non-small-cell lung cancers, and several phase II and III trials are ongoing in different adult tumors, including those of the CNS.\(^15\)

In the field of pediatric neurooncology, little improvement has been gained in the area of targeted therapies, and treatment relies mostly on relatively nonspecific conventional modalities. Only phase I clinical studies of gefitinib have been concluded in children, and these show a safety profile similar to that observed in adults.\(^16\) Phase II clinical studies are currently ongoing for the treatment of pediatric solid tumors, including those of the brain.\(^16\)

So far, there is scant literature on the growth-inhibitory effects of gefitinib in preclinical models of pediatric tumors. Gefitinib has been shown to inhibit the proliferation of neuroblastoma cell lines at submicromolar concentrations.\(^17\) However, as a single agent, it does not markedly inhibit the growth of neuroblastoma or rhabdomyosarcoma xenografts.\(^18\) To our knowledge, no study has been performed on the response of MB cell lines to gefitinib. In the present study, we examined the expression and function of the EGFR pathway in MB cell lines and the growth-inhibitory effects of gefitinib both in vitro and in vivo. In addition, we also investigated whether HER2 signaling is involved in modulating the sensitivity of MB cells to gefitinib.

### Materials and Methods

#### Cell Cultures

We used three human MB cell lines (D283, D341, and Daoy; American Type Culture Collection, Rockville, MD, USA) and the supratentorial primitive neuroectodermal tumor (PNET) cell line PFSK (Dr. Alberto Gulino, University La Sapienza, Rome, Italy). D283 and D341 or Daoy and PFSK cells were maintained in 10% fetal bovine serum (FBS)/Dulbecco’s modified Eagle’s medium or 10% FBS/RPMI, supplemented with 100 µg/ml streptomycin and 2 mM L-glutamine (Sigma, Dorset, UK). Primary MB cell cultures were established from children’s biopsy material and cultured in 10% FBS/RPMI.

For overexpression of HER2, Daoy cells were transfected with HER2 cDNA (kindly provided by Dr. Maurizio Alimandi, University La Sapienza, Rome, Italy) and cloned into the pcDNA3.1 vector (Invitrogen Corp., Paisley, UK) using the Lipofectamine reagent (Invitrogen). Separate clones of HER2 and empty vector alone were selected under 100 µg/ml of G418 (Geneticin; Gibco BRL Technologies, Inc., Grand Island, NY, USA), and HER2 expression was verified by immunoblotting.

#### Growth Inhibition Assay

Cells were seeded at the appropriate concentrations to prevent confluence throughout the experiment. After 24 h, cells were treated with drug or vehicle on days 1 and 4. Viable cells were counted daily for up to 5 days. Half-maximal inhibitory concentration (IC	extsubscript{50}) values were determined. The sensitivity of MB cells to gefitinib.

#### Cell Cycle Analysis by Flow Cytometry

Cells were exposed to serial concentrations of gefitinib and collected after 24, 48, and 72 h. Nuclei were isolated and stained using a solution containing 0.1% sodium citrate, 0.1% Nonidet-P40, 4 mM EDTA, and 50 µg/ml propidium iodide. Flow cytometric DNA ploidy analysis was performed by acquiring a minimum of 15,000 nuclei using a FACSScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and quantified by LYSYS II software (Becton Dickinson). DNA fragmentation in the sub-G1 region of DNA histograms was used to evaluate apoptosis.

#### Quantitative Real-Time Reverse Transcriptase PCR

Total RNA was extracted from cells using TRIzol (Invitrogen) and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (RT) and random primers (Promega, Southampton, UK). Gene expression...
assays for EGFR, HER2, HER3, EGF, transforming growth factor α (TGFα), amphiregulin (AREG), epiregulin (EGFR), and neuregulin 1 (NRG1), as well as for the reference normalization gene (gluceraldehyde-3-phosphate dehydrogenase [GAPDH]) were obtained from Applied Biosystems (Foster City, CA, USA). Each amplification reaction was performed in triplicate on a 7500 Real-Time PCR System (Applied Biosystems), and the average of the three threshold cycles was used to calculate the amount of transcript in the sample. All values were normalized to the endogenous control GAPDH.

**Ligand Stimulation and Western Blot Analysis**

For growth factor treatments, 80% confluent cells were starved overnight in 0.5% FBS-containing medium. Cells were then exposed to gefitinib (0.1–10 μM) for 2 h and subsequently stimulated for 30 min with 25 ng/ml EGF (Sigma). At the end of the treatment, cells were scraped in lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride]. Total lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Amersham Pharmacia, Buckinghamshire, UK), and probed with antibodies to EGFR, phosphorylated EGFR (pEGFR), HER3, actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pHER2 (Upstate Biotechnology, Lake Placid, NY, USA), HER2, pHER3, pAKT, AKT, pMAPK, and MAPK (Cell Signaling Technology, Beverly, MA, USA). The membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Vector, Burlingame, CA, USA), and the immunoblots were visualized using the ECL detection system (Amersham Pharmacia).

**Xenograft Experiments**

Approximately 15 × 10⁶ Daoy, Daoy/HER2 transfectant, or D341 cells were subcutaneously inoculated into both abdominal flanks of 5-week-old male CD1 nu/nu nude mice (Charles River, Calco, Italy), which were maintained under pathogen-free conditions. Mice (n = 8–10) were orally administered vehicle (0.5% polyoxyethylene sorbitan; Sigma) or 150 mg/kg gefitinib daily five times per week for 4 weeks. Tumor volume (TV) and total weight were monitored every 3 days. TVs were calculated by the formula TV = d² × D/2, where d and D represent the shortest and the longest diameter, respectively. The antitumor activity was evaluated by the tumor volume inhibition (TVI) in treated (T) versus control (C) mice according to the formula TVI = 100 – (T/C × 100). Two-tailed Student’s t-tests were applied to compare tumor growth between treated and control groups; p-values < 0.05 were considered significant.

For Western blot analysis, tumor xenografts were excised at the end of treatment and immediately frozen at −80°C. Samples were then processed as previously described. All experimental animal investigations complied with the guidelines of the Istituto Superiore di Sanità (Rome, Italy) on experimental neoplasia in animals.

**Histology and Immunohistochemistry**

Xenograft specimens were fixed with phosphate-buffered formalin (pH 7.2) for 12–18 h. They were collected on 3-aminopropyltriethoxysilane (Sigma) or naturally charged slides (Dako Autostainer, Milano, Italy) and allowed to dry overnight at 37°C to ensure optimal adhesion. The sections were dewaxed, rehydrated, and treated with 0.3% H₂O₂ in methanol for 10 min to block endogenous peroxidase, followed by incubation at room temperature with normal rabbit serum for 15 min. The slides were stained with hematoxylin and eosin.

**Results**

**Effects of Gefitinib on Proliferation and Survival of MB/PNET Cell Lines and Primary Cultures**

Gefitinib inhibited the growth of four human MB/PNET cell lines in a time- and concentration-dependent manner: D283, PFSK, and Daoy cells showed a similar sensitivity to the inhibitor (IC₅₀ ~ 3.5 μM at 5 days of treatment), while the D341 cell line was the less responsive, with an IC₅₀ of 6.6 μM (Fig. 1, Table 1). At a concentration equal to 1 μM—which is reported to be specific for EGFR inhibition—gefitinib reduced the growth of three out of the four cell lines (D283, PFSK, and Daoy) by 20%–30% at 5 days, whereas a minimal effect was observed in D341 cells, suggesting that in this cell line the antiproliferative effect of gefitinib might be mediated by actions additional to inhibition of EGFR.

We next determined the effects of gefitinib in four MB primary cultures. By immunohistochemical analysis, these cells showed large nuclei with numerous mitoses and a high nuclear:cytoplasmic ratio, which are all features of neoplastic cells. In addition, these cells were negative for glial fibrillary acidic protein, a marker for stromal astrocytes, further confirming that our primary cultures were made up of neoplastic cells (data not shown). The primary cultures tested were overall less responsive, with IC₅₀ ranging from 5.9 to 14.7 μM (Table 1).

To clarify the mechanisms underlying gefitinib’s growth-inhibitory effects, cell cycle profiles after treatment with the agent at equitoxic doses (IC₇₀ values) were analyzed over a 72-h period (Fig. 2A and data not shown). In all cell lines, gefitinib induced an arrest in the G₀/G₁ phase at the expense of the S phase, with minor changes in the G₂/M phase. Such a block was evident at 24 h and lasted throughout the time course.

An estimation of the cell death rate was quantified calculating the amount of cells with hypodiploid DNA content after exposure to 20 and 30 μM gefitinib, concentrations previously reported to induce apoptosis. We observed that cell death was induced dose-dependently only in D283 and D341 cells (Fig. 2B) and was remarkable at 30 μM. These data were consistent with the
caspase activation by gefitinib, as detected by cleaved poly(ADP-ribose) polymerase (PARP) (Fig. 2B, inset). Taken together, our results indicate that gefitinib action is mainly cytostatic in Daoy and PFSK cells, while in D283 and D341 cells, apoptosis contributes to gefitinib-induced cytotoxicity.

**Expression of HER Family Members and Their Ligands in MB Cell Lines and Primary Cultures**

To determine whether there was a correlation between sensitivity of cells to gefitinib and expression of EGFR and/or HER2, we examined the levels of expression of both receptors in all cell lines by quantitative PCR (Q-PCR). EGFR was the receptor less abundantly expressed at the mRNA level in all the established cell lines except Daoy cells (Fig. 3A), while HER2 was expressed at the highest level in D283 and D341 cells.

Collectively, primary cell lines showed a pattern of receptor expression similar to that of Daoy cells, although cell lines from patient 3 showed relatively much higher levels of HER2.

To identify a potential gefitinib-sensitive autocrine loop, we next determined by Q-PCR the levels of some ligands of the HER family: EGF, TGFβ, AREG, EREG, and NRG1 (Fig. 3B). Of the ligands examined, only EGF, AREG, and NRG1 were expressed in all lines, although at variable levels. Daoy cells showed the highest expression of the EGFR-specific ligands EGF and TGFβ, consistent with the high expression of their cognate receptor in these cells. D283 and D341 cells lacked expression of TGFβ, whereas PFSK and D341 cells lacked EREG expression. The four primary cultures displayed a profile of ligand expression similar to that of Daoy, with all ligands examined presenting at relatively high levels.

To further characterize the relevance of an HER-mediated autocrine loop, we determined the levels of expression and activation of the HER receptors in serum-free conditions by Western blot analysis. Total EGFR and HER2 were found to be tightly correlated with receptor mRNA (Fig. 3C). Phosphorylated EGFR (pEGFR) was detectable in both D341 and Daoy cells, whereas pHER2 was present at the highest expression level in D283 and D341. Collectively, we found no correlation between sensitivity to gefitinib and activation of a potential HER autocrine loop, on the basis of receptor and ligand expression and receptor activation.

**Effect of Gefitinib on EGFR-Related Signaling Pathways**

We next compared the effects of gefitinib on the EGF-triggered pathways in Daoy and D341 cells, which
respectively display high expression levels of either EGFR or HER2 and show a differential sensitivity to the inhibitor (IC$_{50}$ values varying by almost 2-fold).

Cells were pretreated with different concentrations of gefitinib 2 h prior to stimulation with EGF. In Daoy cells, EGFR was highly phosphorylated upon addition of EGF and was dose-dependently inhibited by gefitinib, returning to the unstimulated level at 5 μM (Fig. 4A). In spite of low expression levels of total HER2 protein, we detected EGF-induced pHER2, whose pattern of inhibition by gefitinib paralleled that of pEGFR, which suggests that HER2 inhibition was most likely due to the blocking of the transphosphorylating EGFR. AKT was still phosphorylated in serum-starved Daoy cells; however, it was further increased by the addition of EGF, being reduced below the basal level at 1 μM gefitinib. Phosphorylation of MAPK was completely prevented by the same gefitinib concentration.

Serum-starved D341 control cells displayed high levels of constitutively activated EGFR and HER2, and stimulation with EGF did not result in an increase in the phosphorylation of either receptor (Fig. 4A). Gefitinib (1 μM) reduced the activation of EGFR below the basal level, but was ineffective for pHER2, even at the highest doses. AKT and MAPK were only slightly induced upon ligand treatment, with gefitinib being able to decrease the activation of AKT, but not that of MAPK. Overall, D341 cells were less responsive to EGF stimulation, consistent with their lower sensitivity to gefitinib’s growth-inhibitory effects.

The presence of baseline pEGFR and pAKT indicate intrinsic activity of these kinases in Daoy and D341 cells. Therefore, we investigated the effects of gefitinib on the phosphorylation status of EGFR and AKT in basal conditions. Gefitinib coordinately inhibited pEGFR and pAKT in both cell lines. No effect on constitutive phosphorylation of HER2 was found (Fig. 4B).

To address the importance of the EGF-dependent pathway in the presence of a complex mixture of growth factors, we also investigated the effects of gefitinib on phosphorylation of AKT and MAPK in Daoy cells grown in 10% serum-containing medium. Consistent with the
Fig. 4. Dose-dependent inhibition of mitogen-activated protein kinase (MAPK) and AKT kinase pathways by gefitinib in Daoy and D341 cell lines. (A) Serum-starved cells were incubated with the indicated concentrations of gefitinib and then stimulated with epidermal growth factor (EGF) for 30 min. Membranes were immunostained for phosphorylated EGFR (pEGFR), pHER2, pMAPK, and pAKT. Actin was used as a loading control. (B) Effects of gefitinib on baseline EGFR, HER2, and AKT phosphorylation in Daoy and D341 cells. Serum-starved cells were incubated with the indicated concentrations of gefitinib for 2 h. (C) Dose-dependent inhibition by gefitinib of AKT and MAPK phosphorylation in Daoy cells treated with 1, 5, and 10 μM gefitinib for 24 h in 10% fetal bovine serum medium. Relative changes of the phosphorylation of AKT or MAPK (A), EGFR and AKT (B), and AKT and MAPK (C) were quantified by densitometric analysis and normalized to the densitometric value of actin. One representative experiment is shown.
Experiment in serum-free conditions, gefitinib inhibited the activation of both AKT and MAPK in a dose-dependent manner after a 24-h exposure (Fig. 4C). These data indicate that the EGFR signaling is relevant for MB cell lines even in the presence of various growth factors.

Effects of Gefitinib on the Growth of MB in Nude Mice

We examined the antitumor activity of gefitinib in mice bearing Daoy and D341 tumor xenografts. Antitumor efficacy was expressed as TVI on the final treatment day. Gefitinib effectively inhibited the growth of Daoy and D341 xenografts to a similar extent (Fig. 5A, B), with a TVI of 49% and 51%, respectively. Treatment was generally well tolerated by mice, with no signs of acute or delayed toxicity. An approximate 10%–15% reduction in body weight was observed at the end of treatment (Fig. 5C, D).

To address the impact of HER2 overexpression on sensitivity to gefitinib in MB, we stably transfected HER2 in the Daoy cell line, which has no detectable HER2 protein. A number of clonal derivatives were obtained, which expressed elevated levels of basal HER2 protein, which were strongly reduced after gefitinib treatment, indicating that HER2 might be an additional, yet important, determinant of sensitivity (Fig. 6C). The decrease in the expression of the gefitinib targets at the end of treatment is suggestive of a change in the cellular composition of the mass, made up mostly of neoplastic HER2- and/or EGFR-overexpressing cells in the control tumors, and of giant cells and fibrous tissue in the treated ones. Indeed, histological examination and immunohistochemical analysis of xenografts confirmed the disappearance of the neoplastic cells in the treated animals, associated with an intense infiltration of foamy, often multinucleate, macrophages (Fig. 6D).

Discussion

The EGFR-TK inhibitor gefitinib is currently being used in clinical trials against a number of tumors, including those of lung, prostate, and breast. To address whether gefitinib might have a therapeutic potential in MB, which expresses the gefitinib target EGFR, we investigated the effects of the inhibitor on proliferation of human MB cell lines in vitro and in vivo.

The MB cell lines and primary cultures used expressed variable levels of EGFR and HER2. pEGFR was detected in both D341 and Daoy cells, whereas pHER2 was present at the highest expression level in D283 and D341, consistent with the constitutive activation through homodimerization reported in HER2-overexpressing cell lines.
Of the HER ligands examined, only EGF, AREG, and NRG1 were expressed in all cell lines, although at different levels. Sensitivity to gefitinib varied across lines and was not associated with the expression of HER receptors and ligands or with the activation status of the receptors. The correlation of gefitinib growth-inhibitory effects and EGFR expression of cells is controversial. However, our results in MB cells are in agreement with a number of other preclinical studies in different tumors, including breast, lung, and bladder.12,22,23

The effects of gefitinib on malignant cells of several origins have been described as mainly cytostatic, with apoptosis occurring at the higher doses.12,13,19 In all of our cell lines, gefitinib at IC_{70} values resulted in a retention of the cells in G_{0} phase, a sharp decrease in the S-phase population, but no or minor changes in the G_{2}/M fraction. Apoptosis increased in a dose-dependent manner only in D283 and D341 cells lines. The cell cycle perturbations that we observed are consistent with previous publications, which have characterized the effects of gefitinib as primarily due to G_{0} arrest and impaired G/S phase transition.14,24 In the context of HER signaling, HER2 overexpression seems to protect tumor cells from death by downregulating proapoptotic proteins and upregulating survival factors.24,25 Consistently, the blockade of the EGFR/HER2 signaling through TK inhibitors, including gefitinib, results in coordinate modulation of pro/antiapoptotic proteins in HER2-overexpressing breast cancer cells, associated with induction of apoptosis. However, although gefitinib-induced apoptosis was a relevant phenomenon only in the two HER2-overexpressing MB cell lines, the HER2 pathway is unlikely involved, because gefitinib was not able to inhibit phosphorylation of HER2 in D341 and D283 cells (data not shown). Alternatively, p53 status might dictate the susceptibility of MB cells to gefitinib-induced apoptosis, since both D283 and D341 cells display wild-type p53, whereas Daoy cells harbor a mutant p53.26 Indeed, a recent study shows that ectopically expressed p53 enhances growth inhibition and apoptosis by gefitinib in p53-null non-small-cell lung cancer through the upregulation of Fas,27 and we found that gefitinib induced modulation of p53 in D283 and D341 cells, but not in Daoy cells (data not shown).
To investigate the molecular mechanisms underlying the response of MB cells to gefitinib, we focused on Daoy and D341 lines, which displayed a different pattern of receptor expression and sensitivity to the inhibitor in vitro. We found that the more gefitinib-sensitive Daoy cell line showed a much higher activation of the EGF-dependent pathway compared to the less responsive D341 line. In addition, a different pattern of inhibition of proteins downstream from EGFR was seen. Indeed, in Daoy cells, both AKT and MAPK were inhibited by gefitinib, while in D341 cells only AKT was inhibited, correlating with the less responsiveness of the latter cell line to the agent. In agreement with our results, previous studies have found an association between the level of activation of the EGFR-triggered pathway and the response to gefitinib of tumor cell lines. Persistent MAPK and/or AKT activity has been reported to represent a potentially relevant mechanism of resistance to gefitinib in glioma, lung, and breast cancer cell lines, with sensitivity being linked to the ability of the inhibitor to downmodulate both pathways. Therefore, also in MB lines the extent of the antiproliferative effects of gefitinib might be related to its ability to effectively inhibit the pathway of MAPK in addition to that of AKT.

The MB cells tested expressed high levels of either EGFR or HER2, indicating that EGFR might form homodimers with itself or heterodimers with other members of this family. Gefitinib was able to decrease EGF-induced EGFR phosphorylation in both Daoy and D341 cells, whereas phosphorylation of HER2 was reverted only in Daoy cells. These data indicate that gefitinib is able to inhibit the EGFR-mediated transphosphorylation of HER2, but not the phosphorylation of HER2/HER2 homodimers, which spontaneously arise in HER2-overexpressing cells. Our results are consistent with previous publications, which indicate that inhibition of the HER2 kinase in intact cells by gefitinib is not attributable to a direct competition for ATP binding to HER2, but rather to some indirect mechanism mediated by EGFR. Indeed, higher concentrations of gefitinib were required to inhibit HER2 in EGFR-negative breast cancer cells. Since the levels of EGFR were low compared to HER2 in D341 cells, it is possible that the EGFR-mediated transmodulation of HER2 was minimal and not detectable in these cells.

Tumor cell lines that express HER2 in addition to EGFR show increased sensitivity to gefitinib in vitro and in vivo, most likely because gefitinib is able to disrupt EGFR/HER2 heterodimers, which function as an onco- genic unit. In MB, HER2 upregulates the transcription of prometastatic genes, promotes cell proliferation, and confers a poor clinical outcome. Although gefitinib is mainly targeted against EGFR, it affects signal transduction through different HER receptors. Therefore, HER2-overexpressing MB could be a potential target for antitumor molecular therapy with gefitinib. We found no correlation between responsiveness to gefitinib and HER2 overexpression within the panel of MB cell lines as a whole, perhaps because of highly diverse genetic backgrounds. Therefore, to further address whether HER2 might modulate the response to gefitinib in MB, we compared isogenic Daoy cells, which ectopically overexpress HER2. In vitro, HER2-transfected clonal lines exhibited a sensitivity to gefitinib similar to that of wild-type cells, whereas a significant improvement of antitumor activity was observed in vivo. The discrepancy between our in vitro and in vivo results suggests that gefitinib might affect host-dependent processes promoting cancer growth more than tumor cells themselves.

One such process might be angiogenesis, since gefitinib is reported to dose-dependently inhibit the production of angiogenic factors, such as TGFα, basic fibroblast growth factor, and vascular endothelial growth factor (VEGF). Interestingly, HER2 has been implicated in the regulation of VEGF, and HER2-overexpressing breast tumors tend to be more angiogenic than other breast tumors. The HER2-overexpressing D341 line, which is characterized by a more aggressive phenotype, was as responsive to gefitinib as the Daoy line, bringing indirect evidence that HER2 might sensitize cells to gefitinib antitumor activity in vivo. Knocking down HER2 expression in cells that endogenously overexpress HER2 might help further define the role for HER2 in modulating gefitinib sensitivity in MB.

In conclusion, our data indicate that gefitinib is able to inhibit the growth of MB cells in vitro and in vivo. No molecular correlates of gefitinib responsiveness were found in MB cell lines, although HER2 overexpression in the same genetic background markedly increased sensitivity to gefitinib in vivo. These data lend experimental support for the therapeutic use of gefitinib in the treatment of MB; however, the identification of gefitinib-sensitive targets in MB might be useful to prospectively identify tumor cells that are most likely to respond to gefitinib therapy.

Acknowledgments

This work was supported by Fondazione per l’Oncoologia Pediatrica. We thank AstraZeneca for supplies of gefitinib (ZD1839, Iressa). Iressa is a registered trademark of the AstraZeneca group of companies.
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