EGFR Inhibition Using Gefitinib Is Not Active in Neuroblastoma Cell Lines

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Abstract. Background: Inhibition of the epidermal growth factor receptor (EGFR), using a tyrosine kinase inhibitor such as gefitinib, was suggested to be a new treatment approach for neuroblastoma. Material and Methods: EGFR expression and gene mutation were studied by reversetranscriptase-polymerase chain reacting, fluorescence-activated cell sorting and gene sequencing in six neuroblastoma cell lines. In vitro cytotoxicity of gefitinib 0.1-10 μM alone or in combination with topotecan, vincristine and 9-cis retinoic acid (9cisRA) was determined by MTT proliferation assay. Results: EGFR overexpression and gene mutations were absent in all cell lines tested. Inhibition of cell viability of 62-85% was found at 10 μM gefitinib, concentrations that, however, can clinically not be reached. In addition, gefitinib increased inhibitory effects of topotecan, vincristine and RA by 10-15%. Conclusion: Our in vitro data do not support the use of gefitinib as monotherapy in neuroblastoma and its chemosensitizing effects appear minor.

New drugs targeting specific pathways of cancer cells have entered adult oncology with an enormous success (1). In this regard, imatinib mesylate, the tyrosine kinase inhibitor (TKI) of the ABL transcript, KIT and the platelet-derived growth factor receptors, was the first agent that targets specifically and selectively molecular pathways in malignant cells (2). In addition, further TKIs such as gefitinib and erlotinib targeting endothelial growth factor receptor (EGFR) tyrosine kinase have been developed and showed efficiency in non-small cell lung cancer (NSCLC), particularly in adenocarcinoma of women and non-smokers bearing EGFR gene mutations in exons 19 and 21 (3, 4).

Neuroblastoma, the second most frequent solid tumor of childhood, still has an unacceptably poor outcome, notably at advanced stages (5). Therefore, new therapeutic approaches have to be urgently explored. Within this context, TKIs are under investigation for the treatment of neuroblastoma (6). For example, sensitivity of imatinib has been studied extensively in vitro and in vivo (7-10). These pre-clinical evaluations suggest a potential of imatinib in combination with chemotherapy, retinoic acid (RA) or radiotherapy (11). However, first clinical data on imatinib used as a single agent in pediatric malignancies showed only minor effects, including in neuroblastoma patients (12), and combination treatments are under discussions. Furthermore, EGFR inhibition has been suggested for neuroblastoma. Ho et al. reported inhibition of EGFR autophosphorylation in vitro using gefitinib by blocking the phosphoinositide-3-kinase (PI3K)/AKT pathway (13) and Tamura et al. showed induction of apoptosis (14). We investigated if gefitinib has cytotoxic activity as a single agent or in combination with currently used chemotherapeutics, topotecan and vincristine, as well as the differentiating agent 9cisRA.

Materials and Methods

Cell culture. The neuroblastoma cells SH-EP, SH-SY5Y, Kelly and Lan-5 were kindly provided by Professor M. Schwab, DKFZ Heidelberg, Germany, and SK-N-LO and SK-N-FI by Dr. A. Voigt, Department of Pediatrics, University of Jena, Germany. The lung cancer cells HCC-827 (control) were received from the DSMZ (Braunschweig, Germany). All cells were cultured in RPMI-1640 medium (Gibco BRL, Eggenstein, Germany) with 10% fetal serum (FCS) and 1% penicillin/streptomycin under 5% CO2 in atmospheric air at 37°C.

MTT proliferation assay. Gefitinib was kindly provided by AstraZeneca (Macclesfield, Cheshire, UK). A total of 1x10⁴ cells per well were plated in a 96-well-plate and cultured overnight. On
Reverse transcriptase-polymerase chain reaction. Total RNA was isolated using PeqGOLD TriFast reagent (PeqLab, Erlangen, Germany). First-strand cDNA synthesis was performed with 5 μg of total RNA using Ready-To-Go T-Primed First-Strand Kit (Amersham Bioscience Europe GmbH, Freiburg, Germany). For PCR amplification 1 μl of cDNA (100 ng) was used. The primers used for PCR were as follows: for EGFR, sense: 5'-GAG AGC TAT GAG AGT GAG GAA-3' (nucleotides 1186-1206) and antisense 5'-GAG TCA CCC CTA AAT GCC A-3' (nucleotides 1385-1367); for β-actin, sense: 5'-CCA AGG CCA ACC GCG AGA AGA TGA C-3' (nucleotides 409-432) and antisense 5'-AGG GTA CAT GGT GGT GCC GCC AGA C-3' (nucleotides 972-995). The PCR conditions were as follows: initial denaturation for 5 minutes at 94°C, amplification at 35 cycles: 30 seconds at 94°C of denaturation, 30 seconds at 60°C of annealing, 1 minute at 72°C of extension and a final elongation for 10 minutes at 72°C. β-Actin as a constitutively expressed gene product was used as control. The PCR products were analyzed after electrophoretic separation in 1% agarose gel and visualized by ethidium bromide staining.

Fluorescence-activated cell sorting analysis. Sub-confluent neuroblastoma cells were harvested from culture flasks and washed twice in phosphate-buffered saline. Approximately 5×10⁵ cells were stained with a polyclonal rabbit anti-EGFR (1:2252; Cell Signaling Technologies, Inc., Danvers, USA) followed by a polyclonal goat anti-rabbit fluorescein (FITC)-conjugated antibody (Jackson Immunoresearch Laboratories, Newmarket, UK) for surface receptor detection. Data from a minimum of 1×10⁴ cells were acquired and analyzed with CellQuest software (BD Biosciences Immunocytometry Systems, San Jose, USA).

Inhibition of cell viability by gefitinib. Cell viability significantly decreased after treatment for 72 hours only at 10 μM gefitinib, the highest concentration tested (Figure 2A). No antiproliferative effect was seen in Kelly or SK-N-LO. The treatment of HCC-827 cells showed a distinct stronger growth inhibition (Figure 2B). In conclusion, the IC₅₀ of gefitinib in HCC-287 cells is <0.1 μM while no IC₅₀ was reached in the 4 most gefitinib-sensitive neuroblastoma cell lines using concentrations up to 10 μM.

Inhibition of cell viability by gefitinib and topotecan. Cell viability measured by MTT test after 72 hours significantly decreased in all neuroblastoma cells tested at therapeutically relevant topotecan concentrations up to 10 ng/ml (Table I).
SK-N-FI showed decreased cell viability in a dose-dependent manner, with a maximum inhibition of 48±4% at 10 ng/ml topotecan. Simultaneous administration of 1 μM gefitinib and topotecan in SK-N-FI resulted in increased inhibitory effects with 79±8% viability for the combination compared to 91±6% for 0.5 ng/ml topotecan alone (Figure 3A). Enhanced inhibition was also observed for 0.5 and 1 μM gefitinib with 1 ng/ml topotecan with 89±7% viability for topotecan alone and 78±6% and 76±5%, respectively, for the combinations.

Inhibition of cell viability by gefitinib and vincristine. Treatment with 10 ng/ml vincristine alone inhibited cell viability in all neuroblastoma cells tested, except in Lan-5 cells (Table I). Gefitinib at concentrations of 0.5 and 1 μM sensitized Lan-5 for the treatment with 10 ng/ml vincristine, resulting in 79±5% and 67±5% viability compared to 96±7% with vincristine alone (Figure 3B). Simultaneous administration of 0.5 or 1 μM gefitinib with 1 and 10 ng/ml vincristine resulted in an increased inhibitory effect over single vincristine treatment in all other neuroblastoma cells tested (data not shown).
Inhibition of cell viability by gefitinib and 9cisRA. Treatment with 1 μM 9cisRA showed no effects on the neuroblastoma cell lines Kelly, SK-N-LO and SK-N-FI, while significantly reduced viability was found in SH-EP, SH-SY5Y and Lan-5 (Table I) cells. Co-administration of 1 μM 9cisRA and 1 or 0.5 μM gefitinib increased the inhibitory effects on cell viability of the RA-sensitive Lan-5 cells from 84±4% viability for RA alone to 75±6% and 74±8% for the combinations (Figure 4). Similar effects were observed in SH-SY5Y cells. However, no additional effects on the inhibitory activity of gefitinib with RA were observed in SH-EP and the 9cisRA-resistant cell lines (data not shown).

Discussion

EGFR is an interesting survival signalling pathway in neuroblastoma (16-18) and was used successfully as a target to enhance the oncolytic potency of conditionally replicative...
Adenoviruses (19). To date, two in vitro studies reported on a potential therapeutic approach using gefitinib for EGFR targeting. Based on EGFR and EGF receptor-ligand interaction, Ho et al. suggested a role of this pathway for cell proliferation although most of the neuroblastoma cells analyzed did not express EGFR levels higher than that of normal fetal brain (13). In the second report, EGFR protein expression was shown for 8 out of 10 cell lines at various levels, however much below those of the A431 epidermoid carcinoma cell line (14). Our own study confirms that RNA expression of EGFR does not always lead to detectable EGFR surface protein expression. Four out of six cell lines expressed EGFR mRNA, but only SH-SY5Y showed EGFR protein expression, suggesting that EGFR expression in neuroblastoma underlies post-transcriptional regulation and that EGFR overexpression is rare. Moreover, we extended the findings on the absence of gene mutations in the tyrosine kinase domain of this receptor in neuroblastoma.

For in vitro cytotoxicity effects of gefitinib, we tested all neuroblastoma cell lines under normal growth conditions (10% FCS), including the EGFR non-expressing cells. We found significant inhibitory effects only at a high gefitinib concentration (10 μM) which was independent of the presence of EGFR expression. In contrast, Ho et al. found an IC50 of 0.05 μM gefitinib in the EGFR-expressing NLF and SH-SY5Y cell lines when they were cultured with 10% FCS (13). Tamura et al. demonstrated the IC50 of gefitinib in the EGFR positive KP-N-TK and KP-N-SIFA cell lines to be 1.2 μM when they were cultured with 2% FCS, with IC50s of 10 and 12 μM, respectively, when cells were cultured at 10% FCS. Moreover, apoptotic effects of gefitinib were only found at concentrations greater than 30 μM for the two cell lines (14). It is important to note that the clinically achievable plasma concentration in patients receiving gefitinib at the maximum tolerated dose was 1 μM (20), thus the in vitro effects observed in our studies may not be relevant for neuroblastoma patients.
Since the EGFR pathway is a major cell survival pathway in response to chemotherapy, we intended to evaluate the potential of gefitinib in combination with cytotoxic agents currently used in neuroblastoma. We found an increase of approximately 10% of the inhibitory effects when gefitinib was administered simultaneously with topotecan and vincristine compared to inhibition with these agents alone. In the vincristine-resistant Lan-5 cell line, vincristine sensitivity was achieved by addition of 0.5 or 1 µM gefitinib. Sensitizing effects of gefitinib in neuroblastoma has previously been shown for cisplatin with enhanced growth inhibition of 5-15% when gefitinib was combined (14). The chemosensitizing effect of gefitinib could be due to activation of the EGFR signaling by chemotherapeutics (21), or due to down-regulation of the ATP-binding cassette transporter BCRP and the multiple drug resistance MDR1 membrane proteins that regulate cellular drug efflux by gefitinib (22). Furthermore, we showed that gefitinib increased the cytotoxic effect of cisRA by 10-15% in RA-sensitive but not in RA-resistant neuroblastoma cells. EGFR down-regulation has been described during neuronal differentiation and could explain this effect of RA as a differentiating agent (23). We looked for EGFR expression after cisRA treatment of the six neuroblastoma cell lines, which was not altered (data not shown). In summary, gefitinib enhanced moderately cytotoxic effects of chemotherapeutics and RA, and even reduced chemoresistance in one case.

Limited activity of gefitinib as a single agent in vivo has been shown in a panel of 10 pediatric tumor xenografts, including three neuroblastoma models (24). However, the use of gefitinib in combination with the topoisomerase I inhibitor irinotecan resulted in more than additive activity in an EGFR-negative neuroblastoma xenograft model. A clinical chemosensitizing effect for gefitinib has been suggested in a heavily pre-treated neuroblastoma xenograft model. A clinical chemosensitizing result in more than additive activity in an EGFR-negative neuroblastoma over 1 year of age. Cancer Lett 197: 11-17, 2003.

Targeting EGFR using gefitinib as a single agent appears to have a minor interest for the treatment of neuroblastoma. The moderate sensitizing effect of EGFR targeting with topotecan, vincristine and cisRA in vitro needs to be further explored for its clinical relevance. In addition, pathways other than EGFR signaling should be explored in preclinical studies to bring forward new treatment protocols for neuroblastoma.

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References


