

## Cisplatin-Resistant Neuroblastoma Cells Express Enhanced Levels of Epidermal Growth Factor Receptor (EGFR) and Are Sensitive to Treatment with EGFR-Specific Toxins

Martin Michaelis,<sup>1</sup> Jennifer Bliss,<sup>1</sup> Sonja C. Arnold,<sup>2</sup> Nora Hinsch,<sup>3</sup> Florian Rothweiler,<sup>1</sup> Hedwig E. Deubzer,<sup>5</sup> Olaf Witt,<sup>5</sup> Klaus Langer,<sup>2</sup> Hans W. Doerr,<sup>1</sup> Winfried S. Wels,<sup>4</sup> and Jindrich Cinatl, Jr.<sup>1</sup>

**Abstract Purpose:** Neuroblastomas frequently show expression of the epidermal growth factor receptor (EGFR) and may therefore be susceptible to EGFR-targeted therapies. Here, EGFR expression and functionality was investigated in parental chemosensitive neuroblastoma cell lines (UKF-NB-3, IMR-32, NLF, SH-SY5Y) and their cisplatin-resistant sublines (UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, IMR-32<sup>r</sup>CDDP<sup>1000</sup>, NLF<sup>r</sup>CDDP<sup>1000</sup>, and SH-SY5Y<sup>r</sup>CDDP<sup>500</sup>). Moreover, the EGFR antibody cetuximab, the EGFR tyrosine kinase inhibitor Tyrphostin B46, and recombinant EGFR-targeted toxins were investigated for their influence on the viability and growth of neuroblastoma cells.

**Experimental Design:** EGFR expression and function was measured by flow cytometry or Western blot. Cell viability was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis was examined by immunostaining for active caspase-3 or cleaved poly(ADP-ribose) polymerase. Cellular binding of FITC-labeled immunotoxins was studied by flow cytometry, and cellular uptake was studied by confocal laser scanning microscopy.

**Results:** The EGFR-targeted antibody and growth factor toxins scFv(14E1)-*Pseudomonas* exotoxin A (ETA) and TGF- $\alpha$ -ETA exerted anti-cancer effects in neuroblastoma cell lines that were insensitive to cetuximab or EGFR tyrosine kinase inhibitors. Furthermore, adaptation of chemosensitive neuroblastoma cells to cisplatin increased EGFR expression and sensitivity to both recombinant toxins. Treatment of chemosensitive neuroblastoma cells with cisplatin reversibly increased EGFR expression, whereas cisplatin-resistant cells showed enhanced EGFR expression independent of the presence of cisplatin. Combination treatment with scFv(14E1)-ETA or TGF- $\alpha$ -ETA and cisplatin exerted significantly improved anticancer effects compared with either single treatment in parental neuroblastoma cells, cisplatin-resistant sublines, and primary cultures.

**Conclusions:** EGFR-targeted cytotoxic reagents such as scFv(14E1)-ETA and TGF- $\alpha$ -ETA represent promising candidates for further development as antineuroblastoma agents, especially in combination with cisplatin.

The epidermal growth factor receptor (EGFR, also HER1 or ErbB1) is a 170-kDa transmembrane protein tyrosine kinase that plays a crucial role in signal transduction pathways that regulate key cellular functions such as survival and

proliferation. In normal tissues, ligands that can activate EGFR include EGF, amphiregulin, and transforming growth factor- $\alpha$  (TGF- $\alpha$ ),  $\beta$ -cellulin, heparin-binding EGF, and epiregulin (1, 2).

EGFR is normally expressed in all epithelial and stromal cells, but more selectively expressed in glial and smooth muscle cells. High levels of EGFR have been observed in many tumors of epithelial origin, including head and neck, non-small cell lung, prostate, breast, gastric, colorectal, and ovarian cancers (1, 2). High EGFR expression has also been observed in glioblastoma multiforme (3). Because EGFR is easily accessible extracellularly, EGFR-specific antibodies and immunotoxins have been developed (2, 4, 5). In addition, the EGFR tyrosine kinase inhibitors gefitinib and erlotinib have been approved for the treatment of non-small cell lung cancer and head and neck cancer. Moreover, they are under clinical examination for other cancer entities (2, 6).

Neuroblastoma cells have been shown to express EGFR (7, 8), and increased chemotherapy-induced EGFR expression has previously been described in different chemoresistant cancer cells (9–13), including vincristine- or actinomycin-D-resistant neuroblastoma cells (14). Here, we investigated

**Authors' Affiliations:** <sup>1</sup>Institut für Medizinische Virologie, Klinikum der J.W. Goethe Universität; <sup>2</sup>Institut für Pharmazeutische Technologie, Johann Wolfgang Goethe-Universität; <sup>3</sup>Senckenbergisches Institut für Pathologie, Klinikum der J.W. Goethe Universität; <sup>4</sup>Chemotherapeutisches Forschungsinstitut Georg-Speyer-Haus, Frankfurt am Main, Germany; and <sup>5</sup>Deutsches Krebsforschungszentrum (DKFZ), Klinische Kooperationseinheit Pädiatrische Onkologie (G340) and Pädiatrie III, Zentrum für Kinder- und Jugendmedizin, Heidelberg, Germany  
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**Requests for reprints:** Jindrich Cinatl, Jr., Institut für Medizinische Virologie, Paul Ehrlich-Str. 40, 60596 Frankfurt/Main, Germany. Phone: 49-69-6301-6409; Fax: 49-69-6301-4302; E-mail: Cinatl@em.uni-frankfurt.de.

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### Translational Relevance

The finding that cisplatin increases epidermal growth factor receptor (EGFR) expression in cancer cells may be relevant for the planning of future clinical studies. The finding that (EGFR-targeted) *Pseudomonas* exotoxin A resensitizes cisplatin-resistant cells to cisplatin may represent a novel treatment option for cisplatin-resistant tumors.

EGFR expression and functionality in the neuroblastoma cell lines UKF-NB-3, IMR-32, NLF, and SH-SY5Y, and their cisplatin-resistant sublines UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, IMR-32<sup>r</sup>CDDP<sup>1000</sup>, NLF<sup>r</sup>CDDP<sup>1000</sup>, and SH-SY5Y<sup>r</sup>CDDP<sup>500</sup>. Moreover, we examined their sensitivity to the EGFR-specific antibody cetuximab, the EGFR tyrosine kinase inhibitor Tyrphostin B46, and EGFR-targeted recombinant toxins. The effects of the combination of cisplatin with EGFR-targeted toxins were studied in cell lines and in primary neuroblastoma cells.

### Materials and Methods

**Materials.** Cetuximab (Erbix) was received from Merck Pharma GmbH. Cisplatin was obtained from GRY-Pharma. The immunotoxins scFv(14E1)-*Pseudomonas* exotoxin A (ETA) and TGF- $\alpha$ -ETA were produced as described before (15). The EGFR tyrosine kinase inhibitor Tyrphostin B46 (AG555) was purchased from Merck Biosciences.

**Cells.** A431 and the parental neuroblastoma cell lines IMR-32 and SH-SY5Y were obtained from the American Type Culture Collection. UKF-NB-3 was established as described (16). NLF cells were kindly provided by Dr. Angelika Eggert of Universitäts-Kinderklinik Essen, Essen, Germany. The adaptation of cells to cisplatin was done as described before (16). The parental cell lines UKF-NB-3, IMR-32, and NLF were adapted to the growth in the presence of 1,000 ng/mL cisplatin. The resulting cisplatin-resistant sublines were named UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, IMR-32<sup>r</sup>CDDP<sup>1000</sup>, and NLF<sup>r</sup>CDDP<sup>1000</sup>. SH-SY5Y was adapted to the growth in the presence of 500 ng/mL cisplatin and named SH-SY5Y<sup>r</sup>CDDP<sup>500</sup>. The IC<sub>50</sub> values (concentrations that cause a 50% decrease in cell viability) for cisplatin in the cell lines showing cisplatin resistance of adapted cells are shown in Table 1.

Fresh neuroblastoma cells (MYCN-amplified) were isolated from the bone marrow aspirate of two patients with metastasized International

Neuroblastoma Staging System stage 4 neuroblastoma following informed consent. All cell lines were grown in Iscove's modified Dulbecco's medium supplemented with 10% FBS, 100 IU/mL penicillin, and 100 mg/mL streptomycin at 37°C.

**Viability assay.** Cell viability was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay modified after (17) as described before (18).

**Flow cytometry.** The expression of EGFR was evaluated by flow cytometry using primary anti-EGFR antibody (R&D Systems) and FITC-conjugated secondary antibody (BD Biosciences) as described before (16). Fluorescence was determined using Becton Dickinson FACScan, CellQuest software (BD Biosciences) and expressed as well as quantified in terms of relative fluorescence units (RFU).

**Western blot.** Cells were lysed in Triton X sample buffer and separated by SDS-PAGE, as described (18). Proteins were detected using specific antibodies against  $\beta$ -actin (Sigma), EGFR, phosphorylated EGFR (Upstate Biotechnology), extracellular single-regulated kinase 1 and 2 (ERK 1/2), or the phosphorylated forms of ERK 1/2 (each from New England Biolabs), and were visualized by enhanced chemiluminescence using a commercially available kit (Amersham).

**Confocal laser scanning microscopy.** Labeling of immunotoxins was done using the Alexa Fluor 488 Microscale Protein Labeling Kit (A30006; Invitrogen) following the manufacturer's manual. Confocal laser scanning microscopy was done as described before (19).

**Immune staining.** Activated caspase-3 and the 85-kDa fragment of cleaved poly(ADP-ribose) polymerase (PARP) were detected by immune staining. The following primary antibodies were used: active caspase-3 (R&D Systems) and PARP p85-fragment (Promega). Biotin-conjugated secondary monoclonal antibodies were used and visualization was done with streptavidin-peroxidase complex with AEC as a substrate.

For the staining of EGFR, cell pellets were fixed in 4% formalin, paraffin embedded, and cut in 5- $\mu$ m slices. EGFR immune staining was done with the EGFR pharmDx-Kit (Dako, GmbH), following the manufacturer's instructions.

**Statistics.** Results represent the mean  $\pm$  SD of at least three experiments. Comparisons between two groups were done using Student's *t* test; three and more groups were compared by ANOVA followed by the Student-Newman-Keuls test. *P* values lower than 0.05 were considered to be significant.

### Results

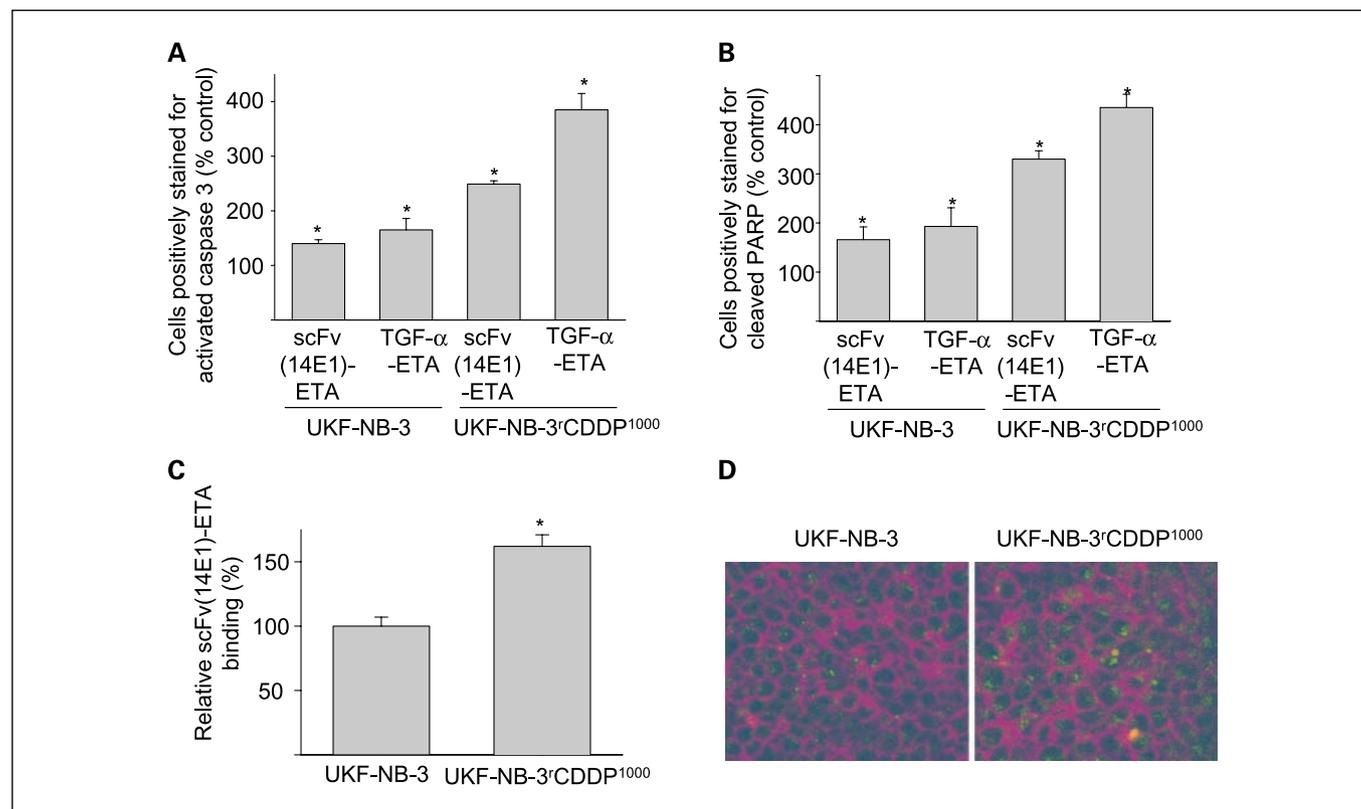
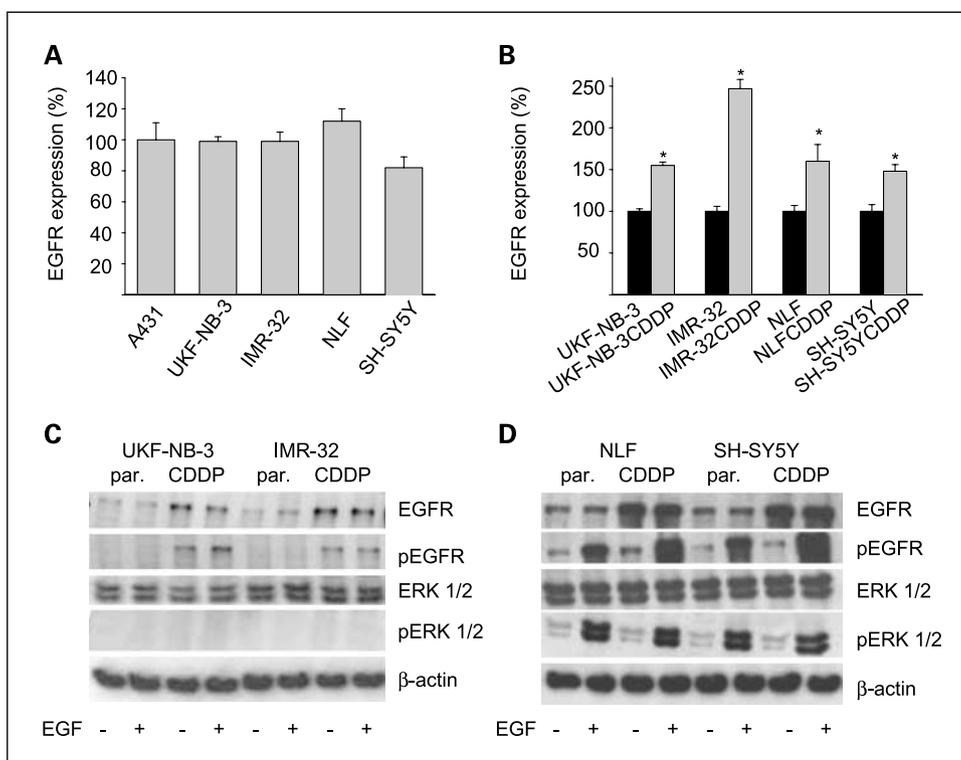
**Increased EGFR expression in cisplatin-resistant neuroblastoma cells.** EGFR protein expression was investigated in four different neuroblastoma cell lines (UKF-NB-3, IMR-32, NLF, and SH-SY5Y) in comparison with their cisplatin-resistant sublines (UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, IMR-32<sup>r</sup>CDDP<sup>1000</sup>, NLF<sup>r</sup>CDDP<sup>1000</sup>, and

**Table 1.** Cytotoxicity of the EGFR tyrosine kinase inhibitor tyrphostin B46 or the EGFR-targeted recombinant toxins TGF- $\alpha$ -ETA and scFv(14E1)-ETA toward neuroblastoma cells

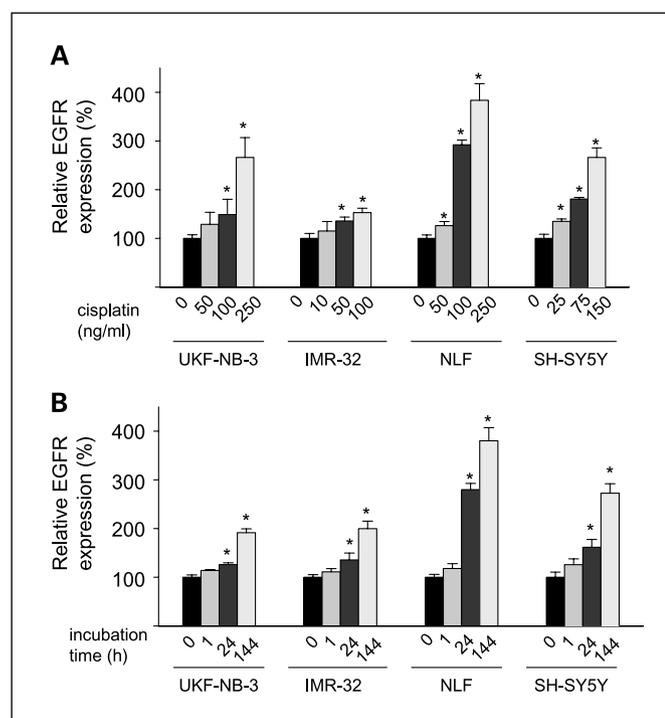
Cell line	IC <sub>50</sub> (ng/mL), mean $\pm$ SD			IC <sub>50</sub> ( $\mu$ mol/L), mean $\pm$ SD
	Cisplatin	TGF- $\alpha$ -ETA	scFv(14E1)-ETA	Tyrphostin B46*
UKF-NB-3	76.6 $\pm$ 37.3	192.9 $\pm$ 31.1	745.1 $\pm$ 221.9	7.7 $\pm$ 1.9
UKF-NB-3 <sup>r</sup> CDDP <sup>1000</sup>	1,489.6 $\pm$ 262.6	14.7 $\pm$ 9.4	155.9 $\pm$ 41.2	11.5 $\pm$ 0.8
IMR-32	37.0 $\pm$ 17.7	83.9 $\pm$ 5.0	52.8 $\pm$ 10.5	5.4 $\pm$ 0.8
IMR-32 <sup>r</sup> CDDP <sup>1000</sup>	2,800.7 $\pm$ 136.3	44.8 $\pm$ 8.6	25.0 $\pm$ 7.3	10.0 $\pm$ 0.9
NLF	141.5 $\pm$ 27.9	25.0 $\pm$ 4.5	51.1 $\pm$ 7.1	9.5 $\pm$ 3.0
NLF <sup>r</sup> CDDP <sup>1000</sup>	2,269.1 $\pm$ 959.9	7.1 $\pm$ 3.2	14.6 $\pm$ 1.9	32.9 $\pm$ 5.2
SH-SY5Y	121.4 $\pm$ 54.3	192.7 $\pm$ 35.7	987.9 $\pm$ 21.0	11.6 $\pm$ 1.6
SH-SY5Y <sup>r</sup> CDDP <sup>500</sup>	558.2 $\pm$ 65.1	20.89 $\pm$ 3.54	105.6 $\pm$ 20.00	21.0 $\pm$ 4.1

\*IC<sub>50</sub> for EGFR tyrosine kinase inhibition = 0.7  $\mu$ mol/L.

**Fig. 1.** Expression and phosphorylation status of EGFR and ERK 1/2 in neuroblastoma cell lines. **A**, EGFR expression (determined by flow cytometry) in UKF-NB-3, IMR-32, NLF, or SH-SY5Y relative to A431 cells that served as positive control (100%). **B**, EGFR expression (determined by flow cytometry) in the cisplatin-resistant neuroblastoma cell lines UKF-NB-3' CDDP<sup>1000</sup> (UKF-NB-3CDDP), IMR-32' CDDP<sup>1000</sup> (IMR-32CDDP), NLF' CDDP<sup>1000</sup> (NLF CDDP), and SH-SY5Y' CDDP<sup>500</sup> (SH-SY5Y CDDP) relative to their corresponding parental cell lines (100%). \*,  $P < 0.05$  relative to EGFR expression of parental cell line. **C** and **D**, representative Western blots showing expression and phosphorylation status of EGFR and ERK 1/2 in neuroblastoma cells. Confluent cells were kept for 24 h in serum-free cell culture medium and then stimulated for 30 min with EGF (100 ng/mL).  $\beta$ -Actin served as loading control. **C**, UKF-NB-3, UKF-NB-3' CDDP<sup>1000</sup> (UKF-NB-3CDDP), IMR-32, IMR-32' CDDP<sup>1000</sup> (IMR-32CDDP) cells. **D**, NLF, NLF' CDDP<sup>1000</sup> (NLF CDDP), SH-SY5Y, SH-SY5Y' CDDP<sup>500</sup> (SH-SY5Y CDDP) cells.



**Fig. 2.** Apoptosis induction by EGFR-targeted toxins (**A** and **B**), cell binding (**C**), and internalization (**D**). **A**, UKF-NB-3 or UKF-NB-3' CDDP<sup>1000</sup> cells were treated with scFv(14E1)-ETA (100 ng/mL) or TGF- $\alpha$ -ETA (100 ng/mL) for 72 h and stained for activated caspase-3. \*,  $P < 0.05$  relative to untreated cells. **B**, UKF-NB-3 or UKF-NB-3' CDDP<sup>1000</sup> cells were treated with scFv(14E1)-ETA (100 ng/mL) or TGF- $\alpha$ -ETA (100 ng/mL) for 72 h and stained for cleaved PARP. \*,  $P < 0.05$  relative to untreated cells. **C**, flow cytometric analysis of UKF-NB-3 and UKF-NB-3' CDDP<sup>1000</sup> cells incubated with fluorescence-labeled scFv(14E1)-ETA (10  $\mu$ g/mL). \*,  $P < 0.05$  relative to parental cell line. **D**, representative images showing UKF-NB-3 and UKF-NB-3' CDDP<sup>1000</sup> cells (red) incubated with fluorescence-labeled (green) scFv(14E1)-ETA (1  $\mu$ g/mL) for 6 h.



**Fig. 3.** EGFR expression in cisplatin-treated parental neuroblastoma cell lines. *A*, neuroblastoma cell lines were treated with nontoxic, moderately toxic, or highly toxic concentrations of cisplatin (UKF-NB-3: 50, 100, 250 ng/mL; IMR-32: 10, 50, 100 ng/mL; NLF: 50, 100, 250 ng/mL; SH-SY5Y 25, 75, 150 ng/mL) for 144 h. *B*, neuroblastoma cell lines were treated for 1, 24, or 144 h with cisplatin (UKF-NB-3: 250 ng/mL; IMR-32: 100 ng/mL; NLF: 250 ng/mL; SH-SY5Y: 150 ng/mL). EGFR expression was measured by flow cytometry. \*,  $P < 0.05$  relative to untreated cells.

SH-SY5Y<sup>r</sup>CDDP<sup>500</sup>) and with the epidermoid carcinoma cell line A431, which served as a positive control for EGFR expression (20). The parental neuroblastoma cell lines showed EGFR expression similar to A431 (Fig. 1A). All cisplatin-resistant sublines showed increased EGFR expression (1.5- to 2.5-fold) compared with their parental cell lines (Fig. 1B). Similar results were obtained by immune staining (data not shown).

**Influence of cetuximab or EGFR tyrosine kinase inhibitors on the growth of neuroblastoma cells.** The anti-EGFR antibody cetuximab is approved for the treatment of metastasized colorectal cancer and locally advanced head and neck squamous cell carcinomas.<sup>6</sup> Moreover, it is under clinical examination for a number of additional forms of cancer (21). Steady state serum levels in humans have been determined at around 150  $\mu$ g/mL.<sup>6</sup> Neuroblastoma cell lines were incubated with cetuximab at concentrations up to 200  $\mu$ g/mL, and cell viability was determined in MTT assays. NLF<sup>r</sup>CDDP<sup>1000</sup> was the only cell line in which the IC<sub>50</sub> was lower than 200  $\mu$ g/mL (25.93  $\pm$  2.09  $\mu$ g/mL). Cetuximab at 200  $\mu$ g/mL did not significantly influence the growth of IMR-32 (cell viability relative to untreated control as indicated by MTT assay, 111.84  $\pm$  3.26%), IMR-32<sup>r</sup>CDDP<sup>1000</sup> (114.65  $\pm$  10.12%), and SH-SY5Y cells (93.73  $\pm$  1.89%). The viability of UKF-NB-3 (80.34  $\pm$  1.52%), UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> (74.46  $\pm$  8.00%), and NLF (81.00  $\pm$  4.53%) cells was moderately

influenced, whereas the viability of SH-SY5Y<sup>r</sup>CDDP<sup>500</sup> cells (53.3  $\pm$  5.36%) was more strongly affected. No statistically significant differences were detected for the effects of cetuximab on the parental cell lines IMR-32 and UKF-NB-3 and their cisplatin-resistant sublines IMR-32<sup>r</sup>CDDP<sup>1000</sup> and UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>. In contrast, SH-SY5Y<sup>r</sup>CDDP<sup>500</sup> and NLF<sup>r</sup>CDDP<sup>1000</sup> cells were more sensitive to cetuximab than their parental cell lines SH-SY5Y and NLF.

The IC<sub>50</sub> values for the effects of the EGFR tyrosine kinase inhibitor Tyrphostin B46 on the proliferation of the investigated neuroblastoma cell lines are shown in Table 1. The values are clearly higher (5.44-32.91  $\mu$ mol/L) than those described for specific EGFR tyrosine kinase inhibition (700 nmol/L). Therefore, the antiproliferative effects caused by Tyrphostin B46 are more likely to be caused by unspecific effects than by EGFR tyrosine kinase inhibition. Moreover, cisplatin-resistant cell lines are less sensitive to EGFR tyrosine kinase inhibitor treatment than the parental cell lines although cisplatin-resistant cells show increased EGFR expression.

**EGF-induced EGFR signaling in neuroblastoma cells.** After activation by ligands such as EGF, EGFRs dimerize, resulting in receptor autophosphorylation and internalization (1, 2). Downstream EGFR effects are mediated by several signal transduction pathways including the Ras/Raf/MEK/ERK pathway. To investigate ligand-induced EGFR signaling in neuroblastoma cells, the cells were kept for 24 hours in serum-free medium, before EGF (100 ng/mL) was added for 30 minutes and cellular levels of EGFR, phosphorylated EGFR (pEGFR), ERK 1/2, and phosphorylated ERK 1/2 were examined by Western blot (Fig. 1C and D). UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup> and IMR-32<sup>r</sup>CDDP<sup>1000</sup> showed increased basal expression of EGFR and pEGFR compared with their parental cell lines UKF-NB-3 and IMR-32. No differences were found in ERK 1/2 and phosphorylated ERK 1/2 expression. EGF did not induce EGFR phosphorylation or ERK 1/2 phosphorylation in UKF-NB-3, UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, IMR-32, or IMR-32<sup>r</sup>CDDP<sup>1000</sup> cells (Fig. 1C). NLF<sup>r</sup>CDDP<sup>1000</sup> and SH-SY5Y<sup>500</sup> had increased EGFR levels compared with their parental cell lines NLF and SH-SY5Y. EGF induced strong phosphorylation of EGFR and ERK 1/2 in NLF, NLF<sup>r</sup>CDDP<sup>1000</sup>, SH-SY5Y, and SH-SY5Y<sup>r</sup>CDDP<sup>500</sup> cells (Fig. 1D).

**Increased efficacy of EGFR-targeted toxins against cisplatin-resistant neuroblastoma cells.** EGFR-targeted toxins exert their cytotoxic activity after receptor binding and intracellular uptake independent of EGFR signaling. Hence, we investigated the effects of the two EGFR-targeted toxins, scFv(14E1)-ETA and TGF- $\alpha$ -ETA (15, 22-24), on neuroblastoma cell viability. TGF- $\alpha$ -ETA is a recombinant fusion protein consisting of the natural EGFR ligand TGF- $\alpha$  and a truncated form of ETA devoid of the toxin's endogenous cell binding domain. ScFv(14E1)-ETA is a similar single-chain antibody-toxin derived from the EGFR-specific monoclonal antibody 14E1 (15).

The neuroblastoma cells were incubated for 5 days with increasing concentrations of the recombinant toxins, and cell viability was determined in MTT assays. The resulting IC<sub>50</sub> values are shown in Table 1. The cisplatin-resistant sublines were significantly more sensitive to the toxins than the parental cell lines.

Toxins carrying truncated ETA as a cytotoxic domain were shown to exert anticancer effects via induction of apoptosis as indicated by caspase-3 activation and PARP cleavage (25, 26). To investigate the induction of apoptosis in neuroblastoma cells,

<sup>6</sup> <http://www.emea.europa.eu>

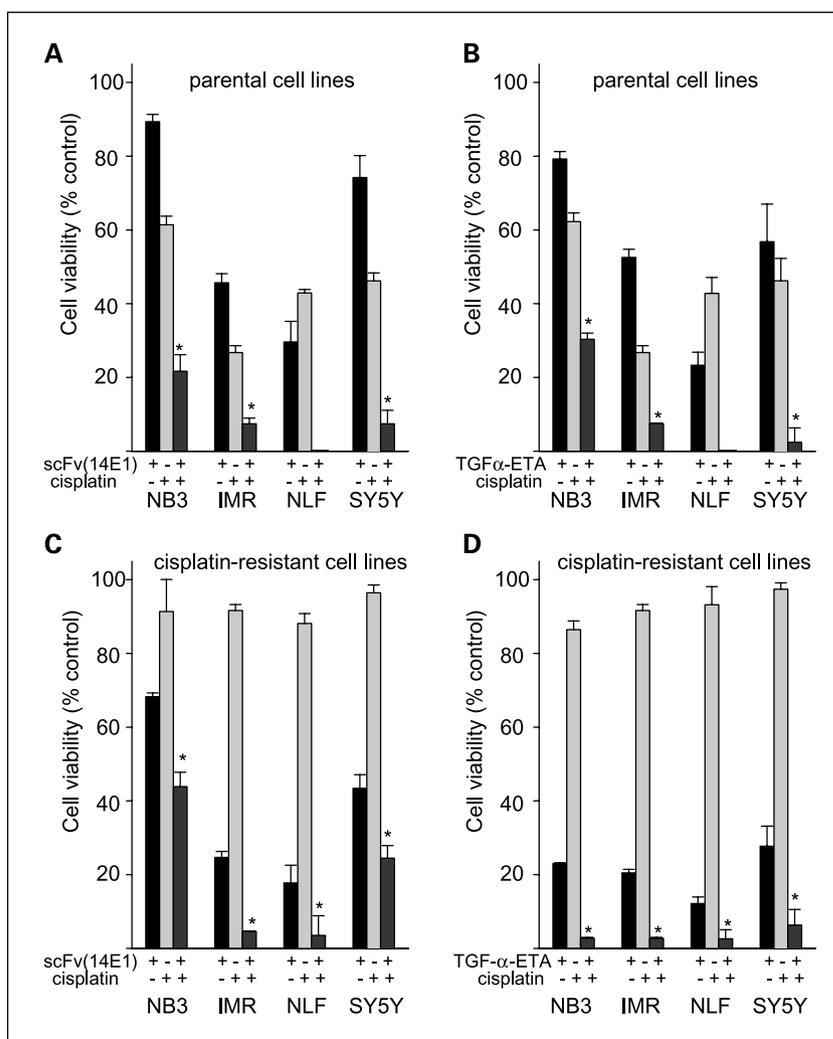
UKF-NB-3 and UKF-NB-3<sup>CDDP</sup>1000 cells were incubated for 72 hours with 100 ng/mL of scFv(14E1)-ETA or TGF- $\alpha$ -ETA. Apoptotic cells were detected by immune staining for activated caspase-3 or cleaved PARP. Whereas the toxins induced apoptosis in both cell lines, toxin-induced apoptosis was enhanced in cisplatin-resistant UKF-NB-3<sup>CDDP</sup>1000 cells (Fig. 2A and B).

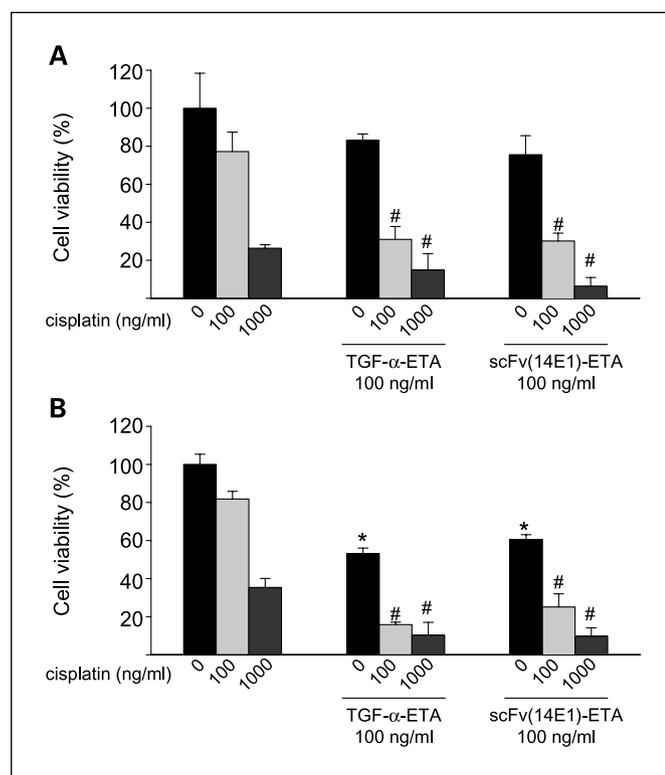
**Increased binding and uptake of scFv(14E1)-ETA by cisplatin-resistant UKF-NB-3 cells.** The binding and uptake of scFv(14E1)-ETA was studied in UKF-NB-3 and UKF-NB-3<sup>CDDP</sup>1000 cells by flow cytometry and confocal laser scanning microscopy using fluorescence-labeled antibody toxin. Flow cytometry analysis revealed increased binding of fluorescence-labeled scFv(14E1)-ETA to UKF-NB-3<sup>CDDP</sup>1000 cells compared with UKF-NB-3 cells (Fig. 2C). Similar results were obtained when uptake was investigated by confocal laser scanning microscopy (Fig. 2D).

**Dependence of EGFR expression on the presence of cisplatin.** To investigate whether acute cisplatin treatment increases EGFR expression in neuroblastoma cells, the influence of cisplatin on EGFR expression was investigated in parental chemosensitive cells. MTT assays were done to determine cisplatin concentrations that did not induce cytotoxic effects, that induced moderate cytotoxic effects, or that had strong

cytotoxic effects in the individual cell lines (data not shown). The cells were then treated with these three different cisplatin concentrations for 144 hours. Cisplatin induced a concentration-dependent increase in EGFR expression (Fig. 3A). Incubation of parental neuroblastoma cell lines with the highest chosen cisplatin concentrations for 1, 24, or 144 hours showed that cisplatin-induced EGFR expression increased with prolonged incubation periods (Fig. 3B). To investigate whether cisplatin-induced EGFR expression is reversible in parental cells, neuroblastoma cell lines were incubated for 144 hours with the highest chosen cisplatin concentrations, washed, and incubated for a further 48 hours with cisplatin-free medium. Thereby, in all cell lines investigated EGFR expression levels returned to those of untreated controls (data not shown). This indicates that cisplatin induces EGFR expression in a reversible manner. In contrast, the passing of all cisplatin-resistant neuroblastoma cells for four passages without cisplatin did not result in a decrease in EGFR expression. UKF-NB-3<sup>CDDP</sup>1000 and IMR-32<sup>CDDP</sup>1000 were cultivated for 16 passages without cisplatin, and EGFR expression remained constant (data not shown), indicating that EGFR expression in cisplatin-resistant cells no longer depends on the continuous presence of cisplatin.

**Fig. 4.** Combined effects of EGFR-targeted toxins and cisplatin against cisplatin-sensitive neuroblastoma cells and cisplatin-resistant sublines. **A**, the parental cell lines UKF-NB-3 (NB3), IMR-32 (IMR), NLF, and SH-SY5Y (SY5Y) were treated with 100 ng/mL scFv(14E1)-ETA [scFv(14E1)], 100 ng/mL cisplatin, or a combination of scFv(14E1)-ETA and cisplatin. No viable cells were detected in NLF cells treated with the combination of scFv(14E1)-ETA and cisplatin. **B**, the parental cell lines NB3, IMR, NLF, and SY5Y were treated with TGF- $\alpha$ -ETA (100 ng/mL), cisplatin (100 ng/mL), or a combination of TGF- $\alpha$ -ETA and cisplatin. No viable cells were detected in NLF cells treated with the combination of TGF- $\alpha$ -ETA and cisplatin. **C**, the cisplatin-resistant cell lines UKF-NB-3<sup>CDDP</sup>1000 (NB3), IMR-32<sup>CDDP</sup>1000 (IMR), NLF<sup>CDDP</sup>1000 (NLF), and SH-SY5Y<sup>CDDP</sup>500 (SY5Y) were treated with scFv(14E1)-ETA (100 ng/mL), cisplatin (1,000 ng/mL), or a combination of scFv(14E1)-ETA and cisplatin. **D**, the cisplatin-resistant cell lines NB3, IMR, NLF, and SY5Y were treated with TGF- $\alpha$ -ETA (100 ng/mL), cisplatin (1,000 ng/mL), or a combination of TGF- $\alpha$ -ETA and cisplatin. Cell viability was measured after a 5-d incubation period by MTT assay relative to untreated controls. \*,  $P < 0.05$  relative to either single treatment.





**Fig. 5.** Combined effects of EGFR-targeted toxins and cisplatin against primary neuroblastoma cells. Primary neuroblastoma cells from the bone marrow of two patients with metastasized relapsed International Neuroblastoma Staging System stage 4 neuroblastoma (A and B) were treated with scFv(14E1)-ETA (100 ng/mL), TGF- $\alpha$ -ETA (100 ng/mL), cisplatin (100 ng/mL or 1,000 ng/mL), or combinations of scFv(14E1)-ETA or TGF- $\alpha$ -ETA and cisplatin. \*,  $P < 0.05$  relative to untreated control; #,  $P < 0.05$  relative to either single treatment.

**EGFR-targeted toxins increase the antitumoral activity of cisplatin.** EGFR-targeted toxins and cisplatin were combined to investigate the effects of the combinations in comparison with either single treatment. The parental cell lines UKF-NB-3, IMR-32, NLF, and SH-SY5Y were treated with 100 ng/mL of the recombinant toxins scFv(14E1)-ETA or TGF- $\alpha$ -ETA, 100 ng/mL of cisplatin, or a combination of toxin plus cisplatin. The cisplatin-resistant cell lines UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, IMR-32<sup>r</sup>CDDP<sup>1000</sup>, and NLF<sup>r</sup>CDDP<sup>1000</sup> were treated with 100 ng/mL of the toxins, 1,000 ng/mL of cisplatin, or a combination of toxin plus cisplatin. SH-SY5Y<sup>r</sup>CDDP<sup>500</sup> was treated with 100 ng/mL of the toxins, 500 ng/mL of cisplatin, or a combination of toxin plus cisplatin. Survival of cells was determined after a five-day incubation period by MTT assay relative to untreated controls. The results are shown in Fig. 4. In all cell lines investigated, the combination of EGFR-targeted toxins with cisplatin decreased cell viability significantly more than single treatment with toxins or cisplatin. Similar results were obtained in primary neuroblastoma cells isolated from the bone marrow of patients with metastasized International Neuroblastoma Staging System stage 4 neuroblastoma (Fig. 5).

## Discussion

In this report, we found that neuroblastoma cell lines adapted to growth in the presence of 1,000 ng/mL of cisplatin (UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, IMR-32<sup>r</sup>CDDP<sup>1000</sup>, NLF<sup>r</sup>CDDP<sup>1000</sup>), or

500 ng/mL cisplatin (SH-SY5Y<sup>r</sup>CDDP<sup>500</sup>) display enhanced EGFR expression compared with their cisplatin-sensitive parental cell lines.

Contradictory data have been published concerning the influence of EGF on neuroblastoma cells. EGF has been shown to stimulate, inhibit, or not influence human neuroblastoma cell growth (7, 8, 27–35). Here, the cell lines NLF and SH-SY5Y and their corresponding cisplatin-resistant sublines (NLF<sup>r</sup>CDDP<sup>1000</sup>, SH-SY5Y<sup>r</sup>CDDP<sup>500</sup>) displayed EGF-induced EGFR signaling indicated by EGFR autophosphorylation and ERK 1/2 phosphorylation. Among these cell lines, only the cisplatin-resistant NLF<sup>r</sup>CDDP<sup>1000</sup> and SH-SY5Y<sup>r</sup>CDDP<sup>500</sup> cells, but not the parental cell lines NLF and SH-SY5Y, showed significant sensitivity to cetuximab in concentrations up to 200  $\mu$ g/mL, a concentration in the range of therapeutic plasma concentrations in patients<sup>7</sup> (36). In contrast, EGF did not induce EGFR signaling in UKF-NB-3, IMR-32, UKF-NB-3<sup>r</sup>CDDP<sup>1000</sup>, or IMR-32<sup>r</sup>CDDP<sup>1000</sup> cells that were insensitive to cetuximab. In agreement with our data, previous reports showed EGFR signaling in NLF and SH-SY5Y cells (7). In contrast to our results, however, a mitogenic response to EGF was described for IMR-32 cells in another study (28). Hence, culture conditions may influence the response of neuroblastoma cells to stimulation by EGF or specific inhibition of EGFR signaling. Furthermore, mitogenic effects and proapoptotic actions of EGF may be concentration-dependent (34).

The EGFR tyrosine kinase inhibitor gefitinib was shown to induce apoptosis in different neuroblastoma cell lines (KP-N-TK, KP-N-SIFA; ref. 8). In contrast, for the cell lines investigated in our study, we did not observe specific effects by the small molecular weight EGFR tyrosine kinase inhibitor Tyrphostin B46. Unlike antibodies and EGFR tyrosine kinase inhibitors, EGFR-targeted toxins should be active against cancer cells expressing EGFR for as long as they are internalized after toxin binding (22). We investigated the effects of the EGFR-targeted toxins scFv(14E1)-ETA and TGF- $\alpha$ -ETA on the growth of neuroblastoma cells. Cisplatin-resistant neuroblastoma cells showed an increased sensitivity to treatment with EGFR-targeted toxins when compared with the corresponding cisplatin-sensitive parental neuroblastoma cells. This is most likely due to enhanced cell binding and intracellular uptake as indicated by flow cytometry and confocal laser scanning microscopy.

Acute cisplatin treatment of parental chemosensitive neuroblastoma cells also induced increased EGFR expression. This effect was reversible and depended on the presence of cisplatin. In contrast, the cisplatin-resistant variants showed enhanced EGFR expression also after cultivation for several passages in the absence of cisplatin, indicating that adaptation of neuroblastoma cells to cisplatin results in stable changes that cause elevated EGFR expression. Combined treatment of parental cisplatin-sensitive cells with either one of the two EGFR-targeted toxins plus cisplatin showed significantly stronger anticancer effects than either single treatment alone. Surprisingly, this was also the case for cisplatin-resistant sublines. Whereas the cisplatin concentrations used had no significant effect on the growth of these cells, combinations of

<sup>7</sup> <http://www.emea.europa.eu>

EGFR-targeted toxins with cisplatin were significantly more effective than treatment with toxin as a single modality. This suggests that the inhibition of protein synthesis by the recombinant toxins and/or their proapoptotic activity can at least in part restore the cytotoxic effects of cisplatin. Notably, the combination of EGFR-targeted toxins with cisplatin also showed significantly enhanced antineuroblastoma effects in comparison with either single treatment in primary cell cultures. The systemic use of EGFR-targeted toxins has been limited by liver toxicity in clinical trials (37). Combination with cisplatin may decrease the doses needed to exert anticancer effects in neuroblastoma and therefore reduce adverse events.

In conclusion, our data show that the EGFR-targeted toxins scFv(14E1)-ETA and TGF- $\alpha$ -ETA exert potent anticancer effects against neuroblastoma cells that are insensitive to cetuximab or

EGFR tyrosine kinase inhibitors. Adaptation of chemosensitive neuroblastoma cells to cisplatin enhanced EGFR expression and increased their sensitivity to EGFR-specific toxins. Combinations of EGFR-specific toxins with cisplatin exerted improved anticancer effects when compared with either single treatment in parental neuroblastoma cell lines, their cisplatin-resistant sublines, and primary neuroblastoma cells.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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