Simultaneous inhibition of EGFR signalling and enhanced activation of TRAIL-R-mediated apoptosis induction by an scFv:sTRAIL fusion protein with specificity for human EGFR.

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Abstract
EGFR-signalling inhibition by monoclonal antibodies (MAb) and EGFR-specific tyrosine kinase inhibitors has shown clinical efficacy in cancer by restoring susceptibility of tumour cells to therapeutic apoptosis induction. TRAIL is a promising anti-cancer agent with tumour-selective apoptotic activity. Here we present a novel approach that combines EGFR-signalling inhibition with target cell-restricted apoptosis induction using a TRAIL fusion protein with engineered specificity for EGFR. This fusion protein, scFv425:sTRAIL, comprises the EGFR-blocking antibody fragment scFv425 genetically fused to soluble TRAIL (sTRAIL). Treatment with scFv425:sTRAIL resulted in the specific accretion to the cell surface of EGFR-positive cells only. EGFR-specific binding rapidly induced the dephosphorylation of EGFR- and downstream mitogenic signalling, which was accompanied by c-FLIPL down-regulation and BAD dephosphorylation. EGFR-specific binding converted soluble scFv425:sTRAIL into a membrane-bound form of TRAIL that crosslinked agonistic TRAIL receptors in a paracrine manner, resulting in potent apoptosis induction in a series of EGFR-positive tumour cell lines. Co-treatment of EGFR-positive tumour cells with the EGFR tyrosine kinase inhibitor Iressa resulted in a potent synergistic pro-apoptotic effect, caused by the specific down-regulation of c-FLIPL. Furthermore, in mixed culture experiments binding of scFv425:sTRAIL to EGFR-positive target cells conveyed a potent apoptotic effect towards EGFR-negative bystander tumour cells. The favourable characteristics of scFv425:sTRAIL, alone and in combination with Iressa, as well as its potent anti-tumour bystander activity indicate its potential value for treatment of EGFR-expressing cancers.

Introduction
The Epidermal Growth Factor Receptor (EGFR) is a transmembrane receptor tyrosine kinase comprising an extra-cellular ligand binding domain, a transmembrane lipophilic segment, and an intra-cellular tyrosine kinase domain\(^1\). Binding of its ligand EGF or TGF\(\alpha\) results in EGFR-dimerization and activates the intrinsic protein tyrosine kinase. Activated EGFR concomitantly triggers signalling by the downstream mitogenic signal transduction pathways p44/42 MAPK and PI3K\(^3,4\). Normal EGFR signalling plays a pivotal role in organ development and repair. The important role of EGFR in the regulation of cell survival is underscored by the fact that aberrant EGFR activity strongly contributes to tumorigenesis in various tumour types. aberrant activation of EGFR is associated with reduced recurrence-free or overall survival rates\(^5,6\) and can arise from EGFR gene amplification, leading to high cell surface expression of over 10\(^6\) EGFR molecules per cell, or alternatively, oncogenic mutation of EGFR. One
of the most frequent tumour specific mutant forms is the EGFRvIII, a mutant receptor commonly found in glioblastoma multiforme\textsuperscript{7,8}. EGFRvIII possesses ligand-independent tyrosine kinase activity\textsuperscript{9} and is associated with enhanced tumorigenicity in mice\textsuperscript{10,11}. Very recently, mutations have been identified in the intra-cellular tyrosine kinase domain of EGFR in lung cancer patients that appear to activate anti-apoptotic pathways. Several strategies have been developed to specifically inhibit aberrant EGFR signalling. Monoclonal antibodies, e.g. MAb C225 (Cetuximab\textsuperscript{TM}) and MAb 425\textsuperscript{12,13}, competitively inhibit the binding of natural ligands to the extracellular ligand-binding domain. Small molecule tyrosine kinase inhibitors, e.g Iressa (ZD1839 or Gefitinib\textsuperscript{TM})\textsuperscript{14,15}, competitively inhibit with ATP for binding to the intracellular tyrosine kinase domain. The clinical efficacy of these agents appears to rely on multiple anti-cancer mechanisms, including inhibition of cell cycle progression, inhibition of metastasis, and an increase in the susceptibility of cells to apoptosis.

However, despite promising anti-tumour activity in clinical trials\textsuperscript{16-19}, both classes of EGFR-signalling antagonists do not appear to be curative. Therefore, additional EGFR-targeted strategies or combination with other therapeutic approaches are warranted. In this respect, strong synergistic tumoricidal effects have been reported for strategies in which EGFR-signalling antagonists are combined with radiation- or chemotherapy\textsuperscript{17,19,20}, and more recently, with the cytokine TRAIL\textsuperscript{21}.

TRAIL is normally present as a trimeric type II transmembrane protein (memTRAIL) on various immune effector cells. TRAIL specifically induces apoptosis in cancer cells\textsuperscript{22} and virus-infected cells\textsuperscript{23}, without apparent apoptotic activity towards normal human cells. Homotrimeric memTRAIL initiates apoptosis by crosslinking of the agonistic receptors TRAIL-R1 and TRAIL–R2\textsuperscript{24-27}, leading to activation of the extrinsic apoptotic pathway via the Death Inducing Signalling Complex (DISC)\textsuperscript{28-35}. Assembly of the DISC sequentially activates initiator caspases (caspase-8 or -10) and effector caspases (e.g. caspase-3, and -7) and ultimately ends in apoptotic cell death.

MemTRAIL can be proteolytically cleaved into a soluble form (sTRAIL). Several recombinant forms of sTRAIL have been generated that show strong tumoricidal activity in vitro and in xenografted mouse models without toxic side-effects\textsuperscript{36-38}. Pharmacokinetic studies in cynamolgous monkeys and chimpanzees revealed no toxicity, thus further establishing the potential for clinical application of sTRAIL in cancer therapy.

However, TRAIL receptors are expressed in various tissues and, thereby, may potentially compete with tumour tissue for binding of i.v. applied sTRAIL. In addition, several papers described apoptotic activity of sTRAIL on various normal cells, including primary human hepatocytes\textsuperscript{39}, keratinocytes\textsuperscript{40}, prostate epithelial cells\textsuperscript{41} and brain tissue\textsuperscript{42}. Fortunately, the binding characteristics of sTRAIL for its receptors have typical “fast on/fast off” rates. Previously, we and others showed that sTRAIL can be genetically fused to a tumour
specific antibody fragment with “fast on/slow off” rates\textsuperscript{43,44}, resulting in the preferential binding to the pre-selected target antigen. In addition, the selected target antigen was selectively over-expressed compared to TRAIL-receptors on the tumour cell surface, thereby, further optimizing tumour cell-specific binding.

An additional advantage of antibody fragment targeted sTRAIL over conventional sTRAIL is its acquired capacity to activate TRAIL-R2. Conventional sTRAIL can efficiently activate TRAIL-R1 but not TRAIL-R2, the high affinity receptor that is activated only by membrane-bound TRAIL or sTRAIL secondarily crosslinked by antibodies\textsuperscript{45,46}. Consequently, sTRAIL induces apoptosis less effectively in the many tumour types that predominantly express TRAIL-R2. Importantly, antibody fragment-mediated binding converts soluble scFv:sTRAIL into an artificial membrane bound form of TRAIL. Subsequently, a surplus of sTRAIL domains is available on the target cell surface for crosslinking of TRAIL-R2 on proximal tumour cells, resulting in enhanced target antigen-restricted reciprocal apoptosis.

Here, we report on a novel fusion protein, scFv425:sTRAIL, designed to combine EGFR-signalling inhibition with tumour-specific apoptosis induction by sTRAIL. ScFv425:sTRAIL consists of an antibody fragment derived from EGFR-blocking monoclonal antibody MAb 425\textsuperscript{12} genetically fused to sTRAIL. Binding of scFv425:sTRAIL via the high affinity antibody fragment leads to specific accretion to the cell surface of EGFR-positive tumour cells. EGFR-specific binding of scFv425:sTRAIL was designed to rapidly inhibit EGFR signalling and, thereby, to sensitize cells to apoptosis\textsuperscript{47}. EGFR-restricted binding of scFv425:sTRAIL restores full signalling capacity of scFv425:sTRAIL for both TRAIL-R1 and TRAIL-R2. Here we present a new therapeutic strategy combining EGFR-signal inhibition with simultaneous target antigen-restricted apoptosis induction by TRAIL. Taken together, the data described here warrant further clinical development of this novel fusion protein.

**Materials and methods**

**Cell lines**

The following cell lines were purchased from the ATCC (Manassas, USA); Jurkat (ALL T-cell line), A431 (epidermoid vulva carcinoma), A172, Hs683 (glioblastoma), SW948, and WiDr (colon carcinoma). Jurkat.EGFRvIII was generated by electroporation of Jurkat cells with plasmid pHßApr-1-neo/EGFRvIII (kind gift of Dr. D. Bigner, Duke University Medical Center, NC, USA), after which transfectants were selected by G418 selection (500 µg/ml, Gibco Life Technologies b.v. Breda, The Netherlands). Cell lines were cultured at 37°C, in a humidified 5% CO\textsubscript{2} atmosphere. Jurkat, SW948, and WiDr were cultured in RPMI 1640 (Cambrex Bio Science, Verviers, France) supplemented with 15% FCS. A431, HS683 and A172 were cultured in DMEM, 10% FCS, 4 mM L-glutamine (Cambrex Bio Science).
Monoclonal antibodies and inhibitors
TRAIL neutralizing MAb 2E5 was purchased from Alexis (10P's, Breda, The Netherlands). MAb 425 (kindly provided by Merck, Darmstadt, Germany) is a murine IgG2a with high binding affinity for the extra-cellular domain of EGFR and the mutant tumour specific variant EGFRvIII. MAb 425 blocks EGF binding to EGFR and competes with scFv425 for binding to the same epitope. Total caspase inhibitor Z-VAD-FMK, caspase-8 inhibitor Z-IETD-FMK, and caspase-9 inhibitor Z-LEHD-FMK were purchased from Calbiochem (San Diego, CA, USA). EGFR tyrosine kinase inhibitor Iressa was kindly provided by AstraZeneca Inc (Macclesfield, Cheshire, UK). PI3K inhibitor Wortmannin was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Final working concentrations of inhibitors were diluted in serum free medium from a stock of 10 mM in DMSO.

Production of scFv425:sTRAIL
Fusion protein scFv425:sTRAIL was constructed and produced essentially as described previously \(^43\). Briefly, In the first MCS of vector pEE14, the high-affinity antibody fragment scFv425 (Vh-(G4S)\(^3\)-Vl format) \(^48\) was directionally inserted using the unique SfiI and NotI restriction enzyme sites. In the second MCS, a PCR-truncated 593 bp DNA fragment encoding the extracellular domain of human TRAIL (sTRAIL) was cloned in frame using restriction enzymes XhoI and HindIII, yielding plasmid pEE14-scFv425:sTRAIL. Expression plasmid pEE14-scFv425:sTRAIL was transfected into CHO-K1 cells using Fugene 6 reagent (Roche Diagnostics, Almere, The Netherlands) according to manufacturer’s recommendations, after which transfectants were selected by the glutamine synthetase system as described \(^49\). Single cell sorting using the MoFlo high speed cell sorter (Cytomation, Fort Collins, USA) established clone 100F1, stably secreting 2,4 µg/ml scFv425:sTRAIL into the culture medium.

EGFR-specific binding of scFv425:sTRAIL
EGFR-specific binding of scFv425:sTRAIL was assessed by flow cytometry using the EGFR-positive tumour cell line A431 and the EGFR-negative cell line Jurkat. In short, 1-10\(^6\) cells were incubated with scFv425:sTRAIL (300 ng/ml) in the presence or absence of MAb 425 (3 µg/ml). Specific binding of scFv425:sTRAIL was detected using PE-conjugated anti-TRAIL MAb B-S23 (Diaclone SAS, Besançon, France) and subsequent FACS analysis using an EPICS ELITE flow cytometre (Beckman Coulter, Mijdrecht, The Netherlands). Incubations were carried out for 45 min at 0°C and were followed by two washes with serum free medium.

Target cell-restricted induction of apoptosis by scFv425:sTRAIL
Target cell-restricted induction of apoptosis by scFv425:sTRAIL was assessed by analysis
of tumour cell viability, loss of mitochondrial membrane potential (Δψ), caspase-8 and -3 activation, and PARP cleavage/DFF degradation, as described in more detail below. Where indicated, treatment with scFv425:sTRAIL was performed in the presence or absence of MAb 425 (3 µg/ml) or MAb 2E5 (1 µg/ml).

**Apoptosis assessed by viability assay**
Cells were pre-cultured in a 96-well plate at a density of 3·10^4 cells/well. Subsequently, cells were treated for 16 h with the various experimental conditions in a final volume of 200 µl. Cell viability of adherent cell lines was determined by crystal violet staining (Sigma, Germany) as described previously. Cell viability of suspension cell lines was determined using MTS assay (Promega Benelux b.v., Leiden, The Netherlands) according to manufacturer’s recommendations. Experimental apoptosis induction was quantified as the percentage of apoptosis induction compared to base-line apoptosis in medium control, which was set at 0% apoptosis. Each experimental condition consisted of six independent wells.

**Apoptosis assessed by loss of Mitochondrial Membrane Potential (Δψ)**
Δψ was analyzed using the stain DiOC6 (Eugene, The Netherlands) as previously described. Briefly, cells were pre-cultured in a 24-well plate at a concentration of 0.5·10^6 cells/well. Subsequently, cells were treated for 16 h with the various experimental conditions, after which cells were harvested and incubated for 20 minutes with DiOC6 (0,1 µM) at 37°C, harvested (300xg; 5 min.), resuspended in PBS, and analyzed by flow cytometry.

**Immunoblot analysis**
Cells were pre-cultured at 1.5·10^6 cells/well in a 6-well plate, after which cells were incubated with scFv425:sTRAIL in the presence or absence of MAb 425 or MAb 2E5 for the indicated time-points. Cell lysates were prepared as described previously. Subsequently, 30 µg of lysate was separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose by electro blotting. Apoptosis signalling: Caspase activation was detected using antibodies directed against caspase-8 and active caspase-3 (Cell signalling, Beverly, MA, USA). PARP cleavage and DFF degradation was assessed using anti-PARP MAb (Santa Cruz Biotechnology Inc., Santa Cruz, Ca, USA) and anti-DFF MAb (Santa Cruz). Expression of c-FLIP_L and BAD phosphorylation was determined using anti-c-FLIP_L MAb clone NF6 (Alexis) and anti-phospho BAD antibody (Cell signalling). EGFR signalling: Expression levels of total and active EGFR were assessed using anti-total EGFR (Cell Signalling) and anti-activated EGFR (Tyr1173) (Santa Cruz). The different signal transduction pathways controlled by EGFR were analyzed with the phosphoERK1/2
sampler kit (Cell Signalling) and phospho-AKT sampler kit (Cell Signalling). Equal protein loading was assessed using anti-actin MAb (Boehringer Mannheim, Germany). Specific binding was visualized using appropriate secondary HRPO-conjugated antibody (DAKO Cytomation, Glostrup, Denmark) and chemoluminescence (Roche).

**Differential quantification of apoptosis in target and bystander cells during mixed culture experiments**

Differential cell membrane labelling of target and bystander cells was achieved using the Vybrant Multicolor Cell-Labeling kit (Molecular probes). Briefly, Jurkat.EGFRvIII target cells were labelled with the red fluorescent dye DiI. Labelling was performed by incubation of Jurkat.EGFRvIII cells (1⋅10^6 cells/ml in serum free medium) with 5µM DiI (37°C, 5 min), followed by three washes with standard medium. DiI-labelled Jurkat.EGFRvIII target and non-labelled Jurkat bystander cells were mixed at the indicated ratios at a final concentration of 0.5⋅10^6 cells/well in a 24-well plate. After treatment, differential fluorescent characteristics of target cells and bystander cells were used to separately evaluate induction of apoptosis in both populations by Δψ as described above.

**Synergistic induction of apoptosis by scFv425:sTRAIL and Iressa**

Jurkat.EGFRvIII cells and A431 cells were simultaneously treated with suboptimal concentrations of scFv425:sTRAIL (80 ng/ml) and Iressa (250 and 2000 nM, respectively), unless indicated otherwise. Where indicated, cells were co-incubated with MAb 425 (3 µg/ml), MAb 2E5 (1 µg/ml), Z-VAD-FMK (1 µg/ml), Z-IETD-FMK (1 µg/ml), Z-LEHD-FMK (1 µg/ml), or PI3K inhibitor Wortmannin (10 µM). After 16 h treatment, apoptosis was assessed by Δψ as described above. Synergy was determined using the cooperativity index (CI), in which the sum of apoptosis induced by single-agent treatment is divided by apoptosis induced by combination-treatment. When CI<1, treatment was termed synergistic. The effect of single-agent and co-treatment of scFv425:sTRAIL and Iressa on apoptotic signalling and EGFR-signal transduction by PI3K and MAPK was assessed by immunoblot as described above.

**Results**

**EGFR-specific binding of scFv425:sTRAIL**

To assess whether scFv425:sTRAIL displayed specific and enhanced binding to EGFR-positive cells, A431 cells were incubated with scFv425:sTRAIL and analyzed for binding by flow cytometry. Strong binding of scFv425:sTRAIL was detected to the cell surface (Fig.1A, solid line), which could be specifically inhibited by pre-incubation with parental EGFR-blocking MAb 425 (Fig.1A, dashed line). In contrast, binding of scFv425:sTRAIL to
TRAIL-receptors on the cell surface of EGFR-negative Jurkat cells was barely detectable (Fig.1B). The intensity of scFv425:sTRAIL binding directly correlated to the amount of cell surface expressed EGFR (data not shown).

**EGFR-restricted induction of apoptosis by scFv425:sTRAIL**

Treatment of EGFR-positive tumour cell lines with scFv425:sTRAIL (300 ng/ml) potently induced apoptosis (Fig.2A: A431; 66%, HS683; 85%, WiDr; 68%, SW948; 78%, A172; 70%, Jurkat.EGFRvIII; 82%), whereas EGFR-negative Jurkat cells were fully resistant to treatment (3%). Apoptosis was specifically inhibited when cells were co-incubated with MAb 425 or TRAIL-neutralizing MAb 2E5 during treatment with scFv425:sTRAIL (Fig.2B). Binding of scFv425:sTRAIL to EGFR and subsequent reciprocal activation of agonistic TRAIL-receptors in a paracrine fashion should also lead to apoptotic activity towards neighbouring EGFR-negative tumour cells. To investigate the presence of such a bystander effect, Jurkat.EGFRvIII target cells were mixed with Jurkat bystander cells (ratio 1:1) and treated with scFv425:sTRAIL. After treatment, bystander and target cells were separately assessed for apoptosis, which identified a potent bystander effect of 64% apoptosis in Jurkat bystander cells (Fig.2C). Apoptosis in Jurkat.EGFRvIII target cells reached approximately 50%. In both target cells and bystander cells, apoptosis was specifically inhibited by co-treatment with MAb 425 (4%) or MAb 2E5 (1%) (Fig.3C).

**Inhibition of EGFR-signalling and subsequent sensitization to apoptosis by scFv425:sTRAIL treatment**

Since scFv425:sTRAIL primarily binds via its EGFR-blocking antibody fragment scFv425,
the effect of scFv425:sTRAIL treatment on EGFR-signalling was determined. In A431 cells, scFv425:sTRAIL induced a rapid dephosphorylation of EGFR at Tyr 1173 within 10 min, while total EGFR levels remained constant (Fig.3A). The phosphorylation of EGFR observed during normal culture conditions is most likely due to a previously described TGFα-induced autophosphorylation loop. Specific inactivation of EGFR signalling was accompanied by a small decrease in MAPK pathway activity, which was detected for MAPK
Fig. 3. Inhibition of EGFR signaling and sensitization to apoptosis by scFv425:sTRAIL. A431 cells were challenged with 300 ng/ml scFv425:sTRAIL in the presence of 1 µg/ml cycloheximide for 10 min, 1 h, and 3 h. At elapsed time point 3 h, cells were additionally treated with scFv425:sTRAIL in the presence or absence of MAb 425 or MAb 2E5. A; Cell lysates were analyzed for the amount of total and phosphorylated EGFR (pTyr1173). B; Cell lysates were analyzed for MAPK and PI3K pathway activity by measurement of phosphorylated p44/42 MAPK, total and phosphorylated Akt. Actin levels were determined to confirm equal protein loading. C; Cell lysates were analyzed for the apoptosis associated features of caspase-8 activation, the expression level of c-FLIP\textsubscript{L} and the phosphorylation status of BAD at residue Ser136.

at 1 and 3 h of treatment (Fig. 2B). In addition, the PI3K pathway was markedly inhibited after 1 and 3 h of treatment, as measured by dephosphorylation of Akt at residues Tyr308 and Ser473 (Fig. 2B), while total Akt levels remained constant.

Resistance to apoptosis by EGFR-signalling is mediated in part by its effect on the anti-apoptotic protein c-FLIP\textsubscript{L} and the phosphorylation of BAD via PI3K signalling. Since PI3K
signalling was inactivated by scFv425:sTRAIL treatment, c-FLIP L expression and BAD phosphorylation were investigated. At early time-points of 1 and 3 h of treatment, a decrease was detected in expression of the anti-apoptotic caspase 8 homologue c-FLIP L (Fig.3C), which coincided with the activation of caspase 8 (Fig.3C). Additionally, a marked decrease was observed in phosphorylation of BAD (Fig.3C), sensitizing the mitochondria to apoptosis.

Fig 4. Synergistic target-cell restricted apoptosis induction by scFv425:sTRAIL and Iressa. 
A; A431 cells and B; Jurkat.EGFRvIII cells were treated with increasing concentrations of the EGFR-TKI Iressa in the presence or absence of a fixed concentration of scFv425:sTRAIL (80 ng/ml). C; Jurkat.EGFRvIII cells and A431 cells were treated with increasing concentrations of scFv425:sTRAIL in the presence of a fixed concentration of Iressa (250 nM and 2000 nM, respectively). D; Jurkat. EGFRvIII, A431, and Jurkat cells were treated alone, or with a combination of scFv425:sTRAIL and Iressa in the presence or absence of MAb 425. In all experiments, apoptosis was assessed by Δψ
Synergistic induction of apoptosis by scFv425:sTRAIL and Iressa

Previously EGFR-signalling inhibition was shown to synergistically enhance TRAIL-activity\(^2\). Therefore, potential synergistic effects of scFv425:sTRAIL with the EGFR-TKI Iressa was assessed on A431 cells and Jurkat cells positive for the mutant receptor EGFRvIII. Treatment of A431 cells with increasing concentrations of Iressa (250 - 2000 nM) and a fixed concentration of scFv425:sTRAIL (100 ng/ml) resulted in a dose-dependent synergistic increase in apoptosis (Fig.4A). Similar results, but with lower concentrations of Iressa (50 - 250 nM) and scFv425:sTRAIL (80 ng/ml), were obtained for Jurkat.EGFRvIII (Fig.4B). Dose-response curves of treatment with a fixed concentration of Iressa (250 and 2000nM, respectively) and increasing concentrations of scFv425:sTRAIL (up to 100 ng/ml) revealed a potent dose-dependent increase in apoptosis in both A431 and Jurkat.EGFRvIII cells already at 20 ng/ml of scFv425:sTRAIL (Fig.4C). The synergistic pro-apoptotic activity of scFv425:sTRAIL and Iressa was potently inhibited by co-treatment with MAb 425 (Fig.4C). Target antigen-negative Jurkat cells, subjected to the same experimental conditions, were fully resistant to treatment (Fig.4D). In control experiments with DMSO, alone or in combination with scFv425:sTRAIL, no significant induction of apoptosis was detected (data not shown).

Synergistic induction of apoptosis by scFv425:sTRAIL and Iressa is caspase 8-mediated

Next, the mechanism underlying the synergistic pro-apoptotic effect was investigated. Treatment of A431 cells and Jurkat.EGFRvIII cells with scFv425:sTRAIL and Iressa did not significantly alter TRAIL receptor expression (data not shown). Using specific caspase inhibitors, induction of apoptosis was found to be largely caspase-8 dependent, since the specific caspase-8 inhibitor Z-IETD-FMK inhibited apoptosis to levels observed for Iressa alone (Fig.5A). On the other hand, caspase-9 inhibition using Z-LEHD-FMK only had a minimal effect. Immunoblot analysis further revealed a strong activation of both caspase 8 and 3, resulting in PARP cleavage within 3 h of treatment with scFv425:sTRAIL and Iressa (Fig.5B). Single agent treatment only marginally activated caspase-8 and caspase-3 (Fig. 5B). Similar results were obtained when A431 cells were treated with scFv425:sTRAIL and Iressa (Fig.5C). The appearance of apoptotic features was specifically inhibited when treatment was performed in the presence of MAb 425 (Fig.5B and C).

Inhibition of EGFR signalling by co-treatment with scFv425:sTRAIL and Iressa

Simultaneous treatment of Jurkat.EGFRvIII with scFv425:sTRAIL and Iressa resulted in PI3K inactivation within 2 h in Jurkat.EGFRvIII, as measured by Akt dephosphorylation at Ser 473 (Fig.6A). No inhibition of MAPK signalling was observed in Jurkat.EGFRvIII, which is in line with a previous report showing that EGFRvIII specifically regulates PI3K activity\(^5\). At the concentrations used, single agent treatment had no effect on mitogenic
signalling in Jurkat.EGFRvIII (Fig.6A). The role of PI3K inhibition in the synergistic pro-apoptotic effect on Jurkat.EGFRvIII was confirmed by simultaneous treatment of Jurkat.EGFRvIII with the specific PI3K inhibitor Wortmannin and scFv425:sTRAIL, resulting in levels of apoptosis comparable to treatment with scFv425:sTRAIL and Iressa.
(Fig.6B). For A431 cells, no effect of single agent and co-treatment was detected on PI3K signalling (Fig.6A). On the other hand, single agent treatment with Iressa already markedly inhibited MAPK signalling, while scFv425:sTRAIL treatment alone only had a

**Fig.6. Inhibition of EGFR signaling by co-treatment with scFv425:sTRAIL and Iressa.** Jurkat.EGFRvIII and A431 cells were treated either alone or with a combination of scFv425:sTRAIL and Iressa, in the presence or absence of MAb 425. After 2h treatment, PI3K pathway and MAPK pathway activity in A; Jurkat.EGFRvIII and B; A431 was assessed by immunoblot analysis of total Akt and active phosphorylated Akt, and phosphorylated MAPK, respectively. Equal protein loading was confirmed by actin staining. C; Jurkat.EGFRvIII was treated with scFv425:sTRAIL and either Iressa or the specific PI3K inhibitor Wortmannin, after which apoptosis induction was assessed by ∆ψ. D; Cell lysates of A431 and Jurkat.EGFRvIII, treated alone or with a combination of scFv425:sTRAIL and Iressa, were analyzed for expression of the anti-apoptotic caspase 8 homologue c-FLIPL.
minimal effect. Co-treatment of A431 also inhibited MAPK signalling, but to a similar extent as Iressa treatment alone (Fig.6B).

**Treatment with scFv425:sTRAIL and Iressa induces c-FLIP\_L down regulation**

Simultaneous treatment with scFv425:sTRAIL and Iressa markedly reduced the expression of c-FLIP\_L in both Jurkat.EGFRvIII and A431 cells (Fig.6D). To a lesser extent, treatment with Iressa alone down-regulated c-FLIP\_L in A431 cells, whereas in Jurkat.EGFRvIII no effect of single agent treatment was seen. Treatment in the presence of MAb 425 prevented down regulation of c-FLIP\_L in both cell lines.

**Discussion**

EGFR-signalling inhibition by EGFR-blocking monoclonal antibodies and small molecule tyrosine kinase inhibitors is a promising therapeutic approach that can restore the susceptibility of tumour cells to apoptosis induction. Here we describe a novel therapeutic approach in which EGFR-signalling inhibition is combined with target cell-restricted apoptosis induction using the new fusion protein scFv425:sTRAIL. Fusion protein scFv425:sTRAIL, comprising EGFR-blocking antibody fragment scFv425 genetically fused to sTRAIL, clearly accreted at the cell surface of EGFR-positive cells, which was specifically abrogated by pre-incubation with parental EGFR-blocking MAb 425. Together with the barely detectable binding of scFv425:sTRAIL to cognate TRAIL receptors on EGFR-negative cells, these data provide strong evidence for the enhanced binding specificity of scFv425:sTRAIL for EGFR-positive tumour cells.

Interestingly, high concentrations of parental MAb 425 were required to competitively inhibit binding of scFv425:sTRAIL, implying a high affinity of scFv425:sTRAIL for EGFR. Previously, we demonstrated that eukaryotically expressed scFv:sTRAIL is produced as a soluble homogeneous trimer\textsuperscript{43}. Although not investigated here, such a stable trimeric form would provide a logic explanation for the strong binding observed on A431 cells. Trimeric scFv425:sTRAIL contains three identical antibody fragment domains and will, therefore, benefit from an associated enhanced avidity effect. Enhanced avidity has been shown to improve the in vivo tumour-targeting efficacy in several antibody-based strategies\textsuperscript{52,53}. The above-described enhanced binding specificity and avidity of scFv425:sTRAIL may help increase tumour cell retention and reduce the total dose required to obtain a therapeutic effect.

Treatment with scFv425:sTRAIL potently induced apoptosis in EGFR-positive tumour cells that was specifically abrogated by co-incubation with parental MAb 425. When combined with the absence of apoptotic activity on EGFR-negative Jurkat cells, this established EGFR-specific binding of scFv425:sTRAIL as a critical component of its apoptotic activity. Interestingly, the appearance of apoptotic features, such as processing of caspase 8, was
preceded by the specific dephosphorylation of EGFR, and coincided with dephosphorylation of the PI3K signal transduction pathway and to a lesser extent the MAPK signal transduction pathway.

This rapid inactivation of EGFR-signalling clearly points to a role for EGFR inhibition in scFv425:sTRAIL-induced apoptosis. One of the main regulators of TRAIL sensitivity, the anti-apoptotic caspase-8 homologue c-FLIP<sub>L</sub>, has previously been shown to be regulated by PI3K signalling<sup>57,58</sup>. In A431 cells, inactivation of PI3K signalling was accompanied by a decrease in expression of c-FLIP<sub>L</sub> after 1 and 3 h of treatment. Besides regulating c-FLIP<sub>L</sub> expression, PI3K signalling also influences the phosphorylation status of BAD<sup>59,60</sup>. In A431 cells, a marked dephosphorylation of BAD was detected after 1 and 3 h. Therefore, inhibition of PI3K signalling appears to facilitate caspase 8 activation, by down-regulating c-FLIP<sub>L</sub>, and sensitizes the mitochondria to induction of apoptosis, by dephosphorylation of BAD.

In addition to PI3K inhibition, dephosphorylation of the MAPK signal transduction pathway was detected after 1 and 3 h of treatment with scFv425:sTRAIL. Previously, MAPK activation was shown to protect against TRAIL-induced apoptosis by a mechanism occurring at or above the level of caspase-8 processing, which did not involve c-FLIP<sub>L</sub><sup>47</sup>. Conversely, although not formally proven here, MAPK inhibition could sensitize tumour cells towards scFv425:sTRAIL-induced apoptosis at or above the level of caspase 8 processing.

From the above-discussed data, a model for the apoptotic activity of scFv425:sTRAIL can be formulated (for schematic representation see Fig.7). First, binding of scFv425:sTRAIL leads to accretion at the cell surface of EGFR-positive tumour cells only. Subsequently, EGFR-specific binding inhibits EGFR mitogenic signalling via PI3K and MAPK and, thereby, sensitizes tumour cells to apoptosis by e.g. down-regulation of c-FLIP<sub>L</sub> and BAD dephosphorylation. Concomitantly, membrane-bound scFv425:sTRAIL induces apoptosis by reciprocal crosslinking of agonistic TRAIL-receptors on neighbouring EGFR-positive tumour cells.

Paracrine activation of TRAIL-receptors by scFv425:sTRAIL is not necessarily restricted to EGFR-positive tumour cells but can also be directed towards neighbouring tumour cells devoid of target antigen. In a recent report, we described a potent anti-tumour bystander effect for an scFv:sTRAIL fusion protein with specificity for the carcinoma-associated cell surface target antigen EGP2<sup>61</sup>. Here, we show that scFv425:sTRAIL also potently induced apoptosis in EGFR-negative bystander Jurkat cells during mixed culture experiments with Jurkat.EGFRvIII cells. This pro-apoptotic bystander effect might help reduce the appearance of therapy-resistant recurrences emerging after seemingly successful treatment, as has been reported for conventional MAb-based therapy<sup>62,63</sup>.

In a recent report, the synergistic effect of combined EGFR-targeting with the anti-EGFR monoclonal antibody cetuximab and the EGFR-specific tyrosine kinase inhibitor
Iressa was described\textsuperscript{64}, Together with the fact that TRAIL has been shown to synergize with anti-EGFR agents\textsuperscript{20}, this provided a strong rationale for the combination of scFv425:sTRAIL treatment with Iressa. Potent synergistic induction of apoptosis was observed in both wild-type EGFR-positive A431 cells and EGFRvIII-positive Jurkat.EGFRvIII cells upon treatment with scFv425:sTRAIL and the Iressa. The synergistic pro-apoptotic effect of scFv425:sTRAIL and Iressa was fully EGFR-restricted and TRAIL-mediated and did not involve modulation of TRAIL receptor expression. Interestingly, inhibition of caspase 8 activity, by a specific caspase 8 inhibitor, reduced apoptosis induction by scFv425:sTRAIL and Iressa to the levels observed during Iressa treatment alone. On the other hand, caspase 9 inhibition only had a minimal effect on apoptosis induction. These data point to an increased processing of caspase 8 as the main cause for the synergistic pro-apoptotic effect with no or only minimal involvement of the mitochondrial route of apoptosis.

When cells were subsequently analyzed for expression of c-FLIP\textsubscript{L}, the expression level of which is an important regulator of caspase 8 processing, a marked down regulation in both Jurkat.EGFRvIII and A431 was observed within 3 h of combination treatment with scFv425:sTRAIL and Iressa. Down-regulation of c-FLIP\textsubscript{L} coincided with the time of caspase-8 activation and was preceded by inactivation of the PI3K pathway in Jurkat. EGFRvIII cells. In A431 cells, combination treatment significantly inhibited MAPK-signalling but only to a similar degree as that observed during Iressa treatment alone.

**Fig 7. Schematic model of the apoptotic activity of scFv425:sTRAIL.** Antibody fragment binding of scFv425:sTRAIL to EGFR inhibits mitogenic signaling by this receptor and its downstream signaling pathways and, thereby, sensitizes tumor cells to apoptosis. Furthermore, antibody fragment binding to EGFR immobilizes soluble scFv425:sTRAIL on the cell surface of EGFR-positive tumor cells and converts soluble scFv425:sTRAIL into a membrane bound form that can efficiently initiate apoptosis by crosslinking of the agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2.
Based on these results, it can be concluded that the synergistic pro-apoptotic effect largely depends on the specific down-regulation of c-FLIPL. For EGFRvIII positive Jurkat cells, down-regulation of c-FLIPL is a consequence of PI3K inhibition. In A431 cells, MAPK dephosphorylation may play a role but the exact mechanism remains to be elucidated.

In conclusion, we report for the first time on a recombinant fusion protein that combines the tumoricidal effect of EGFR-signal inhibition with target cell-restricted apoptosis induction. The unique characteristics of scFv425:sTRAIL described here indicate its potential therapeutic value, alone and in combination with EGFR tyrosine kinase inhibitor Iressa, for the treatment of EGFR and EGFRvIII expressing human cancers.

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