The novel Raf inhibitor Raf265 decreases Bcl-2 levels and confers TRAIL-sensitivity to neuroendocrine tumour cells

Kathrin Zitzmann*, Enrico de Toni*, Janina von Rüden, Stephan Brand, Burkhard Göke, Rüdiger P Laubender† and Christoph J Auernhammer

Department of Internal Medicine II, University-Hospital Munich-Grosshadern, University of Munich, Marchioninistr.15, 81377 Munich, Germany

†Institute of Medical Informatics, Biometry and Epidemiology, University of Munich, 81377 Munich, Germany

(Correspondence should be addressed to C J Auernhammer; Email: christoph.auernhammer@med.uni-muenchen.de)

*(K Zitzmann and E de Toni contributed equally to this work)

Abstract
The tumour-selective death receptor ligand tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising agent for the treatment of human cancer. However, many tumours have evolved mechanisms to resist TRAIL-induced apoptosis. A number of studies have demonstrated that aberrant PI(3)K–Akt–mTOR survival signalling may confer TRAIL resistance by altering the balance between pro- and anti-apoptotic proteins. Here, we show that neuroendocrine tumour (NET) cell lines of heterogeneous origin exhibit a range of TRAIL sensitivities and that TRAIL sensitivity correlates with the expression of FLIP_S, caspase-8, and Bcl-2. Neither single mTOR inhibition by everolimus nor dual mTOR/PI(3)K inhibition by NVP-BEZ235 was able to enhance TRAIL susceptibility in any of the tested cell lines. In contrast, dual PI(3)K–Akt–mTOR and Raf–MEK–Erk pathway inhibition by the IGF-1R inhibitor NVP-AEW541 effectively restored TRAIL sensitivity in NCI-H727 bronchus carcinoid cells. Furthermore, blocking Raf–MEK–Erk signalling by the novel Raf inhibitor Raf265 significantly enhanced TRAIL sensitivity in NCI-H727 and CM insulinoma cells. While having no effect on FLIP_S or caspase-8 expression, Raf265 strongly decreased Bcl-2 levels in those cell lines susceptible to its TRAIL-sensitizing action. Taken together, our findings suggest that combinations of Raf–MEK–Erk pathway inhibitors and TRAIL might offer a novel therapeutic strategy in NET disease.

Introduction
Response rates for currently available antiproliferative strategies against neuroendocrine tumours (NETs) of the gastroenteropancreatic (GEP) system are extremely poor. Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily, which was originally identified by sequence homology to Fas ligand (FasL) and TNF. TRAIL binds to four distinct receptors: death receptor (DR)4, DR5, decoy receptor (DcR)1 and DcR2. While DcR1 and DcR2 lack functional domains, engaged DR4 or DR5 initiate the apoptotic machinery by triggering formation of the death-inducing signalling complex (DISC; Mahalingam et al. 2009). In type I cells, DISC-mediated activation of caspase-8 is sufficient to cleave effector caspases-3, -6, and -7 (extrinsic apoptotic pathway). In contrast, type II cells use the proapoptotic Bcl-2 family member Bid to amplify low caspase-8 activity. Caspase-8-mediated cleavage of Bid leads to the formation of a Bid/Bax complex, which induces permeabilization of the outer mitochondrial membrane. The resulting release of cytochrome c enables the formation of a heptameric apoptosome complex. Once formed, the apoptosome recruits and activates caspase-9, which subsequently cleaves caspases-3, -6, and -7 (intrinsic apoptotic pathway).

In recent years, evidence has accumulated that TRAIL plays an important role in tumour surveillance.
For instance, TRAIL-knock-out mice have been shown to develop increased primary tumours and metastasis formation. Concordantly, the review of clinical data suggests a correlation between loss of TRAIL sensitivity, dedifferentiation and size of tumours, poor prognosis as well as tumour recurrence (Walczak et al. 2008). In numerous preclinical models, TRAIL has been shown to selectively induce apoptosis in cancer cells while sparing normal cells, suggesting its use in cancer therapy (Wiley et al. 1995, Griffith et al. 2009). However, cancer cells tend to have profound defects in apoptosis regulation and a significant percentage of human cancers are insensitive to TRAIL-induced apoptosis. This opens perspectives for novel tailor-made cancer therapies, as defining and targeting the genetic aberration underlying apoptosis resistance may restore tumour sensitivity to TRAIL and other apoptotic stimuli. While complete loss of TRAILR1 or TRAILR2 may result in TRAIL resistance (Horak et al. 2005, Elias et al. 2009), there is no clear correlation between TRAIL sensitivity and the expression level of TRAIL receptors. Instead, TRAIL resistance seems to be the result of alteration in the pathway linking TRAIL receptors to the apoptotic machinery. Several studies have revealed that overexpression of the caspase-8 inhibitor c-FLIPs is a common reason for decreased TRAIL sensitivity. Similarly, decreased levels of caspase-8 or increased levels of anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-XL, and Mcl-1) may confer TRAIL resistance (Shivapurkar et al. 2002, Zhang & Fang 2005). Often, such alterations seem to be the result of constitutively activated survival signalling through PI(3)K–Akt–mTOR. Pharmacological inhibition of PI(3)K restored TRAIL sensitivity in numerous cancer cells by reducing expression levels of c-FLIP and Bcl-2 or by up-regulating expression levels of Bax and Bak (Uchida et al. 2007, Opel et al. 2008). Similarly, inhibition of mTOR was shown to sensitize glioblastoma multiforme cells to TRAIL by reducing expression levels of c-FLIP (Panner et al. 2005). However, so far, the effects of TRAIL have not been investigated in NET cells. In this study, we examine four human NET cell lines of pancreatic-, midgut-, and bronchus origin with respect to TRAIL sensitivity and the TRAIL-sensitizing potential of PI(3)K-, mTOR-, insulin-like growth factor (IGF)-1R-, and Raf inhibition. We found that the extent of TRAIL sensitivity was highly variable among the examined NET cell lines. While inhibition of PI(3)K or mTOR had no effect, inhibition of IGF-1R or Raf could partially restore TRAIL sensitivity.

Materials and methods

Reagents

Everolimus (RAD001), NVP-BEZ235, RAF265, and NVP-AEW541 were purchased from Novartis Pharma. Recombinant human TRAIL was purchased from R&D Systems (Minneapolis, MN, USA). All kinase inhibitors were dissolved in DMSO; TRAIL was dissolved in water. In all experiments, DMSO was added to the control in the concentration used for the highest inhibitor dose.

Cell culture

Human pancreatic neuroendocrine BON1 tumour cells were provided by R Göke (Marburg, Germany) and were cultured in DMEM/F12 (1:1) medium (Gibco/Invitrogen). Human insulinoma CM cells and human midgut carcinoid GOT1 cells were provided by P Pozzilli (Rome, Italy) and Ola Nilsson (Göteborg, Sweden), respectively. Human bronchopulmonary neuroendocrine NCI-H727 tumour cells were purchased from ATCC (Manassas, VA, USA). CM, GOT1, and NCI-H727 cells were cultured in RPMI medium (PAA, Pasching, Austria). All media were supplemented with 10% FCS (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Gibco BRL/Life Technologies), and 0.4% amphotericin B (Biochrom). GOT1 culture medium was additionally supplemented with 0.135 IU/ml insulin and 5 μg/ml apo-transferrin. All cells were cultured at 37 °C in a 5% CO2 atmosphere.

RT-PCR

Total RNA was isolated using Trizol reagent (Gibco). For reverse transcription (RT)-PCR, RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (TURBO DNA-free-Kit, Ambion, Austin, TX, USA) to remove contaminating genomic DNA. Total RNA was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. The following pairs of primers were used for amplification: DR4 forward (F), 5'-AGAGAGAAGTTCCCTGCACCA-3'; DR4 reverse (R), 5'-GGCTCATG-CTCCAGGGCGTACAAT-3'. RT-PCR was performed with the following conditions: 95°C for 5 min, then 40 cycles of 95°C for 30 s, 56°C for 30 s, 68°C for 30 s, and a final extension at 68°C for 5 min. PCR products were resolved on 1% agarose gels (Eurogencells, Germany), and bands of the expected size were excised and purified using the QIAquick Gel Extraction Kit (Qiagen). Purified products were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and analysed on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems). The sequence was compared to the human database by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Assessment of cell viability

BON1, CM, GOT1, and NCI-H727 cells were seeded into 96-well plates at densities of 3000 (BON1 and CM), 50 000 (GOT1), and 4000 (NCI-H727) cells/well.
and grown for 24 h. Next, the cells were incubated with various concentrations of NVP-BEZ235, everolimus, Raf265, or NVP-AEW541 alone or in combination with TRAIL in medium containing 10% FCS. Metabolic activity was measured with Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) after 24 h of incubation, according to the manufacturer's instructions. Following 3 h of incubation with Cell Titer 96 solution, absorbance at 492 nm was determined using an ELISA plate reader.

Protein extraction and western blotting
Protein extraction and western blotting were done as previously described in detail (Zitzmann et al. 2006). Primary antibodies used were PARP, caspase-3, cleaved caspase-3, caspase-8, cleaved caspase-9, Bcl-XL, Bid (Cell Signaling, Danvers, MA, USA), Bcl-2 (BD, Franklin Lakes, NJ, USA), and β-actin (Abcam, Cambridge, UK).

Quantification of DNA fragmentation
The rate of apoptotic cell death was quantified by determining DNA fragmentation according to Nicoletti et al. (1991). Briefly, cells were incubated for 24 h in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide) and analyzed by flow cytometry on a FACSCalibur (BD), using CellQuest software (BD). Nuclei to the left of the "G1-peak" containing hypodiploid DNA were considered apoptotic.

Statistical analysis
Dunnett’s test was used for the comparison of several TRAIL dose levels with a zero dose control with regard to cell viability (in percentage of the control). Two-way ANOVA was used to model synergisms (antagonisms) between a substance and TRAIL with regard to cell viability (in percentage of the control). The factors employed were the type of substances (substance versus substance plus TRAIL) and the dose (treated as a factor with ordered levels along with orthogonal polynomial contrasts). Statistical significance of synergism between a substance dose and TRAIL was detected using the F-test. As two-way ANOVA was performed for each combination of the four cell types (BON1, CM, GOT1, and H727) and four substances (RAF265, RAD001, BEZ235, and AEW541), 16 interaction effects at a significance level of 0.05 (two-sided) were tested. To account for multiple testing, the familywise error rate was controlled by using the Bonferroni–Holm method. Analogously, two-way ANOVA followed by the Bonferroni–Holm method was used for modelling synergisms between a substance and TRAIL with regard to apoptosis (in percentage of the control) for each combination of two cell types (GOT1 and H727) and substances (RAF265 and AEW541). The data so obtained on cell viability and apoptosis were transformed by adequately selected power transformation to gain homogeneity of variance.

Results
Expression of TRAIL receptors in NET cells
It has been shown that the expression level of TRAIL receptors does not necessarily correlate with TRAIL sensitivity. However, promoter hypermethylation may result in complete loss of TRAIL receptors and TRAIL resistance (Horak et al. 2005, Elias et al. 2009). Using RT-PCR, we could show that all NET cells (pancreatic BON1 and CM cells, bronchus NCI-H727 cells, and midgut GOT1 cells) express mRNA of DR4 and DR5 (Fig. 1A). Specificity of both amplificates was proven by full-length sequencing.

Identification of TRAIL-sensitive and TRAIL-resistant NET cell lines
In order to determine the TRAIL sensitivity of NETs, BON1, CM, GOT1, and NCI-H727 cells were incubated with rising concentrations of TRAIL. Measurement of cell viability after a 24 h incubation showed that the pancreatic tumour cell lines BON1 and CM were most sensitive to TRAIL, responding to concentrations as little as 1 ng/ml with significantly decreased cell viability (Fig. 1B). While NCI-H727 bronchus carcinoid cells were susceptible to concentrations ≥5 ng/ml, GOT1 midgut carcinoid cells were resistant to any concentration within the tested range (Fig. 1B).

Association of TRAIL sensitivity with overexpression of FLIPs and Bcl-2 and with low levels of caspase-8
FLIPs, caspase-8, Bcl-2, and Bcl-XL are known modulators of TRAIL receptor signalling towards effector caspase activation and apoptosis. In order to determine whether the expression of one of these modulators can be associated with the differential response of NET cells to TRAIL, we assessed the protein levels of FLIPs, caspase-8, Bcl-2, and Bcl-XL in BON1, CM, NCI-H727, and GOT1 cells. As demonstrated in Fig. 1C, GOT1 and NCI-H727 cells,
which exhibited a remarkably lower sensitivity to TRAIL, showed increased levels of FLIPs and Bcl-2 compared with TRAIL-sensitive BON1 and CM cells. Furthermore, GOT1 cells showed the lowest caspase-8 expression among the examined cell lines (Fig. 1C).

**Effects of mTOR inhibition on TRAIL sensitivity**

Several studies have demonstrated that hyperactivation of mTOR may confer TRAIL resistance, which can be overcome by the use of mTOR inhibitors (Panner et al. 2005). We have previously shown that everolimus is a potent inhibitor of mTOR in NET cells (Zitzmann et al. 2007). To explore the role of mTOR in the regulation of TRAIL-induced apoptosis, we incubated NET cells with everolimus and TRAIL in a final concentration of 100 nM and 10 ng/ml, respectively. However, everolimus did not synergize with TRAIL to decrease cell viability in the examined NET cell lines (Fig. 2A). Consistently, additional treatment with everolimus had no effect on the cleavage of caspases-8, -9 and -3, Bid, or PARP (Fig. 3A).

**Effects of combined mTOR/PI(3)K inhibition on TRAIL sensitivity**

In previous studies, we have demonstrated that in BON1, GOT1, and NCI-H727 cells, mTOR inhibition results in negative feedback activation of Akt (Zitzmann et al. 2010). In order to examine whether dual mTOR/PI(3)K inhibition is superior over single mTOR inhibition, we employed NVP-BEZ235, a novel dual mTOR/PI(3)K inhibitor which was previously shown to effectively abrogate the negative feedback loop on Akt (Zitzmann et al. 2010). As for everolimus, NVP-BEZ235 failed to synergize with TRAIL’s effect on cell viability (Fig. 2B). Again, additional treatment with NVP-BEZ235 had no effect on the cleavage of caspases-8, -9 and -3, Bid, or PARP (Fig. 3A).

**Effects of IGF-1R inhibition on TRAIL sensitivity**

In addition to PI(3)K–Akt–mTOR signalling, Raf–MEK–Erk signalling has been shown to play a major role for cell survival. We have previously demonstrated that treatment of NET cells with the novel, selective IGF-1R inhibitor NVP-AEW541 effectively inhibits both PI(3)K–Akt–mTOR and Raf–MEK–Erk survival signalling (Zitzmann et al. 2010). In the next step, we aimed at examining the effects of NVP-AEW541 in respect to its TRAIL-sensitizing properties. As depicted in Fig. 2C and 3A, NVP-AEW541 potently increased TRAIL sensitivity in NCI-H727 cells, while
having no significant effect on the TRAIL susceptibility of BON1, CM, and GOT1 cells. FACS analysis of NCI-H727 cells confirmed the data obtained by cell viability assays and western blot analysis (Fig. 3B).

**Effects of Raf inhibition on TRAIL sensitivity**

As inhibition of PI(3)K–Akt–mTOR could not re-establish TRAIL sensitivity, we questioned whether the TRAIL-sensitizing effect of NVP-AEW541 in NCI-H727 cells was due to Raf–MEK–Erk pathway inhibition. Indeed, treatment with the novel Raf inhibitor Raf265 significantly decreased the viability of CM- and NCI-H727 cells in response to TRAIL, while having no effect on the TRAIL susceptibility of BON1 and GOT1 cells. Notably, the extent of cell viability reduction correlated with the activation of caspases-8, -9, -3, and PARP (Fig. 3A). In addition, Raf265 significantly increased the number of subG1 events (Fig. 3B).

**Raf inhibition decreases Bcl-2 expression in CM- and NCI-H727 cells**

To identify mechanisms responsible for synergistic interactions between NVP-AEW541/Raf265 and TRAIL, expression patterns of FLIPs, Bcl-2, and Bcl-XL were examined by western blot analysis. In those
cell lines susceptible to its TRAIL-sensitizing action (NCI-H727 and CM), Raf265 markedly reduced the protein level of Bcl-2 (Fig. 4). In contrast, NVP-AEW541 did not affect Bcl-2 levels (Fig. 4). However, in NCI-H727 cells, NVP-AEW541 synergized with TRAIL to suppress Bcl-2 expression. No significant changes in Bcl-XL and FLIPS expression were observed upon treatment with NVP-AEW541 or Raf265 (Fig. 4).

Discussion
Most available anticancer regimens primarily aim at inducing apoptosis in cancer cells. As an endogenous inducer of apoptosis, TRAIL plays a key role in tumour surveillance by the immune system and numerous studies suggest that exogenously administered TRAIL might have enormous potential in cancer therapy (Smyth et al. 2003). However, a significant number of human tumours are considered TRAIL resistant. In the present study, we investigated the TRAIL sensitivity of four NET cell lines of heterogeneous origin. GOT1 midgut carcinoid cells were resistant to TRAIL’s pro-apoptotic effect. Accordingly, as we evaluated the expression of known regulators of apoptosis, we found that GOT1 cells – compared with the other NET cell lines – exhibited low levels of pro-apoptotic caspase-8 and extremely high levels of FLIP S and Bcl-2. In recent years, numerous studies have associated aberrant activation of the PI(3)–Akt–mTOR pathway with apoptosis resistance and adverse clinical outcome. Akt is known to confer protection against t-Bid-induced apoptosis by up-regulating anti-apoptotic Bcl-2 and Bcl-XL and by antagonizing activation of pro-apoptotic Bax and Bak at the mitochondria (Majewski et al. 2004). Furthermore, mTOR has been shown to be a potent regulator of FLIPS translation (Panner et al. 2005). In numerous tumour models, inhibition of PI(3)K, Akt, or mTOR were shown to restore the sensitivity to a number of apoptotic stimuli including TRAIL by suppressing levels of FLIP S, Bcl-2, or Bcl-XL (Panner et al. 2005, Zauli et al. 2005, Tazzari et al. 2008, Opel et al. 2008). However, in our study, both the mTOR inhibitor everolimus and the dual mTOR/PI(3)K inhibitor NVP-BEZ235 failed to synergize with TRAIL in any of the tested NET cell line. Interestingly, no correlation was obvious between basal levels of pAkt and FLIPS, Bcl-2 or Bcl-XL; Akt phosphorylation in GOT1 cells was even slightly lower than in BON1, CM, and NCI-H727 cells (K Zitzmann, unpublished observations).
In recent years, several studies have revealed that cell survival is not exclusively dependent on PI(3)K–Akt–mTOR signalling but is also strongly supported by the Raf–MEK–Erk pathway. For instance, Raf–MEK–Erk signalling has been shown to increase the expression of several pro-survival Bcl-2 proteins by down-regulating pro-apoptotic Bcl-2/Bcl-XL-associated death promoter (Bad) and by promoting \textit{de novo} gene expression of Bcl-2, Bcl-XL, and Mcl-1 (Boucher et al. 2000, Boisvert-Adamo & Aplin 2008, Balmanno & Cook 2009). Consistently, inhibition of Raf–MEK–Erk signalling induced apoptosis in numerous tumour models (Balmanno & Cook 2009).

IGF1 is a major regulator of PI(3)K–Akt–mTOR and Raf–MEK–Erk signalling in NET cells (von Wichert et al. 2000). We have previously demonstrated that treatment of NET cells with the novel, selective IGF-1R inhibitor NVP-AEW541 effectively inhibits both PI(3)K–Akt–mTOR and Raf–MEK–Erk survival signalling (Zitzmann et al. 2010). By doing so, NVP-AEW541 induces potent antitumour effects which involve the induction of apoptosis as well as G0/G1 arrest (Höpfner et al. 2006, Zitzmann et al. 2010). These data led us to examine the effects of NVP-AEW541 in respect of its TRAIL-sensitizing properties. Using two-way ANOVA, we could demonstrate that the pro-apoptotic effects of combined treatment with NVP-AEW541 and TRAIL were highly synergistic in NCI-H727 bronchus carcinoid cells. To prove whether the TRAIL-sensitizing effects were due to Raf–MEK–Erk pathway inhibition, we selectively inhibited Raf by the novel small-molecule inhibitor Raf265. Interestingly, selective Raf inhibition by Raf265 showed even higher synergism with TRAIL than dual PI(3)K–Akt–mTOR and Raf–MEK–Erk pathway inhibition by NVP-AEW541. In addition to NCI-H727 cells, Raf265 and TRAIL synergized to induced apoptosis in CM insulinoma cells. To our knowledge, this is the first report of TRAIL sensitization by NVP-AEW541 and Raf265. However, several recent studies could demonstrate that the Raf-inhibitor sorafenib was able to restore TRAIL sensitivity by down-regulating Mcl-1 (Meng et al. 2007, Ricci et al. 2007, Rosato et al. 2007). A clinical phase Ib study is currently evaluating the safety and tolerability of escalating doses of mapatumumab (a human monoclonal antibody to TRAILR1) in combination with sorafenib in patients with advanced hepatocellular carcinoma (ClinicalTrials.gov Identifier: NCT00712855). In our study, while having no effect on FLIPs or caspase-8 expression, Raf265 strongly decreased Bcl-2 levels in those cell lines susceptible to its TRAIL-sensitizing action. These data

Figure 4 The TRAIL-sensitizing effects of Raf265 correlate with Bcl-2 suppression. NET cells were incubated with 1 µM NVP-AEW541 or 10 µM Raf265 alone or in combination with 10 ng/ml TRAIL for 8 h. Subsequently, the expression of FLIP\textsubscript{s}, Bcl-2, and Bcl-XL was evaluated by western blot analysis. For each cell line, one representative blot out of three performed experiments is shown.
suggest that Raf265 might exert its TRAIL-sensitizing effect through down-regulation of t-Bid binding Bcl-2. The resulting release of t-Bid might then facilitate TRAIL-induced permeabilization of the outer mitochondrial membrane. However, further studies using siRNA against Bcl-2 will be needed to prove this hypothesis. Taken together, our findings suggest that combinations of Raf–MEK–Erk pathway inhibitors and TRAIL might offer a novel therapeutic strategy in NET disease.

Declaration of interest
C J Auernhammer has received research contracts, lecture honorarium and advisory board honorarium from Novartis. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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