# Scatter factor protects tumor cells against apoptosis caused by TRAIL

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**Scatter factor (SF) and its receptor c-Met are overexpressed in various tumor types, and their expression often correlates with a poor prognosis. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), is a proposed tumor-specific chemotherapy agent, but its clinical usage is limited by acquisition of TRAIL resistance by tumors. The goals of this study were to determine whether and how SF protects tumor cells against TRAIL and whether SF-induced TRAIL resistance could be reversed. We used MTT assays, trypan blue dye exclusion assays, apoptosis assays, RNA interference, luciferase reporter assays, immunoprecipitation/western blotting, and other cell biological techniques to study SF protection of cultured human tumor cells against TRAIL. SF conferred resistance to TRAIL in various human prostate carcinoma and breast carcinoma cell lines. SF inhibited TRAIL-induced caspase-3 activation, poly (ADP-ribose) polymerase cleavage, and cell death. SF protection against TRAIL required c-Akt; but unlike protection against adriamycin, it did not require Src signaling or the classical pathway of nuclear factor-kappaB activation. Protection against TRAIL was blocked by knockdown of X-linked inhibitor of apoptosis or FLICE-inhibitor protein (FLIP) (a component of the death-inducing signaling complex). We found that c-Met physically associates with several TRAIL receptors and SF regulates their protein stability. Protection against TRAIL was blocked by a novel small molecule inhibitor of c-Met (PHA665752) and by an inhibitor of cyclooxygenase 2. In conclusion, these findings elucidate potential mechanisms of TRAIL resistance in tumors that overexpress the SF/c-Met and identify possible means of reversing this resistance. *Anti-Cancer Drugs* 21:10–24 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Scatter factor (SF) (also called hepatocyte growth factor) is a pleiotrophic cytokine that can stimulate motility, mitogenesis, and morphogenesis in a variety of cell types, in a context-specific manner [1,2]. Its cognate receptor is c-Met, a tyrosine kinase that is encoded by a proto-oncogene [3]. The oncogenic form of c-Met, a rearranged transforming gene called Tpr-Met, was so named because it can promote tumor metastasis [4]. SF and c-Met are involved in multiple biological processes, including normal development, tissue repair and remodeling, tumorigenesis, and angiogenesis [1,2,5–8]. A variety of studies have identified roles for the SF/c-Met axis in tumorigenesis and tumor progression (reviewed in Refs [1,2,5–7]). Roles for aberrant SF/c-Met signaling in human tumor development and progression are suggested by the findings that SF and c-Met are often overexpressed in many tumor types (e.g. breast, prostate, lung, bladder, brain, and others) [5–14]. SF and c-Met expression are usually significantly greater in invasive than in non-invasive forms of the same cancer. Within invasive cancers (e.g. breast, prostate, lung, and salivary cancers), high expression of SF and/or c-Met is correlated with a poor prognosis [7–12], suggesting an association between SF/c-Met signaling and the metastatic phenotype and/or resistance to therapy. In addition, hereditary papillary renal carcinoma type 1 is linked to germ-line mutations of the tyrosine kinase domain of c-Met [15]. c-Met mutations also occur in sporadic papillary renal carcinoma cells. c-Met mutations are found in other sporadic cancer types [16,17], but the role of the mutations in these cancers is not clear.

We have been studying the mechanisms by which SF protects carcinoma and glioma cells against DNA-damaging agents as a model for understanding chemoresistance in tumors that overexpress SF and c-Met [18–21]. In the course of these studies, we observed that SF also protects these cell types against cytotoxicity caused by the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). The death receptor ligand TRAIL binds to several receptors – including death-inducing receptors (DR4 and DR5) and two 'decoy' receptors (DCR1 and DCR2) – and activates the extrinsic pathway of apoptosis [22,23]. Endogenous TRAIL is a cell surface-expressed type II membrane

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protein; but an engineered soluble form of TRAIL can activate DR4 and DR5 and induce apoptosis [22]. TRAIL has been of considerable interest because tumor cells are often significantly more sensitive to it than normal cells; and as a result, it has been proposed as a cancer chemotherapy agent, either alone or in combination with other agents, such as adriamycin (ADR), a DNA topoisomerase IIα inhibitor [22–24]. However, its clinical utilization has been limited because of the occurrence of TRAIL resistance in tumors [25,26].

Although loss of death receptors (DR4 and DR5) expression is one possible mechanism of TRAIL resistance, these receptors are completely lost in only a minority of cancer cell lines. Other proposed mechanisms of TRAIL resistance include overexpression or constitutive activation of survival pathways, including those involving c-Akt, nuclear factor-kappaB (NF-κB), nitric oxide synthase, FLICE-inhibitor protein (c-FLIP), inhibitor of apoptosis proteins, Bcl-2, Bcl-$X_L$, osteoprotegerin (another putative TRAIL decoy receptor), and others (reviewed in Refs [25,26]). Interestingly, some of the genes implicated as mediators of TRAIL resistance are antiapoptotic target genes for NF-κB [e.g. c-FLIP, cIAP-1, cIAP-2, X-linked inhibitor of apoptosis (XIAP), Bcl-2, and Bcl-$X_L$], a transcription factor that is activated by SF and is required for SF-mediated cell protection against the DNA-damaging agent ADR [18].

In this paper, we report on studies documenting SF-mediated TRAIL resistance in cancer cells, some of the mechanisms of this resistance, and several means by which SF-induced resistance to TRAIL may be overcome. Based on these considerations, we propose that the overexpression and constitutive activation of the SF/c-Met signaling pathways in tumors is a potentially reversible mechanism for tumor cell resistance to TRAIL.

## Methods

### Cell lines and culture

All cell lines, including human prostate carcinoma (DU-145, LNCaP), human breast carcinoma (T47D, MDA-MB-435), and Madin–Darby canine kidney epithelial cells were originally obtained from the American Type Culture Collection (Manassas, Virginia, USA). The cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 5 or 10% fetal calf serum (v/v), nonessential amino acids (100 mmol/l), L-glutamine (5 mmol/l), streptomycin (100 μg/ml), and penicillin (100 units/ml) (all obtained from BioWhittaker, Walkersville, Maryland, USA). The cells were grown at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ and were subcultured approximately at weekly intervals using trypsin.

A set of DU-145 cell clones stably transfected with a full-length SF cDNA in the pcDNA3.1 vector and control clones transfected with empty vector were created to study the effects of autocrine SF signaling. The clones were screened for SF expression using an enzyme-linked immunosorbent assay. Here, we have studied one SF-expressing cell clone (DU-145/SF) and one empty vector-transfected cell clone (DU-145/control).

### Reagents

Recombinant human two-chain SF was a gift from Genentech Inc. (South San Francisco, California, USA). Genetically engineered soluble recombinant human TRAIL was obtained from R&D Systems (Minneapolis, Minnesota, USA). ADR (doxorubicin hydrochloride), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, and propidium iodide were obtained from the Sigma Chemical Co. (St. Louis, Missouri, USA). The selective IKK-β inhibitor Bay 11-7092 was obtained from Marligen Biosciences Co. (Ijamsville, Maryland, USA). The selective c-Met tyrosine kinase inhibitor PHA665752 [(3*Z*)-5-[(2,6-dichlorobenzyl) sulfonyl]-3-[(3,5-dimethyl-4-{[(2*R*)-2-(pyrrolidin-1-ylmethyl) pyrrolidin-1-yl] carbonyl}-1*H*-pyrrol-2-yl) methylene]-1,3-dihydro-2*H*-indol-2-one] was obtained from Pfizer Inc. (La Jolla, California, USA). The cyclooxygenase-2 (COX-2) inhibitor NS-398 and the proteasome inhibitor MG132 were each purchased from Calbiochem (San Diego, California, USA).

### Expression vectors

A dominant negative (DN) kinase-dead mutant Akt (K179A) [DN-Akt] and a constitutively active Akt (which contains a myristoylation sequence from pp60c-Src) [Akt-myr] in the pCIS2 vector were provided by Dr Michael J. Quon, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA [27]. A cDNA encoding the DN kinase-dead mutant Src (Src-K295) expression vector was provided by Dr D. Shalloway (Cornell University, Ithaca, New York, USA) [28]. The Src-K295R cDNA was cloned into the pcDNA3 vector (Clontech, Mountain View, California, USA) using a PCR-based cloning strategy; and the success of cloning was confirmed by sequence analysis. The pCMV4-IκB-α mutant (S32,36A) expression vector has been described earlier [29]. It encodes a nonphosphoryl-atable non-degradable mutant IkB-α in which serines 32 and 36 are mutated to alanines. The IκB-α (S32,36A) protein acts as a 'super-active' IkB-α and a 'super-repressor' of NF-κB activity. A full-length wild-type FLIP cDNA in the pcDNA3 vector was kindly provided by Dr Wafik S. El-Deiry (University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA).

### RNA interference

Human XIAP-siRNA (#6446) was purchased from Cell Signaling Technology (Boston, Massachusetts, USA); and nontargeting siRNA pools (control-siRNA, #D-001206-13-01) were obtained from Upstate Biotechnology

(Charlottesville, Virginia, USA). A FLIP-siRNA (sc-35388) was purchased from Santa Cruz Biotechnology, Santa Cruz, California, USA). siRNAs were transfected into cells using Oligofectamine (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. After 24 h, the cells were transiently transfected overnight with indicated vector(s). The transfected cells were then incubated in serum-free medium containing 100 ng/ml of SF for 48 h; treated with TRAIL for another 24 h; and then harvested for MTT assays to determine cell viability (see below).

#### Flow cytometry to determine cell cycle distribution

Asynchronously proliferating DU-145 cells were pretreated ± SF (100 ng/ml) for 48 h, treated ± TRAIL (50 ng/ml) for 24 h, using trypsin, washed with phosphate-buffered saline (PBS), and fixed in cold 70% ethanol. The samples were then treated with RNase A, stained with propidium iodide (50 μg/ml), and analyzed by FACSort (Becton Dickson, San Jose, California, USA), using the ModFit software (Verity Softwarehouse, Topsham, Maine, USA). At least 20 000 events were collected and analyzed.

#### Transient transfections

For transient transfections, subconfluent proliferating cells were transfected overnight with the vector of interest or with the empty pcDNA3 vector (Invitrogen) (10 μg of plasmid DNA per 100-mm dish or 5 μg per well in 6-well dishes) using Lipofectamine (Life Technologies, Gaithersburg, Maryland, USA) and then washed to remove the excess vector and Lipofectamine. To quantify the transfection efficiency, cultures were co-transfected with plasmid pRSV-β-gal (Promega Corporation, Madison, Wisconsin USA) to allow staining with X-gal reagent and visualization of transfected (blue-staining) cells.

#### Measurement of nuclear factor-kappaB transcriptional activity

NF-κB transcriptional activity was measured as described earlier [19]. The NF-κB-Luc reporter plasmid (NF-κB-Luc) (Stratagene, La Jolla, California, USA) is composed of five copies of the NF-κB enhancer element upstream of a TATA box and the luciferase gene. Briefly, subconfluent proliferating cells in 2-$cm^2$ wells were transfected overnight with 0.25 μg of NF-κB-Luc reporter and 0.25 μg of each indicated vector, using Lipofectamine 2000 (Invitrogen). The cells were then washed to remove lipofectamine and excess vectors, allowed to recover for several hours, treated ± SF (100 ng/ml) for 24 h, and harvested for the measurement of luciferase activity, using the Dual Luciferase Reporter Assay System (Promega, Piscataway, New Jersey, USA). Relative transfection efficiency was determined using the Galacto-Star Mammalian Reporter Gene Assay System (Applied Biosystems, Foster City, California, USA) according to the manufacturer's protocol. Luciferase values were means ± SEMs of quadruplicate wells. Each experiment was performed at least twice to assure that the findings were reproducible.

#### MTT cell viability assay

This assay is based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide], into an insoluble formazan precipitate, which is dissolved in dimethyl sulfoxide and quantitated by spectrophotometry [30]. After the indicated treatment, cells in 96-well dishes were tested for MTT dye conversion. The cell viability was calculated as the amount of MTT dye conversion relative to sham-treated control cells.

#### Trypan blue dye exclusion assay

This assay measures the ability of intact cell membranes of viable cells to exclude trypan blue dye. After the indicated treatment, cells were trypsinized, resuspended in 0.5 ml of Hanks' balanced salt solution, and stained by adding 0.5 ml of 0.4% trypan blue for 5 min. All the stained and unstained cells were counted in 10 squares of a hemocytometer; and the percentage of viable (unstained) cells was calculated. Values are means of three experiments for each cell line tested.

#### Measurement of IKK-β enzymatic activity

After the indicated cell treatments described in the text or figure legends, human IKK-β kinase activity was measured using the HTScan IKK-beta Kinase Assay Kit (catalog no. 7547, Cell Signaling Technology Inc., Danvers, Massachusetts, USA). This assay measures the ability of the cell lysate being tested to phosphorylate a biotinylated IκB-α (Ser32) substrate peptide. A phospho-IκB-α (Ser32/36) specific mouse monoclonal antibody was used to detect the phosphorylated form of the substrate peptide. As a positive control and for standardization of the assay, the kit provides an active IKK-β kinase in the form of a glutathione-*S*-transferase fusion protein. Each assay condition was tested in five replicate wells; and the values of IKK-β kinase activity were expressed relative to non-SF non-TRAIL-treated control cells. Each experiment was performed at least twice to assure reproducibility of the results.

#### Assays of caspase activity

After the indicated cell treatment, cell extracts were prepared and assayed using the CaspACE Assay System, Fluorometric (Cat# G3540; Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions. This assay system utilizes fluorogenic substrates and inhibitors to quantify ICE (caspase-1) and CPP32 (caspase-3/DEVDase) protease activity in cell extracts. These assays were performed in triplicate in 96-well flat-bottom plates by incubating 10 μg of cell protein per sample with the specific fluorigenic substrates provided. After 1 h of

incubation, the reaction products were measured by using a plate reader. Increases in fluorescence were linear over the time period and the extract concentration studied.

### Immunoprecipitation

Asynchronously proliferating cells were washed with serum-free medium and postincubated in serum-free medium containing 100 ng/ml SF for 20 min. The cells were then scraped into 1 ml of lysis buffer [20 mmol/l Tris–HCl (pH 7.5), 150 mmol/l NaCl, 10% glycerol, 1% NP-40, and protease inhibitor cocktail set I (Calbiochem)] per 100-mm culture dish. After incubating on ice for 30 min, the lysate was centrifuged at 17 000*g* for 15 min at 4°C; and the supernatants were collected and their protein concentrations were determined using the BioRad colorimetric protein assay (Bio-Rad Laboratories, Hercules, California, USA). For immunoprecipitation (IP), 500 μg of whole-cell lysate protein was precleared for 1 h by adding Bio-Mag beads (QIAGEN, Valencia, California, USA). The antibody (5 μg) was added to the precleared lysates and kept at 4°C for 1 h. BioMag beads were added to each tube and kept at 4°C overnight. The beads were washed four times with 0.5 ml of lysis buffer, and 1x loading buffer [25 mmol/l Tris–HCl (pH 6.5), 5% glycerol, 1% SDS, 1% 2-mercaptoethanol, and 0.05% bromophenol blue] was added. The samples were boiled for 3 min and analyzed by using SDS–polyacrylamide gel electrophoresis, followed by western blotting.

The antibodies utilized for the IPs were the following: c-Met [rabbit polyclonal antibody (Cat# ab14570; Abcam, Inc., Cambridge, Massachusetts, USA)]; DR4 [mouse monoclonal (Cat# ab59047; Abcam)]; DR5 [mouse monoclonal (Cat# ab47179; Abcam)]; DCR1 [mouse monoclonal antibody (Cat# ab59871; Abcam)]; and DCR2 [mouse monoclonal (Cat# ab59511; Abcam)].

### Western blotting

Western blotting was performed as described earlier [18,19]. For straight western blotting of whole-cell extracts, the cells were collected using trypsin, washed twice with PBS, and pelleted by centrifugation. The pellet was re-suspended in RIPA buffer (1x PBS, 1% NP-40, 0.5% sodium desoxycholate, and 0.1% SDS), placed on ice for 30 min, and spun for 15 min at 14 000 rpm at 4°C to remove the insoluble material. Equal aliquots of whole-cell protein (100 μg per lane) were electrophoresed through SDS–polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, Massachusetts, USA). Alternatively, the immunoprecipitated proteins (see above) were subjected to SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was then blocked with a solution of 5% nonfat dried milk dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h and then incubated with the indicated primary antibody overnight at 4°C. The membrane was washed three times with TBST and incubated with the appropriate secondary antibody for 1 h. After washing in TBST, the protein bands were detected using the enhanced chemiluminescence detection system (Amersham, Piscataway, New Jersey, USA).

The primary antibodies utilized in this study were as follows: Src [mouse monoclonal antibody (AH01152, 1 : 1000 dilution; Biosource International, Camarillo, California, USA)]; Akt [rabbit polyclonal (Cat# P8103S, 1 : 200; New England Biolabs, Beverly, Massachusetts, USA)]; c-Met [rabbit polyclonal (Cat# sc-8307, 1:1000; Santa Cruz Biotechnology, Santa Cruz, California, USA)]; phosphorylated-c-Met (pY1230/4/5; Biosource); DR4 [mouse monoclonal (Cat# ab13890, 1 : 500; Abcam)]; DR5 [mouse monoclonal (Cat# ab22044, 1 : 500; Abcam)]; DCR1 [rabbit polyclonal (Cat# ab13863, 1 : 500; Abcam)], DCR2 [rabbit polyclonal (Cat# ab2019, 1 : 500; Abcam)]; FLIP [polyclonal anti-FLIP antibody (Cat # F9800, 1 : 500, Sigma)]; XIAP [rabbit polyclonal (Cat# 6446, 1 : 1000; Cell Signaling Technology, Boston, Massachusetts, USA)]; and actin (goat polyclonal, 1:1000; Santa Cruz)). Polyclonal antibodies against the active fragment of caspase-3 and full-length caspase-3 were obtained from BD PharMingen (San Diego, CA). An anti-poly (ADP-ribose) polymerase (PARP) antibody was purchased from Upstate Biotechnology.

### Statistical analyses

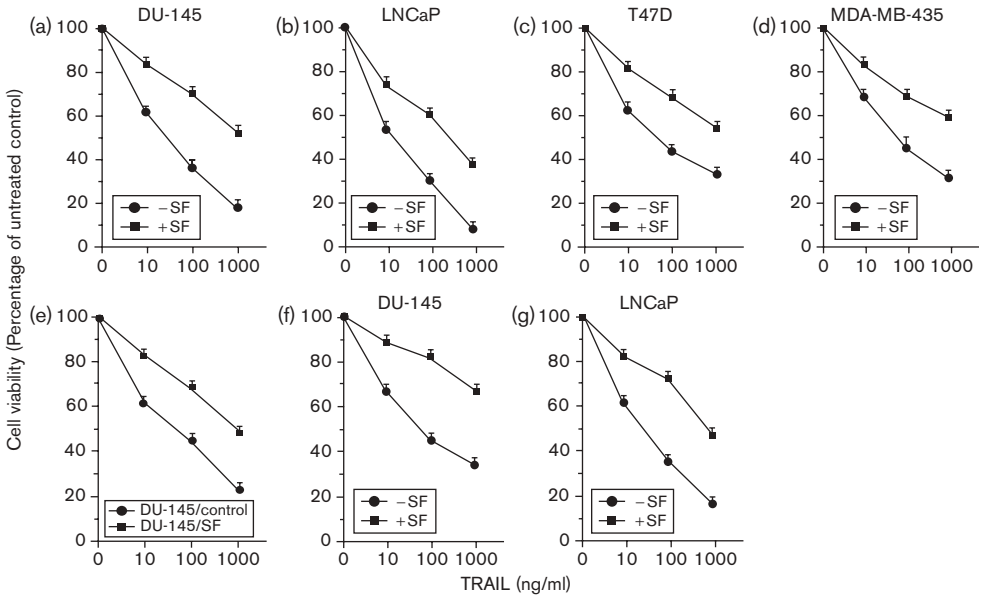
Values plotted are means ± SEMs. Where appropriate, statistical comparisons were made using the two-tailed Student's *t*-test.

## Results

### Scatter factor protects various cell types against TRAIL-induced cytotoxicity

We tested the effects of pretreatment of various cell lines with recombinant human SF (100 ng/ml × 48 h) on the subsequent cytotoxicity caused by a 24 h exposure to recombinant soluble TRAIL. Cell viability was determined using MTT assays, as described earlier [30]. As illustrated in Fig. 1a–d, TRAIL caused dose-dependent reductions in cell viability that were significantly attenuated by SF in both prostate (DU-145 and LNCaP) and breast (T47D and MDA-MB-435) carcinoma cell types. In these four cell lines, pretreatment with SF increased the percentage of viable cells from 16 to 34% ($P < 0.001$–$0.01$, two-tailed *t*-tests). In these assays, $ED_{50}$ values (concentrations of TRAIL required to produce 50% cell death) ranged from about 12 to 50 ng/ml in the absence of SF to about 200 to more than 1000 ng/ml in the presence of SF. Similar results were observed when the sensitivity to TRAIL was compared in a clone of DU-145 cells transfected with a full-length human SF cDNA versus a control cell clone transfected with the empty vector ($P < 0.001$) (Fig. 1e). As compared with the

**Fig. 1**




Scatter factor (SF) protects against tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cytotoxicity. (a–e). Subconfluent proliferating cells were pretreated ± SF (100 ng/ml) for 48 h; exposed to the indicated concentration of recombinant human TRAIL for 24 h; and then assayed for cell viability using MTT assays. Values are means ± SEM of 10 replicate wells. Dose response data are shown for human prostate carcinoma cell lines DU-145 (a) and LNCaP (b) human breast carcinoma cell lines T47D (c) and MDA-MB-435 (d); and DU-145 cell clones transfected with a human SF expression vector (DU-145/SF) versus empty vector (DU-145/control) (e). (f and g) DU-145 cells were treated with SF and TRAIL as described above and then assayed for cell viability using the trypan blue dye exclusion assay. Values are means ± SEM of three separate experiments. For each panel (a–g), at each dose of TRAIL, cell viability was higher for cells treated with SF + TRAIL than for cells treated with TRAIL alone ($P < 0.001$–$0.01$, two-tailed *t*-tests).

DU-145/control clone, DU-145/SF cells exhibited similar total levels of c-Met but much higher levels of phosphorylated (activated) c-Met; and the DU-145/SF cell clone was also considerably more resistant to the DNA-damaging agent ADR than was the DU-145/control cell clone (data not shown).

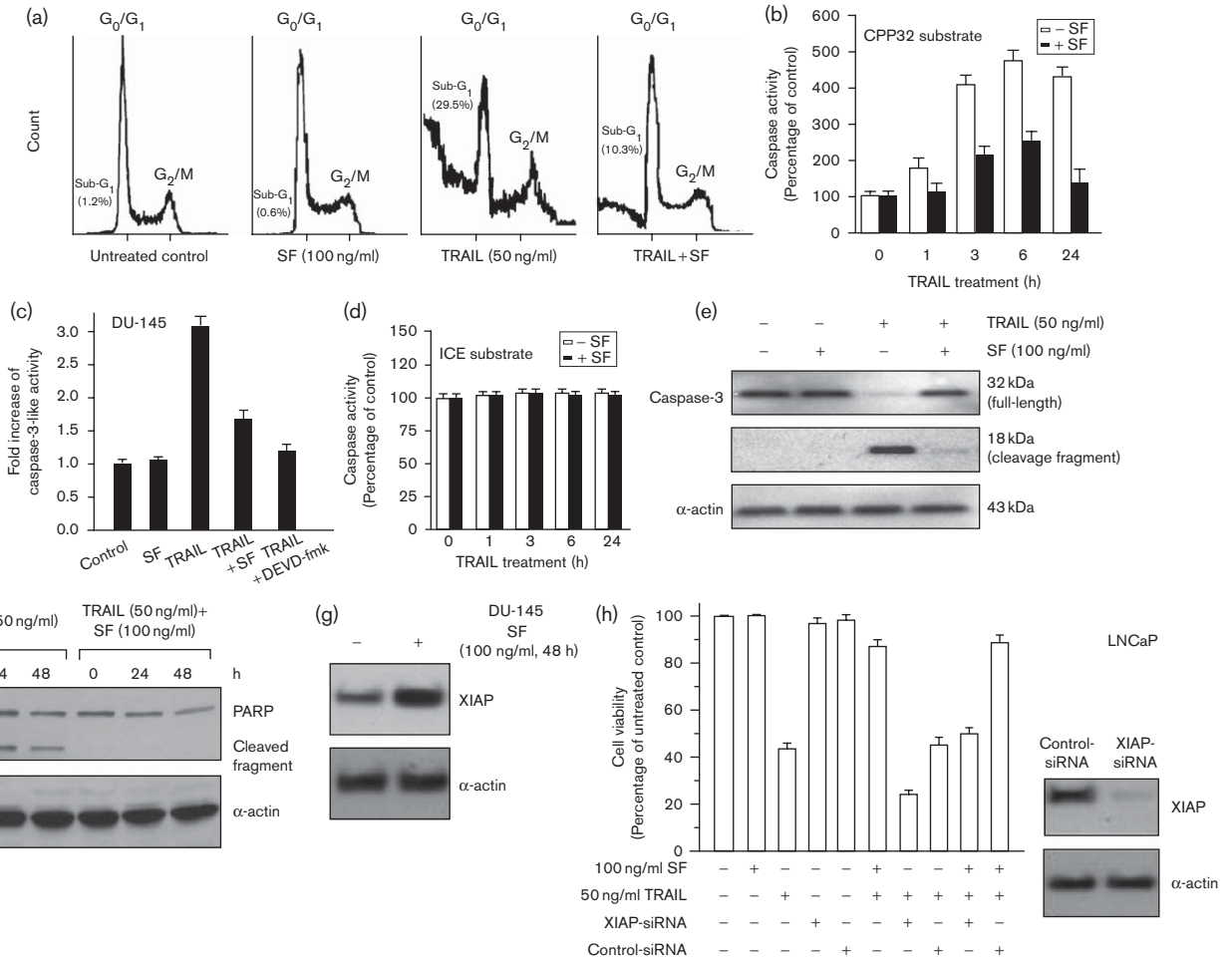
Similar to results obtained using MTT assays, trypan blue dye exclusion assays also yield high degrees of protection by SF against TRAIL in both DU-145 (Fig. 1f) and LNCaP (Fig. 1g) cells ($P < 0.001$ at all concentrations of TRAIL). It was noted that cell viability as determined by trypan blue dye exclusion was somewhat higher than that determined using MTT assays. This may reflect the presence of 'nonviable' cells with decreased ability to reduce MTT dye that have not yet lost membrane integrity and are thus still able to exclude trypan blue dye.

### Scatter factor inhibits TRAIL-mediated apoptosis

As demonstrated by the flow cytometry of propidium iodide-stained nuclei, pretreatment with SF significantly reduced the proportion of TRAIL-exposed DU-145 cells with sub-$G_1$ (apoptotic) DNA content (from 30 to 10% in the experiment shown) (Fig. 2a). Based on a fluorimetric substrate assay, SF strongly attenuated the time-dependent increase in caspase-3 (CPP32)-like enzymatic activity caused by exposure to 50 ng/ml of TRAIL (Fig. 2b). Most of the increase in 'caspase-3-like' activity caused by TRAIL was blocked when the fluorimetric assays were carried out in the presence of the selective caspase-3 inhibitor acetyl-DEVD-fmk (Fig. 2c), suggesting that the measured caspase-3-like activity was, in fact, because of caspase-3. In contrast to caspase-3, treatment with TRAIL for up to 24 h did not cause any increase in caspase-1 (ICE)-like activity (Fig. 2d). Consistent with the caspase-3 activity assays, exposure to TRAIL caused the cleavage of the full-length pro-caspase-3 (32 kDa) to the active 18 kDa caspase-3; and pretreatment with SF blocked this cleavage (Fig. 2e). In addition, in agreement with these findings, SF blocked the TRAIL-induced cleavage of the caspase-3 substrate PARP (Fig. 2f).

The X-linked inhibitor of apoptosis (XIAP) is an NF-κB target gene that has previously been found to inhibit TRAIL-induced apoptosis [31,32]. Here, we showed that treatment of DU-145 cells with SF for 48 h significantly increased the cellular XIAP protein levels (Fig. 2g). To test the role of XIAP in SF protection against TRAIL, we tested the effect of XIAP-siRNA on SF protection against TRAIL (50 ng/ml), using MTT assays. In the experiment shown in Fig. 2h, pretreatment with XIAP-siRNA (50 nmol/l × 48 h) reduced the viability of TRAIL-treated cells by about 20% ($P < 0.001$), suggesting that the basal levels of XIAP are sufficient to provide some protection against TRAIL. The survival of cells treated with SF + XIAP-siRNA + TRAIL was significantly less than

**Fig. 2**




Scatter factor (SF) blocks tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in DU-145 cells. (a) SF blocks TRAIL-induced apoptosis. Proliferating cells were pretreated ± SF (100 ng/ml) for 48 h, treated ± TRAIL (50 ng/ml) for 24 h, stained with propidium iodide, and analyzed for DNA content by flow cytometry. The percentages of cells with apoptotic (sub-$G_1$) DNA content were as follows: control (1.2%), SF alone (0.6%), TRAIL alone (29.5%), and SF + TRAIL (10%). (b, c) SF inhibits TRAIL-stimulated caspase-3-like activity. Cells were pre-treated with SF (100 ng/ml) for 48 h, exposed to TRAIL (50 ng/ml) for the indicated times, and harvested for measurement of caspase-3 (CPP32) using the fluorometric CaspACE assay system. As a control for specificity of the assay, in (c), the caspase-3 inhibitor Ac-DEVD-fmk (50 μmol/l) was added to the TRAIL-treated cells. Values are means ± SEM of triplicate assays and are expressed relative to the non-TRAIL exposed controls. Comparisons of caspase-3 activity in cells treated −SF versus + SF were significant ($P < 0.001$ to 0.05) at all TRAIL treatment times from 1 to 24 h. (d) TRAIL does not stimulate caspase-1-like activity. Assays were performed as described (b), except using a caspase-1 (ICE) substrate. (e) SF blocks TRAIL-induced caspase-3 cleavage. DU-145 cells were pretreated with SF for 48 h, exposed to TRAIL (50 ng/ml) for 24 h, harvested, and western blotted to detect full-length pro-caspase-3, cleaved (active) caspase-3, or actin (control for loading and transfer) (f). SF blocks TRAIL-induced cleavage of poly (ADP-ribose) polymerase (PARP). DU-145 cells were treated as indicated and harvested for western blotting to detect full-length PARP (a caspase-3 substrate), the cleaved PARP fragment, or actin (loading control). (g) SF stimulates expression of the X-linked inhibitor of apoptosis (XIAP). Cells were treated ± SF for 48 h and harvested for western blotting for XIAP and actin. (h) Knockdown of XIAP sensitizes cells to TRAIL and blocks SF protection against TRAIL. LNCaP cells were pretreated ± SF for 48 h in the presence of control-siRNA or XIAP-siRNA (50 nmol/l), exposed to TRAIL (50 ng/ml) for 24 h, and harvested for MTT assays. Values are means ± SEM of 10 replicate wells. Western blotting was performed to confirm XIAP knockdown. Statistical comparisons: XIAP-siRNA + TRAIL versus control-siRNA + TRAIL, $P < 0.001$; SF + XIAP-siRNA + TRAIL versus SF + control-siRNA + TRAIL, $P < 0.001$.

that of cells treated with SF + control-siRNA + TRAIL ($P < 0.001$). However, the survival of cells treated with SF + XIAP-siRNA + TRAIL was significantly greater than that of cells treated with XIAP-siRNA + TRAIL, indicating that SF can still protect the cells (although to a lesser degree), when XIAP levels have been knocked down. The efficacy of the XIAP-siRNA in knocking down XIAP protein levels is shown in the western blot included in Fig. 2h.

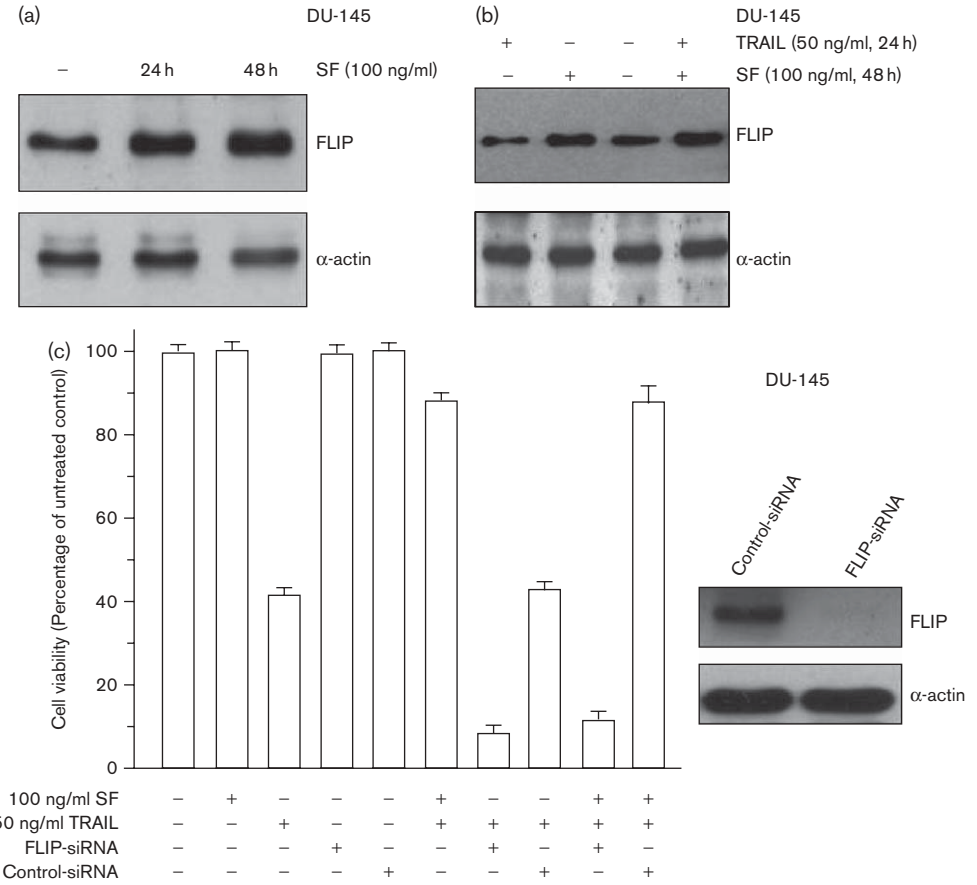
As noted earlier, c-FLIP is another protein that has been implicated as an antiapoptotic mediator of TRAIL resistance. Here, we investigated the potential role of FLIP in SF-mediated protection against TRAIL, using DU-145 cells. As illustrated in Fig. 3a, treatment with SF for 24 or 48 h caused an increase in the FLIP protein levels. The ability of SF to increase FLIP protein levels was maintained in cells treated with TRAIL; stated otherwise, TRAIL did not attenuate SF stimulation of

TRAIL expression (Fig. 3b). As shown in Fig. 3c, knockdown of FLIP had little or no effect on cell viability by itself, but it significantly enhanced TRAIL-induced cell killing ($P < 0.001$). It was further noted that the survival of cells treated with SF + FLIP-siRNA + TRAIL was similar to that of cells treated with FLIP-siRNA + TRAIL, suggesting that unlike XIAP, SF can no longer protect cells at all in the absence of TRAIL. The efficacy of FLIP-siRNA in reducing FLIP protein levels is shown in the inset western blot in Fig. 3c.

### Scatter factor signal transduction for protection against TRAIL

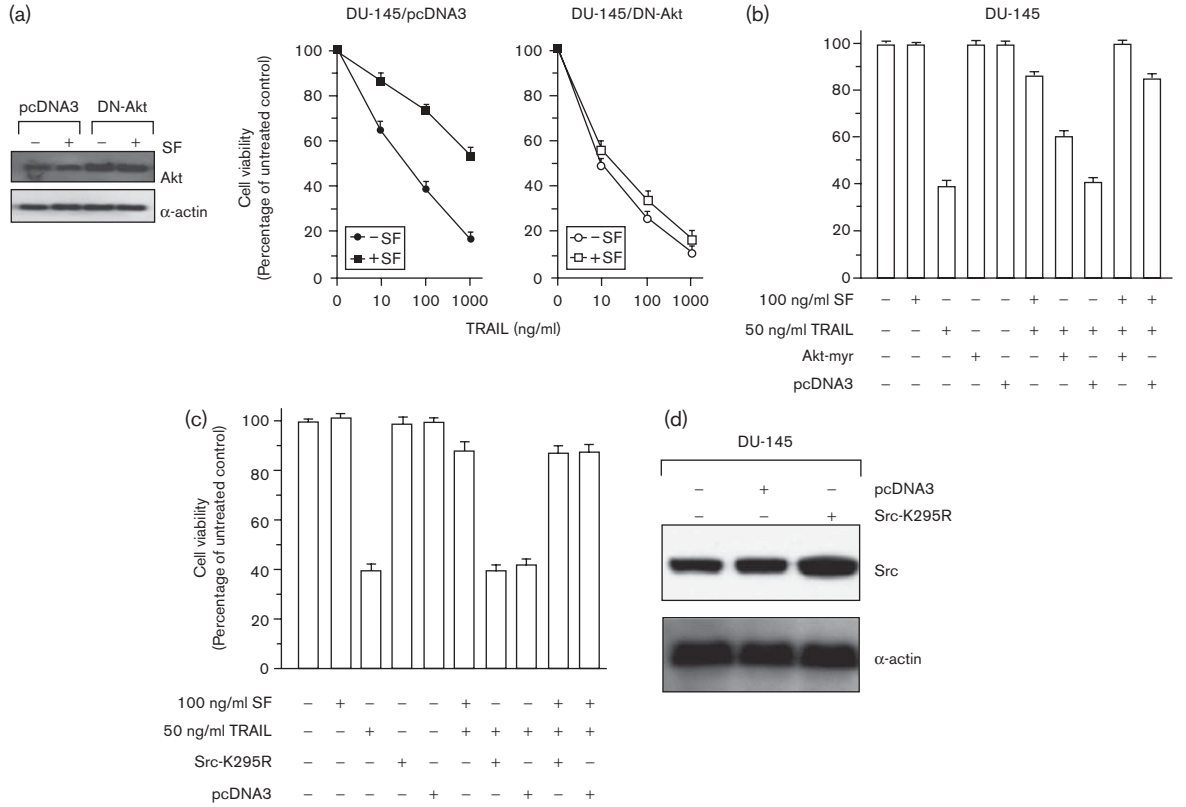
Previously, we reported that Akt is required for SF protection of carcinoma and glioma cells against DNA-damaging agents, such as ADR [18–21,33,34]. Here, we tested whether Akt signaling is required for protection against TRAIL, by the use of a DN-Akt expression vector that encodes a kinase-dead point mutant Akt (K179A). We previously showed that transfection of this vector into DU-145 blocks SF-induced Akt phsophorylation as well as the phosphorylation of several Akt substrates (e.g. GSK3 and mTOR) [20]. As shown in Fig. 4a, the DN-Akt protein was expressed in transiently transfected DU-145 cells. DU-145 cells transfected with the empty pcDNA3 vector showed strong protection against TRAIL by SF, similar to that observed in nontransfected DU-145 cells. Cells transfected with DN-Akt and exposed to TRAIL in the absence of SF treatment showed lower survival than did cells transfected with empty pcDNA3 vector; and there was little or no protection by SF at any dose of TRAIL in the DN-Akt transfected cells. These findings suggest that (i) the endogenous low levels of Akt activity are sufficient to afford some protection against TRAIL; and (ii) SF-stimulated Akt activity is required for protection against TRAIL.

**Fig. 3**




Role of FLICE-inhibitor protein (FLIP) in scatter factor (SF)-mediated protection of DU-145 cells against tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). (a) SF stimulates FLIP protein expression. Subconfluent proliferating cells were treated with SF (100 ng/ml) for the indicated time interval and then harvested for western blotting to detect FLIP or α-actin (control for loading and transfer). (b) TRAIL does not attenuate the SF-mediated increases in FLIP protein levels. Cells were treated ± SF for 48 h; then exposed to TRAIL for 24 h; and then harvested for western blotting as in panel A. (c) Knockdown of FLIP abrogates the SF-mediated protection against TRAIL. Cells were pretreated ± SF for 48 h in the presence of control-siRNA or FLIP-siRNA (50 nmol/l), exposed to TRAIL (50 ng/ml) for 24 h, and harvested for MTT assays. Values are means ± SEM of 10 replicate wells. Western blotting was performed to confirm FLIP knockdown. Comparisons: FLIP-siRNA + TRAIL versus control-siRNA + RAIL, $P < 0.001$; SF + FLIP-siRNA + TRAIL versus SF + control-siRNA + TRAIL, $P < 0.001$.

**Fig. 4**




Scatter factor (SF) protection against tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) requires Akt but not Src signaling. (a) Akt is required for SF protection against TRAIL. Subconfluent proliferating DU-145 cells were transiently transfected overnight with the empty pcDNA3 vector or a kinase-dead dominant negative Akt (DN-Akt), washed, allowed to recover for several hours, harvested using trypsin, and seeded into 96-well dishes. The cells were allowed to attach overnight and then treated ± SF (100 ng/ml) for 48 h and exposed to different concentrations of TRAIL for 24 h, after which cell viability was determined using MTT assays. Values plotted are means ± SEM of 10 replicate wells. (b) Constitutively active Akt (Akt-myr) protects against TRAIL in the absence of SF. Assays were carried out as described in (a), except that the cells were transfected with Akt-myr rather than DN-Akt and were treated with a single concentration of TRAIL (50 ng/ml). Values are means ± SEM of 10 replicate wells. Comparison: Akt-myr + TRAIL versus pcDNA3 + TRAIL, $P < 0.001$. (c) Src is not required for SF protection against TRAIL. Subconfluent proliferating DU-145 cells were transfected overnight with empty pcDNA3 vector or a kinase-dead dominant negative Src (Src-K295R), washed, allowed to recover for several hours, harvested using trypsin, and seeded into 96-well dishes. The cells were allowed to attach overnight and then treated ± SF (100 ng/ml) for 48 h. They were then incubated ± TRAIL (50 ng/ml) for 24 h, after which MTT assays were performed. The values plotted are means ± SEM for 10 replicate wells. Comparison: SF + K295R + TRAIL versus SF + pcDNA3 + TRAIL, *P* not significant. (d) Expression of the dominant negative Src protein (Src-K295R). DU-145 cells were transfected overnight with empty pcDNA3 vector or Src-K295R, washed, postincubated for 48 h, and harvested for western blotting to detect Src and α-actin.

Consistent with a role for Akt in protection against TRAIL, we found that an expression vector encoding a constitutively active (myristoylated) Akt protein (Akt-myr) conferred significant protection against TRAIL (survival increase from 40 to 60%) in the absence of SF (Fig. 4b) ($P < 0.001$). However, Akt-myr was not as effective in protecting the cells against TRAIL as SF. This finding suggests that SF has additional protective actions against TRAIL beyond those because of Akt.

Previously, we showed that Src signaling is required for the activation of NF-κB by SF and that NF-κB signaling is required for protection against ADR [19]. Here, we tested the role of Src in SF protection against TRAIL, using a DN kinase-dead Src expression vector (Src-K295R). Unlike DN-Akt, the Src-K295R vector had little or no effect on cytotoxicity caused by TRAIL (50 ng/ml) either without or with SF treatment (Fig. 4c). In contrast to TRAIL, Src-K295R caused some reduction in the survival of ADR-treated cells in the absence of SF and nearly abrogated the SF protection against ADR (data not shown). The expression of the Src-K295R protein is illustrated in Fig. 4d. These findings suggest that, unlike Akt, Src signaling is not required for protection against TRAIL.

### Role of nuclear factor-kappaB in scatter factor protection against TRAIL

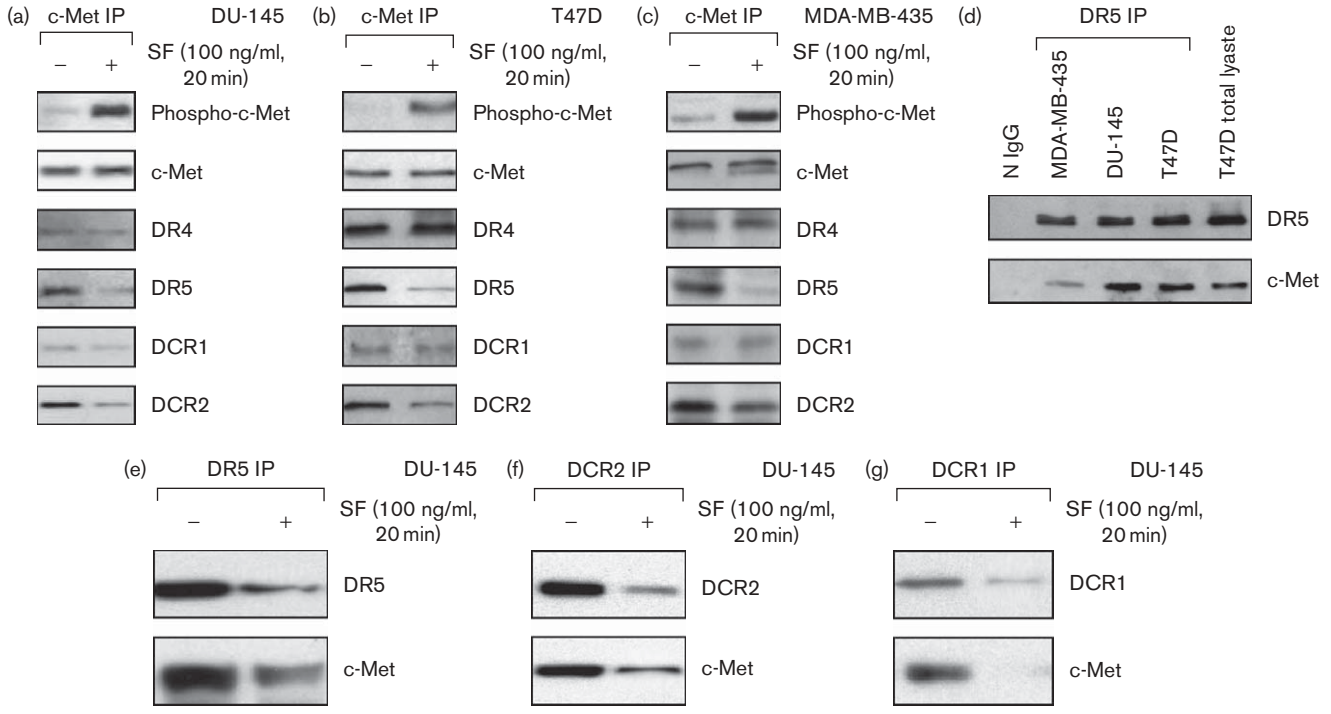
Previously, we reported that SF stimulates NF-κB transcriptional activity and that this activity is required for SF protection against ADR [19]. Here, we tested the effect of TRAIL on NF-κB activity using the NF-κB-Luc

reporter. In both DU-145 and LNCaP cells, exposure to TRAIL alone for 24 h caused a reduction in NF-κB-Luc activity of $\geq$ 80% (supplementary Fig. 1a), although the endogenous NF-κB levels are low, therefore the significance of this decrease is unclear. In the presence of SF, TRAIL caused a modest reduction of NF-κB activity in DU-145 but no reduction of activity in LNCaP cells. Consistent with our previous findings [18], a 20-min exposure to SF stimulated IKK-β kinase activity by 4.4–6.5 fold, whereas a 20-min exposure to TRAIL alone caused a reduction in IKK-β kinase activity to about 10–20% of control (supplementary Fig. 1b and c). When these cells were exposed to SF and TRAIL together, there was only a modest reduction of SF-stimulated IKK-β activity, to 3.7–4.2-fold relative to the control values, suggesting that in the presence of TRAIL, SF maintains the ability to stimulate the IKK-β/NF-κB pathway, but slightly less efficiently.

To validate the NF-κB reporter assays, we tested the effect of expression of the IκB-α 'super-repressor' – a mutant non-phosphorylatable IκB-α (S32,36A) that inhibits the classical pathway of NF-κB activation [32] – on NF-κB-Luc activity. In DU-145 and LNCaP cells, IκB-α (S32,36A) reduced the SF-stimulated NF-κB-Luc activity from 55–60-fold to only 3–5-fold [*P* (supplementary Fig. 1d)]. In the absence of SF, IκB-α (S32,36A) alone reduced the basal NF-κB activity to far less than that because of TRAIL alone. Interestingly, the ability of IκB-α (S32,36A) to block NF-κB activity was somewhat less in the presence of SF + TRAIL than in the presence of TRAIL alone. We also tested the ability of Bay 11-7082, a selective small molecule inhibitor of IKK-β kinase activity [35], to block NF-κB activity. The inhibitor effectively blocked SF-stimulated and basal NF-κB-Luc activity, but the fold inhibition was smaller in the presence of SF + TRAIL than in the presence of SF alone (supplementary Fig. 1e). These findings suggest that although TRAIL inhibits basal NF-κB activity and modestly inhibits SF-stimulated activity, the presence of SF plus inhibitors of the classical NF-κB activation pathway [IκB-α (S32,36A) or Bay 11-7082] may uncover a mechanism by which TRAIL stimulates NF-κB activity.

Finally, we tested the contribution of NF-κB signaling to SF protection against TRAIL. In supplementary Fig. 1f, cells transiently transfected with IκB-α (S32,36A) were tested for sensitivity to TRAIL (50 ng/ml) and protection by SF. IκB-α (S32,36A) had little or no effect on basal sensitivity of DU-145 or LNCaP cells to TRAIL and did

**Fig. 5**




The scatter factor (SF) receptor c-Met physically associates with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors. (a–c) c-Met immunoprecipitation (IP). Subconfluent proliferating cells were treated ± SF (100 ng/ml) for 20 min and subjected to IP for c-Met and western blotting for phospho-c-Met, total c-Met, TRAIL death-inducing receptors (DR4 and DR5), or TRAIL decoy receptors (DCR1 and DCR2). Results are shown for DU-145 (a), T47D (b), MDA-MB-435 (c), cells, and DR5 IP (d). Untreated subconfluent proliferating DU-145, T47D, or MDA-MB-435 cells were subject to IP for DR5 and western blotting for total c-Met; also shown are lanes corresponding to a control (normal IgG) IP of T47D cells and nonprecipitated T47D cell lysate. (e–g) TRAIL receptor IP of DU-145 cells treated ± SF. Subconfluent proliferating DU-145 cells were treated ± SF for 20 min; subjected to IP for DR5 (e), DCR2 (f), or DCR1 (g); and Western blotted to detect that receptor and to detect total c-Met.

not block SF protection against TRAIL. Consistent with that finding, the IKK-β inhibitor Bay 11-7082 had little or no effect on sensitivity to TRAIL ± SF (supplementary Fig. 1g). These findings suggest that the classical pathway for NF-κB activation is not required for SF protection against TRAIL in the cell lines and assay systems that we have studied.

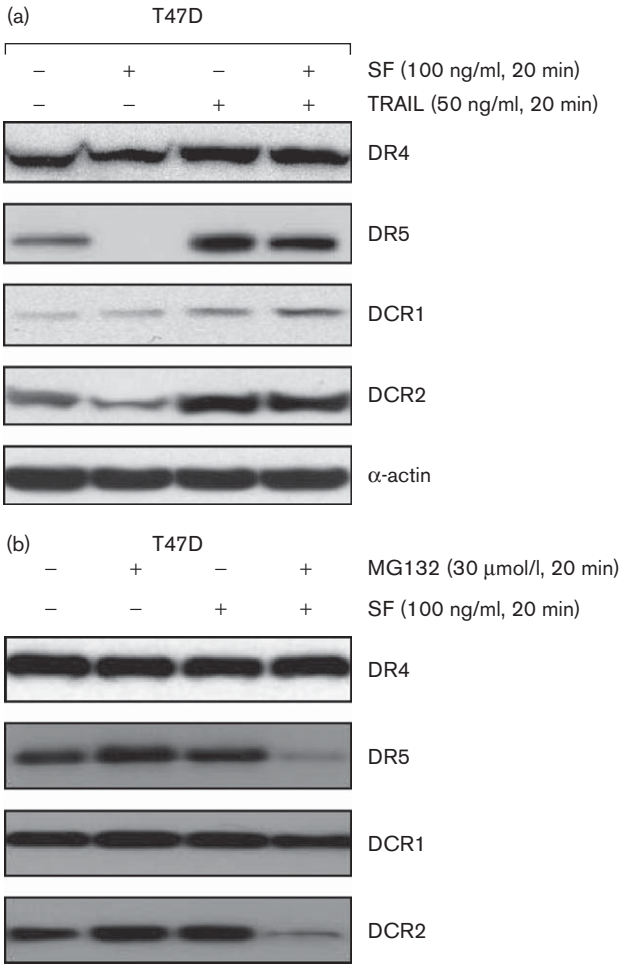
### Association of c-Met with TRAIL receptors

We used IP–western blotting to determine whether the SF receptor c-Met can physically associate with any of the four TRAIL receptors, the death inducting receptors DR4, and DR5 or the 'decoy' receptors DCR1 and DCR2. In one type of experiment, cells were treated ± SF (100 ng/ml) for 20 min and then immunoprecipitated using an anti-Met antibody. As expected, SF caused tyrosine phosphorylation of c-Met but did not cause any change in the total levels of c-Met in all three cell lines tested (DU-145, T47D, or MDA-MB-435) (Fig. 5a–c, respectively). In all three cell types, DR5 and DCR2 were present in the c-Met IP; and the quantities of these receptors were reduced after SF treatment. We also detected DR4 and DCR1 in the c-Met IPs, but the levels of these proteins appeared to be unchanged after SF treatment, except in the case of DCR1 in DU-145 cells. It was noted that the levels of DR4 associated with c-Met were higher in T47D and MDA-MB-435 cells than in DU-145 cells. We confirmed the association of DR5 with c-Met by performing a DR5 IP, in which we found that in the absence of SF, c-Met co-precipitated with DR5 in all three cell lines (Fig. 5d). As a negative control, an IP of T47D cells with an equal quantity of normal (nonimmune) IgG failed to precipitate DR5 or c-Met.

We next performed IP–western blotting of DU-145 cells treated ± SF for 20 min for each of the four TRAIL receptors. For DCR5, DCR2, and DCR1, treatment with SF for 20 min caused significant reductions in the quantities of these receptors that could be precipitated and in the quantity of c-Met associated with them (Fig. 5e–g, respectively). In the case of DR4, DU-145 cells have low basal levels of this receptor; and we were unable to detect this receptor by IP–western blotting.

The reduction in the quantities of DCR5, DCR2, and DCR1 that were precipitated after a 20-min exposure to SF was a somewhat surprising finding; and it suggests that the main reason for the loss of these receptors from the c-Met IP after treatment of DU-145 cells with SF observed in Fig. 5a is a reduction in the total cellular levels of these proteins. We next treated T47D cells with SF and/or TRAIL for 20 min and performed western blotting of whole-cell lysates to detect changes in death receptor protein levels. As illustrated in Fig. 6a, in T47D cells, a 20-min exposure to SF alone reduced the levels of DR5 and DCR2 but had little or no effect on the levels of DR4 and DCR1. In contrast, exposure to TRAIL alone increased the levels of each receptor, to some degree. Interestingly, SF blunted the ability of TRAIL to enhance DR5 levels but had only a modest effect or no effect on the ability of TRAIL to induce the other three receptors. To test the mechanism of the rapid reduction in DR5 protein levels caused by SF, T47D cells were treated with SF for 20 min in the absence or presence of the proteasome inhibitor MG132. The large reductions in DR5 and DCR2 protein levels were because SF were blocked by MG132, which restored the protein levels at least to the basal levels observed in the absence of SF or MG132 (Fig. 6b). In the absence of SF, MG132 caused modest increases in DR5 and DCR2 levels, suggesting

**Fig. 6**




Scatter factor (SF) downregulates death receptor protein levels. (a) Effect of short exposure to SF and/or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on death receptor levels in T47D cells. Subconfluent proliferating cells were treated ± SF and ± TRAIL, as indicated, for 20 min and then harvested for western blotting to detect death-inducing receptors (DR4, DR5), decoy receptors (DCR1, DCR2), and α-actin. (b) Proteasome inhibitor reverses rapid down-regulation of TRAIL receptor proteins by SF. T47D cells were treated ± MG132 (30 μmol/l) and ± SF (100 ng/ml) for 20 min (as indicated); and the cells were harvested for western blotting to detect DR4, DR5, DCR1, and DCR2.

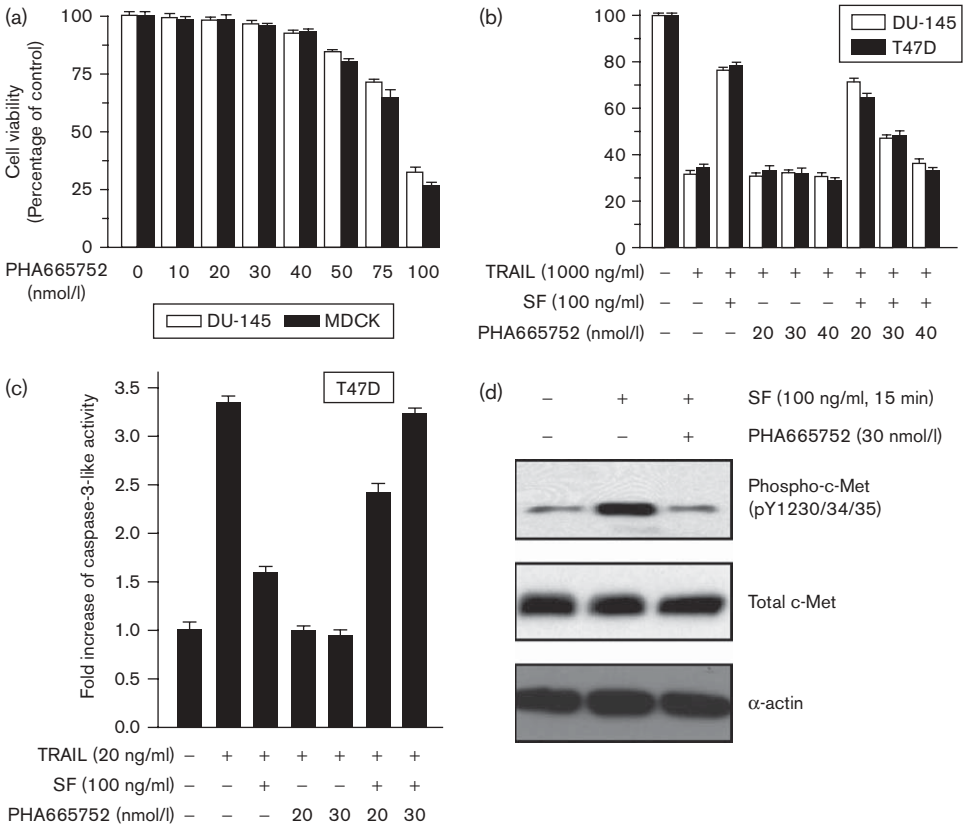
that, to some extent, the basal levels of these TRAIL receptors are also regulated by proteasomal degradation in T47D cells. SF and MG132 had little or no effects on the protein levels of DR4 and DCR1 in these assays.

### c-Met small molecule inhibitor PHA665752 blocks scatter factor protection against TRAIL

Here, we sought to determine whether TRAIL resistance could be reversed by the use of a selective c-Met kinase inhibitor, which has been proposed for clinical use, PHA665752 [36,37]. As a baseline for these experiments, we first tested the effect of inhibitor by itself on cell viability, using MTT assays. As shown in Fig. 7a, cell survival rates were $\geq$ 90% at inhibitor doses of 10–40 nmol/l; and the $ED_{50}$ for loss of cell viability was not reached until somewhere between 75 and 100 nmol/l. Based on these findings, we confined further studies to concentrations of 20–40 nmol/l of PHA665752 to allow conditions in which cell killing by the inhibitor alone was relatively minor. We found that the SF stimulation of NF-κB activity (determined using the NF-κB-Luc reporter) was inhibited by PHA665752 in a dose-dependent manner, with a maximum inhibition of about 80% at 40 nmol/l of PHA665752 (data not shown). In contrast, the reduction of basal NF-κB-Luc activity in the absence of SF was lesser than 20% at all doses of PHA665752, suggesting that the effect of PHA665752 on NF-κB activity was mostly SF specific.

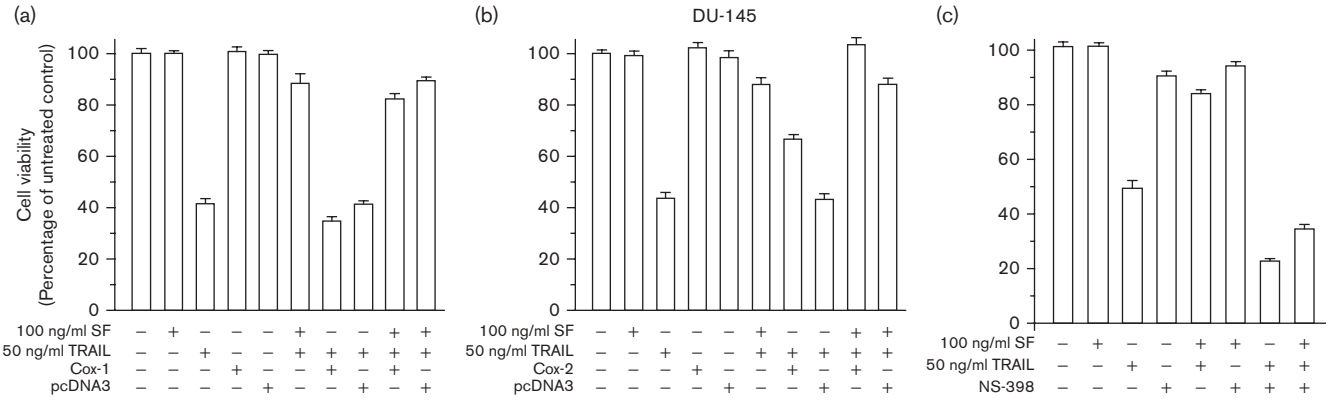
Next, we tested the ability of PHA665752 to block SF-mediated protection against TRAIL (Fig. 7b). Here, DU-145 or T47D cells were pretreated ± SF (100 ng/ml) in the absence or presence of inhibitor (20–40 nmol/l) for 48 h and then exposed to TRAIL and assayed for cell

**Fig. 7**




c-Met inhibitor PHA665752 blocks scatter factor (SF) protection against tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). (a) Effect of PHA665752 on cell viability. Subconfluent proliferating cells were incubated with different concentrations of PHA665752 for 24 h and then assayed for cell viability using MTT assays. Values are means ± SEM of 10 replicate wells. (b) PHA665752 blocks SF protection against TRAIL. Subconfluent proliferating cells in 96-well dishes were pretreated with SF and inhibitor (as indicated) for 48 h. They were then treated with TRAIL (as indicated) for 24 h, and harvested for MTT assays. Values are means ± SEM for 10 replicate wells. Comparisons: SF + PHA665752 + TRAIL versus SF + TRAIL, $P < 0.001$ at PHA665752 concentrations of 30 and 40 nmol/l. (c) PHA665752 inhibits SF protection against activation of caspase-3. DU-145 cells were pretreated ± SF (100 ng/ml) along with the indicated concentration of PHA665752 for 48 h and then treated ± TRAIL (50 ng/ml) for 24 h. The cells were lysed and caspase-3-like activity was determined by measuring cleavage of the substrate Ac-DEVD-pNA. As a control, the caspase-3 inhibitor Ac-DEVD-fmk (50 μmol/l) was added to the TRAIL-treated cells. Comparisons: SF + PHA665752 + TRAIL versus SF + TRAIL, $P < 0.001$ at PHA665752 concentrations of 20 and 30 nmol/l. (d) PHA665752 blocks activation (phosphorylation) of c-Met caused by SF. DU-145 cells were treated with SF (100 ng/ml) in the absence or presence of PHA665752 (30 nmol/l) for 15 min and then harvested for western blotting to detect phospho-c-Met, total c-Met, or actin.

**Fig. 8**




Effect of cycloxygenase (COX) on scatter factor (SF) protection against tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). (a) Effect of wild-type COX-1 on TRAIL cytotoxicity and SF protection against TRAIL. DU-145 cells were transfected overnight with the empty pcDNA3 vector or a wild-type COX-1 expression vector ('Cox-1'), allowed to recover for several hours, harvested using trypsin, and seeded into 96-well dishes. The cells were allowed to attach overnight and then treated ± SF (100 ng/ml) for 48 h and exposed to TRAIL for 24 h, after which cell viability was determined using MTT assays. Values are means ± SEM of 10 replicate wells. (b) Effect of wild-type COX-2 on TRAIL cytotoxicity and SF protection against TRAIL. Assays were carried out as described above for (a), except that a wild-type COX-2 expression vector ('Cox-2') was used instead of the COX-1 expression vector. Comparisons: Cox-2 + TRAIL versus pcDNA3 + TRAIL, $P < 0.001$; SF + Cox-2 + TRAIL versus SF + pcDNA3 + TRAIL, $P < 0.001$. (c) Effect of COX-2 selective inhibitor NS-398 on SF protection against TRAIL. DU-145 cells in 96-well dishes were pretreated with SF and NS-398 (100 μmol/l), as indicated, for 48 h. They were then treated with TRAIL (as indicated) for 24 h, and harvested for MTT assays. Values are means ± SEM for 10 replicate wells. Comparisons: NS-398 + TRAIL versus TRAIL alone, $P < 0.001$; SF + NS-398 + TRAIL versus SF + TRAIL, $P < 0.001$.

viability, as in previous experiments. At 20 nmol/l of PHA665752, there was a modest reduction of SF protection against TRAIL; whereas at 30 and 40 nmol/l, there were marked reductions in the SF-mediated protection ($P < 0.001$). Fig. 7c shows the ability of PHA665752 to reverse the SF-mediated inhibition of TRAIL-induced activation of caspase-3. Here, both 20 and 30 nmol/l concentrations of PHA665752 gave significant increases in caspase-3-like activity in the presence of SF plus TRAIL ($P < 0.001$). Finally, Fig. 7d shows the ability of PHA665752 to block the SF-induced activating tyrosine phosphorylation of the c-Met receptor.

### Effect of cyclooxygenase on scatter factor protection against TRAIL

Previous studies suggest that the COX-2 gene, which mediates prostaglandin synthesis, can inhibit TRAIL-induced apoptosis, in part, by inhibition DR5 expression [38–40]. Here, we investigated the role of the COX genes in protection against TRAIL in DU-145 cells. The overexpression of COX-1 failed to protect cells against TRAIL or to enhance the SF protection (Fig. 8a). In fact, cell survival was slightly lower in cells transfected with wild-type COX-1 and treated with TRAIL ± SF, compared with cells transfected with the empty pcDNA3 vector and treated similarly. In contrast, overexpression of COX-2 conferred protection against TRAIL in the absence of SF and conferred further protection in the presence of SF ($P < 0.001$) (Fig. 8b). Thus, in the absence of SF, COX-2 increased the survival of TRAIL-treated cells from 42 to 65%; and in the presence of SF, COX-2 increased the survival from 87 to 102%. Interestingly, the increase in survival because of SF alone (+ 45%) was greater than that because of COX-2 alone (+ 23%).

Finally, we tested the effect of a selective COX-2 inhibitor (NS-398) on TRAIL sensitivity. In the experiment shown, NS-398 reduced the survival of cells treated with TRAIL alone from 48 to 21% ($P < 0.001$); and it reduced the survival of cells treated with SF + TRAIL from 82% to 34% ($P < 0.001$) (Fig. 8c). It was also noted that at the dose of NS-398 tested (100 μmol/l), there was some reduction in cell survival in the absence of TRAIL (about 7–10%). These findings suggest that endogenous COX-2 provides some degree of protection against TRAIL, which can be enhanced by overexpressing COX-2. They further suggest that COX-2 activity is required for SF protection against TRAIL, or, at least, that SF protection against TRAIL is very weak in the absence of COX-2 activity.

## Discussion

We showed that various prostate and breast carcinoma cell lines are rendered more resistant to the proposed anticancer agent TRAIL by SF. Based on the survival curves, the $ED_{50}$ values for the reduction of survival to 50% were more than 10-fold higher in the presence of SF than in its absence. Flow cytometry and caspase activation assays suggest that TRAIL-mediated cell killing proceeds through classic apoptosis pathways that

lead to the activation of caspase-3 (but not caspase-1), cleavage of caspase-3 targets such as PARP, and apoptotic DNA fragmentation. These pathways are inhibited by SF. Pretreatment of DU-145 cells with SF caused upregulation of the protein levels of XIAP and FLIP; and knockdown of either of these proteins by RNA interference significantly reduced the survival of TRAIL-treated cells in the absence or presence of SF. However, unlike XIAP, FLIP knockdown virtually abrogated all SF-mediated protection against TRAIL, suggesting that FLIP is essential for SF protection, while some protection is still possible in the absence of XIAP. Both FLIP and XIAP are known antiapoptotic target genes for the survival-promoting transcription factor NF-κB [29]. Previously, we identified several other antiapoptotic NF-κB target genes that are induced by SF and which contribute to SF-mediated protection against ADR, including cIAP-1, cIAP-2, and TRAF-2. Inhibition or knockdown of these proteins attenuated or abolished SF-mediated protection against the DNA-damaging agent ADR.

In studies related to the signaling mechanism for SF-mediated protection, we found that inhibition of Akt (using a kinase-dead DN mutant Akt protein) abrogated SF protection against TRAIL in DU-145 cells; and a constitutively active mutant Akt protein (Akt-myr) conferred protection against TRAIL in the absence of SF. These findings suggest that signaling down-stream of Akt is required for protection against TRAIL. Interestingly, although Src signaling was required for protection SF against ADR, it was not required for SF protection against TRAIL, indicating a difference in the signaling pathways for SF protection against apoptosis induced by these two agents.

Another interesting and unexpected finding was that although previous studies indicate that NF-κB signaling is required for SF protection against ADR [18,19], neither the IκB super-repressor nor the IKK-β inhibitor Bay 11-7082 effectively blocked SF protection against TRAIL. Each of these agents inhibited SF-stimulated NF-κB-Luc activity. It is noted that although these agents block the 'classical' pathway of NF-κB activation, an 'alternative' pathway, which involves the activation of IKK-α through the NF-κB-inducing kinase NIK, can also mediate NF-κB-dependent gene expression [41]. Although the classical pathway usually involves activation of RelA (p65)/p50 heterodimers, the alternative pathway involves activation of RelB/p52 dimers. The RelB/p52 dimers preferentially recognize a distinct type of NF-κB binding site that is present in IKK-α-dependent promoters [42]. Our findings indicate that the ability of SF to stimulate the classical pathway of NF-κB activation does not contribute significantly to protection against TRAIL. However, they do not rule out the possibility that this alternative pathway could contribute SF protection against TRAIL, especially because our previous study showed that SF causes the rapid nuclear translocation of RelB in DU-145 cells [19]. Our findings are also consistent with the possibility that SF can upregulate some antiapoptotic genes such as XIAP and FLIP by other pathways unrelated to NF-κB.

Our studies further suggest that SF can induce a rapid loss of some death/decoy receptor proteins (e.g. DR5 and DCR2 in T47D cells) in a 20-min time frame, probably through degradation in the 26S proteasome. Somewhat surprisingly, we detected a physical association of death receptors and the 'decoy receptors' with the c-Met receptor, demonstrated by reciprocal IP–western blotting. Reduced levels of some DR and DCR proteins were associated with the liganded receptor, as compared with the unliganded receptor. However, the reduced quantities of DR/DCR proteins associated with ligation of c-Met could simply be because of a reduction in the total levels of these proteins on account of SF-induced degradation. The significance of the observation that DR and DCR proteins can associate with c-Met is unclear at present, but one possibility is that the receptor, once ligated, assists in the targeting of the bound DR or DCR proteins for degradation. For example, it was shown that after ligation, c-Met is endocytosed and degraded in a manner that requires the proteasome [43,44]. However, c-Met degradation usually requires at least 2 h; and our studies showed no coincident SF-induced reduction of total c-Met protein levels during the same 20-min interval that was sufficient for reduction of DR5 and DCR2 protein levels.

Alternatively, it is possible that SF stimulates posttranslational modification of DR proteins (e.g. phosphorylation), resulting in their being targeted for degradation or that the unliganded c-Met receptor binds to DRs and prevents them from being degraded; and conversely, release of the DR protein by the liganded receptor (e.g. because of reduced affinity of DR binding to phosphorylated c-Met) allows them to be targeted for degradation. Further study will be required to resolve these issues.

These studies suggest or show several different potential approaches to overcoming TRAIL resistance, that is, because of overexpression of SF and c-Met in tumors. Thus, PHA665752, a clinically proposed small molecule inhibitor of c-Met kinase activity, [36,37] was found to inhibit SF-mediated NF-κB activation, protection against TRAIL-induced cytotoxicity, and protection against TRAIL-mediated caspase-3 activation. One possible benefit of this approach is that sensitization to TRAIL can be achieved at doses of PHA665752 that cause only modest or no additional cytotoxicity in the absence of SF, that is, doses that may be insufficient to cause tumor regression by themselves. A second benefit is that it may be possible to achieve sensitization to TRAIL by delivering PHA665752 for a limited time only in conjunction with TRAIL. Lower doses and

intermittent administration would serve to reduce potential normal tissue toxicity, although this remains to be proven. Although we have chosen to study PHA665752, other c-Met small molecule inhibitors are available and may be suitable for a similar application (e.g. PF02341066 and SU11274) [44–47]. Besides the small molecule inhibitors, antibodies have been prepared against SF and against c-Met and are undergoing study for their efficacy against SF/c-Met-driven cancers [48–50].

FLIP is a particularly interesting target for sensitization of cancers to TRAIL, as knockdown of FLIP markedly sensitized the cells to TRAIL both in the absence and presence of SF. XIAP may also serve as a target for sensitization to TRAIL; although in our study, the effect of knocking down FLIP was greater than that of knocking down XIAP. Previous studies suggest that some agents that act to reduce the levels of FLIP or XIAP can sensitize tumor cells to TRAIL [e.g. the proteasome inhibitor PS-341 (Velcade) and the chemotherapy drug actinomycin D (a DNA intercalating agent that inhibits RNA synthesis)] [51,52]. However, these agents have many biochemical actions; and it would be desirable to have available small molecule compounds that more selectively target FLIP and XIAP.

Finally, we documented that overexpression of COX-2 induces resistance to TRAIL and that the COX-2 inhibitor NS-398 could significantly sensitize DU-145 cells to TRAIL in the absence or presence of SF. Interestingly, it has been reported that in some cell types, selective COX-2 inhibitors (including NS-398) can sensitize the cells to several death receptor ligands (including TRAIL) in a manner that is independent of COX-2 inhibition [53]. Regardless of the mechanism, COX-2 inhibitors are attractive agents for sensitizing tumors to TRAIL because they are currently in clinical use and they are currently under investigation for cancer prevention.

## Acknowledgement

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