Enhanced Antitumor Efficacy of a DR5-Specific TRAIL Variant over Recombinant Human TRAIL in a Bioluminescent Ovarian Cancer Xenograft Model

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Abstract

**Purpose:** Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) is clinically evaluated as a novel anticancer drug. rhTRAIL-DR5, a rhTRAIL variant that specifically binds to DR5 receptor, has recently been developed. We investigated whether rhTRAIL-DR5 is more efficient than rhTRAIL in combination with cisplatin in DR5-expressing human A2780 ovarian cancer cells.

**Design:** Effect of cisplatin alone or in combination with rhTRAIL or rhTRAIL-DR5 on DR5 surface expression, apoptosis, and cell survival of A2780 was measured. Biodistribution analysis was done in mice with 125I-rhTRAIL administered intravenously versus intraperitoneally. Antitumor efficacy of rhTRAIL-DR5 versus rhTRAIL was determined in an intraperitoneally growing bioluminescent A2780 xenograft model.

**Results:** Cisplatin strongly enhanced DR5 surface expression. Both rhTRAIL and rhTRAIL-DR5 in combination with cisplatin induced high levels of caspase-3 activation, apoptosis, and cell kill, with rhTRAIL-DR5 being most potent. Intraperitoneal administration of 125I-rhTRAIL resulted in a 1.7-fold higher area under the curve in serum, increased tumor exposure, and more caspase-3 activation in the tumor than intravenous administration. Intraperitoneal administration of rhTRAIL-DR5 delayed A2780 tumor progression, reflected in a mean light reduction of 68.3% (P = 0.015), whereas rhTRAIL or rhTRAIL-DR5 plus cisplatin resulted in 85% (P = 0.003) and 97% (P = 0.002) reduction compared with A2780 tumor progression in vehicle-treated animals. Combination of rhTRAIL-DR5 with cisplatin was more effective than cisplatin alone (P = 0.027).

**Conclusion:** rhTRAIL-DR5 was superior over rhTRAIL in vitro and in vivo against DR5-expressing ovarian cancer also in combination with cisplatin. Intraperitoneal administration of rhTRAIL-DR5 warrants further exploration in ovarian cancer.

In developed countries, ovarian cancer is the fifth leading cause of deaths related to cancer in women (1). Although initial response rates to first-line treatment are up to 80% in advanced stage patients, the overall 5-year survival is low due to the occurrence of drug resistance (2). A reduced tendency of cancer cells to undergo apoptosis is due to defects in the intrinsic apoptosis pathway, which contributes to drug resistance (3, 4). Therefore, an attractive strategy for targeting cancer cells involves shifting cellular balance in favor of cell death. Such a shift can be achieved by targeting the extrinsic apoptotic pathway. This pathway is activated after binding of death ligands of the tumor necrosis factor family to their respective receptors at the cell membrane (5). The recombinant human form of the death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is regarded as the most promising death ligand due to its selective toxicity against tumor cells while sparing most normal tissues (6). rhTRAIL (Apo2L) is currently evaluated in clinical trials. A recent phase I study showed that rhTRAIL can be administered safely and is well tolerated (7). This ligand binds four membrane-bound receptors, of which death receptor 4 (DR4) and death receptor 5 (DR5) act as agonistic receptors and decoy receptor 1 (DcR1) and decoy receptor 2 (DcR2) act as antagonist receptors (8).

Besides rhTRAIL, we and others have developed alternative strategies for targeting death receptors such as agonistic antibodies (9) and TRAIL variants that selectively activate DR4 or DR5 (10, 11). By avoiding competition with other
TRAIL receptors, we showed that specific targeting of DR5 using DR5-selective TRAIL variants resulted in enhanced apoptosis in several cancer cell lines, including the A2780 ovarian cancer cell line. In these cell lines, rhTRAIL-induced apoptosis was primarily mediated by DR5 (11). The DR5-selective rhTRAIL variants possess increased binding capacities for the designated receptor, which may enhance efficacy and improve the therapeutic window (11). The role of DR5 has been implied in ovarian cancer, because hypermethylation of the DR4 promoter and reduced expression of DR4 frequently occur in ovarian cancers (12). Moreover, univariate analysis showed an association for high DR5 expression with decreased survival in ovarian cancers (13). In addition, use of rhTRAIL combined with cisplatin enhanced apoptosis and growth inhibition in several ovarian cancer cell lines, which was related to cisplatin-induced DR4 and DR5 cell surface expression (14). Taken together, these results present DR5 as an interesting target in ovarian cancer. Thus far, studies combining chemotherapeutic drugs with DR5 targeting drugs have not been done in ovarian cancer models.

Clinical efficacy of death receptor targeted therapies in ovarian cancer depends on their biological activity and pharmacologic behavior. As the peritoneal cavity is the main site of disease in ovarian cancer, intraperitoneal drug administration may result in increased tumor penetration and drug exposure with reduced systemic toxicity (15). Intraperitoneal cisplatin administration increases survival compared with intravenous administration in advanced-stage ovarian cancer patients (16). As intravenous rhTRAIL administration in humans results in rapid renal clearance with a half-life of ~30 min (17), intraperitoneal administration may delay its clearance and lead to increased antitumor activity.

The aim of the present study was to compare the in vitro and in vivo efficacy of a novel rhTRAIL variant directed at DR5 (rhTRAIL-DR5) with that of rhTRAIL alone and in combination with cisplatin in a bioluminescent human A2780 intraperitoneal ovarian cancer model. The rhTRAIL variant, obtained by computational design, contains two amino acid mutations, D269H and E195R, which ensure high-affinity binding specifically to DR5 (11). The optimal route of in vivo variant or rhTRAIL administration, intravenously or intraperitoneally, was evaluated by biodistribution analysis with radiolabeled rhTRAIL. In vivo efficacy was determined by bioluminescence.

**Materials and Methods**

**Cell lines and transfection procedure.** The human ovarian cancer cell line A2780, a kind gift from Dr. Hamilton (Fox Chase Cancer Center), forms intraperitoneal xenografts mimicking peritonitis carcinomatosis in nude mice (18). The A2780-Luc cell line was generated as follows: the luciferase gene was excised from pGL3-basic (Promega) with HindIII and XbaI restriction enzymes (Roche Applied Science) and ligated into a pcDNA3 vector under the control of the cytomegalovirus promoter. A2780 cells were cultured to 70% confluency and transfected by incubation with 2.5 µg plasmid DNA and 5 µL Fugene6 (Roche) in 250 µL Opti-MEM (Invitrogen). Two days after transfection, transfectants were selected by adding genetin (1 mg/mL) (Roche Applied Science). Stable transfectants were obtained with a clonogenic assay followed by subcloning of positive clones by limiting dilution. The cell lines were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS and 0.1 mol/L l-glutamine in a humidified atmosphere with 5% CO2 at 37°C. Genetin was added once a month to the A2780-Luc culture. Luciferase expression was regularly tested with the luciferase assay (Promega) and the Bio-Rad ChemiDoc XRS system (Bio-Rad).

**Cytotoxicity assays and determination of apoptosis.** The microculture tetrazolium assay, done as described earlier (19), was used to measure cytotoxicity. The cells were cultured in Ham’s/F-12 and DMEM supplemented with 20% FCS and 0.1 mol/L l-glutamine. rhTRAIL and rhTRAIL-DR5 were produced as we have described earlier (11, 20). Binding capacity to DR4 and DcR1 is virtually absent for TRAIL-DR5, whereas affinity for DcR2 is reduced (11). Treatment consisted of continuous incubation with 0 to 100 ng/mL rhTRAIL-DR5 or rhTRAIL. In cell viability assays assessing combination treatment with cisplatin, the cells were preincubated for 4 h with 2.5 µmol/L cisplatin (inhibitory concentration 20%, IC20), before addition of 0 to 25 ng/mL rhTRAIL or rhTRAIL-DR5.

Caspase-3/7 activity was used as an early apoptosis marker. Caspase-3/7 activity was determined with a caspase-3/7 fluorometric assay (Zebra Biosciences). For the fluorometric detection of DEVDase activity, cells were plated in 6-well plates and left to adhere overnight. The cells were exposed to 2.5 µmol/L cisplatin for 4 h, after which cisplatin was washed away with PBS [6.4 mmol/L Na2HPO4, 1.5 mmol/L KH2PO4, 0.14 mmol/L NaCl, 2.7 mmol/L KCl (pH 7.2)] and fresh medium was added to the cells. Twenty hours later, 50 ng/mL rhTRAIL-DR5 or rhTRAIL was added for various times. Thereafter, the cells were harvested with trypsin and washed twice with ice-cold PBS. Before performing the caspase-3 activity assay according to the manufacturer’s protocol, protein content of the lysates was determined with Bradford analysis (21).

The acidine orange staining served as a marker for end-stage apoptosis. For the apoptosis assay, 10,000 cells were incubated in 96-well tissue culture plates. The cells were exposed to 2.5, 10, or 30 µmol/L cisplatin for 4 h, after which they were washed with PBS twice and incubated in regular culture medium. Twenty hours thereafter, cells were incubated in regular culture medium with or without 100 or 250 ng/mL rhTRAIL-DR5 or rhTRAIL for an additional 4 h. The same procedure was done in the presence of 2.5 µg/mL mouse anti-DcR2 antibody (R&D Systems), with the exception that
1 h preincubation with the blocking antibody preceded rhTRAIL-DR5 and rhTRAIL incubation. With this anti-Dr2 antibody, an enhanced effect of rhTRAIL was observed in Colo205 human colon carcinoma cells. After drug incubation, acridine orange was added to each well to distinguish apoptotic cells from viable cells. Staining intensity was determined by fluorescence microscopy. Apoptosis was defined by the appearance of apoptotic bodies and/or chromatin condensation and expressed as the percentage of apoptotic cells counted in three fields containing minimally 300 cells.

To quantitatively express the efficacy of combination therapy (cisplatin + rhTRAIL or rhTRAIL-DR5) compared with both agents alone, we calculated enhancement ratios for cell kill and apoptosis as follows: enhancement ratio = [% induced by combination therapy / (% induced by cisplatin alone + % induced by ligand)].

**Flow cytometry.** Analysis of TRAIL receptor membrane expression was done by fluorescence-activated cell sorting analysis as described previously (22). For death receptor expression after cisplatin exposure, cells were exposed for 4 h, washed with PBS, and incubated for 20 h in regular culture medium, after which fluorescence-activated cell sorting analysis was done. Cells were subsequently washed twice with cold PBS containing 2% FCS and 0.1% sodium azide and incubated with phycoerythrin-conjugated mouse monoclonal antibodies against DR4, DR5, Dr2, and Dr2. Mouse phycoerythrin-labeled IgG1 and IgG2B were used as isotype controls. All phycoerythrin-labeled antibodies were purchased from R&D Systems. Membrane receptor expression was analyzed with WinList and WinList 32 software (Verity Software House) and shown as mean fluorescent intensity of all analyzed cells.

**DR5 RNA interference and Western blotting.** Small interfering RNA (siRNA) specific for human DR5 was designed and synthesized by Eurogentec. The double-stranded siRNA specific for human DR5 was 5'-GACCCUUGUGCUCGUUGUC-dTdT-3' (sense) and 5'-GACACAGACAGACACAGAGGCGC-dTdT-3' (antisense). Double-stranded luciferase siRNA sequence was 5'-CUUUAGCCUGGAAUGUCCAUGA-dTdT-3' (sense) and 5'-UCGAAGUACUCGGUUAGG-dTdT-3' (antisense). A2780 cells were transfected in 6-well plates (at 50-60% confluency) with siRNA duplexes (133 nmol/L) using Oligofectamine transfection reagent according to the manufacturer’s instructions (Invitrogen/Life Technologies). After 24 h, medium was aspirated and cells were harvested and plated. Then, cells were exposed to various cisplatin concentrations for 4 h, washed with PBS, and incubated for 20 h in regular culture medium. Finally, cells were harvested and used for flow cytometry or Western blotting. For the apoptosis assay, cells were incubated in regular culture medium with or without rhTRAIL-DR5 or rhTRAIL (100 ng/ml) for an additional 4 h and apoptosis was determined with the acridine orange assay.

For Western blotting, cells were washed in ice-cold PBS and lysed in SDS sample buffer (4% SDS, 20% glycerol, 0.5 mol/L Tris-HCl (pH 6.8), 0.002% bromophenol blue) containing 10% 2-β-mercaptoethanol by boiling for 5 min in a water bath. Proteins were separated on a SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore) by wet blotting. Western blotting was done using skim milk as blocking agent (19). The following antibodies were applied: rabbit anti-DR5 antibody (Cell Signaling Technology) and mouse-anti-actin as the control for equal protein loading (ICN Biomedicals). Secondary antibodies conjugated with horseradish peroxidase were obtained from DAKOCytomation. Chemiluminescence was detected using ECL Chemiluminescence Blotting Substrate (POD) or Lumi-LightPLUS Western blotting substrate (Roche Diagnostics).

**Animals and bioluminescence imaging procedure.** Female nude mice (Hsd:athymic nude-nu) were obtained from Harlan Nederland at age 6 to 8 weeks (~21 g). Incubation was done 10 days after acclimatization. All animal studies were conducted in accordance with the Law on Animal Experimentation and local guidelines and approved by the local ethical committee.

Imaging was conducted with the IVIS 100 series (Xenogen) composed of a cooled charge-coupled device camera connected to a light-tight black chamber. Before in vivo imaging, animals were anesthetized with 4% isoflurane and injected intraperitoneally with α-luciferin (150 mg/kg; Xenogen) reconstituted in sterile PBS. Mice were placed in prone position on a warmed stage (37°C) in the imaging chamber, and grayscale reference images were obtained under dim illumination. Pseudocolour images representing bioluminescent intensity were acquired with LivingImage software (version 2.50; Xenogen) 10 and 15 min after α-luciferin injection in complete darkness. These images were superimposed on the grayscale images for analysis with Igor Pro software (version 4.09; WaveMetrics). All bioluminescence imaging (BLI) data are depicted in radiance units (photons s/cm²/sr) enabling absolute comparisons between bioluminescent images and represent final data obtained after subtraction of the background signal.

**Characterization of the intraperitoneal bioluminescent model.** The A2780-Luc peritonitis carcinomatosis model, characterized in 45 mice, showed exponential tumor growth from 5 days after intraperitoneal inoculation with 2 x 10⁶ A2780-Luc cells. Approximately 10 days later, the increment of the bioluminescence signal was delayed, characterized by a flattening of the BLI log-growth curve that evolved in an almost flat slope. This flattening preceded the development of macroscopic disease, bloody ascites formation, and deterioration of general condition. Animal survival based on clinical condition was, on average, 4 weeks after inoculation. Flattening was most likely due to superposed tumor tissue that absorbed and scattered light emitted from tumor cells situated deeply in the peritoneal cavity. To employ flattening of the BLI growth curve in the definition of a uniform endpoint, we used logistic regression analysis. The exponential curve was represented by: y = a + b(e⁻ᵃx), where y stands for the bioluminescent signal (in radiance), a for the intercept with the y axis (BLI signal at day 5), e for the uniform mathematical constant, x for time (in days), and b for the equation-specific constant, which is calculated by logistic regression. The equation can be used to predict BLI signal on consecutive imaging days. Definition of flattening, which serves as a surrogate endpoint for survival, was a bioluminescent signal of <50% as the expected signal based on the equation. We used this endpoint in the efficacy studies with one refinement. Treatment induced alterations in the log growth, which did not allow determining an equation representing the BLI regression curve for each mouse during treatment. The time from cessation of treatment to flattening was too short to reliably employ logistic regression analysis. Therefore, the mean signal at flattening in the vehicle-treated group minus 1 x SD was defined as absolute cutoff value (3.1 x 10⁶ photons/s/cm²/sr), which was valid as a uniform survival endpoint in 85% of the mice.

**In vivo biodistribution with 125I-rhTRAIL.** Radioiodination of rhTRAIL was done with a 125I-TRAIL solution of 1 mg/ml in Tris (pH 7.4), containing 100 μmol/L zinc sulfate and 10% glycerol. rhTRAIL (45 μg) and chloramphenicol T (50 μg; Merck) were allowed to react with 70 MBq 125I-NaI in 0.05 mol/L NaOH (pH 9.0; GE Healthcare) during 3 min at pH 8.0. The labeling reaction was terminated with sodium metabisulfite (Acros Organics). Nonbound 125I was removed by gel filtration chromatography. The PD-10 column (Sephadex G-25M; Amershams Biosciences) was eluted with Tris containing 100 μmol/L zinc sulfate, 10% glycerol, and 0.5% human serum albumin. The biodistribution study was conducted in 50 mice after establishment of A2780-Luc intraperitoneal xenografts. 125I-trTRAIL (0.15 mL; 150 kBq, 0.5 μg) was administered intravenously through retro-orbital injection in 25 mice and intraperitoneally in 25 mice. At t = 15, 30, 60, 90, and 360 min, groups of 5 mice were sacrificed and organs and tissues were excised, rinsed for residual blood, and weighed. Tumor tissue was additionally fixed in 10% buffered formalin for histologic assessment. Samples were counted for radioactivity in a calibrated well-type LKB-1282-CompuGamma counter. Tissue activity was expressed as %ID/g. Tumor-to-blood and tumor-to-muscle ratios were also calculated. All data were corrected for physical decay and compared with a known standard sample. Pharmacokinetic parameters...
were derived using the KINFIT module of the MW/PHARM computer program package (version 3.50; MediWare). Clearance rates of 125I-rhTRAIL from the circulation were calculated using nonlinear regression analysis.

**Immunohistochemistry.** Tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut in 4 μm sections, which were mounted on APES-coated glass slides and deparaffinized in xylene. Antigen retrieval was done by microwave treatment for 8 min in 0.01 mol/L citrate buffer (pH 6.0). For active caspase-3 staining, the slides were incubated overnight at 4°C with a polyclonal rabbit anti-cleaved caspase-3 antibody (Asp175, 1:200; Cell Signaling Technology). The antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175. Biotinylated swine anti-rabbit (DAKO) was used as a secondary antibody (1:300 dilution), after which streptavidin/horseradish peroxidase (DAKO; 1:300 dilution) was applied. Negative controls were obtained by omission of the primary antibody and by incubation with normal rabbit IgG1. Slides were counterstained with hematoxylin. Immunohistochemical staining for cleaved caspase-3 was semiquantitatively scored as follows: 0, no positive staining cells; +/-, focal staining in one small field; +, scattered staining of few cells/focal staining in a larger field; and ++, scattered staining of multiple cells/focal staining in several large fields.

Histologic assessment of liver tissue was carried out on H&E-stained slides.

**In vivo imaging of antitumor activity.** A2780-Luc cells (2 × 10^6) were injected intraperitoneally into 60 nude mice. Five days after inoculation, the mice were randomized in groups of 10 mice per treatment arm. Treatment consisted of intraperitoneal injections with vehicle (NaCl, 5 mice, days 5 and 12, and rhTRAIL-buffer, 5 mice, days 5-10 and 12-16), cisplatin (4 mg/kg at days 5 and 12), rhTRAIL or rhTRAIL-DR5 (5 mg/kg, days 5-10 and 12-16), or the combination of cisplatin with rhTRAIL or rhTRAIL-DR5. Cisplatin in the combination therapy was administered 4 h before rhTRAIL or rhTRAIL-DR5 injections. We determined the maximum tolerated dose of cisplatin (4 mg/kg intraperitoneally, weekly × 2; ref. 23) in a pilot study based on maximum 15% weight loss in tumor-bearing mice. In rat, a single intraperitoneal dose of 4 mg/kg resulted in a total platinum peak concentration of ~10 μmol/L in plasma and ~100 μmol/L in the peritoneal cavity (23), whereas a peak serum concentration of ~50 μmol/L was reached in patients at the maximum tolerated dose (intravenous dose 100 mg/m²; ref. 24). Mice were monitored daily for general condition and weight. BLI was done at 2- to 3-day intervals. When the signal reached a value ≥3.1 × 10^8 photons/s/cm²/sr, mice were sacrificed. Tumor and liver tissue samples were excised for histologic assessment.

**Statistical analysis.** In vitro data and biodistribution results were assessed for differences with unpaired two-tailed Student's *t* test or χ² analysis. Results from the in vivo efficacy study, with bioluminescence signals depicted in radiance (photons/s/cm²/sr), are represented as

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**Fig. 1.** A, levels of TRAIL receptor membrane expression in A2780 before and after exposure to 2.5 μmol/L determined by flow cytometry analysis. Cells were incubated with cisplatin for 4 h and cultured in normal medium for an additional 20 h. TRAIL receptor expression is expressed as the fluorescence intensity (phycocerythrin). B, survival of A2780 assessed with a cytotoxicity assay after 96 h exposure to 0 to 100 ng/mL rhTRAIL and rhTRAIL-DR5, *P = 0.008. C, survival of A2780 determined with a cytotoxicity assay. Cells were incubated for 4 h with 2.5 μmol/L cisplatin, after which the cells were washed, cultured in medium for 20 h, and exposed to 0 to 25 ng/mL rhTRAIL or rhTRAIL-DR5 for an additional 72 h, *P < 0.01, rhTRAIL versus cisplatin and rhTRAIL, *P < 0.01, rhTRAIL-DR5 versus cisplatin and rhTRAIL-DR5, *P < 0.001, cisplatin and rhTRAIL versus cisplatin and rhTRAIL-DR5. Mean ± SD of at least three independent experiments.
mean ± SE. Percent signal reduction compared with vehicle-treated mice at the end of treatment (day 16) was calculated according to the formula: 100 - (signal intensity at day 16) / (mean signal intensity vehicle group at day 16) × 100. One-way ANOVA was done to determine differences in signal intensity between groups and between differences in percent signal reduction; significant differences were subjected to post hoc analysis with Tamhane's T2 and Dunnett’s T3 tests assuming unequal variances. Survival (days) was estimated by 

Fig. 2. A, induction of apoptosis after exposure to 50 ng/mL rhTRAIL or rhTRAIL-DR5 alone or after preincubation with 2.5 μmol/L cisplatin for 4 and 20 h before rhTRAIL or rhTRAIL-DR5 administration. Caspase activation was determined with a caspase-3 activity assay after 1, 3, and 5 h treatment. B, cells were preincubated with medium and 2.5, 10, and 30 μmol/L cisplatin for 4 h and washed. After 20 h, the cells were exposed for 4 h to 100 or 250 ng/mL rhTRAIL or rhTRAIL-DR5, and apoptosis was assessed by means of acridine orange staining. For clarity, only significance between apoptosis levels in cells treated with 30 μmol/L cisplatin in combination with rhTRAIL-DR5 versus 30 μmol/L cisplatin in combination with rhTRAIL was indicated. Apoptosis levels were also significantly higher for rhTRAIL-DR5 versus rhTRAIL (P < 0.001) for 100 and 250 ng/mL. Apoptosis levels were also significantly higher (P < 0.01) for 2.5 and 10 μmol/L cisplatin in combination with rhTRAIL-DR5 versus 2.5 and 10 μmol/L cisplatin in combination with rhTRAIL. C, DR5 membrane expression in A2780 cells transfected with siRNA against luciferase (Luc siRNA) or siRNA against DR5 (DR5 siRNA). Cells were preincubated with cisplatin for 4 h, washed, and cultured in medium for 20 h and DR5 membrane expression was determined with flow cytometry. Mean fluorescence intensities were corrected for staining with a nonspecific isotype control. D, DR5 cellular protein expression in A2780 cells transfected with siRNA against luciferase or siRNA against DR5 (DR5 siRNA). Cells were preincubated with cisplatin for 4 h, washed, and cultured in medium for 20 h and DR5 protein expression was determined with Western blotting using actin as a control for protein loading. E, A2780 cells transfected with siRNA against luciferase (Luc siRNA) or siRNA against DR5 (DR5 siRNA) were preincubated with medium or 30 μmol/L cisplatin for 4 h and then washed. After 20 h, the cells were exposed for 4 h to 250 ng/mL rhTRAIL or rhTRAIL-DR5, and apoptosis was assessed by means of acridine orange staining. Mean ± SD of three independent experiments.
Kaplan-Meier analysis and compared with log-rank tests. $P < 0.05$ was considered significant. Statistical analyses were generated using GraphPad Prism software version 4.0 (GraphPad software) and SPSS 14.0 for Windows (SPSS).

### Results

**In vitro activity of rhTRAIL-DR5, rhTRAIL, and cisplatin on A2780.** A2780 cells express DR5 and low DcR2 cell surface levels, whereas DR4 and DcR1 are undetectable (Fig. 1A). Long-term exposure (96 h) of A2780 cells to relatively low concentrations of rhTRAIL and rhTRAIL-DR5 induced a loss of viability, reaching a maximum effect between 25 and 100 ng/mL (Fig. 1B). rhTRAIL-DR5 was more effective than rhTRAIL at the highest ligand concentration. Short treatment with cisplatin for 4 h resulted in a clearly detectable increase in DR5 and DcR2 cell surface expression after 24 h without substantially affecting cell growth in a survival assay (Fig. 1A and C). Therefore, we preincubated cells with cisplatin (2.5 μmol/L) for 4 h and cultured the cells for an additionally 20 h, allowing enhanced DR5 cell surface expression. Cells were continuously treated with rhTRAIL or rhTRAIL-DR5 for an additional 72 h (Fig. 1C). rhTRAIL-DR5 decreased cell survival more effectively than rhTRAIL both as a single agent ($P < 0.01$) and in combination with cisplatin ($P < 0.001$). Cisplatin sensitized A2780 cells to rhTRAIL-DR5- or rhTRAIL-induced cell kill with enhancement ratios of 1.6 and 1, respectively.

Taking into account the short half-life of rhTRAIL in mice and human (17), we assessed apoptosis using a caspase-3 activity assay in A2780 cells following short exposure to rhTRAIL and rhTRAIL-DR5. Caspase activity was enhanced within 1 h after exposure of the cells to rhTRAIL or TRAIL-DR5 (Fig. 2A). Preincubation with 2.5 μmol/L cisplatin for 4 h further enhanced caspase-3 activation. Cisplatin followed by rhTRAIL-DR5 was more effective than cisplatin followed by rhTRAIL after 1 h ($P < 0.003$). The fast activation of caspase-3, detectable within 1 h after start of treatment with rhTRAIL or TRAIL-DR5, probably resulted in an underestimation of caspase-3 activation at the last time point (5 h) due to degradation of active caspase-3 in late apoptotic cells. With an acridine orange assay detecting late apoptotic cells, we showed that rhTRAIL-DR5 was more effective than rhTRAIL following pretreatment with cisplatin for 4 h with clinically relevant concentrations up to 30 μmol/L (Fig. 2B; ref. 24). Cisplatin (30 μmol/L) combined with rhTRAIL or rhTRAIL-DR5 gave enhancement ratios of 8.2 and 6, respectively.

The involvement of the DR5 in inducing apoptosis in these cells was further assessed in cells in which the DR5 had been knocked down by siRNA. A strong cisplatin concentration-dependent increase in DR5 surface expression, as well as DR5 cellular protein expression, was observed in the luciferase siRNA-treated cells, whereas DR5 siRNA resulted in complete down-regulation of DR5 in the presence of cisplatin up to 30 μmol/L (Fig. 2C and D). In addition, DR5 siRNA completely protected A2780 cells against TRAIL- and TRAIL-DR5-induced apoptosis also in the presence of cisplatin (Fig. 2E). The apoptosis assays for rhTRAIL and rhTRAIL-DR5 were also done with coincubation of a DcR2 blocking antibody, which had no effect on the apoptosis levels (Supplementary Figure).

These data show that the DR5 pathway is important for rhTRAIL- and rhTRAIL-DR5-induced apoptosis, which is further activated by cisplatin in ovarian cancer cells.

**$^{125}\text{I}-\text{rhTRAIL} \text{ biodistribution in tumor-bearing mice.}** Tissue biodistribution and tumor uptake of intravenously (Supplementary Table S1A) and intraperitoneally (Supplementary Table S1B) administered $^{125}\text{I}-\text{rhTRAIL}$ were compared in nude mice with intraperitoneal A2780-Luc xenografts. The administration route influenced the disposition of $^{125}\text{I}-\text{rhTRAIL}$. Blood activity (％ID/g) was higher at 15 min (43.29 ± 11.04 versus 25.30 ± 5.04) and 30 min (30.51 ± 12.40 versus 15.33 ± 3.78) after intravenous versus intraperitoneal injection, whereas it was lower at 90 min (7.52 ± 1.22 versus 23.74 ± 6.85) and 360 min (2.63 ± 0.56 versus 8.26 ± 1.74). The blood kinetics of $^{125}\text{I}-\text{rhTRAIL}$ in blood could be described by a two-compartment model. The resulting blood activity versus time profiles (Fig. 3A) showed a higher area under the time curve...
After intraperitoneal administration than after intravenous administration. After intraperitoneal injection, the peak blood activity is lower than after intravenous injection but remains higher for a longer period. Kidney uptake (%ID/g) showed the same pattern as blood pool activity, with higher activity after intravenous versus intraperitoneal administration at 15 min (199.2 ± 40.69 versus 19.83 ± 1.38) and 30 min (126.6 ± 49.68 versus 21.45 ± 1.90) and lower activity at 90 min (12.73 ± 2.47 versus 17.87 ± 1.28) and 360 min (2.06 ± 0.56 versus 4.45 ± 0.31). Activity in well-perfused organs such as lung, liver, and spleen displayed similar kinetics as the blood pool activity in both administration routes. Stomach activity increased over time, which can be attributed to in vivo dehalogenation. Intraperitoneal administration resulted in high tumor activity at 15 min (11.31 ± 1.51) and 60 min (12.91 ± 3.29) with a gradual decrease to 360 min, whereas after intravenous administration tumor activity remained largely unchanged up to 60 min (6.85 ± 1.29) and then gradually decreased to 360 min. At all time points, tumor uptake (%ID/g) was higher after intraperitoneal administration versus intravenous administration but only reached significance at 90 min. The tumor-to-blood ratios were higher after intraperitoneal versus intravenous administration at 15 min (0.48 ± 0.03 versus 0.13 ± 0.02) and 60 min (0.55 ± 0.06 versus 0.38 ± 0.04). Tumor-to-blood ratios remained constant over time after intraperitoneal injection, whereas tumor-to-blood ratios after intravenous administration gradually increased to ratios observed with intraperitoneal administration (Fig. 3B). These results indicate that intraperitoneal administration of rhTRAIL may have advantages compared with intravenous administration in this intraperitoneally growing tumor model.

Assessment of caspase-3 activity in tumors. To determine whether intraperitoneal and/or intravenous administration resulted in 125I-rhTRAIL-induced cleavage of procaspase-3 into active caspase-3, paraffin-embedded tumor tissues obtained at 15, 30, 60, 90, and 360 min after 125I-rhTRAIL injection were stained for active caspase-3. Whereas almost no active caspase-3 was detected in samples obtained at 15 min, tumors obtained between 30 and 360 min showed low but clearly visible active caspase-3-positive tumor cells following either intraperitoneal or intravenous administration of 125I-rhTRAIL. Focal staining as well as scattered staining of tumor cells often just below the tumor surface was observed following intraperitoneal administration (Fig. 4A), whereas focal staining or scattered staining throughout the tumor was observed following intravenous administration. Active caspase-3 staining was found near blood vessels in two tumors from the intravenous group (Fig. 4B). Semiquantitative analyses of the tumors, taking together the staining in tumors from sacrificed mice between 30 and 360 min after administration of 125I-rhTRAIL, revealed that scattered staining of multiple cells or focal staining in several fields was more often observed in tumors following intraperitoneal than intravenous administration (7 of 15 evaluable tumors in the intraperitoneal group versus 1 of 16 evaluable tumors in the intravenous group; \( P = 0.01 \)). The low levels of active caspase-3 in both intraperitoneal and intravenous groups were probably due to the relatively low concentration of 125I-rhTRAIL (0.5 μg/mice) used in the biodistribution study.

In vivo efficacy of rhTRAIL, rhTRAIL-DR5, and cisplatin on intraperitoneal xenografts. The response of intraperitoneal A2780-Luc xenografts to treatment with rhTRAIL, rhTRAIL-DR5, and cisplatin or a combination of either rhTRAIL or rhTRAIL-DR5 with cisplatin was assessed by BLI. Tumor regression was not visible within the first 48 h after treatment initiation at day 5 but was clearly evident at the end of the first treatment period (day 9), with the largest signal reduction seen after combination of rhTRAIL or rhTRAIL-DR5 with cisplatin (Fig. 5A). Signals rose in the days between both treatments. All treatment groups, except the rhTRAIL-treated arm, had significantly smaller tumors at day 16 than the vehicle-treated group. This is reflected in the mean signal reduction as to vehicle-treated mice, whereas rhTRAIL alone did not result in a significant decrease (48.8%; range, 32.8-64.6%; \( P = 0.097 \)); rhTRAIL-DR5 and cisplatin gave a reduction of 68.3% (range, 61.8-74.8%; \( P = 0.015 \)) and 72.3% (range, 59.8-84.9%; \( P = 0.009 \)), respectively. Combination therapies were highly effective; rhTRAIL plus cisplatin caused a decline in signal intensity of 84.8% (range, 73.5-96.1; \( P = 0.003 \)) and
Fig. 5. Visualization of response to rhTRAIL, rhTRAIL-DR5, cisplatin, and the combination of either ligand with cisplatin by means of BLI. Nude mice were inoculated intraperitoneally with $2 \times 10^6$ A2780-Luc cells. After 5 days, treatment was initiated; cisplatin (4 mg/kg intraperitoneally) or vehicle at days 5 and 12, rhTRAIL and rhTRAIL-DR5 (5 mg/kg intraperitoneally) or vehicle at days 5 to 10 and 12 to 16, or a combination of rhTRAIL or rhTRAIL-DR5 with cisplatin. CP, cisplatin; TR, rhTRAIL/rhTRAIL-DR5. A, change in light emission (in radiance units) over time per treatment arm. Bioluminescent signals at each time point were averaged per treatment group. Mean ± SE. Significant differences in signal intensities at day 16 were observed between vehicle ($4.6 \times 10^8 \pm 6.7 \times 10^7$) vs. rhTRAIL ($2.3 \times 10^8 \pm 3.1 \times 10^7$) $P = 0.007$, vehicle vs. rhTRAIL-DR5 ($1.4 \times 10^8 \pm 1.3 \times 10^7$) $P = 0.015$, vehicle vs. cisplatin ($1.3 \times 10^8 \pm 2.4 \times 10^7$) $P = 0.009$, vehicle vs. cisplatin and rhTRAIL ($6.7 \times 10^7 \pm 2.1 \times 10^7$) $P = 0.003$, and vehicle vs. cisplatin and rhTRAIL-DR5 ($1.6 \times 10^7 \pm 4.6 \times 10^7$) $P = 0.002$. B, bioluminescent images at the end of treatment (day 16) of each 4 mice representative for 10 mice per experimental arm. Images are displayed and quantified in log radiance (photons/s/cm²/sr). C, Kaplan-Meier survival analysis of all mice. A bioluminescent signal $1 \times 10^9$ was used as surrogate endpoint for survival as described in Materials and Methods.
rhTRAIL-DR5 plus cisplatin resulted in 96.5% (range, 93.7-99.4%; $P = 0.002$) signal reduction. The decline in signal intensity after rhTRAIL-DR5 plus cisplatin was higher than the mean light reduction after cisplatin alone ($P = 0.027$). Thus, all therapies, except rhTRAIL monotherapy, exhibited significant antitumor activity at the end of treatment, with the combination therapies displaying the highest activity.

In general, light intensity at the end of treatment was inversely associated with survival (Fig. 5B). Animals were sacrificed when a bioluminescent signal $\geq 3.1 \times 10^8$ photons/s/cm$^2$/sr was reached as a surrogate marker for survival. The median survival of the vehicle controls was 16 days, with no mice surviving after 18 days (Fig. 5C). Monotherapy with rhTRAIL and rhTRAIL-DR5 prolonged median survival to 21 days ($P < 0.001$) and with cisplatin to 28 days ($P < 0.0001$). rhTRAIL-DR5 in combination with cisplatin resulted in a median survival of 31 days ($P < 0.0001$), and rhTRAIL plus cisplatin resulted in a median survival of 32.5 days ($P < 0.0001$). The latter was also significant compared with cisplatin monotherapy ($P = 0.038$). Liver histology at sacrifice did not show any signs of liver damage.

**Discussion**

In this study, we show that rhTRAIL-DR5, a rhTRAIL variant designed to specifically bind DR5, induced higher levels of apoptosis and growth inhibition in ovarian cancer cells than rhTRAIL. Pretreatment with cisplatin strongly enhanced apoptosis and cytotoxicity induced by rhTRAIL-DR5 or rhTRAIL, with the combination of cisplatin and rhTRAIL-DR5 being most effective. Intraperitoneal administration of these drugs in an orthotopic bioluminescent mouse model of human ovarian peritonitis carcinomatosis delayed tumor growth, with superior efficacy of cisplatin combined with rhTRAIL-DR5 over cisplatin alone.

Whereas rhTRAIL can bind to DR4 and DR5, agents that specifically target one death receptor are in various stages of development. Using receptor-selective rhTRAIL variants, we and others have recently defined that cancer cells can display a preference for either DR4 or DR5 for apoptosis signaling, resulting in enhanced apoptosis when the dominant receptor is targeted. Colon and breast cancer cell lines were reported to signal primarily through DR5 (10, 11), whereas primary lymphoid malignancies do so through DR4 (25, 26). Interestingly, DR5-selective TRAIL variants, including rhTRAIL-DR5, caused higher levels of apoptosis than wild-type rhTRAIL in DR5-expressing Jurkat and A2780 cells as well as in BJAB cells when DR5 was reexpressed (10, 11). In the present study, we used the rhTRAIL variant that contains two amino acid mutations, D269H and E195R, with high-affinity binding for DR5 and almost no affinity for DR4 (11). We showed that pretreatment of A2780 cells with cisplatin augmented rhTRAIL-DR5- or rhTRAIL-induced cytotoxicity and apoptosis, with the highest efficacy of cisplatin combined with rhTRAIL-DR5. Combinations of anticancer agents with receptor targeted drugs are often more effective than the single agents in preclinical models. Death receptor up-regulation induced by chemotherapeutic drugs or irradiation in a p53-dependent (27–29) or p53-independent (30) matter is regarded as one of the mechanisms contributing to enhanced effects of combinatorial regimens. In our model, cisplatin induced DR5 and DcR2 up-regulation. Drug-induced DR5 up-regulation is more frequently reported than DR4 up-regulation (22, 27, 31). Several other studies have reported the important role of DR5 in ovarian cancer (12, 13). Moreover, in ovarian cancer specimens obtained before and after cisplatin treatment, DR5 expression increased from 37% to 74% after chemotherapy, whereas DR4 staining remained unaltered (32). This might imply that combinatorial strategies with DR5 targeted agents are more effective than combinational regimes with DR4 targeted drugs in ovarian cancer.

Additionally, agents binding only one receptor will cause exclusively the formation of DR4 or DR5 homotrimers, whereas TRAIL binding may cause homotrimers and heterotrimer formation. Whether this affects apoptosis induction is not well established. Immunoprecipitation of death-inducing signaling complexes after treatment with TRAIL showed fewer heterotrimeric than homotrimeric complexes, which might indicate that homotrimers are favored over heterotrimers (33). Moreover, targeting a single receptor may induce enhanced apoptosis due to a lack of competition with decoy receptors (34, 35). A2780 cells express DR5 and low levels of DcR2. Blocking antibodies against DcR2 did not enhance rhTRAIL-induced apoptosis, indicating that another mechanism is responsible for the superior efficacy of rhTRAIL-DR5 over rhTRAIL. Increased binding capacities for DR5 of the mutant over rhTRAIL might be involved (11), although the exact kinetics of receptor binding of rhTRAIL-DR5 need to be established in further detail. Other TRAIL mutants showed that increased affinity for the targeted receptor might play a role (10). Alternatively, a recent study showed that the extracellular domains of DR4, but not of DR5, can interact with the extracellular domain of DcR1 and DcR2 (36). The absence of DR4 in A2780 may then explain why DcR2 antibodies had no effect on rhTRAIL in these cells. This indicates that, in cell lines positive for DR4 and DR5 surface expression, the differences in apoptosis induction between rhTRAIL and rhTRAIL-DR5 might be larger. Loss of affinity for decoy receptors may result in toxicity of rhTRAIL-DR5. However, a clear correlation between decoy receptor expression and resistance to rhTRAIL in normal cells has not been established (37) and monoclonal antibodies devoid of binding capacity to decoy receptors can be safely administered in clinical trials.

The rationale behind intraperitoneal drug administration is to increase local drug exposure while lowering plasma clearance (38). We show that intraperitoneal rhTRAIL administration resulted in a higher area under the curve and a reduced clearance. The high kidney activity confirms the function of the kidney as main site of rhTRAIL clearance, which is not influenced by intraperitoneal administration. Activity in most organs followed that of blood pool activity, suggesting that distribution to normal tissues was limited, which corresponds to previous studies (39). Our results indicate that intraperitoneal administration of rhTRAIL may result in favorable tumor uptake in intraperitoneally growing tumors. Moreover, we found active caspase-3 staining after intraperitoneal injection at this nontherapeutic dose of rhTRAIL. Whether this is caused by local rhTRAIL penetration into the tumor, a limiting factor for intraperitoneal administration of antibodies (40), needs to be established. To further investigate the efficacy of intravenous versus intraperitoneal administration on intraperitoneally growing tumors, therapeutic doses of rhTRAIL or rhTRAIL variants have to be used and related to tumor effects.
responses. Additionally, those experiments should focus on the relation between caspase-3 activation and tumor responses and the molecular characteristics of the surviving tumor cells.

In the present study, we used BLI to assess tumor response, because no reliable methods based on clinical features exist to accurately evaluate intraperitoneal tumor proliferation over time. The BLI data clearly visualized the differences in response to the applied treatments. Furthermore, we used bioluminescence to define an endpoint for survival. As survival of mice with intraperitoneal xenografts is mostly based on assessment of clinical condition, our method ensured the definition of a uniform and objective early endpoint. The response to treatment in each arm reflected the in vitro results with high accuracy. At the end of treatment, tumor burden in mice treated with low-dose cisplatin together with rhTRAIL-DR5 was lower than after cisplatin alone. These results were associated with a survival advantage, although this advantage was limited. This may be due to the cell line model we used, which is not extremely sensitive to cisplatin combined with ligands in vitro and which is growing extremely rapidly in vivo. Our study comprising two cycles of therapy was, however, not designed to primarily assess survival but to show a proof of concept. The cisplatin dose used in mice is comparable with clinically achievable doses (24), but no more cycles could be given to these mice due to toxic side effects. Further studies in mice and finally in patients are warranted to define optimal dosage schedules for maximal survival benefit.

In summary, our data indicate that a receptor selective variant of rhTRAIL, rhTRAIL-DR5, displays better antitumor efficacy than rhTRAIL. The combination of rhTRAIL-DR5 together with cisplatin might offer a new strategy for more effective ovarian cancer treatment.

Disclosure of Potential Conflicts of Interest

W.J. Quax, A. Samali, founders/directors, Triskel Therapeutics.

References

Enhanced Antitumor Efficacy of a DR5-Specific TRAIL Variant over Recombinant Human TRAIL in a Bioluminescent Ovarian Cancer Xenograft Model
