Novel in vivo imaging shows up-regulation of death receptors by paclitaxel and correlates with enhanced antitumor effects of receptor agonist antibodies

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Abstract

Susceptibility to apoptosis by tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is mediated through cognate death receptor signaling. We hypothesized that auto-amplification of this apparatus would enhance antitumor effects in vivo and could be optimized using the results obtained from novel imaging techniques. We therefore imaged mice bearing human colorectal cancer (Colo205) tumor xenografts with HGS-ETR1 and HGS-ETR2 agonist antibodies to TRAIL receptor-1 (TRAIL-R1) and TRAIL-R2, respectively, after radiolabeling the antibodies. Paclitaxel significantly increased in vivo expression of TRAIL-R1 and TRAIL-R2 in a time-dependent manner. The imaging results were confirmed by immunoblots for steady-state protein levels (>20-fold increase in TRAIL-R1 and TRAIL-R2 levels in tumor xenografts by 48 h after paclitaxel administration). TRAIL-R1 and TRAIL-R2 mRNA expression did not change, suggesting that these effects were posttranscriptional. Sequential treatment with paclitaxel followed by HGS-ETR1 or HGS-ETR2 after 48 h resulted in markedly enhanced antitumor activity against Colo205 mouse xenografts. Our experiments suggest that sequential taxane treatment followed by TRAIL-R agonist antibodies could be applied in the clinic, and that novel imaging techniques using radiolabeled receptor antibodies may be exploitable to optimize sequence timing and patient selection. [Mol Cancer Ther 2006;5(12):2991–3000]

Introduction

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), also known as Apo2L, is a potent promoter of programmed cell death in diverse tumor types (1). TRAIL binds to a family of receptors, including death receptors 4 and 5 [TRAIL receptor-1 and -2 (TRAIL-R1 and TRAIL-R2)]. Ligand/receptor interaction leads to apoptosis via a conserved cytoplasmic death-signaling module. The pronounced antitumor activity of the death receptors and their ligand has raised significant interest in these molecules for the treatment of cancer patients. Recently, clinical trials have been initiated with a recombinant TRAIL and with HGS-ETR1 (mapatumumab) and HGS-ETR2 (lexatumumab) and TRAIL-R1– and TRAIL-R2–specific agonist monoclonal antibodies (mAb) that mimic the activity of native TRAIL (2–5). Clinical experience suggests that combinations of anticancer agents are often markedly more potent than single agents. This is not surprising because most cancer cells have numerous aberrant survival pathways, and it is unlikely that any signal agent will completely eradicate advanced tumors. However, the myriad of agents available makes it imperative that combinations be based on a solid biological rationale for optimization of antitumor effects. Drugs, such as taxanes, are often used in combination chemotherapy. These compounds include paclitaxel and docetaxel and are among the most commonly given and most potent agents for a variety of malignancies. Although they have several mechanisms of cytotoxic action, the primary one involves direct binding to β-tubulin and inhibition of microtubule depolymerization, the latter being a requirement for mitosis and cell proliferation (6).

In the current study, we show that the antitumor activity of the TRAIL-R agonist antibodies HGS-ETR1 and HGS-ETR2 against human colorectal cancer xenografts can be remarkably enhanced by prior administration of paclitaxel. The mechanism by which this occurs seems to be due to a striking, time-dependent up-regulation of TRAIL-R1 and TRAIL-R2, as shown by in vivo tumor imaging using radiolabeled HGS-ETR1 and HGS-ETR2 and by analysis of steady-state protein levels from the colorectal tumor xenografts. These results have important clinical implications in that they suggest that pretreatment with taxanes could substantially augment the antitumor activity of TRAIL/TRAIL-R machinery–derived molecules, and that new imaging techniques may be exploitable to gain important data on agent uptake, tumor pharmacokinetics, and optimal timing of these sequential anticancer compounds in patients.
Materials and Methods

Chemicals and Analysis
Sulfo-N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl were purchased from Pierce (Radford, IL). All other chemicals were purchased from Aldrich (Milwaukee, WI). 99mTc-pertechnetate was obtained from a commercial 99mTc generator (Mallinckrodt Diagnostics, Houston, TX), and 111In was purchased from DuPont NEN (Boston, MA).

Cell Lines
Colo205 and PA-1 were purchased from the American Type Culture Collection (Rockville, MD). PA-1 is a human ovarian cancer line, which is maintained in MEM with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Woodland, CA). Colo205 is a human colorectal tumor cell line maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum.

Antibodies
HGS-ETR1 (mapatumumab) and HGS-ETR2 (lexatumumab) are agonistic human mAbs specific for TRAIL-R1 or TRAIL-R2 (Human Genome Science, Inc., Rockville, MD; refs. 2–5). Isotype mAb was used as a control (Human Genome Science). Anti–TRAIL-R1 or rabbit polyclonal anti–TRAIL-R2 and anti–actin antibody (Sigma, St. Louis, MO) was used to ensure equal protein loading. Anti–poly(ADP-ribose) polymerase antibody (anti-PARP; Calbiochem, San Diego, CA) recognizes full-length PARP and the cleaved fragment during apoptosis. Horseradish peroxidase–conjugated secondary antibody (anti– actin antibody, Santa Cruz Biotechnology, Santa Cruz, CA) was used for visualization. The antibody was labeled with technetium (99mTc) or indium (111In). 99mTc and 111In have half-lives of 6.01 h and 2.805 days, respectively. Ethylenedicysteine (EC) was prepared in a two-step manner according to a published method (7–9). Synthesis of 99mTc-EC-HGS-ETR and 111In-EC-HGS-ETR Antibodies

Imaging with Labeled TRAIL-R mAb

To determine the effect of HGS-ETR antibodies on Colo205 cell line, several concentrations (300, 600, and 1,200 ng/mL) and time point (24 h) were tested. Cells were isolated by centrifugation and lysed in radioimmunoprecipitation assay buffer. Immunoblots were done as above. Antibodies used were anti–PARP (Calbiochem).

Annexin V/Propidium Iodide and Fluorescence-Activated Cell Sorting Analysis to Determine Apoptosis
Cells were tested for apoptosis by using the Annexin V-Fluor Staining kit (Roche Diagnostics GmbH, Mannheim, Germany). To determine the effect of HGS-ETR antibodies, several concentrations (75, 150, 300, and 600 ng/mL) and time point (24 h) were tested. Cells were then incubated with 20 µL goat anti-mouse immunoglobulin FITC at dark for 30 min, washed twice, and resuspended in 500 µL of PBS. Labeled cells were quantitated by flow cytometry (Coulter Epics XL, Miami, FL).

Radiosynthesis of 99mTc-EC-HGS-ETR and 111In-EC-HGS-ETR Antibodies
The antibodies were labeled with technetium (99mTc) or indium (111In). 99mTc and 111In have half-lives of 6.0 h and 2.805 days, respectively. Ethylenedicysteine (EC) was selected as a chelator because EC-drug conjugates could be labeled with 99mTc or 111In easily and efficiently with high radiochemical purity and stability (7–9). Synthesis of EC was prepared in a two-step manner according to a
method described (10–12). EC was conjugated to HGS-ETR1 or HGS-ETR2 or isotype control mAb or bovine serum albumin (BSA) using sulfo-N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl as coupling agents. HGS-ETR1 and HGS-ETR2 and an isotype control mAb obtained from Human Genome Sciences or BSA was stirred with EC, sulfo-N-hydroxysuccinimide, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl at room temperature for 17 h. After dialysis, 2.3 to 3.4 mg of EC antibody was obtained. The Na99mTcO4 was added into a vial containing EC antibody and SnCl2 to yield 99mTc-EC-HGS-ETR1, 99mTc-EC-HGS-ETR2, 99mTc-EC-isotype control mAb, or 99mTc-EC-BSA. The procedures for radiosynthesis of 111In-labeled antibodies were the same as above, except without adding SnCl2, and 111In was substituted for 99Tc. Radiochemical purity for EC antibodies (Rf = 0.1) were >95% as determined by using radio-TLC (Bioscan, Washington, DC) eluted with saline or acetone.

Scintigraphic Imaging and Autoradiography Studies

The animal experiments were approved by the University of Texas M.D. Anderson Institutional Animal Care and Use Committee. All experiments involved four or five animals per subgroup. Six- to 8-week-old female nude mice (National Cancer Institute, Washington, DC) were inoculated i.m. into the hind legs with 0.1 mL of tumor cell suspensions (1 × 107 per mouse). Animals were divided into four subgroups (group 1 = 111In-EC-BSA, group 2 = 111In-EC-isotype control mAb, group 3 = 111In-EC-HGS-ETR1, and group 4 = 111In-EC-HGS-ETR2). The imaging studies were done 12 days after inoculation when tumor had grown to 1 cm in greatest diameter. Each group of animal was injected i.v. with 100 μCi 111In-labeled compounds as above. In other experiments, 1 mCi 99mTc-labeled compounds were used. At 2, 24, and 48 h following administration of the radiotracers, the scintigraphic images, using a γ camera (Siemens, Hoffman, IL) equipped with a medium-energy (111In) or low-energy (99mTc), parallel-hole collimator, were obtained. Animals were then treated with paclitaxel (60 mg/kg i.v.) and, 24 h later, were injected with 100 μCi 111In-labeled compounds or 1 mCi 99mTc-labeled compounds as above. Again, the scintigraphic images were obtained 2, 24, and 48 h after administration of the radiotracer. Computer-outlined regions of interest (counts per pixel) of the tumor lesion site and symmetrical normal muscle site were used to determine tumor/muscle count density ratios. The ratios were used to compare dynamic tumor uptake pretreatment and posttreatment of paclitaxel. Whole-body autoradiograms were also obtained by a quantitative analyzer (Cyclone Storage Phosphor System, Meridian, CT). Following the imaging, one animal from each group was sacrificed, and the body was fixed in carboxymethyl cellulose (4%) as described (13, 14). The frozen body was mounted on to a cryostat and cut into 100-μm coronal sections. Each section was thawed and mounted on a slide. The slide was then placed in contact with the multipurpose phosphor storage system screen and exposed for 16 h.

Immunoblots to Detect TRAIL-R, p53, and Erk1/2 Expression and Phosphorylation Level in Mouse Xenograft Tumors after Paclitaxel Treatment

Six- to 8-week-old nude female mice (National Cancer Institute) were inoculated i.m. with Colo205 cell suspensions (1 × 105 per mouse). Paclitaxel treatment (60 mg/kg i.v. in tail vein) was done 10 to 14 days after inoculation when tumor had grown to 1 cm in greatest diameter.

Figure 1. Immunoblot of steady-state level TRAIL-R and cell surface TRAIL-R. A, cell lysate were loaded in each well. Monoclonal anti–TRAIL-R1 and rabbit polyclonal anti–TRAIL-R2 were used to detect the presence of TRAIL-R1 and TRAILR2. To ensure equal protein loading, the blot was also exposed to anti–β-actin antibodies. Colo205 cells express both TRAIL-R1 and TRAIL-R2, whereas PA-1 cells have lower level of expression. B, cell surface TRAIL-R expression were measured on Colo205 and PA-1 cells using flow cytometry (black line, control IgG; green line, TRAIL-R1 antibody; red line, TRAIL-R2 antibody).

Figure 2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide assay for survival/proliferation of cancer cell lines after exposure to anti–TRAIL-R antibodies. Colo205 (A) and PA-1 (B) cells were incubated with antibodies for 24 h. Expressed as a percentage of survival. Similar experiments were done with an isotype control antibody, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide assays were then done. Concentration-dependent antiproliferative effect for HGS-ETR1 and HGS-ETR2 on Colo205 but not on PA-1 cells.
Tumors were incised 24 or 48 h after paclitaxel treatment. Tissue was diced into very small pieces using a clean razor blade and thawed in immunoprecipitation assay buffer. Further disruption and homogenization of tissue was done with a dunce homogenizer. Immunoblots were done as described previously with antibodies as appropriate: anti–TRAIL-R1, anti –TRAIL-R2, anti-p53, anti –phospho-p53 (Ser15), anti-Erk1/2, and anti–phospho-Erk1/2 (Thr 202/Tyr204).

**Real-time Reverse Transcription-PCR to Quantitate TRAIL-R Transcripts**

RNA was extracted from Colo205 tumor established in the mouse model using RNeasy Mini kit from Qiagen (Valencia, CA). Random hexamer primer was used to amplify cDNA sequence using Transcriptor First-Strand cDNA Synthesis kit from Roche Diagnostics. Transcript quantification was done with ABI 7500 Sequence Detection System using the Taqman Universal PCR Master Mix according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The relative efficiency of amplification of each cDNA sample (TRAIL-R1 and TRAIL-R2) was monitored in parallel, separate wells by analysis of glyceraldehyde-3-phosphate dehydrogenase cDNA using primer for TRAIL-R1 (Hs00269492_m1), TRAIL-R2 (Hs00366272_m1), and glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1) purchased from Applied Biosystems.

**In vivo Evaluation of the Antitumor Effect of Antibody to TRAIL-R after Paclitaxel Treatment**

Six- to 8-week-old nude female mice (National Cancer Institute) were injected with Colo205 cell suspensions (5 × 10⁶ per mouse) at the right flank area ~ 1 cm cranial to the hind limb (n = 5 per group). Body weight and tumor growth were monitored thrice per week. Palpable tumors were measured using a vernier caliper. Tumor volume (mm³) was calculated using the formula: volume = 0.5 (L × W²). When the tumors grew to 8 to 10 mm in average diameter, the mice were randomized according to tumor size (small, medium, and large) into treatment groups. Mice were treated for 3 weeks i.v. (tail vein) injection with either single-agent treatment or paclitaxel followed 2 days later by antibodies HGS-ETR1 or HGS-ETR2 or isotype control mAb. Animal studies were conducted in the veterinary facilities of the M.D. Anderson Cancer Center in accordance with institutional guidelines.

**Statistical Analysis**

All statistical analyses are done by our biostatistician L.B. (see author list). The change in tumor volume from baseline

![Image](https://example.com/image.png)

**Figure 3.** Apoptosis as determined by fluorescence-activated cell sorting analysis of Annexin V/propidium iodide study. Cells were cultured for 24 h with increasing concentration of isotype control and HGS-ETR1 and HGS-ETR2 antibodies. Apoptosis was quantified by the Annexin V-Fluos stain followed by fluorescence-activated cell sorting analysis. Expressed as the percentage of apoptotic cells. A and C, Annexin V/propidium iodide test for apoptosis of Colo205 and PA-1 cells. B and D, corresponding fluorescence-activated cell sorting analysis results. HGS-ETR1 and HGS-ETR2 induce apoptosis of Colo205 but not PA-1 cells.

![Image](https://example.com/image.png)

**Figure 4.** Anti–TRAIL-R antibodies induce apoptosis as shown by PARP cleavage. Colo205 cells incubated with HGS-ETR1, HGS-ETR2, or isotype control antibody for 24 h. Concentration-dependent PARP cleavage was shown in HGS-ETR1– and HGS-ETR2–treated samples but not in isotype control antibody–treated samples.
across time was computed. Because the baseline tumor volumes were significantly different, a one-way analysis of covariance was done to test for differences in the average change in tumor volume between the five groups, adjusting for the baseline tumor volume. In addition, a Lowess plot was produced to graph the change in tumor volume (relative to background) for the five groups.

Results

TRAIL-R Are Expressed in Colo205 Cells and to a Lesser Extent in PA-1 Cells

Steady-state protein levels of TRAIL-R1 and TRAIL-R2 in Colo205 and PA-1 cell lines were studied by Western blot analysis (Fig. 1A). Colo205 cancer cells expressed higher levels of TRAIL-R1 and TRAIL-R2 than PA-1 cells. Cell surface TRAIL-R expressions were also measured on Colo205 and PA-1 cells using flow cytometry (Fig. 1B). Colo205 cells express TRAIL-R twice as high as PA-1 cells. TRAIL-R2 is expressed more than TRAIL-R1 in both of cell lines.

TRAIL-R Antibody Induced Growth Inhibition in Colo205 Cells, but not in PA-1 Cells

Sensitivity of Colo205 and PA-1 cell lines to the growth inhibition of HGS-ETR1 and HGS-ETR2 for 24 h was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay (Fig. 2). IC_{50} of Colo205 was 800 ng/mL for HGS-ETR1 and 150 ng/mL for HGS-ETR2. PA-1 cells were resistant to HGS-ETR1 and HGS-ETR2 with no detectable growth inhibition at concentration as high as 800 ng/mL. These results are consistent with the immunoblot analysis, which showed higher levels of TRAIL-R1 and TRAIL-R2 in Colo205 compared with PA-1 cells (Fig. 1). Because not all cell lines that express TRAIL-R1 and TRAIL-R2 are sensitive to their growth suppressive effects (15), these results are consistent with the notion that the presence of TRAIL-R in some cell lines are necessary but not sufficient for growth suppression by HGS-ETR1 and HGS-ETR2.

TRAIL-R Antibodies Induce Apoptosis in Colo205 Cells

As shown by Annexin V/propidium iodide stain of fluorescence-activated cell sorting analysis in Fig. 3, HGS-ETR1 induced 25% of Colo205 cells to undergo apoptosis after exposure to concentrations of 300 ng/mL for 24 h, whereas HGS-ETR2 induced ~60% of cells to undergo apoptosis at the same concentrations. To confirm the presence of programmed cell death in HGS-ETR1- and HGS-ETR2–treated Colo205 cells, we analyzed the cleavage of PARP (Fig. 4), a major substrate of the effector caspase-3 during apoptosis (16). As shown in Fig. 4, in Colo205 cells, HGS-ETR1 and HGS-ETR2 caused PARP cleavage after 24 h of treatment.

Tumor Uptake of 111In-Labeled TRAIL-R Antibodies in Colo205 Mouse Xenograft Model Is Optimal at 24 to 48 h

We used radiotracer labeling of HGS-ETR1 and HGS-ETR2 antibodies to determine in vivo tumor uptake. As shown in Figs. 5 and 6, optimal tumor uptake of 111In-EC-HGS-ETR1 and 111In-EC-HGS-ETR2 occurred at 24 to 48 h after injection of 111In-labeled compounds. At 2 h, uptake of 111In-EC-HGS-ETR1 or 111In-EC-HGS-ETR2 did not differ from that of control (111In-EC-BSA and 111In-EC-isotype control mAb; Fig. 6). These results were similar to those with the use of 99mTc-labeled compounds (data not shown).

Paclitaxel Enhances Tumor Uptake of 99mTc-EC–Labeled TRAIL-R Antibodies in the Colo205, but not in PA-1 Mouse Xenograft Model

Computer-outlined region of interest showed that the tumor/muscle ratios of HGS-ETR1 and HGS-ETR2 uptake before and after paclitaxel treatment were not significantly different in PA-1 cells (Fig. 7). This is not surprising in light of the relative low level of expression of TRAIL-R in PA-1 cells as shown by Western blots (Fig. 1). In contrast, paclitaxel increased uptake of 99mTc-EC–labeled HGS-ETR1 and HGS-ETR2 antibodies in Colo205 tumor tissue 24 h after paclitaxel treatment (Fig. 8). Scintigraphic and autoradiography studies of 111In-EC-HGS-ETR1 and 111In-EC-HGS-ETR2 also showed good tumor uptake of radiolabeled compounds in vivo for Colo205 cells (Fig. 9).

Paclitaxel Increases Expression of TRAIL-R, Phospho-Erk1/2, and Phospho-p53 Level In vivo

Steady-state protein levels of TRAIL-R1 and TRAIL-R2 in Colo205 tumors mouse model were evaluated by Western

![Figure 5.](image-url) Tumor/muscle count density ratios of 111In-labeled compounds in Colo205 mouse xenograft model. Colo205 tumor-bearing nude mice were injected with 100 µCi 111In-EC-HGS-ETR1 and 111In-EC-HGS-ETR2, respectively. Images were obtained 1, 1.5, 2, 24, and 48 h after injection of 111In-labeled antibodies. Binding of TRAIL-R1 or TRAIL-R2 progressively increased with time up to 24 to 48 h.

![Figure 6.](image-url) Tumor/muscle count density ratios of 111In-labeled compounds in Colo205 mouse xenograft model. Colo205 tumor-bearing nude mice were injected with 100 µCi 111In-EC-HGS-ETR1 or 111In-EC-HGS-ETR2, respectively. Images were obtained 2, 24, and 48 h after injection of 111In-labeled antibody. Binding of HGS-ETR1 or HGS-ETR2 does not differ from that of controls at 2 h but is significantly increased at 24 and 48 h.
activity of HGS-ETR1 system, Applied Biosystems; data not shown). 

Transcripts remained unchanged as determined by quantification of protein level; expression of TRAIL-R1 and TRAIL-R2 phospho-p53 and phospho-Erk1/2 as it occurred at the paclitaxel is unlikely to be due to the increased levels of tumor uptake after paclitaxel treatment (Figs. 8 and 9).

The status of phospho-Erk1/2 and phospho-p53 were evaluated, and both were also up-regulated (Fig. 11). Paclitaxel had no effect on tumor uptake antibodies. These results suggest that TRAIL-R antibodies did not bind to PA-1 tumor xenograft and is consistent with the less of TRAIL-R1 and TRAIL-R2 expression in PA-1 as shown by Western blot.

Figure 7. Tumor/muscle count density ratios of 99mTc-EC–labeled compounds before and after paclitaxel treatment in PA-1 mouse xenograft model. Columns, mean tumor/muscle count density ratios of 99mTc-EC–HGS-ETR1, 99mTc-EC HGS-ETR2, and 99mTc-EC-BSA in PA-1-bearing nude mice (n = 3) before and after treatment with paclitaxel (60 mg/kg i.v.); bars, SE. Imaging was prepared 2 and 24 h after injection of radiolabeled compound. Paclitaxel had no effect on tumor uptake antibodies. These results suggest that TRAIL-R antibodies did not bind to PA-1 tumor xenograft and is consistent with the less of TRAIL-R1 and TRAIL-R2 expression in PA-1 as shown by Western blot.

Blot analysis (Fig. 10). Results showed that 24 and 48 h after the paclitaxel, there is a marked up-regulation of TRAIL-R1 and TRAIL-R2 in Colo205 cells at the protein level. These results are consistent with the imaging studies that showed low levels of radiotracer uptake of 111In-EC– or 99mTc-EC–labeled HGS-ETR1 and HGS-ETR2 in untreated mice bearing Colo205 xenografts but significantly enhanced tumor uptake after paclitaxel treatment (Figs. 8 and 9). The status of phospho-Erk1/2 and phospho-p53 were evaluated, and both were also up-regulated (Fig. 11). Erk1/2 acts both upstream and downstream of TRAIL-R (17, 18). In addition, it has been reported that TRAIL-R2 is a DNA damage-inducible, p53-regulated death receptor gene (19). Nonetheless, death receptor up-regulation after paclitaxel is unlikely to be due to the increased levels of phospho-p53 and phospho-Erk1/2 as it occurred at the protein level; expression of TRAIL-R1 and TRAIL-R2 transcripts remained unchanged as determined by quantitative real-time reverse transcription-PCR (RT-PCR 7500 System, Applied Biosystems; data not shown).

Pretreatment Paclitaxel Enhanced the Antitumor Activity of HGS-ETR1 In Vivo

In the mouse Colo205 xenograft tumor model, treatment with either paclitaxel (30 mg/kg) or HGS-ETR1 (10 mg/kg) resulted in tumor growth inhibition but not regression (Fig. 12). HGS-ETR2 was more effective than HGS-ETR1 and resulted in marked tumor regression when given to mice (10 mg/kg i.v. weekly; Fig. 13). Lowering the dose of HGS-ETR2 to 0.625 mg/kg i.v. suppressed tumor growth but did not induce regression (Fig. 13A). However, treatment with paclitaxel (30 mg/kg) on day 1 followed by HGS-ETR1 (10 mg/kg) on day 3 weekly for 3 weeks resulted marked tumor regression, an effect that was significantly more potent than the antitumor effect of single-agent therapy (P < 0.05; Fig. 12B). Indeed two of five mice treated with paclitaxel followed by HGS-ETR1 showed complete tumor regression within 3 weeks. Similar synergy was seen using paclitaxel followed by HGS-ETR2 (Fig. 13B), although the dose of HGS-ETR2 had to be lowered from 10 to 0.625 mg/kg to detect synergy because HGS-ETR2 itself at 10 mg/kg caused substantial tumor kill. The effect of paclitaxel followed by control IgG (isotype antibody) treatment did not differ from that of paclitaxel alone (Fig. 12B).

Figure 8. Tumor/muscle count density ratios of 99mTc-EC–labeled compounds before and after paclitaxel treatment in Colo205 mouse xenograft model. Columns, mean tumor/muscle count density ratios of 99mTc-EC–HGS-ETR1 and 99mTc-EC–HGS-ETR2 in Colo205-bearing nude mice (n = 3) before and 24 h after treatment with paclitaxel (60 mg/kg i.v.); bars, SE. Imaging was done 2 and 24 h after injection of radiolabeled compound. Paclitaxel significantly increased tumor uptake of HGS-ETR1 and HGS-ETR2 at 24 h after injection of 99mTc-EC–labeled compounds.

Discussion

In the present study, we determined the effect of the anti-microtubule taxane (paclitaxel) on TRAIL-R1 and TRAIL-R2 expression and on receptor agonist antibody (HGS-ETR1 and HGS-ETR2)–induced regression of human colorectal (Colo205) xenograft tumors. We show that treatment with tolerable doses of paclitaxel increased TRAIL-R1 and TRAIL-R2 protein levels substantially in a time-dependent manner. This was shown both by in vivo imaging using a novel radiolabeling technique that allowed...
imaging of HGS-ETR1 and HGS-ETR2 binding to tumor in vivo (Fig. 9) and by analysis of steady-state protein levels of TRAIL-R1 and TRAIL-R2 derived from the treated tumors (Fig. 10). Quantitative real-time reverse transcription-PCR showed that treatment of Colo205 xenografts with paclitaxel did not induce TRAIL-R1 and TRAIL-R2 transcripts, suggesting that the in vivo induction of TRAIL-R1 and TRAIL-R2 protein levels by paclitaxel is posttranscriptional.

HGS-ETR1 has completed phase 1 and 2 single-agent studies; HGS-ETR2 is currently being examined in phase 1 studies. Preliminary reports indicate that these antibodies are well tolerated, and, whereas evidence of tumor regression has been seen with HGS-ETR1 as a single agent in patients with non-Hodgkin’s lymphoma, their administration has primarily been associated with stabilization of disease (20, 21). This finding, however, is not unexpected considering the fact that the treated patients had advanced metastatic disease and had generally failed to respond to multiple prior therapies (22). Even so, because many if not most patients with metastatic tumors are resistant to single-agent therapy, strategies to potentiate the antitumor properties of individual drugs are very appealing. Indeed, curative regimens for diseases such as lymphomas depend on the use of multiple anti-neoplastic drugs given together.

Deciding which drugs to combine in the management of cancer is a complex problem, and the number of possible combinations is daunting. Clinical strategies often involve using two or more drugs with proven salutary effects and distinct toxicity profiles. Mechanism-based combinations are also highly desirable and may be sensitive to...
sequencing and other factors. Of interest, taxanes, such as paclitaxel, are among the most active antitumor compounds available, and their use in a broad range of tumors, including but not limited to breast, lung, and ovarian cancer, is well established (6). Despite their potency, however, these drugs are rarely curative.

Our experiments showing marked antitumor synergy in vivo when paclitaxel treatment is followed by HGS-ETR1 or HGS-ETR2 is therefore of substantial clinical relevance. When this sequence was given, tumor regressions were marked or complete (Figs. 12 and 13). The basis of this synergy seems to be a striking up-regulation of HGS-ETR1 and HGS-ETR2 expression by paclitaxel in the tumor but not normal tissue in vivo. The sequence of paclitaxel followed by ETR1 and ETR2 showed antitumor synergy without enhanced toxicity. As discussed, this is probably related to specific up-regulation of TRAIL-R of tumor cell but not normal cell, as detected by imaging. This specificity indicates that antitumor synergy without enhanced toxicity may be seen in patents.

Of interest, this up-regulation is apparent at 24 h (Fig. 8) but is considerably further enhanced by 48 h and reaches levels of >20-fold (Fig. 10). Such results emphasize the importance of finding ways to ascertain and to up-regulate expression of the death receptors in tumors in vivo in the clinic to select patients for treatment and to augment activity of TRAIL-related compounds.

Our current results confirm and extend those of Nimmanapalli et al. (23) who showed up-regulation of TRAIL-R1 and TRAIL-R2 by paclitaxel in vitro in prostate cancer cell lines, accompanied by enhanced apoptosis when these cell lines were pretreated with paclitaxel before exposure to TRAIL. Our studies suggest that these in vitro findings can be extrapolated to the in vivo setting and thus support bringing this sequential therapy to the patient care setting. Similarly, it has been reported that treatment with a different class of anticancer drugs (the DNA-damaging agents: etoposide, doxorubicin, and CPT-11) and radiation can induce p53 and/or nuclear factor-κB, which can up-regulate TRAIL-R1 and TRAIL-R2 expression, hence enhancing TRAIL-mediated apoptosis (24–29). The mechanism of death receptor up-regulation by DNA-damaging agents seems to be distinct from that of the microtubule inhibitor paclitaxel because the former increase death receptor transcripts, whereas paclitaxel increases protein expression.

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The sequence of paclitaxel followed by ETR1 and ETR2 showed antitumor synergy without enhanced toxicity. As discussed, this is probably related to specific up-regulation of TRAIL-R of tumor cell but not normal cell, as detected by imaging. This specificity indicates that antitumor synergy without enhanced toxicity may be seen in patents.

Of interest, this up-regulation is apparent at 24 h (Fig. 8) but is considerably further enhanced by 48 h and reaches levels of >20-fold (Fig. 10). Such results emphasize the importance of finding ways to ascertain and to up-regulate expression of the death receptors in tumors in vivo in the clinic to select patients for treatment and to augment activity of TRAIL-related compounds.

Our current results confirm and extend those of Nimmanapalli et al. (23) who showed up-regulation of TRAIL-R1 and TRAIL-R2 by paclitaxel in vitro in prostate cancer cell lines, accompanied by enhanced apoptosis when these cell lines were pretreated with paclitaxel before exposure to TRAIL. Our studies suggest that these in vitro findings can be extrapolated to the in vivo setting and thus support bringing this sequential therapy to the patient care setting. Similarly, it has been reported that treatment with a different class of anticancer drugs (the DNA-damaging agents: etoposide, doxorubicin, and CPT-11) and radiation can induce p53 and/or nuclear factor-κB, which can up-regulate TRAIL-R1 and TRAIL-R2 expression, hence enhancing TRAIL-mediated apoptosis (24–29). The mechanism of death receptor up-regulation by DNA-damaging agents seems to be distinct from that of the microtubule inhibitor paclitaxel because the former increase death receptor transcripts, whereas paclitaxel increases protein expression.

The sequence of paclitaxel followed by ETR1 and ETR2 showed antitumor synergy without enhanced toxicity. As discussed, this is probably related to specific up-regulation of TRAIL-R of tumor cell but not normal cell, as detected by imaging. This specificity indicates that antitumor synergy without enhanced toxicity may be seen in patents.
levels without a change in mRNA expression (ref. 26; data not shown). Of interest in this regard is the observation that paclitaxel administration was associated with an increase in phospho-p53 and phospho-Erk1/2 in our colorectal tumor xenografts. The role of p53 activation in taxane-induced apoptosis is not clear-cut, and both p53-dependent and p53-independent pathways have been shown (30, 31). Taxane-induced microtubule damage also triggers signaling cascades that involve Erk1/2 (30, 31). Erk1/2 acts both upstream and downstream of TRAIL-R; therefore, it is also possible that activation of Erk1/2 occurs as a downstream effect of up-regulation of the TRAIL-R (17, 18). Although p53 is known to induce the death receptors, it is unlikely that this mechanism is operative here as p53 up-regulation of these receptors occurs at the transcriptional level (19), and our experiments showed that paclitaxel induced TRAIL-R via a posttranscriptional mechanism (because mRNA levels were not increased).

In summary, the presence of death receptors is a necessary (although not always sufficient) prerequisite for antitumor activity induced by TRAIL and related molecules. Using novel imaging techniques, we show a profound, time-dependent in vivo up-regulation of death receptors after paclitaxel treatment associated with marked antitumor synergy. In the clinic, imaging-based ascertainment of binding of receptor agonist antibodies HGS-ETR1 or HGS-ETR2 to tumor could conceivably be used to determine if cancers with poor binding are less likely to respond to these antibodies. Although a correlation between up-regulation of TRAIL-R and antitumor activity is implicated by our experiments, it has been reported that up-regulation of TRAIL-R is not required for enhanced cytotoxicity in other systems (32). The effect of up-regulation of TRAIL-R in patients’ tumors, therefore, needs investigation. Optimization of timing of drug sequence in patients may be necessary in the clinic and could be elicited with the use of imaging of radiolabeled HGS-ETR1 and HGS-ETR2, as done in the current animal studies (Fig. 9). These imaging techniques could be a noninvasive alternative to the use of tumor biopsies, which are painful and risky, and also often inaccurate because of i.t. variability. Ultimately, a wealth of clinical experience supports the bench-side observations of the complexity of survival pathways in human malignancies and indicates that mechanism-based combinations of treatments, such as those described herein, need to be applied in the clinic to eradicate a malignancy.

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Molecular Cancer Therapeutics

Novel *in vivo* imaging shows up-regulation of death receptors by paclitaxel and correlates with enhanced antitumor effects of receptor agonist antibodies

Jing Gong, David Yang, Saady Kohanim, et al.

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