Dual combination therapy targeting DR5 and EMMPRIN in pancreatic adenocarcinoma

Hyunki Kim1,2,4,5, Guihua Zhai1, Sharon L. Samuel1, Christopher J. Rigell1, Heidi R. Umphrey1, Samir Rana1, Cecil R. Stockard4, Naomi S. Fineberg3, Kurt R. Zinn1,4

Departments of Radiology1, Biomedical Engineering2, Biostatistics3, and the Comprehensive Cancer Center4

University of Alabama at Birmingham, Birmingham, Alabama 35294

Grant support: Research Initiative Pilot Award from the Department of Radiology at UAB, AACR-PANCAN Career Development Award, HSF-GEF Scholar Award, NIH grants 5P50CA89019, P20CA10195, and 5P30CA013148.

Requests for reprints: Hyunki Kim (E-mail: Hyunki@uab.edu), VH G082C5, University of Alabama at Birmingham, Birmingham, AL, 35294-0012.

Running title: Anti-DR5 and anti-EMMPRIN therapy

Keywords: Pancreatic cancer, DR5, EMMPRIN, Imaging
Abstract

The study goal was to assess the efficacy of combined EMMPRIN and DR5 targeted therapy for pancreatic adenocarcinoma in orthotopic mouse models using multi-modal imaging. Cytotoxicity of anti-EMMPRIN antibody and anti-DR5 antibody (TRA-8) in MIA PaCa-2 and PANC-1 cell lines was measured by ATPlite assay in vitro. The distributions of Cy5.5-labeled TRA-8 and Cy3-labeled anti-EMMPRIN antibody in the two cell lines were analyzed by fluorescence imaging in vitro. Groups 1-12 of SCID mice bearing orthotopic MIA PaCa-2 (groups 1-8) or PANC-1 (groups 9-12) tumors were used for in vivo studies. DCE-MRI was applied in group 1 (untreated) or group 2 (anti-EMMPRIN antibody). The tumor uptake of Tc-99m-labeled TRA-8 was measured in group 3 (untreated) and group 4 (anti-EMMPRIN antibody). PET/CT imaging with \(^{18}\)F-FDG was applied in groups 5-12. Groups 5-8 (or groups 9-12) were untreated or treatment with anti-EMMPRIN antibody, TRA-8, and combination, respectively. TRA-8 showed high killing efficacy for both MIA PaCa-2 and PANC-1 cells in vitro, but additional anti-EMMPRIN treatment did not improve the cytotoxicity. Cy5.5-TRA-8 formed cellular caps in both the cell lines, while the maximum signal intensity was correlated with TRA-8 cytotoxicity. Anti-EMMPRIN therapy significantly enhanced the tumor delivery of the MR contrast agent, but not Tc-99m-TRA-8. Tumor growth was significantly suppressed by the combination therapy, and the additive effect of the combination was demonstrated in both MIA PaCa-2 and PANC-1 tumor models.
Introduction

Pancreatic cancer is a highly malignant disease and the fourth leading cause of cancer death in the United States (1). Due to the nonspecific symptoms, pancreatic cancer is typically detected at the very late stages (2), and therefore only 15% of patients are eligible for curable operation at diagnosis (3). Gemcitabine is the first-line therapeutic agent for unresectable pancreatic cancer, but offers only modest benefit (4). Radiation or erlotinib (a small molecule targeting epidermal growth factor receptor) combined with gemcitabine delivered better efficacy than gemcitabine alone (5, 6), but the routine clinical application is not recommended because of the minimal benefit. More recently, Conroy et al reported that FOLFIRINOX (quadruple therapy with oxaliplatin, irinotecan, leucovorin, and fluorouracil) extended the patient life significantly, but the median survival time was still less than a year (7).

A monomeric monoclonal antibody, TRA-8, specifically targets only death receptor 5 (DR5) among five TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) receptors (8). TRA-8 has been considered as a promising novel drug for pancreatic cancer (9, 10). Since DR5 is present in most cancer cells, but limited in normal cells, TRA-8 enables selective killing of cancer cells without causing severe side effects. TRA-8 induces DR5 aggregation triggering apoptosis (11) and suppressing cell proliferation (12). Because pancreatic cancer stem cells express higher level of DR5, TRA-8 will be able to suppress pancreatic-tumor regrowth efficiently (13). The phase I clinical trial of the humanized TRA-8, tigatuzumab, was completed, and no adverse side effects were identified (14).
A monomeric monoclonal antibody targeting extracellular matrix metalloprotease inducer (EMMPRIN) was recently developed, and a significant anti-cancer effect was demonstrated in orthotopic pancreatic-cancer murine models (15). EMMPRIN is a membrane-bound glycoprotein expressed in pancreatic cancer with high incidence (16). Matrix metalloproteinases (MMPs), stimulated by EMMPRIN, are essential to degrade extracellular matrix components and thereby to invade tissue boundaries (17-20). EMMPRIN also affects tumor neovascularization by stimulating VEGF isoforms and VEGFR-2 (21), and therefore anti-EMMPRIN therapy is capable of suppressing tumor angiogenesis as well as cancer-cell invasion and metastasis. The anti-angiogenic effect may induce the normalization of tumor microvasculature, reducing interstitial pressure and thereby improving drug delivery, which may lead to a better treatment (22). In fact, we recently demonstrated that anti-EMMPRIN therapy induced a synergy when used with gemcitabine in a pancreatic cancer model (23).

Antibody-based therapies for cancer are attractive because of minimal systemic toxicity compared with chemotherapy. Since a therapeutic antibody is specific for a target in one pathway, there is the potential for combining antibody therapies for additive or synergistic benefits. The current study targeted both DR5 and EMMPRIN to maximize the overall therapeutic effect by directly inducing cancer-cell apoptosis via the TRA-8 antibody while simultaneously suppressing tumor invasion, metastasis, and angiogenesis via the anti-EMMPRIN antibody. The efficacy of the combination approach was followed over time using multi-modal imaging.
Materials and Methods

Reagents and cell lines. All reagents were from Fisher (Pittsburg, PA) unless otherwise specified. Dr. Tong Zhou (UAB, Birmingham, AL) provided purified monomeric monoclonal anti-EMMPRIN antibody (mouse origin IgG1 kappa) and TRA-8. Cy5.5 and Cy3 were purchased from GE Healthcare Inc (Princeton, NJ). Fresh Tc-99m pertechnetate was purchased from Birmingham Nuclear Pharmacy (Birmingham, AL). 18F-FDG was purchased from PETNET Solutions (Birmingham, AL). Two human pancreatic cell lines, MIA PaCa-2 and PANC-1, were obtained from Dr. Donald Buchsbaum (UAB, Birmingham, AL) more than 6 months ago, and have not tested for authentication in our laboratory. DR5 and EMMPRIN expressions in both MIA PaCa-2 and PANC-1 cells were validated by immunoblot analysis (24, 25). MIA PaCa-2 and PANC-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech Inc, Herndon VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). OmnipaqueTM (iohexol, 350 mg/ml, GE Healthcare Inc., Princeton, NJ) and prohance® (gadoteridol, an MR contrast agent; Bracco Diagnostics Inc., Princeton, NJ) were purchased from the University of Alabama at Birmingham Hospital Pharmacy.

Cell Viability Assay. In vitro viability assays for MIA PaCa-2 and PANC-1 cells were conducted with TRA-8 alone or in combination with anti-EMMPRIN antibody. For each cell line, a total of 1000 cells were added to each well of 96-well plates (4 columns x 18 rows). TRA-8 was diluted to four different concentrations (0, 10, 50, 500 ng/ml) and was added to 18 wells per TRA-8 concentration (same concentration at each column). Anti-EMMPRIN antibody was diluted to three different concentrations (0, 50, 100 ng/ml) and was added to the 6 rows (24 wells) of cells per anti-EMMPRIN
concentration. After 24 hours of incubation at 37 °C in 5% CO₂, the ATP level was determined using the ATPlite assay (Perkin-Elmer, Boston, MA). The light emission from the wells of the plates was measured using an IVIS-100 imaging system (Caliper Life Sciences, Hopkinton, MA) and quantified using the vendor software. The luminescent exposure time was 60 seconds, while binning was 8. Regions of interest (ROIs) were drawn manually around the area of each individual well in the well plate, and the intensity of light emitted from each ROI was measured. Data were normalized to light emission of an equal number of untreated cells otherwise incubated under the same conditions as the treated cells.

**HYNIC Conjugation and Radiolabeling.** HYNIC conjugation and radiolabeling were conducted for a biodistribution study. A fresh 1.8 mM solution of succinimidyl 6-hydrazinonicoinate (HYNIC; courtesy of Dr. Gary Bridger, AnorMED Inc., Langley, British Columbia) in dimethylformamide was prepared. Forty picomoles was transferred to glass vials, followed by freezing at -90°C, then the solutions were vacuum-dried using Advantage Benchtop Freeze Dryer (Virtis Co. Inc., Gardiner, NY) with the shelf temperature at -75°C and trap at -90°C. A vial was reconstituted with 1.0 ml of sodium phosphate buffer [0.15 mol/L (pH 7.80)] containing 1 mg of TRA-8 (HYNIC/antibody molar ratio = 6). After a 3-hour incubation at room temperature, the mixture was transferred to Slide-A-Lyzer dialysis cassette having molecular weight cutoff of 10,000 (Pierce, Rockford, IL) and then immersed into 1,000 ml of phosphate-buffered saline (PBS; pH 7.4) overnight at 4°C. The HYNIC-modified antibody was labeled with Tc-99m using SnCl₂/tricine as the transfer ligand (26), and the unbound Tc-99m was removed by G-25 Sephadex size exclusion chromatography. The protein
concentrations of the collected fractions were measured by Lowry assay (27). The Tc-99m-labeled TRA-8 was greater than 95%, as measured by TLC using separate strips developed with saline and methyl ethyl ketone.

**Fluorescence Imaging.** Fluorescence imaging was performed to analyze the cellular distributions of the antibodies. Monofunctional dyes Cy5.5 or Cy3 were conjugated to TRA-8 or anti-EMMPRIN monoclonal antibody, respectively, at a 3:1 molar ratio according to manufacturer’s instructions. Each of MIA PaCa-2 and PANC-1 cell lines (1 x 10^5 cells/plate) was seeded onto 35-mm glass bottom culture dishes (MatTek Corp., Ashland, MA) and incubated in complete cell culture medium. At 16 hours after seeding, the cells were washed with PBS and then treated with Cy5.5-TRA-8 (5 μg/ml), Cy3-CD147 (5 μg/ml), a combination of the two, or control. At 24 hours post treatment, the cells were washed with PBS and then 3 images (X20) were taken per each treatment with a Leica DMIRE2 inverted microscope equipped with a Nuance camera (CRI Inc., Woburn, MA) for randomly selected areas.

To quantify the distributions of Cy5.5-TRA-8 and Cy3-anti-EMMPRIN antibody, a line was drawn on a randomly selected cell (n=10 per each image), and the signal intensities of the fluorophores located on the line were measured. The maximum and minimum signal values together with variance per size were determined on each cell line when the two fluorophore labeled antibodies were used individually or combined. Variance per size (var/size) was calculated by

\[
\text{Var/Size} = \frac{\sum_{i=1}^{N} \left( \text{Sig}_i - \langle \text{Sig} \rangle \right)^2 \times PDF_i}{N}
\]

where \(\text{Sig}_i\) is the signal intensity of the fluorophore at location \(i\), \(\langle \text{Sig} \rangle\) is the average signal intensity of all locations, \(PDF_i\) is the probability density function at location \(i\), and \(N\) is the number of locations.
, where $\text{Sig}_i$ is the signal intensity on the pixel $i$, $\langle \text{Sig} \rangle$ is the mean signal intensity of all pixels on the line, $PDF_i$ is the probability density function on the pixel $i$, and $N$ is the total number of pixels on the line. $PDF_i$ was calculated by

$$PDF_i = \frac{\text{Sig}_i / \text{Sig}_{\text{max}}}{\sum_{i=1}^{N} (\text{Sig}_i / \text{Sig}_{\text{max}})}$$

, where $\text{Sig}_{\text{max}}$ is the maximum signal intensity on the line. Variance per size presents how randomly the fluorophore labeled antibodies are distributed on the cell membrane, and is the smaller when the signal intensity is the more focused on a restricted region. The signal intensities of fluorophores were measured by ImageJ, version 1.40g (National Institutes of Health, Bethesda, MD).

**Animal modeling:** Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at UAB. Twelve groups of female SCID BALB/c mice (NCI-Frederick Animal Production Program, Fredrick, MD, 4~6 weeks old, $n=5$ for groups 1-4, $n=6$ for groups 5-12) were used. The procedure for intrapancreatic tumor implantation was the following; a 1-cm incision was made in the left upper quadrant of the abdomen of anesthetized mice, and a solution of $2.5 \times 10^6$ MIA PaCa-2 (groups 5-8) or PANC-1 (groups 9-12) cells in 30 μL of DMEM was injected into the tail of the pancreas. The skin and peritoneum were closed in 1 layer with 3 interrupted 5-0 Prolene sutures. Drug dosing started at 21 days after cell implantation for groups 5-8, or 28 days after cell implantation for groups 9-12, to model established pancreatic tumors for all groups. For groups 1 and 2, a vascular access port (PennyPort, Access Technologies, Skokie, IL) was subcutaneously implanted on the right shoulder of each mouse and the catheter connected to the port was inserted into a jugular vein to
facilitate repeated intravenous gadoteridol injections, at 4 days prior to dosing (day 17), as described in our previous studies (28, 29). Groups 1 and 2 were intraperitoneally injected with PBS (serving as control) and anti-EMMPRIN antibody (0.2 mg) respectively on day 21, and DCE-MRI was applied on days 21 (prior to dosing; baseline imaging) and 24. Groups 3 and 4 were intraperitoneally injected with PBS (serving as control) and anti-EMMPRIN antibody (0.2 mg), respectively, twice for a week. At 1 week after therapy initiation, groups 3 and 4 were intravenously injected with Tc-99m-TRA-8 (0.40±0.02 MBq; 2.6±0.1 μg), and biodistribution study was followed at 24 hours thereafter. Groups 5-8 were intraperitoneally injected with PBS (serving as control), anti-EMMPRIN antibody (0.2 mg, semiweekly), TRA-8 (0.1 mg, weekly), and combination, respectively, for 2 weeks, while \(^{18}\)F-FDG PET/CT imaging was applied weekly. Same dosing and imaging schedules were applied for groups 9-12. All tumors of groups 5-12 were collected for Ki67 and CD31 staining at the end of the imaging study. All animals were anesthetized using isoflurane gas (1~2%) during imaging.

**MR imaging.** Small animal MRI was conducted with a Bruker BioSpec 9.4T system (Bruker BioSpin Corp., Billerica, MA), to assess the changes of microvasculature. The tumor was imaged using a combination of a \(^1\)H volume resonator/transmitter and a surface coil receiver (Bruker BioSpin Corp., Billerica, MA). Respiratory rate of animals was monitored using an MR-compatible small-animal respiratory monitoring device (SA Instrument, Inc., Stony Brook, NY) during imaging. A 27-gauge needle connected to a sterilized polyurethane tube (Strategic Applications Inc., Libertyville, IL) was inserted into the lumen of each port, to deliver gadoteridol. Anatomical MRI to measure tumor-volume was performed using a relaxation
enhancement (RARE) T2-weighted spin-echo sequence with the following acquisition parameters: repetition time (TR)/echo time (TE) = 2000/34 milliseconds, 128x128 matrix, and a 30x30-mm field of view. Continuous 1-mm thick slices were used to cover the entire tumor region. Then, a T1 map was acquired with a gradient-echo multflip-angle approach with the following parameters: repetition time (TR)/echo time (TE) = 115/3 milliseconds, 128x128 matrix, a 30x30-mm field of view, NEX=4, and seven flip angles of 10, 20, 30, 40, 50, 60, and 70°. A total of five 1-mm thick slices were acquired to cover tumor regions of interest in an interlaced mode. DCE-MRI employed the same acquisition parameters as those above but with the fixed flip angle of 30°. Five baseline images were acquired before gadoteridol injection, and then 40 images were acquired after gadoteridol injection of 0.0267 mmol/ml over a period of 15 seconds with a total injection volume of 0.15 ml. A syringe pump (NE-1600, New Era Pump Systems, Inc., Wantagh, NY) was used to inject gadoteridol at a constant rate (0.01 ml/sec).

The reference region model was employed to calculate volume transfer constant, $K^{\text{trans}}$ (transfer rate from plasma to the extravascular-extracellular space (EES)), and rate constant, $k_{ep}$ (rate parameter for transport from the EES to plasma), as described in our previous study (28). The tumor area was segmented from the anatomical MR images using the signal-intensity difference between the ROI and background, while the intensity thresholds were determined manually. The $K^{\text{trans}}$, $k_{ep}$, and $C_{40\text{min}}$ (gadoteridol concentration at 40 minutes after dosing) values were averaged in the whole tumor region. Tumor volume was calculated by summing all voxels inside the tumor boundary of the anatomical MR images. Segmentation of the whole tumor area was performed using ImageJ, version 1.40g (National Institutes of Health, Bethesda, MD). The $K^{\text{trans}}$,...
k_{ep}, and C_{40min} quantification were implemented using computer software developed using Labview, version 8.5 (National Instruments Co., Austin, TX).

**Biodistribution Study.** Tumor and blood were collected from each animal of groups 3 and 4 at 24 hours after radioactivity injection. Those samples were weighed and the Tc-99m activity was measured using a calibrated gamma-ray counter (MINAXIγ Auto-gamma® 5000 series Gamma Counter manufactured by Packard Instrument Company, Downers Grove, IL), and corrected for radioactive decay to the time of injection. The administered activity for each mouse was determined by measurements of the dosing syringe before and after injection with an Atomlab 100 dose calibrator (Biodex Medical Systems, Shirley, NY). Tissue activity concentrations (in percent of the injected dose per gram, %ID/g) and tumor-to-blood activity concentration ratios were derived.

**PET/CT Imaging.** PET/CT imaging study was conducted using Triumph, a PET/CT dual-modality imaging system (GE, Northridge, CA), to monitor tumor response following TRA-8 administration with/without anti-EMMPRIN therapy. For PET imaging, this system provided a 2.2-mm axial spatial resolution and 5.9% sensitivity at the center of field of view, while the axial field of view was 37.5 mm (30). Animals were fasted overnight (17-21 hours) prior to $^{18}$F-FDG injection. $^{18}$F-FDG (7.19±0.07 MBq in 200 μl PBS) was administered intravenously, and a 7-minute scan was performed at 100 minutes after injection. Animals were under isoflurane anesthesia during dosing (~5 minutes) and imaging (~10 minutes); the animal cages were heated with a heating pad (T/Pump, Gaymar Industries, Inc., Orchard Park, NY) set to 42°C and started at 1 hour before $^{18}$F-FDG injection and continued until the imaging of all animals was completed.
The temperature of the animal bed in the Triumph scanner was maintained to 37°C during imaging. PET images were reconstructed with maximum likelihood expectation maximization algorithm (5 iterations) in high-resolution mode. For contrast-enhanced CT imaging, 0.2 ml of iohexol was intraperitoneally injected right before PET imaging. The voltage of X-ray tube was 75 kV$_{p}$ and the anode current was 0.11 mA. The axial field of view was set to 78.9 mm, while 256 projections were acquired in fly gantry-motion mode. The CT acquisition time was 1.07 minutes per each animal. The coregistration of PET and CT images was performed using ImageJ version 1.40g (National Institutes of Health, Bethesda, MD). The tumor area was manually segmented from the co-registered contrast-enhanced CT images based on the signal-intensity difference between the region of interest (ROI) and background. In PET images, the standardized uptake value (SUV) was calculated by $SUV = (C \times W)/D$, where $C$ is tissue activity concentration (MBq/ml), $W$ is animal body weight (g), and $D$ is the administered dose (MBq). Prior to dosing, the averaged tumor SUV$_{\text{mean}}$ of groups 5-8 (MIA PaCa-2 model) was 1.04±0.07 g/ml, and the averaged tumor volume was 91±8 mm$^3$, without statistical difference among groups, while the averaged tumor SUV$_{\text{mean}}$ of groups 9-12 (PANC-1 model) was 0.87±0.04 g/ml, and the averaged tumor volume was 31±2 mm$^3$, without statistical difference among groups.

**Histologic analyses:** Ki67 and CD31 staining were performed for tumor tissues of groups 5-12, with the same procedure as reported previously (29, 31); no tumor cells were remained in one of the samples of the TRA-8 treated group (group 7), so only 5 samples were analyzed for group 7. Two digital microphotographs (X400) were randomly selected for each tumor slice that had undergone Ki67 or CD31 staining,
using SPOT camera on an Olympus 1X70 microscope (Olympus Optical Co., Tokyo, Japan), interfaced with personal computer and SPOT software. The proliferating (Ki67 expressing) cells were segmented by the signal-intensity difference between the target cells and background in each photograph, while the intensity and minimum particle-size thresholds were determined manually. Then the target cells were counted, and the cell density (cell-number(N)/mm²) was calculated. The cell densities of the two photographs for each tumor slide were averaged. The CD31-stained area was segmented in the same way, and the area fraction (CD31-stained area/total area), considered as endothelial-cell density, was calculated. The image analysis was performed using ImageJ, version 1.40g (National Institute of Heath, Bethesda, MD).

**Statistical analysis.** One-way ANOVA (32) was carried out using SAS, version 9.2 (SAS Institute Inc., Cary, NC) to compare the fluorescence signal amplitudes, the variance of the fluorescence signal distributions, and %ID/g of tumor and blood together with tumor-to-blood ratio. ANOVA was also used to compare the changes of the $K_{trans}$, $k_e$, and $C_{40min}$, and the densities of Ki67 and CD31 staining cells in tumors. SPSS version 16.0 (SPSS Inc., Chicago, IL) was employed to compare tumor SUV$_{mean}$ and volume changes over 2 weeks of groups 5-8 (or groups 9-12) using two-way repeated measures analysis of variance (RM ANOVA) (33), while the Pearson correlation coefficient was used to analyze the relationships between two variables (34). $p$ values less than 0.05 were considered significant. Data are presented as means±standard error.
Results

TRA-8 demonstrated high killing efficacy for both MIA PaCa-2 and PANC-1 cell lines in an *in vitro* ATPlite assay, while additional anti-EMMPRIN therapy did not improve the cytotoxicity. Figure 1 presents the *in vitro* viability of (A) MIA PaCa-2 and (B) PANC-1 cells following overnight treatment of TRA-8 with/without anti-EMMPRIN antibody in escalating concentration. TRA-8 monotherapy induced 91% and 64% killing of MIA PaCa-2 and PANC-1 cells, respectively, at 500 ng/ml, but the cytotoxicity was not improved by adding anti-EMMPRIN antibody. TRA-8 killing efficacy for MIA PaCa-2 was 87±28% better than that for PANC-1, when averaged over the three TRA-8 concentrations (10, 50, 500 ng/ml).

Cy5.5-TRA-8 generated cellular caps in both MIA PaCa-2 and PANC-1 cells, while Cy3-anti-EMMPRIN antibody was more uniformly distributed on the cell membrane. Figures 2A and 2B show representative fluorescence images of Cy5.5-TRA-8 (red) and Cy3-anti-EMMPRIN antibody (green) distributed on MIA PaCa-2 and PANC-1 cells, when the two antibodies were used (A) separately or (B) combined. Figures 2C-2F present the signal intensities of Cy5.5-TRA-8 and Cy3-anti-EMMPRIN antibody on the yellow lines shown in the images of (C,E) MIA PaCa-2 or (D,F) PANC-1 cells, when the two antibodies were used (C,D) separately or (E,F) combined. The maximum and minimum signal intensities together with variance per size were summarized in table 1. When added separately, the maximum signal intensity of Cy5.5-TRA-8 was 2.6-fold higher than that of Cy3-anti-EMMPRIN antibody in MIA PaCa-1 cells, while the variance per size was 3.2-fold smaller. However, for PANC-1 cells, the maximum signal value of Cy5.5-TRA-8 was not statistically different from that of Cy3-
anti-EMMPRIN antibody, while the variance per size was significantly smaller. Similar
distribution patterns were observed when combined. However, of interest, Cy3-anti-
EMMPRIN antibody was observed in clusters as found for Cy5.5-TRA-8 in MIA PaCa-2
cells; the capping region of Cy3-anti-EMMPRIN antibody corresponded to that of Cy5.5-
TRA-8 (Fig. 2B). The maximum signal intensity of Cy3-anti-EMMPRIN antibody was
significantly higher when combined with Cy5.5-TRA8 than when alone (p=0.003), while
the variance per size was significantly smaller in the combined treatment also
(p<0.001).

**Anti-EMMPRIN therapy enhanced the tumor uptake of MR contrast agent,**
but not Tc-99m-TRA-8. Figure 3A shows the concentration of MR contrast
(gadoteridol) of two representative mice bearing orthotopic pancreatic tumor xenografts
at 0 (baseline), 5, and 40 minutes after gadoteridol injection, when the animals were
untreated (control) or treated with anti-EMMPRIN antibody at 3 days prior to imaging.
The tumor boundary is indicated with the white dotted circle. Figure 3B shows the
contrast-enhancement curves averaged in the region of interest (ROI) indicated with the
white square (2x1 window: 2 pixels located in the tumor) in the baseline images of Fig.
3A, together with the best-fit 5th order polynomial curves. The MR contrast
concentration in tumor of the control animal was markedly higher than that of the treated
animal immediately after injection, but was rapidly reduced over time. On the other
hand, the MR contrast concentration in the treated tumor was lower than control tumor
immediately after injection, but gradually increased and was maintained higher than that
of control tumor by 20 minutes post injection. Figures 3C and 3D show the tumor $K_{\text{trans}}$
and $k_{\text{ep}}$ maps of the two animals untreated or treated with anti-EMMPRIN antibody,
respectively. The changes of intratumoral $K^{\text{trans}}$, $k_{\text{ep}}$, and $C_{40\text{min}}$ values were -36±9%, -18±12%, and 153±44%, respectively, for 3 days after drug dosing, whereas those of the control group were 38±8%, 10±7%, and 14±25%, respectively, during the same time (p values were <0.001, 0.085, and 0.025, respectively). The tumor-volume change of the treated group was 22±6%, while that of the control group was 52±21% without statistical difference (p=0.316). In the biodistribution study, the %ID/g of Tc-99m-TRA-8 in tumor, blood, and tumor to blood ratio for the anti-EMMPRIN treated group were 28±5%, 17±1%, and 1.8±4 respectively, while those of the control group were 20±5%, 19±1%, and 1.1±0.3 respectively. No statistically significant differences were observed between the two groups for any of these parameters.

18F-FDG PET/CT imaging validated the additive therapeutic efficacy of combined anti-EMMPRIN antibody and TRA-8 for pancreatic cancer. Figure 4A shows representative contrast-enhanced CT (CE-CT), 18F-FDG PET, and PET/CT fused images of a mouse bearing an orthotopic MIA PaCa-2 pancreatic tumor indicated with a white arrow in each sub-figure. Figures 4B and 4C show the changes of MIA PaCa-2 tumor volume and SUV$_{\text{mean}}$ for 2 weeks post therapy initiation when groups 5-8 were untreated or treated with anti-EMMPRIN antibody, TRA-8, and the combination, respectively. The different Greek letters represent statistically significant differences among groups; that is, group labeled with $\alpha$ is significantly different from that with $\beta$, but not from that with $\alpha$, $\beta$. At day 28, the combination therapeutic efficacy was fairly comparable with the sum of the effects of the two monotherapies relative to the control group in volume assessment (decrease of 187% vs. 185%), showing the additive effect of the combination. At the end of the imaging study, the mean tumor volumes of groups
5-8 were 308±28, 274±43, 16±6, and 8±4 mm$^3$, respectively. The correlation between the changes of tumor volume and intratumoral SUV$_{mean}$ over 2 weeks of therapy was statistically significant (p<0.001). The animal body weights of groups 5-8 were not different during 2 weeks of therapy.

Figures 4D and 4E show the changes of PANC-1 tumor volume and SUV$_{mean}$ for 2 weeks post therapy initiation when groups 9-12 were untreated or treated with anti-EMMPRIN antibody, TRA-8, and the combination, respectively. The different Greek letters represent statistically significant differences among groups. The combination therapy presented additive efficacy in volume assessment, but not as marked as that in MIA PaCa-2 model (decrease of 86% vs. 111% for a week). At the end of the imaging study, the mean tumor volumes of groups 9-12 were 79±12, 47±7, 45±9, and 38±7 mm$^3$, respectively. The tumor volume and SUV$_{mean}$ changes over 2 weeks were significantly correlated (p=0.004). The animal body weights of groups 9-12 were not different during 2 weeks of therapy.

Histologic analysis verified that anti-EMMPRIN antibody significantly suppressed the endothelial cell and proliferating cell densities in pancreatic tumors. Figure 5A shows the representative microphotographs of Ki67 and CD31 stained tumor slices (5-μm thickness) of groups 5-8 (or groups 9-12) untreated or treated with anti-EMMPRIN antibody, TRA-8, and the combination, respectively for 2 weeks. The proliferating (Ki67 stained) and endothelial (CD31 stained) cells are indicated with black arrows in each sub-figure. Figures 5B and 5C show the averaged Ki67 and CD31 expressed tumor-cell densities of groups 5-12, respectively; the different Greek letters or alphabets above bars represent the statistical difference among groups.
5-8 (MIA PaCa-2 model) or groups 9-12 (PANC-1 model), respectively. The mean tumor-cell proliferations of groups 6-8 were reduced 34%, 37%, and 26% relative to the control (group 5), respectively, but the statistical significance was detected only between groups 5 and 6 (p=0.035). Similarly, the mean intratumoral endothelial cell densities of groups 6-8 were reduced 46%, 37%, and 17% relative to the control, respectively, but only that of group 6 was significantly lower (p=0.009). But, in PANC-1 tumors, the proliferation cell density was significantly suppressed by either anti-EMMPRIN therapy (p=0.044) or combination therapy (p=0.016), and the endothelial cell density was significantly reduced by any of the three treatments (p<0.05).

Discussion

The super-aggregation, or capping, of death receptors has been considered to play a pivotal role to induce cellular apoptosis; the importance of Fas capping for apoptosis induction was validated (35, 36), and it was also demonstrated that DR5 aggregation triggered by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) was essential to drive intracellular signals for death (37, 38). Similarly, it was found that TRA-8 resistance resulted not from the total number of DR5 expressed on the cell membrane or TRA-8 binding affinity, but perhaps from the inefficient TRA-8/DR5 oligomerization (8). The compact binding of Cy5.5-TRA-8 will lead to the local increase of the fluorescence signal, whereas the lack of clustering may cause the signal dispersion. This is consistent with the data presented in this study; the maximum signal intensity of Cy5.5-TRA-8 capping in MIA PaCa-2 cells was about 2~3 fold higher than that in PANC-1, while the variance per size was about 20~30% smaller, with statistical
significance, which is fairly comparable with TRA-8 cytotoxicity to the two cell lines. Therefore, this technique may be applicable for biopsy specimens of pancreatic cancer during a neoadjuvant trial, to identify potential responders to TRA-8. The clustering of Cy3-anti-EMMPRIN antibody was also observed when used together with Cy5.5-TRA-8, although it did not form the capping when used alone. This is presumably because both EMMPRIN and DR5 may be present in the same lipid raft (39); EMMPRIN might be clustered along with DR5 aggregation, which is supported by the regional consistency in the distributions of Cy5.5-TRA-8 and Cy3-anti-EMMPRIN antibody when combined.

Anti-EMMPRIN therapy increased the tumor delivery of gadoteridol, presumably by reducing intratumoral microvasculature and thereby decreasing interstitial pressure. However, the uptake of Tc-99m-TRA-8 into tumor was not enhanced by anti-EMMPRIN therapy. This discrepancy may be caused by the difference in circulation half-lives of the two agents; the plasma half-life of TRA-8 is about 6 days (data not shown), whereas that of gadoteridol is only about 3 hours in healthy mice (24). Therefore, although high interstitial pressure is present in a tumor, TRA-8 may be eventually delivered into the target cells. In contrast, small molecules are rapidly excreted, unless they reach the target cells and then are internalized within the limited time period. These results may explain the additive efficacy in the combination therapy with anti-EMMPRIN antibody and TRA-8, and the synergistic efficacy when anti-EMMPRN antibody used with gemcitabine (23). Of note, the hypervascular metastatic liver tumors from the primary pancreatic cancer are common (40); about 60% of pancreatic cancer patients have metastases to distant organs like liver and lung at diagnosis, and the complications due to local spread is one of the main causes of patient death. Therefore, enhancing drug
delivery into hypervascular metastatic tumors while suppressing further tumor invasion is essential to significantly extend patient lives, and the anti-EMMPRIN antibody may be a good candidate to achieve that goal. Since no adverse side effect was observed during 2 weeks of anti-EMMPRIN monotherapy or combination therapy, anti-EMMPRIN antibody may be readily combined with other chemotherapeutic agents to maximize therapeutic efficacy.

Histologic analysis validated that anti-EMMPRIN antibody reduced the tumor-cell proliferation and endothelial cell densities, but did not confirm the additive efficacy when combined with TRA-8 in MIA PaCa-2 tumor model. This may reflect that TRA-8 (or combination) therapy killed most cancer cells sensitive to the treatment, and thereafter, a small portion of cells inherently resistant (or acquired resistance) remained and could start dividing again. In fact, the averaged volume of PANC-1 tumors after 2 weeks of combination therapy was 5-fold larger than that of MIA PaCa-2 tumors after the same dosing, and the additive efficacy shown in PANC-1 tumor-volume assessment was validated histologically. Therefore, in order to eradicate all tumor cells, the sequential use of several drugs having different therapeutic mechanisms would be ideal.

$^{18}$F-FDG PET/CT imaging enabled longitudinal evaluation of the combination therapy; since it allowed examining the entire tumor region noninvasively, it might be a better approach to assess tumor response than histological analyses. However, because FDG is captured in proliferating cells as well as cancer cells, it would be hard to differentiate pancreatic cancer from pancreatitis based on $^{18}$F-FDG PET imaging. The higher specificity for targeting proliferating cells might be achieved by using $^{18}$F
labeled 3'-deoxy-3'-fluorothymidine (FLT) (41), although its clinical usefulness will need to be validated using more investigations.

Acknowledgements

Authors thank Dr. Lingling Guo to insert mouse vascular port surgically, and Dr. Eben Rosenthal for study consultation. Authors also thank Lee Whitworth and Amber Martin for assistance with growing cells, in vitro assays, animal monitoring, biodistribution study, histologic analysis, and imaging.

References


### Table 1

<table>
<thead>
<tr>
<th></th>
<th>MIA PaCa-2</th>
<th>PANC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max</td>
<td>Min</td>
</tr>
<tr>
<td>Individual use</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRA-8</td>
<td>65.5±8.0</td>
<td>1.7±0.7</td>
</tr>
<tr>
<td>Anti-EMMP</td>
<td>25.3±1.6</td>
<td>7.3±0.5</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Combined use</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRA-8</td>
<td>62.0±4.5</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>Anti-EMMP</td>
<td>36.0±2.8</td>
<td>7.8±0.9</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Table 1.** The maximum and minimum signal intensities and variance per size of Cy5.5-TRA-8 and Cy3-anti-EMMPRIN antibody distributed on MIA PaCa-2 or PANC-1 cells, when the two antibodies were used individually or combinedly. *p* values for statistical comparison are given. Max = maximum signal intensity, Min = minimum signal intensity, Var/Size = variance per size, TRA-8 = Cy5.5 labeled TRA-8, Anti-EMMP = Cy3 labeled anti-EMMPRIN antibody.
Figure legends

Figure 1. *In vitro* ATP lite assay (mean and SE) to measure killing of (A) MIA PaCa-2 or (B) PANC-1 human pancreatic-cancer cells at increasing concentrations of TRA-8 alone or in combination with anti-EMMPRIN antibody.

Figure 2. *In vitro* fluorescence imaging of antibodies and image analysis. (A,B) Representative fluorescence images of Cy5.5-TRA-8 and Cy3-anti-EMMPRIN antibody with MIA PaCa-2 and PANC-1 cells, when the two antibodies were used (A) separately or (B) combined. (C-F) The fluorescence signal intensities of Cy5.5-TRA-8 and Cy3-anti-EMMPRIN antibody along the yellow lines shown in the images of (C,E) MIA PaCa-2 or (D,F) PANC-1 cells, when the two antibodies were used (C,D) separately or (E,F) combined.

Figure 3. *In vivo* DCE-MRI and image analysis. (A) Representative contrast concentration maps acquired from dynamic contrast-enhanced MR images of two animals at 0 (baseline), 5, and 40 minutes after gadoteridol injection using the same intensity scale (from 0.4 to -0.1 mM), when the two animals were untreated (control) or treated with anti-EMMPRIN antibody at 3 days prior to imaging. (B) Contrast-enhancement curves averaged in the region of interest (ROI) indicated with the white square (2x1 window: 2 pixels) shown in Fig. 3A, together with the best-fitting 5th order polynomial curves. The boundaries of the two tumor regions are indicated with dotted circles, in Fig. 3A. (C) $K_{\text{trans}}$ and (D) $k_{\text{ep}}$ maps of the tumors of the two animals untreated (control) or treated with anti-EMMPRIN antibody at 3 days prior to imaging, while the dark central area is necrosis caused by vascular insufficiency.
**Figure 4.** *In vivo* PET/CT imaging and image analysis. (A) Representative contrast enhanced (CE) CT image, $^{18}$F-FDG PET image, and fused PET/CT image of a mouse bearing a MIA PaCa-2 tumor orthotopically (axial view), while tumor location is indicated with a white arrow in each sub-figure. (B,D) Volume and (C,E) $SUV_{mean}$ changes of (B,C) MIA PaCa-2 or (C,E) PANC-1 tumors after untreated (served as control) or treated with anti-EMMPRIN antibody, TRA-8, and combination, respectively (different Greek letters represent the statistical significance among the groups).

**Figure 5.** Histological analysis of tumor response. (A) Representative microphotographs of Ki67 and CD31 staining of MIA PaCa-2 or PANC-1 tumors after untreated (served as control) or treated with anti-EMMPRIN antibody, TRA-8, and combination for 2 weeks, respectively, while the proliferating and endothelial cells are indicated with black arrows in each sub-figure. (B) Proliferating (Ki67 positive) and (D) endothelial (CD31 positive) cells densities in the four groups, while statistical differences among groups are indicated by different Greek letters (MIA PaCa-2) or alphabets (PANC-1) above bars.
FIGURE 4.
FIGURE 5.
Molecular Cancer Therapeutics

Dual combination therapy targeting DR5 and EMMPRIN in pancreatic adenocarcinoma

Hyunki Kim, Guihua Zhai, Sharon Samuel, et al.

Mol Cancer Ther Published OnlineFirst December 27, 2011.

Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-11-0581

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.