Novel tetravalent bispecific antibody, PSMA/TRAIL-R2 REGULGENT™, induces selective tumor cell apoptosis without hepatotoxicity

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Received April 14, 2025; Accepted July 22, 2025

DOI: 10.3892/or.2025.8988

Abstract. Tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 (TRAIL-R2) can induce apoptosis in various tumors through the oligomerization of TRAIL. Several TRAIL-R2 agonistic monoclonal antibodies have been tested in clinical trials but have failed owing to a lack of efficacy or severe hepatotoxicity. Although bispecific constructs have been developed to improve TRAIL-R2 targeting and enhance efficacy against tumors while reducing adverse effects on hepatocytes, the risk of hepatotoxicity still persists. The present study used a TRAIL-R2 antibody, E11, that does not trigger apoptosis in the absence of crosslinking and constructed a novel tetravalent bispecific IgG4-based antibody, REGULGENT™, comprised of E11 and a clone that binds to prostate-specific membrane antigen (PSMA), a specific marker for prostate tumors. PSMA/TRAIL-R2 REGULGENT™ selectively induced death in PSMA/TRAIL-R2 double-positive cells but not in TRAIL-R2 single-positive cells *in vitro* and *in vivo*. By contrast, a bivalent bispecific antibody did not result in tumor cell death, indicating that tetravalent bispecific antibodies have an important role in inducing tumor cell apoptosis by binding to TRAIL-R2 in a bivalent manner. Moreover, the present study demonstrated, for the first time to the best of the authors' knowledge, that PSMA/TRAIL-R2 REGULGENT™ is not hepatotoxic *in vitro* (primary human hepatocytes) or *in vivo* (chimeric human hepatocyte-transplanted PXB mouse model). This finding suggests that tetravalent bispecific therapeutics such as REGULGENT™ can be promising therapeutic agents for TRAIL-R2-positive tumors by exerting tumor-specific activity while avoiding toxicity.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DISC, death-inducing signaling complex; FGC, fast-growing colony; HMWS, high-molecular-weight species; LMWS, low-molecular-weight species; NS-EM, negative stain electron microscopy; NSCLC, non-small cell lung cancer; PSMA, prostate-specific membrane antigen; VH, variable domain of heavy chain; VL, variable domain of light chain; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PBS, phosphate-buffered saline; SPR, surface plasmon resonance; IVIG, intravenous immunoglobulin; UHP-SEC, ultra-high-pressure size exclusion chromatography

Key words: tetravalent bispecific antibody, prostate-specific membrane antigen, tumor necrosis factor-related apoptosis-inducing ligand-receptor 2, apoptosis, no hepatotoxicity

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in various cancer cells and xenograft models by activating TRAIL-receptor 1 and 2 (TRAIL-R1 and TRAIL-R2, respectively) through trimerization (1). Although a number of TRAIL-R2 agonistic antibodies and recombinant TRAILs have entered clinical trials (2) and exhibit potential cytotoxic activity against tumors, no approved therapeutics targeting TRAIL-R2 are currently available. Early TRAIL-R2 antibodies, such as tigatuzumab (3) and conatumumab (4), require secondary crosslinking mechanisms involving FcγR to exhibit antitumor activity, but they have not shown adequate efficacy in clinical trials (2). Therefore, novel antibodies that are directly activated without secondary crosslinking strategies are needed. For example, KMTR2 and TAS266 have shown good efficacy *in vitro* and *in vivo* without secondary crosslinking, but they were toxic to human hepatocytes, PXB mice and humans in clinical trials (5-7). Although several TRAIL-R2 agonistic proteins fused with tumor-targeting elements have been developed to achieve both apoptosis induction and tumor selectivity (8,9), the risk of

inducing liver damage persists. This is because these proteins are capable of inducing apoptosis without relying on secondary crosslinking, including the induction of apoptosis in nontarget tissues expressing TRAIL-R2. Bispecific antibodies, such as RG7386 (FAP/DR5) (10), BaCa (FOLR1/DR5) (11), BI 905711 (CDH17/DR5) (12) and IMV-M (MUC16/DR5) (13), which combine an antibody for tumor targeting with an antibody for TRAIL-R2, reportedly induce tumor cell apoptosis through *trans* or *cis* interactions in a FAP, FOLR1, CDH17 and MUC16 binding-dependent manner, respectively. However, information on whether these tetravalent antibodies can potentially avoid hepatotoxicity *in vitro* and *in vivo* is scarce.

Prostate-specific membrane antigen (PSMA), a transmembrane glycoprotein present on the cell surface, is highly expressed in prostate cancer (14) as well as other cancers, including non-small cell lung cancer (NSCLC) (15) and breast cancers (16,17) and in the neovascular endothelium around various tumors (18), but not in the vasculature of normal tissues. This unique PSMA expression profile makes it an attractive therapeutic target and candidate for imaging (19-21) and PSMA antibody-drug conjugates and radioisotope-labeled antibodies have been used in clinical trials for relapsed/refractory prostate cancer (22-25).

The present study developed REGULGENT™, a novel tetravalent bispecific antibody based on human IgG4, with mutations to reduce FcγR binding. It comprises a TRAIL-R2 antibody, E11, which does not trigger apoptosis independently and a PSMA antibody as the targeting element for treating PSMA-positive cancer and the neovasculature. The aim was to develop a therapeutic agent that effectively induces tumor cell death in PSMA/TRAIL-R2 double-positive cells while avoiding toxicity in normal tissues, particularly the liver.

Materials and methods

Immunohistochemistry. PSMA and TRAIL-R2 expression was analyzed using a prostate cancer tissue microarray (cat. no. 79562483; Tristar Technology Group LLC). The spots were stained using an anti-PSMA antibody (cat. no. EPR6253; Abcam) and an anti-DR5 antibody (cat. no. ab47179; Abcam). The primary antibodies were incubated for 60 min at 25˚C. For secondary detection, Histofine Simple Stain Mouse MAX-PO (R) (cat. no. 414341; Nichirei Biosciences, Inc.) was applied and incubated for 30 min at 25˚C. The stained sections were observed using a light microscope at a magnification of x200. The H-score was calculated as follows: i) The intensity score was determined based on the staining intensity of positive cells among cancer cells (0; no staining, 1; low, 2; middle, 3: high). ii) The positive occupancy rates (%) of cancer cells for each score were calculated in increments of 5%. iii) Each score was multiplied by the positive occupancy rate and the result was used as the H-score=Σ (positive occupancy rates (%) x intensity score).

Construction and expression of antibodies. The variable domains of the heavy chain (VH) and light chain (VL) regions of E11 (26), a non-agonistic antibody TRAIL-R2 clone, were used in the present study. The novel PSMA antibody clones with a VL in common with E11 were generated using phage display with an E11 VL fixed library (27). cDNA extracted from the spleen of PSMA-immunized human antibody transgenic mice (28) was used as the VH gene source in the library by isolating total RNA using ISOGEN (cat. no. 315-02504; Nippon Gene Co., Ltd.), synthesizing cDNA by reverse transcription, and amplifying VH fragments by PCR. M13 phage panning was then performed on PSMAcoated tubes to obtain clones that bound to PSMA. The PSMA-binding clones were in-frame cloned into the tetravalent bispecific antibody expression vector REGULGENT™ that included a signal peptide and human IgG4 (S228P/L235E/R409K) Fc region designed to reduce Fcγ receptor binding, thereby eliminating effector functions (29). The designed gene sequences were synthesized by Azenta Life Sciences and inserted into the expression vector using restriction enzyme digestion and ligation. The tetravalent bispecific antibody PSMA/TRAIL-R2 REGULGENT™ plasmid was generated by fusing anti-TRAIL-R2 Fab to the N-terminus of the anti-PSMA heavy chain. The bivalent bispecific antibody plasmid was constructed based on IgG1 with knobs-into-holes and Fc-silencing (L234A/L235A/G237A) mutations (10,30,31). The two plasmids were transfected into 293F or Expi293F™ (Thermo Fisher Scientific, Inc.) cells using the FreeStyle™ 293 or Expi293™ Expression Systems (cat. no. A14635; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol; briefly, cells were cultured in suspension, transfected with the supplied transfection reagent, and cultured for 5 days before harvesting. Antibodies were purified using Protein A (cat. no. 29048576; Cytiva). After eluting the purified antibodies, the buffer used to dissolve the sample was replaced with phosphate-buffered saline (PBS, cat. no. 14249-24; Nacalai Tesque, Inc.). Subsequently, the monomer fraction was separated from the antibody solution using an NGC™ Chromatography System (Bio-Rad Laboratories, Inc.) with Superdex™ High-Performance Columns (28990944; Cytiva).

Analysis of antibody purity. The purity of the prepared antibodies was determined using microfluidic chip electrophoresis (LabChip GXII Touch; PerkinElmer, Inc.) and ultra-high-pressure size exclusion chromatography (UHP-SEC). The LabChip analysis was performed using a Protein Express Assay Reagent Kit (cat. no. 760499; PerkinElmer, Inc.); all microfluidic chips were primed and prepared according to the manufacturer's protocol, the antibody samples and ladder were loaded and the run was performed on the LabChip GXII Touch system under standard settings. The non-reducing sample buffer contained N-ethylmaleimide at a final concentration of 6.36 mM and the heat treatment conditions were 70˚C for 15 min. The denatured samples were diluted with deionized water and loaded onto a primed chip and the analysis was conducted according to the manufacturer's instructions. Finally, the LabChip® GX Reviewer program (PerkinElmer, Inc.) was used to automatically calculate the size and concentration of each separated peak. The proportions of monomers, high-molecular-weight species (HMWS) and low-molecular-weight species (LMWS) were determined using UHP-SEC on an ACQUITY UPLC Protein BEH SEC Column [200 À 1.7 μm, 4.6x150 mm (Waters Corporation)]. The mobile phase contained 20 mM sodium phosphate (pH 6.8), 500 mM NaCl and 5% ethanol. The flow rate and detection wavelength were 0.4 ml/min and 215 nm, respectively. Peaks indicative of antibody protein

monomers, HMWS and LMWS were analyzed by integrating the area under each eluting peak using LabSolutions Software (Shimadzu Corporation).

Preparation of PSMA-expressing cells. Human lung adenocarcinoma (NCI-H2122; ATCC no. CRL-5985), human prostate cancer (PC-3; ATCC no. CRL-1435) and lymph node carcinoma of the prostate (LNCaP) clone fast-growing colony (FGC; ATCC no. CRL-1740) cells were seeded in RPMI 1640 medium (cat. no. 11875093; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (cat. no. 10099141; Thermo Fisher Scientific, Inc.). Human PSMA cDNA was transfected into the cell lines via lipofection using HilyMax (cat. no. H357; Dojindo Laboratories, Inc.) according to the manufacturer's instructions. The cells were incubated with the transfection mixture at 37˚C and the medium was replaced with fresh growth medium 2 days after transfection. The transfected cells were then cultured under blasticidin selection to establish stable clones, and subsequent experiments were conducted approximately 1 month after transfection. Nontransfected cells were included as a negative control. The transduced cells were detached by adding 0.25% trypsin/1 mM EDTA to the adhered cells and then counted. Thereafter, the cell suspension was diluted and seeded at a density of one cell per well containing 200 μl of drug selection medium in 96-well plates. Wells in which the cells formed a single colony were expanded. Subsequently, PSMA expression was confirmed using a FACSCanto II Flow Cytometer (BD Biosciences).

Western blot analysis. PSMA expression was confirmed using the Wes™ capillary-based automated western system (ProteinSimple) system. Cells were lysed in RIPA buffer (MilliporeSigma) at 100 μl per 10^6 cells, and the lysates were directly analyzed by loading 0.2 μl of each sample into the capillaries. The analysis was performed with the 12-230 kDa Separation Module (cat. no. SM-W001; ProteinSimple) according to the manufacturer's standard protocol. Blocking, separation, immunodetection, and signal development were carried out automatically within the system. For primary detection, an anti PSMA/GCPII (cat. no. 13163-1-AP; Proteintech Group, Inc.) was applied at a concentration of 20 μg/ml and incubated at 25˚C under instrument default settings. The anti-Rabbit Detection Module for Wes, Peggy Sue or Sally Sue (cat. no. DM-001; ProteinSimple) were used for secondary detection and for blocking and chemiluminescent visualization. An anti-β-actin antibody provided in the detection module was used as a loading control. Signal acquisition and quantification were performed using Compass software version 6.1.0 (ProteinSimple).

Biacore™ surface plasmon resonance. The antigen-binding affinities of the antibodies were measured using a Biacore™ T200 surface plasmon resonance (SPR) system (Cytiva) at 25˚C with HBS-EP+ pH 7.4 (Cytiva) as the buffer. Briefly, a Human Antibody Capture Kit (cat. no. BR100839; Cytiva) was used to immobilize the anti-human IgG onto a CM5 sensor chip (Cytiva). Next, the antibodies were injected, followed by TRAIL-R2 (cat. no. 310-19; PeproTech) and PSMA (PSA-H82Qb-25 μg; ACROBiosystems) solution in a single cycle. A dilution series of the antigens was injected for 4 min at a flow rate of 30 μl/min. The dissociation time was 400 sec. After each binding event, the surface was regenerated using 3 M $MgCl_2$. The sensorgrams were analyzed using Biacore Evaluation software (Cytiva), fitting a 1:1 binding model to determine the following binding kinetics: ka, kd and KD.

Flow cytometric analysis. Cells were suspended in PBS (cat. no. 14249-24; Nacalai Tesque, Inc.) supplemented with 5% fetal bovine serum (cat. no. 10099141; Thermo Fisher Scientific, Inc.), 1 mM EDTA and 0.1% sodium azide (staining buffer) and added to a 96-well plate. After centrifugation at 340 x g for 3 min at 4˚C, the supernatant was removed and each antibody was added to the pellet and incubated for 30 min on ice. After washing, a goat F(ab')2 anti-human IgG (γ chain-specific) R-phycoerythrin conjugate (cat. no. 2043-09; SouthernBiotech) was added and the cells were incubated for 30 min at 4˚C. After washing, the cells were suspended in a staining buffer containing 7-aminoactinomycin D staining solution (cat. no. 559925; BD Biosciences) and incubated for 10 min at 25˚C. The fluorescence intensity of each cell was measured using a FACSCanto II Flow Cytometer (BD Biosciences). Data were analyzed using FlowJo software version 9.6.4 (BD Biosciences).

Cell proliferation assay. Cells were seeded in 96-well plates and cultured overnight at 37˚C under 5% CO_2. Anti-2,4-dinitrophenol (DNP), anti-TRAIL-R2 agonistic KMTR2, anti-TRAIL-R2 E11, anti-PSMA (PN7 or PI101) and PSMA/TRAIL-R2 bispecific antibodies diluted with culture medium were added at 50 μl per well and incubated at 37˚C for 2 days. Cell Counting Kit-8 solution (cat. no. CK04; Dojindo Laboratories, Inc.) was added (10 μl per well) to the cells and the mixture was incubated for 4 h at 37˚C under 5% CO_2. Next, 0.1 M HCl (10 μl per well) was added to stop the reaction. The percent cell viability was calculated as follows: (1-([absorbance at each antibody concentration]-[absorbance of medium only])/([absorbance at an antibody concentration of 0 μg/ml]-[absorbance of medium only])) x100. Data were expressed as mean ± SE of quadruplicate experiments.

Flow cytometry apoptosis assay. Antibodies were added to PC-3 and PSMA/PC-3 cells, after which activated caspases in the cells were detected using a FAM-FLICA Poly Caspase Assay kit (cat. no. 92; ImmunoChemistry Technologies, LLC). Thereafter, the cells were suspended in a medium containing antibodies and TRAIL/Apo2 ligand (1 μg/ml) and incubated at 37˚C for 6 h. After centrifugation at 340 x g for 3 min at 4˚C, the supernatant was removed, 50 μl/ml of FLICA supplied with the kit (diluted in medium) were added and the mixture was incubated at 37˚C for 30 min at 4˚C. After incubation, the cells were washed four times with the apoptosis wash buffer provided with the kit and suspended in 100 μl (per well) of apoptosis wash buffer, after which the fluorescence intensity of each cell was measured using a FACSCanto II Flow Cytometer (BD Biosciences). Data were analyzed using FlowJo software version 9.6.4 (BD Biosciences). The apoptotic rate was calculated as the percentage of FAMFLICA-positive cells (early and late apoptotic cells combined) among the total cell population.

Effect of crosslinker and intravenous immunoglobulin (IVIG) on tumor cell death. PSMA/PC-3 cells were seeded in 96-well plates and incubated overnight at 37˚C to attach to the well. The anti-TRAIL-R2 antibody, apomab (32,33) and PSMA/TRAIL-R2 REGULGENT™ were added at final concentrations of 1 μg/ml each. Antibody crosslinkers [anti-human IgG (γ-chain-specific) antibody; cat. no. I3382; MilliporeSigma] were added to the wells and the cells were cultured at 37˚C for 3 days. Thereafter, Cell Counting Kit-8 solution was added and the mixture was incubated for 4 h to determine cell viability. To examine the effect of IVIG on cell death, it was applied before apomab and PSMA/TRAIL-R2 REGULGENT™ were added to the wells.

Mouse xenograft model. SCID mice were purchased from CLEA Japan, Inc. A total of 80 mice were used in the present study. The mice were housed under specific pathogenfree conditions at 23±3˚C with a 12-h light/dark cycle and 50±20% relative humidity. The body weight of the mice at the start of the experiment ranged from 19-24 g. A xenograft mouse model was developed by injecting NCI-H2122 and PSMA/NCI-H2122 transfectant cells ($5x10^6$) subcutaneously into the dorsal flank of 6-week-old male SCID mice. Tumor volume was measured twice a week using the following formula: (length x width^2)/2. When the mean tumor volume reached 100 mm^3 after 7 days, the mice were randomly assigned to treatment groups using a computer-generated randomization method to ensure unbiased distribution. Each group consisted of five mice. Drug administration was initiated with the following treatments: anti-DNP, anti-TRAIL-R2 agonistic antibody KMTR2 and PSMA/TRAIL-R2 REGULGENT™, diluted in saline containing 0.05 mg/ml Polysorbate 80, administered intravenously through the tail vein at doses of 5 ml/kg once a week. After treatment, tumor volume was measured twice a week to verify efficacy. Data are expressed as mean ± SE. Tumor volumes and animal body weights were measured bi-weekly. The experiments were terminated 2 weeks after the start of treatment. No animal reached the predetermined humane endpoint. Mice were anesthetized by inhalation of 3% isoflurane for ~10 min. The depth of anesthesia was confirmed by assessing reflex responses and respiratory patterns to ensure adequate anesthetic depth. Subsequently, the mice were euthanized by cervical dislocation. The mortality of the experimental animals was verified by the cessation of respiration and heartbeat.

Pharmacokinetic profile analysis. The pharmacokinetic profile of REGULGENT™ was assessed in 6-week-old male SCID mice after a single tail vein injection of 1 or 10 mg/kg of anti-TRAIL-R2 antibody E11, anti-PSMA antibody PI101, or PSMA/TRAIL-R2 REGULGENT™ (PI101/E11). Blood samples were collected at 1, 6, 24, 48, 120 and 168 h post-dose. Serum antibody concentrations were measured by electrochemiluminescence immunoassay using streptavidin-coated plates blocked with PBS containing 1% casein. Biotinylated anti-human antibodies, calibration standards, quality control samples and test samples were incubated in duplicate, followed by HRP-labeled anti-human IgG and ruthenium-labeled anti-HRP detection reagents. Luminescence was read on a SECTOR Imager 2400 (Meso Scale Diagnostics, LLC). Between steps, plates were washed with PBS containing 1% Tween-20.

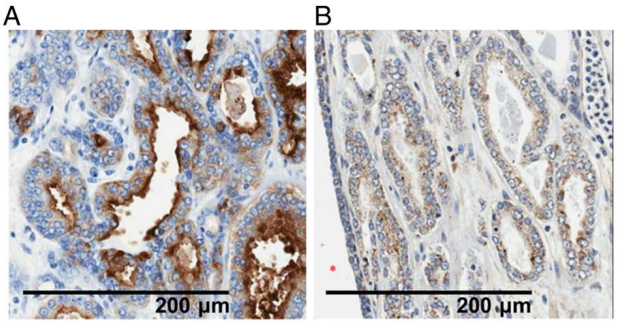
In vitro hepatocyte toxicity assay. Human hepatocytes from six donors (cat. no. M00995-P; BioIVT) were seeded in collagen-coated 96-well plates and cultured overnight at 37˚C under 5% CO_2. The anti-DNP antibody, anti-TRAIL-R2 agonistic antibody KMTR2 and PSMA/TRAIL-R2 REGULGENT™, diluted in culture medium, were added at 50 μl per well and incubated at 37˚C for 4 days. Cell Counting Kit-8 solution was added at 10 μl per well and the plates were incubated for 4 h at 37˚C under 5% CO_2. Next, 0.1 M HCl (10 μl per well) was added to stop the reaction. The percent cell death was calculated as follows: (1-([absorbance at each antibody concentration]-[absorbance of medium only])/([absorbance at an antibody concentration of 0 μg/ml]-[absorbance of medium only])) x100. Data were expressed as mean ± SE of triplicate experiments.

In vivo liver toxicity. Chimeric PXB mice with a humanized liver were purchased from PhoenixBio Co., Ltd. Chimeric PXB mice with a humanized liver were purchased from PhoenixBio Co., Ltd. A total of 16 mice were used in this study. The mice were housed under specific pathogenfree conditions at 23±5˚C with a 12-h light/dark cycle and 55±25% relative humidity. All animals were male, 12-18 weeks old at the start of the study and weighed 18-23 g. The mice were produced by xeno-transplanting human hepatocytes into immunodeficient recipient cDNA-uPA+/-/SCID mice. The PXB mice were assigned randomly to four groups (n=4 per group) and injected intravenously with vehicle, anti-TRAIL-R2 agonistic antibody KMTR2 (1 mg/kg), or PSMA/TRAIL-R2 REGULGENT™ (1 or 10 mg/kg) and monitored for 1 week. Individual body weights were recorded once daily before blood sampling on Day 1, before dosing on Day 1 and before blood sampling on Days 3 and 8. After the completion of serial blood sampling for Day 8, mice were anesthetized by inhalation of 3% isoflurane for ~10 min for induction, followed by maintenance with 2.5% isoflurane. The depth of anesthesia was confirmed by assessing reflex responses and respiratory patterns to ensure adequate anesthetic depth. Subsequently, both the abdominal cavity and the thoracic cavity were opened to access the heart for the terminal blood sampling. The blood was collected from each animal via the heart using disposable needles after which the animals were euthanized by exsanguination. The serum was diluted with saline by a factor of 2.5. The serum human alanine aminotransferase (ALT) concentration was determined using a Human ALT ELISA Kit (cat. no. ab234578; Abcam) (34). Serum ALT/ Aspartate aminotransferase (AST) activities were determined by measuring diaryl imidazole type of leucopigment. A DRI CHEM 7000 (Fujifilm) analyzer was used for these measurements.

Negative stain electron microscopy (NS-EM). For preparation of the antigen–antibody complexes, PSMA/TRAILR2 REGULGENT™, TRAIL/Apo2 ligand, and soluble PSMA were mixed at equal protein amounts to achieve a final concentration of 1 mg/ml and incubated at 4˚C for 16 ho to allow complex formation. Antigenantibody complex fractions were then separated using Superdex™ High-Performance Columns

(Cytiva). The antigen-antibody complexes were diluted to 0.1 μg/ml in phosphate-buffered saline and crosslinked with 0.1% glutaraldehyde at 4˚C for 4 h. Thereafter, sample particles were adsorbed on to a carbon foil and stained with Nano-W® containing methylamine tungstate (Nanoprobes) and the sample grids were imaged using a TF20 microscope (FEI; Thermo Fisher Scientific, Inc.). The particles were selected semi-automatically using COW (https://www.cow-em.de/) and were used for 2D classification using RELION (https://www2.mrc-lmb.cam.ac.uk/relion/index.php/Main_Page).

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 8 (Dotmatics). To evaluate the effect of cross-linker on cytotoxic activity, data were analyzed using one-way ANOVA followed by Dunnett's post hoc test. To assess the combined effects of cross-linker and IVIG on cytotoxic activity, two-way ANOVA with Bonferroni's post hoc correction was applied. For all other analyses in which statistical significance is indicated, repeated-measures one-way ANOVA followed by Tukey's multiple comparisons test was used. $P<0.05$ was considered to indicate a statistically significant difference.



C

H-Score	PSMA 127 (HR+; 19) spots	TRAIL-R2 127 (HR+; 19) spots
0	9(2)	6(1)
1-99	18(2)	27(5)
100-199	15(2)	50(7)
200-300	85(13)	44(6)

D

	PSMA	TRAIL-R2
Overall positive rate (%)	92.9	95.3
HR+ spot positive rate (%)	89.5	94.7

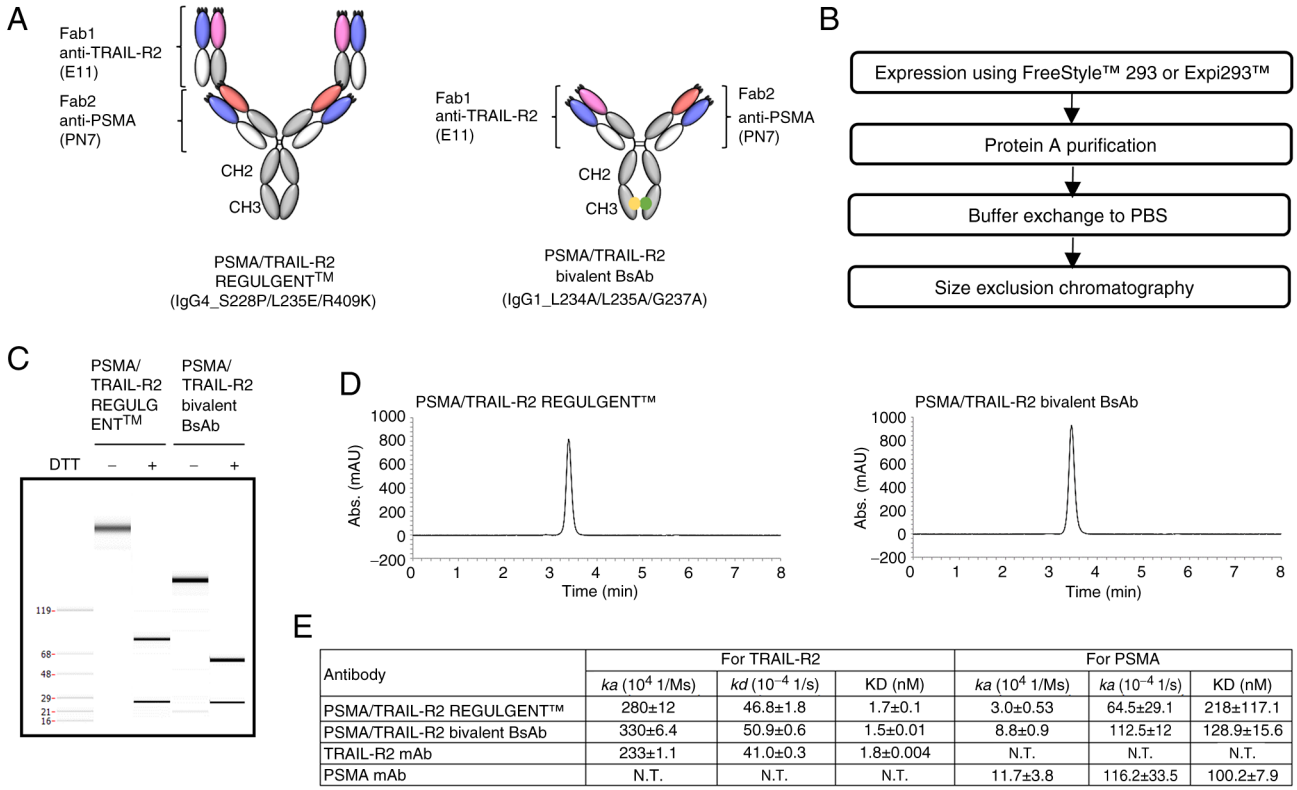
Figure 1. PSMA and TRAIL-R2 expression in patients with prostate cancer. Representative staining of prostate cancer tissue microarrays. (A) PSMA expression and (B) TRAIL-R expression. The H scores were calculated based on percent positivity and intensity score for (C) all spots and (D) spots in hormone-resistant prostate cancers. The red asterisk represents a normal gland/duct. PSMA, prostate-specific membrane antigen; TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand-receptor 2.

Results

PSMA and TRAIL-R2 expression in patients with prostate cancer. The present study first investigated the expression of PSMA and TRAIL-R2 in human prostate cancer, including hormone-resistant cancer, using a tissue microarray. The H-score was calculated as described in the *Materials and methods*. The results showed that >90% of prostate cancers that were tested were positive for both PSMA and TRAIL-R2 (Fig. 1A and B). In addition, ~90% of the samples from hormone-resistant cancers were positive for both PSMA and TRAIL-R2. Collectively, double positivity for PSMA and TRAIL-R2 was present in almost all human prostate cancers, including hormone-resistant cancers ((Fig. 1C and D).

Constructing PSMA/TRAIL-R2 bispecific antibodies. Agonistic TRAIL-R2 antibodies are known to induce apoptotic cell death through peripheral blood mononuclear cells expressing FcγR. However, this poses some adverse risks to normal cells, such as hepatocytes, which also express TRAIL-R2 (26,35). Therefore, a non-agonistic TRAIL-R2 antibody, E11, was selected to construct bispecific antibodies. Additionally, mispairing of antibody light chains can occur if bispecific antibodies are generated using two different antibody clones. The present study used a novel tetravalent and symmetric bispecific antibody, namely REGULGENT™, comprised of a fused Fab in the N-terminus of the IgG4 heavy chain and a common light chain (Fig. 2A, left) (36). The present study successfully constructed some PSMA antibody clones that shared a light chain with the TRAIL-R2 antibody E11 and they were generated via phage display using an E11 VL fixed library. For comparison with PSMA/TRAIL-R2 REGULGENT™, a bivalent bispecific antibody was constructed with a common light chain and IgG1 Fc mutations lacking effector function (Fig. 2A, right) (30). Based on previous reports (10), the present study selected effector function-null IgG variants of IgG4 and IgG1 Fc regions to eliminate nonspecific TRAIL-R2 signaling mediated by Fc-dependent mechanisms. This approach allowed the present study to specifically evaluate activity independent of ADCC against PSMA-expressing cells. Both bispecific antibodies were produced using Expi293F™ or HEK293 cells (Fig. 2B) and their purity was analyzed using LabChip and SEC (Fig. 2C and D). The purity was 97.4% for PSMA/TRAIL-R2 REGULGENT™ and 99.3% for the bivalent bispecific antibody. Subsequently, the PSMA/TRAIL-R2 binding of these bispecific antibodies was evaluated using BIAcore SPR (Fig. 2E). The results indicated that both bispecific antibodies bound to both PSMA and TRAIL-R2, with no marked difference in the binding levels.

Superior tumor cell death-inducing effect of PSMA/TRAIL-R2 REGULGENT™ over the monovalent heterodimeric bispecific antibody. To investigate the biological activities of the bispecific antibodies, the present study evaluated their abilities to induce cell death in some tumor cell lines. First, PSMA-overexpressing variants of the prostate cancer cell line PC-3 and the human lung adenocarcinoma cell line NCI-H2122 were generated, termed PSMA/PC-3 and PSMA/NCI-H2122, respectively. PSMA expression was confirmed by western blot analysis (Fig. S1). Next, PSMA/TRAIL-R2 expression in PC-3 and PSMA/PC-3 cells was verified using flow cytometry (Fig. 3A). The super-agonistic TRAIL-R2 antibody, KMTR2, induced death in both PC-3 and PSMA/PC-3 cells (Fig. 3B and C), whereas the non-agonistic TRAIL-R2 antibody, E11 and PSMA antibody, PN7, did not elicit any cell death. By contrast, PSMA/TRAIL-R2 REGULGENT™ selectively induced death in PSMA/PC-3 but not in PC-3 cells, with a potency superior to that of the KMTR2 antibody. The efficacy of tumor cell death was the same even if another PSMA antibody, clone PI101, was used in REGULGENT™ instead of PN7 (Fig. S2). These findings implied PSMA-dependent biological activity of PSMA/TRAIL-R2 REGULGENT™.



E

Antibody	For TRAIL-R2			For PSMA		
	ka (10^4 1/Ms)	*kd* (10^{-4} 1/s)	KD (nM)	*ka* (10^4 1/Ms)	*ka* (10^{-4} 1/s)	KD (nM)
PSMA/TRAIL-R2 REGULGENT™	280±12	46.8±1.8	1.7±0.1	3.0±0.53	64.5±29.1	218±117.1
PSMA/TRAIL-R2 bivalent BsAb	330±6.4	50.9±0.6	1.5±0.01	8.8±0.9	112.5±12	128.9±15.6
TRAIL-R2 mAb	233±1.1	41.0±0.3	1.8±0.004	N.T.	N.T.	N.T.
PSMA mAb	N.T.	N.T.	N.T.	11.7±3.8	116.2±33.5	100.2±7.9

Figure 2. Design and production of prostate-specific membrane antigen and tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 (PSMA/TRAIL-R2) bispecific antibodies. (A) Schematic of the Fab-based tetravalent bispecific antibody (REGULGENT™) and bivalent bispecific antibody designs. (B) Flowchart of expression and purification of REGULGENT™ and bivalent bispecific antibody. Analytical data of REGULGENT™ and bivalent bispecific antibody after expression in 293F cells and a single-step protein A purification using (C) LabChip and (D) size exclusion chromatography. (E) PSMA or TRAIL-R2 binding of generated antibodies to recombinant proteins as analyzed by Biacore surface plasmon resonance. PSMA, prostate-specific membrane antigen; TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand-receptor 2.

Notably, the bivalent bispecific antibody did not induce death in PSMA/PC-3 cells. Comparable results were obtained for NCI-H2122 cells, PSMA/NCI-H2122 and the LNCaP clone FGC (Fig. 3D-H).

To confirm that the cell death induced by PSMA/TRAIL-R2 REGULGENT™ was apoptotic, activated caspase levels were evaluated using flow cytometry (Fig. 3I). The results showed that PSMA/TRAIL-R2 REGULGENT™ activated caspases only in PSMA/PC-3 cells, suggesting PSMA-dependent apoptotic induction.

Effect of IVIG and crosslinkers on PSMA/TRAIL-R2 REGULGENT™ activity. The first-generation anti-TRAIL-R2 antibody, apomab, requires a crosslinker to induce apoptosis. Apomab markedly decreased cell viability in a crosslinker dose-dependent manner, whereas PSMA/TRAIL-R2 REGULGENTTM slightly increased cell viability at high crosslinker concentrations (Fig. 4A). To examine the effects of these antibodies on cell death, IVIG was used to mimic the *in vivo* environment. The efficacy of Apomab was markedly inhibited by IVIG in a dose-dependent manner. On the other hand, PSMA/TRAIL-R2 REGULGENTTM did not markedly affect its activity in the presence of IVIG (Fig. 4B).

PSMA-dependent in vivo antitumor activity of PSMA/TRAIL-R2 REGULGENT™. To assess whether PSMA/TRAIL-R2 REGULGENT™ exhibited a selective antitumor effect on PSMA-expressing tumors *in vivo*, NCI-H2122 and PSMA/NCI-H2122 cells were implanted subcutaneously into SCID mice (Fig. 5A). Although images of the excised xenograft tumor were not captured, the tumor was visually confirmed to be normal in volume and size. The maximum diameter of the tumor in all mice was 17.9 mm and the maximum volume was 1,195 mm^3. KMTR2 showed a strong antitumor effect in both NCI-H2122- and PSMA/NCI-H2122-bearing mice (Fig. 5B, C and Tables SI-SVI). By contrast, PSMA/TRAIL-R2 REGULGENT™ showed a specific and significant antitumor effect in PSMA/NCI H2122- but not in NCI-H2122-bearing mice. The pharmacokinetic profile of PSMA/TRAIL-R2 REGULGENT™ in wild-type mice was favorable compared with that of monoclonal antibodies (Fig. S3). These results indicated that PSMA/TRAIL-R2 REGULGENT™ exerted a potent antitumor effect in a PSMA-dependent manner *in vivo*.

PSMA/TRAIL-R2 REGULGENT™ does not cause injury to human hepatocytes in vitro or liver toxicity to PXB mice in vivo. Following previous reports, the present study evaluated the toxicity in human hepatocytes and chimeric livers derived from PBX mice, which exhibit higher sensitivity to TRAIL-mediated apoptosis compared to hepatocytes from rodents and non-human primates (6). Therefore, human hepatocytes and PXB mice were used to evaluate whether PSMA/TRAIL-R2 REGULGENT™ exerts liver toxicity. Human hepatocytes did not express PSMA but slightly

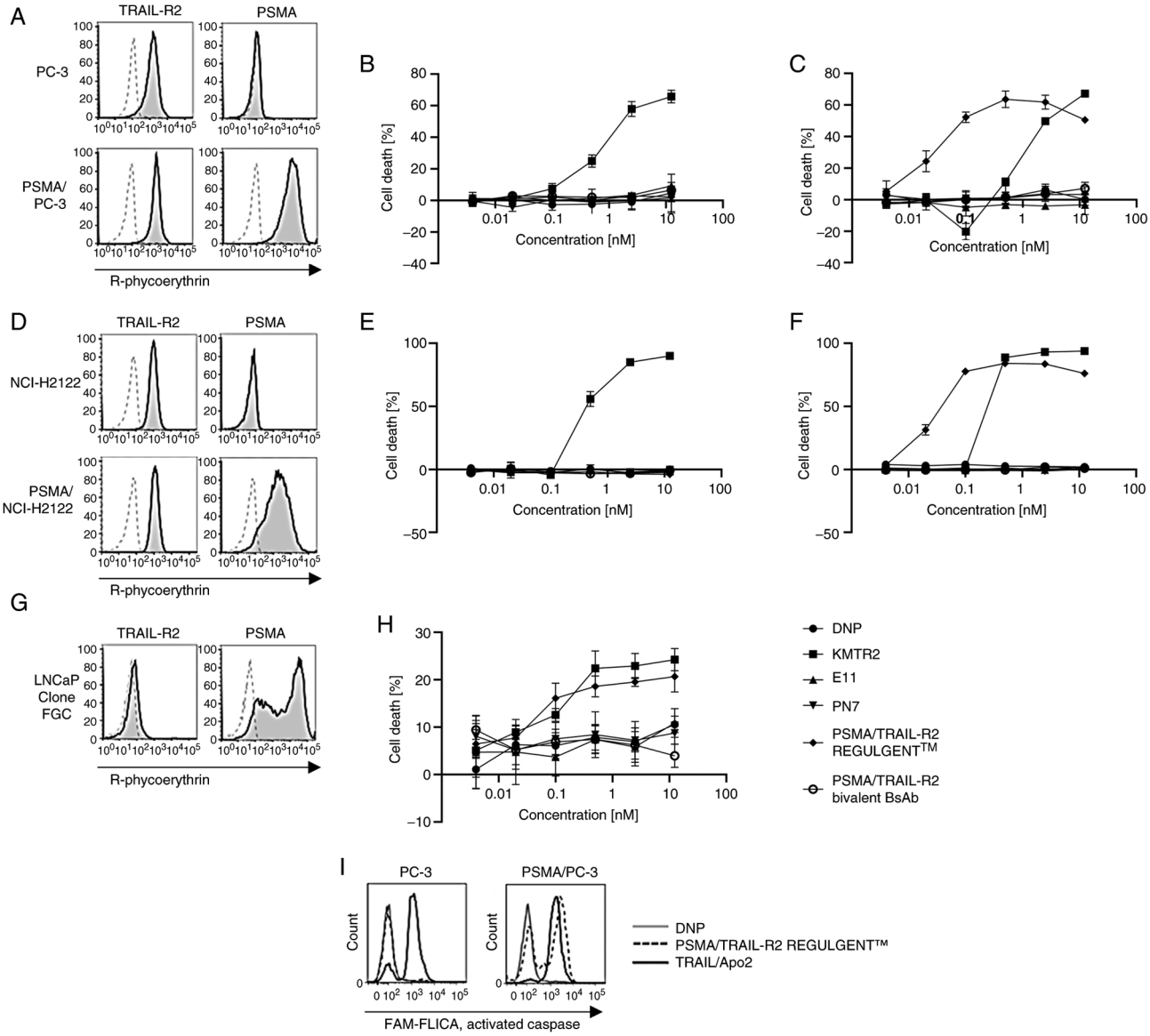


Figure 3. Cancer cell death induced by prostate-specific membrane antigen and tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 (PSMA/TRAIL-R2) bispecific antibodies. (A, D and G) Evaluation of PSMA and TRAIL-R2 expression in various cell lines using flow cytometry. (B, C, E, F and H) Viability of human tumor cell lines in response to treatment with PSMA/TRAIL-R2 bispecific antibodies (REGULGENT™ and bivalent bispecific antibody) in a 96-well cell proliferation assay using the (B) PC-3 and (E) NCI-H2122 cell lines (PSMA$^-$TRAIL-R$^+$), (C) PSMA/PC-3 and (F) a PSMA/NCI-H2122 transfectants and (H) the LNCaP clone FGC cell line (PSMA$^+$TRAIL-R$^+$). Antibodies were tested in fivefold dilutions starting at 12.5 nM to 0.004 nM. Data represent quadruplicate experiments and are shown as means ± SE. The vertical axes represent cell death (%) and the horizontal axes represent the antibody concentration (nM). An anti-DNP antibody was used as the negative control. (I) Caspase activation in PC-3 or PSMA/PC-3 cells by the TRAIL/Apo2 ligand and PSMA/TRAIL-R2 REGULGENT™ using the FAM-FLICA Poly Caspase Assay. The solid line shows caspase activation by the TRAIL/Apo2 ligand, the dotted line shows caspase activation by TRAIL-R2/PSMA REGULGENT™ and the gray line shows caspase activation by the anti-DNP antibody used as the negative control. PSMA, prostate-specific membrane antigen; TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand-receptor 2; DNP, 2,4-dinitrophenol.

expressed TRAIL-R2 on their cell surface (Fig. 6A). KMTR2 induced cell death in human hepatocytes whereas PSMA/TRAIL-R2 REGULGENT™ did not (Fig. 6B). *In vitro* toxicity evaluations were performed using hepatocytes from multiple donors and PSMA/TRAIL-R2 REGULGENT™ did not induce cell death in any of the hepatocytes tested (Fig. 6C).

Subsequently, PXB mice were administered a single dose of KMTR2 or PSMA/TRAIL-R2 REGULGENT™ intravenously to assess liver toxicity (Fig. 6D). Compared with vehicle-treated mice, KMTR2-treated mice displayed statistically significant increase in serum human ALT1 level, an indicator of liver toxicity (Fig. 6E). By contrast, no significant changes in the serum human ALT1 levels were observed in mice administered 1 or 10 mg/kg of TRAIL-R2/PSMA REGULGENT™ (Fig. 6E). Previous studies have reported that human ALT is the optimal indicator of hepatotoxicity in PXB mice (34). In addition to human ALT1, the present study also evaluated the activities of AST and ALT. Significant increases in these values were observed in mice administered KMTR2, whereas no elevations were detected in mice treated with PSMA/TRAIL-R2 REGULGENT™ (Fig. S4). Moreover, the body weights of mice did not differ markedly

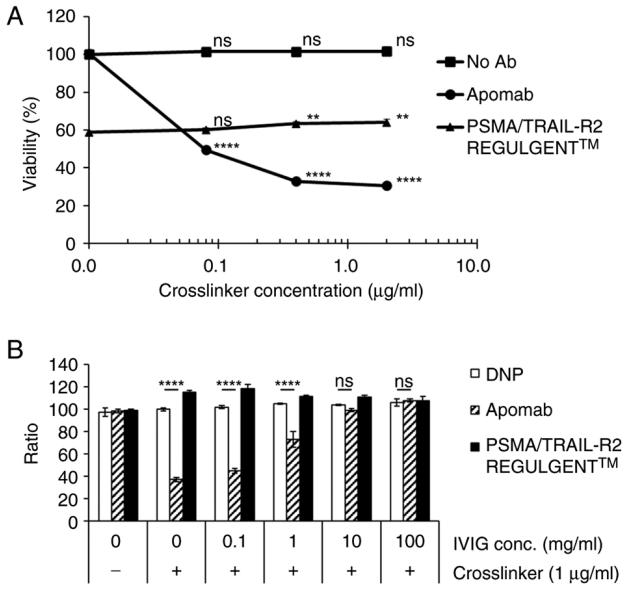


Figure 4. Effect of crosslinker and IVIG on tumor cell death induced by the prostate-specific membrane antigen and the tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 (PSMA/TRAIL-R2) bispecific antibody (REGULGENT™). (A) PSMA/PC-3 cells were seeded onto 96 well plates. Then apomab and PSMA/TRAIL-R2 REGULGENT™ were added and cultured with crosslinkers for 3 days. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test to compare each treatment group at a given concentration with the corresponding no-antibody (no Ab) control group. **P<0.01, ****P<0.0001, ns, no significance. (B) IVIG affected the efficacy of crosslinked apomab and PSMA/TRAIL-R2 REGULGENT™ in a concentration-dependent manner. Statistical analysis was performed using twowayANOVA with Bonferroni post hoc test. ****P<0.0001, ns, no significance. IVIG, intravenous immunoglobulin; PSMA, prostate-specific membrane antigen; TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand-receptor 2; DNP, 2,4-dinitrophenol.

among the treatment groups (data not shown). These results suggested that PSMA/TRAIL-R2 REGULGENT™ does not exhibit hepatotoxic effects in PXB mice when administered intravenously as a single dose of up to 10 mg/kg. By contrast, KMTR2 showed hepatotoxic effects following a single intravenous administration at 1 mg/kg.

Discussion

The present study reported the preclinical antitumor activity of PSMA/TRAIL-R2 REGULGENT™, a novel tetravalent bispecific antibody with common light chains. PSMA/TRAIL-R2 REGULGENT™ binds to PSMA as an anchor to induce TRAIL-R2 activation, leading to tumor cell apoptosis in a PSMA-dependent manner.

Consistent with the findings of a previous study (14), the present study confirmed that PSMA and TRAIL-R2 are expressed in patients with prostate cancer. This finding suggested that PSMA/TRAIL-R2 REGULGENT™ could serve as a therapeutic agent for PSMA/TRAIL-R2 double-positive cancer. Furthermore, PSMA is expressed in NSCLC and breast cancers, suggesting that PSMA/TRAIL-R2 REGULGENT™ could be effective against cancers other than prostate cancer. The expression of PSMA in breast cancers is related to tumor subtype and malignancy, exhibiting heterogeneity (37). Therefore, stratifying patients by types or subtypes of cancer with high PSMA positivity could yield more effective targets. In addition, PSMA/TRAIL-R2 REGULGENT™ may have applications across a broad spectrum of tumors owing to the unique expression of PSMA in the tumor neovasculature.

The present study demonstrated that PSMA/TRAIL-R2 REGULGENT™ exhibited high tumor cell death activity in a PSMA binding-dependent manner without any secondary crosslinkers both *in vitro* and *in vivo*. Additionally, the apoptotic activity remained unaffected in the presence of high IgG levels, suggesting that this bispecific antibody effectively induced tumor cell death *in vivo*. BI 905711, a tetravalent bispecific CDH17/TRAIL-R2 antibody, induces apoptosis specifically in CDH17-positive tumor cells, similar to PSMA/TRAIL-R2 REGULGENT™ (12). However, information on its hepatocytotoxicity is limited, although no cytotoxicity was observed in HepG2 human hepatocellular carcinoma cells (12). The present study detected human ALT1 in the serum of KMTR2-treated PXB mice as reported previously (6), implying that cytotoxic activity against human hepatocytes can be observed directly. Nevertheless, no elevation in the serum human ALT1 level was detected in PSMA/TRAIL-R2 REGULGENT™-treated PXB mice. Thus, it was demonstrated, for the first time to the best of the authors' knowledge, that PSMA/TRAIL-R2 REGULGENT™ lacks hepatotoxicity, based on studies with primary hepatocytes and PXB chimeric mice with humanized livers.

Currently, some biologics (IGM-8444, ABBV-621, INBRX-109 and BI 905711) are being investigated in clinical trials (NCT04553692, NCT04570631 and NCT04137289/NCT05087992, respectively). The first three are TRAIL-R2 antibodies without cancer-targeting molecules, making it difficult to avoid hepatotoxicity. Therefore, their doses should be reduced to lower adverse effects. Cancer-targeting molecules are designed to mitigate hepatotoxicity, as mentioned previously. Recently, Phase 1 data of BI 905711 were released in ASCO2023 (NCT04137289) (38). No dose-limiting toxicities were observed and the maximum tolerated dose was not reached. In heavily pretreated patients, it showed a tolerable safety profile. AST level increased in only 2 of 43 patients, suggesting that the expected mechanism of the tetravalent bispecific antibody, using a TRAIL-R2 non-agonistic antibody clone, might effectively suppress hepatotoxicity when targeted to tumor cells.

Although apoptosis induction by TRAIL-R2 effectively kills tumor cells, not all tumors are sensitive to the TRAIL-R signaling pathway. Molecules that inhibit TRAIL-R signaling have been reported, including cellular FLICE-like inhibitory protein, which competes with caspase-8 for FADD binding, as well as XIAP, cIAp-1 and cIAP-2, which block active caspases (39). In addition, combining TRAIL with small molecules targeting inhibitory molecules against TRAIL-R signaling reportedly induces TRAIL sensitization (40). The present study observed a tendency for c-FLIP to be inversely associated with TRAIL-R2 signaling (data not shown), but no information is available on whether c-FLIP inhibitors synergistically affect PSMA/TRAIL-R2 REGULGENT™-induced tumor cell death. Further analyses will be required to determine which cancers are sensitive to TRAIL-R2 signaling.

Several agents and chemotherapeutic drugs, including paclitaxel, doxorubicin and camptothecin, enhance TRAIL-induced apoptosis in prostate cancer cell lines

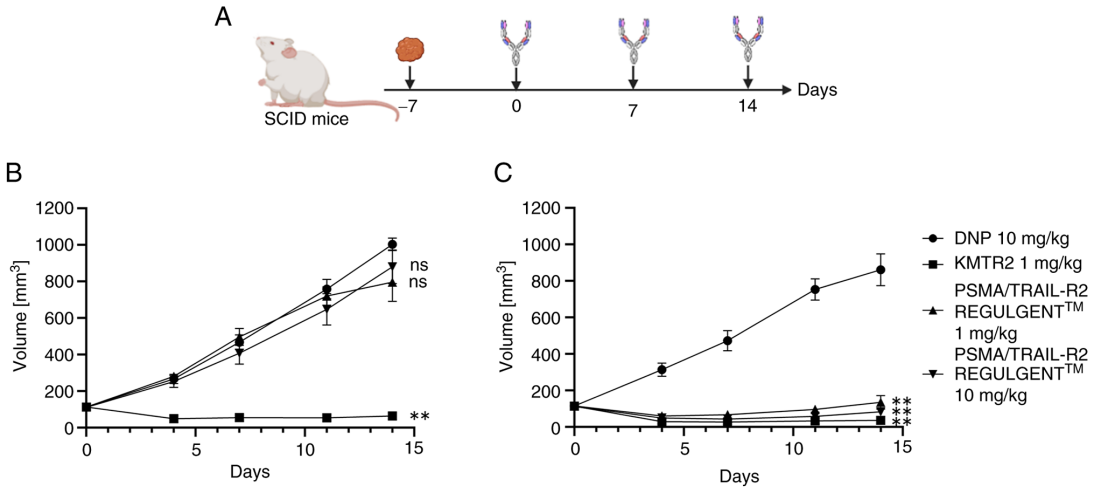


Figure 5. *In vivo* PSMA-dependent antitumor activity of the PSMA/TRAIL-R2 bispecific antibody (REGULGENT™). (A) Schedule of antibody administration and tumor measurement in a xenograft model. (B) NCI-H2122 and (C) PSMA/NCI-H2122 transfectant cells were transplanted subcutaneously into SCID mice. The mice were grouped 7 days after transplantation to start receiving the anti-2,4-dinitrophenol (DNP) antibody, anti-TRAIL-R2 agonistic antibody KMTR2, or PSMA/TRAIL-R2 REGULGENT™. The vertical axes indicate tumor size (mm^3) and the horizontal axes indicate the time (days) after the start of antibody administration. Plotted values are the means (SEM) of duplicates of four independent experiments. To determine the statistical significance, an RM one-way ANOVA with Tukey's multiple comparisons was performed (**P<0.01, ns, no significance). PSMA, prostate-specific membrane antigen; TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand-receptor 2; DNP, 2,4-dinitrophenol.

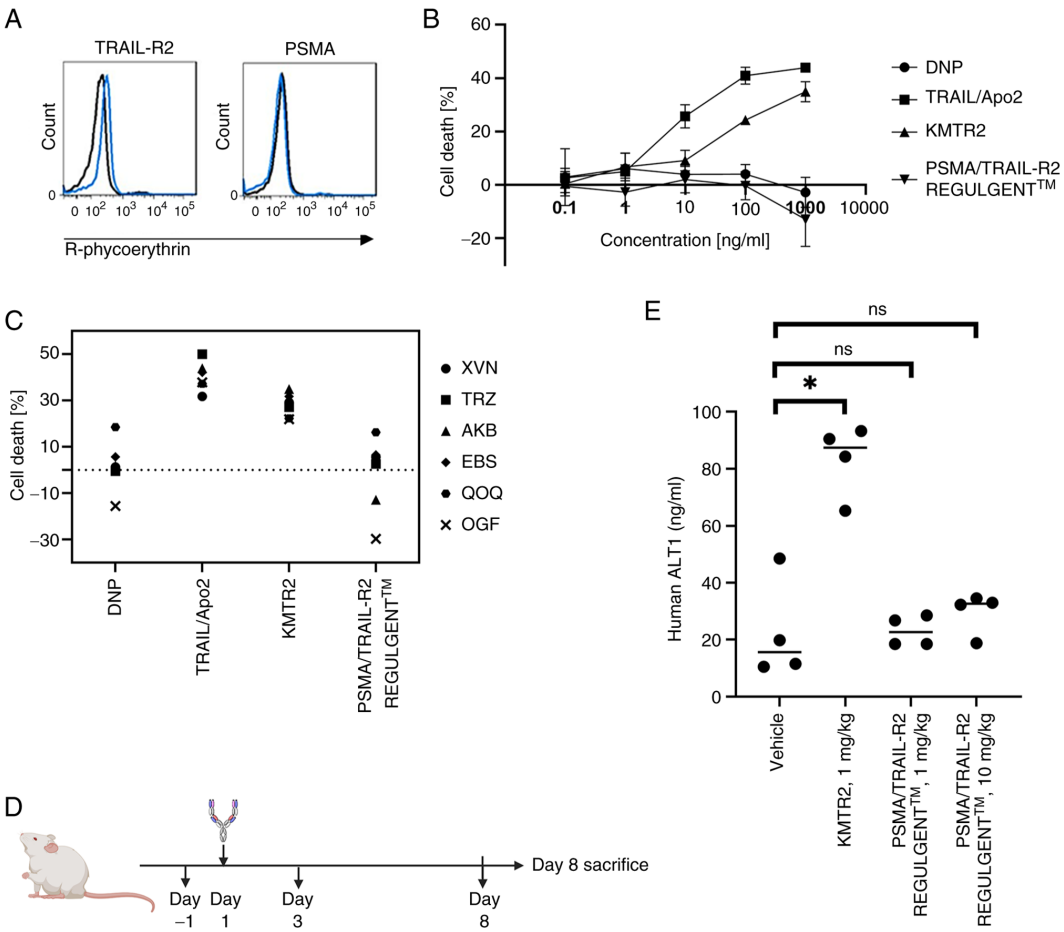


Figure 6. *In vitro*/*in vivo* liver toxicity of prostate-specific membrane antigen and tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 (PSMA/TRAIL-R2) bispecific antibodies. (A) PSMA and TRAIL-R2 expression in hepatocytes was determined using flow cytometry. (B) Hepatocytes were treated with serially diluted antibodies anti-DNP, anti-TRAIL-R2 agonistic KMTR2, anti-TRAIL-R2 non-agonistic E11, or PSMA/TRAIL-R2 REGULGENT™) and incubated for 96 h. Cell viability was determined using Cell Counting Kit-8. (C) Antibody-induced toxicity in human primary hepatocytes from six donors (lot numbers: XVN, TRZ, AKB, EBS, QOQ and OGF). (D) Schedule of *in vivo* safety assessment using PXB mice. (E) Serum human ALT1 concentrations in response to anti-TRAIL-R2 agonistic antibody KMTR2 and PSMA/TRAIL-R2 REGULGENT™ treatment. *P<0.05, ns, no significance. PSMA, prostate-specific membrane antigen; TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand-receptor 2; DNP, 2,4-dinitrophenol; ALT, alanine aminotransferase.

through TRAIL-R2 upregulation. Neferine treatment also enhances the TRAIL-induced apoptosis of human prostate cancer cells via autophagic flux and the c-Jun N-terminal kinase pathway (41). Bortezomib-mediated TRAIL sensitization was facilitated by enhanced formation of the TRAIL death-inducing signaling complex (DISC) and c-FLIP downregulation in the DISC (42-44). The combination of TRAIL with SMAC or BH3 mimetics has been evaluated in various cancers (45,46). CDK9 inhibitors induce tumor cell death in TRAIL-resistant NSCLC cell lines through downregulation of Mcl-1 and c-FLIP expression (47,48). Additionally, elastin and RSL3 might influence TRAIL-R2 sensitivity owing to the accumulation of ROS produced by mitochondria during elastin- and RSL3-induced ferroptosis (49), which purportedly regulates ROS production and affects TRAIL-R2 sensitivity (50). Collectively, these findings suggest that chemotherapeutic drugs might enhance tumor cell death induced by PSMA/TRAIL-R2 REGULGENT™. In the future, it is necessary to screen for agents that exhibit a strong synergistic effect with PSMA/TRAIL-R2 REGULGENT™, tailored to the specific type of cancer being targeted and consider their combined use.

Previous studies have shown that diverse clones within bivalent bispecific antibodies can effectively minimize off-target toxicity (51). By contrast, the current findings indicate that the bivalent bispecific antibody did not demonstrate any tumor cell death-inducing activity, with only the tetravalent format of PSMA/TRAIL-R2 REGULGENT™ exhibiting such activity. For an improved understanding of this difference, the structure of PSMA/TRAIL-R2 REGULGENT™, TRAIL-R2 and the PSMA trimer complex was observed using NS-EM. When the trimeric complex structures of PSMA/TRAIL-R2 REGULGENT™, TRAIL-R2 and PSMA were observed using NS-EM, a structure showing three TRAIL-R2 molecules in proximity was detected (Fig. S5). This may explain why REGULGENT™ induces apoptotic signals more effectively than bivalent bispecific antibodies. In this structure, three PSMA/TRAIL-R2 REGULGENT™ molecules bind through a dimeric PSMA, leading to the aggregation of TRAIL-R2. However, in the bivalent format, aggregation beyond the trimerization of REGULGENT and TRAIL-R2 is not feasible. However, regarding TRAIL-R2, the bivalent format may not allow for the aggregation of REGULGENT and TRAIL-R2 beyond trimerization, potentially impairing signal induction. PSMA/TRAIL-R2 REGULGENT™ slightly decreased cell death at high crosslinker concentrations, assuming that the decrease occurred as a result that PSMA/TRAIL-R2 REGULGENT™ was randomly associated by crossliner. As the trimerization/multimerization of TRAIL-R2 is critical for the induction of apoptosis signaling pathways, it is plausible that the tetravalent format of PSMA/TRAIL-R2 REGULGENT™ was able to efficiently transmit cell death-inducing signals. Moreover, the present study demonstrated that a tetravalent bispecific antibody using a TRAIL-R2 non-agonistic antibody (or a simple binder) was not hepatotoxic. The authors are currently conducting clinical trials to apply this format to different tetravalent bispecific antibodies (NCT06248411, NCT06266299).

In summary, the present study demonstrated that PSMA/TRAIL-R2 REGULGENT™ induced tumor cell apoptosis in a PSMA expression-dependent manner *in vitro* and *in vivo*. Additionally, PSMA/TRAIL-R2 REGULGENT™ did not exert toxicity against human hepatocytes or PXB mice, representing an improvement of the therapeutic window. Taken together, novel non-hepatotoxic tetravalent bispecific antibodies could serve as effective therapeutic agents for cancer treatment. Future studies involving more advanced preclinical evaluations and clinical trials are warranted to further elucidate the therapeutic value of PSMA/TRAIL-R2 REGULGENT™ in patients.

Acknowledgements

The authors thank Dr Shinya Miyamoto and Dr Seiji Saito (Core Research Laboratories, BioPharmaceutical Center, Research Division, Kyowa Kirin Co., Ltd.) for providing experimental information and valuable advice. The authors also thank Mr. Masao Asada, Mr. Ryusei Ogura, and Ms. Kaori Yamazaki (Core Research Laboratories, BioPharmaceutical Center, Research Division, Kyowa Kirin Co., Ltd.) for their cooperation in preliminary studies and experimental work. In addition, Dr Yohei Inai, Dr Junko Iwano, and Dr Kenichirou Nanya (Translational Research Laboratories, BioPharmaceutical Center, Research Division, Kyowa Kirin Co., Ltd.) are acknowledged for their support in experiments, provision of experimental information, and helpful advice. The authors would also like to thank Dr Masakazu Kakuni (Study Service Department, PhoenixBio Co., Ltd.) for his contribution to the PXB mouse experiments.

Funding

The present study was funded by Kyowa Kirin Co., Ltd.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MN, STM, YM, KN and MI designed and performed the experiments. MN and KU confirm the authenticity of all the raw data. MN, STM, YM, KN and MI analyzed the data. MN, NT and KU conceived and supervised the study. MN, STM and KU wrote the manuscript. KU revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal studies were performed in accordance with Standards for Proper Conduct of Animal Experiments at Kyowa Kirin Co., Ltd. under the approval of the company's Institutional Animal Care and Use Committee (approval nos. 15J0057 and 16J1063). Tokyo Research Park of Kyowa Kirin Co., Ltd. is fully accredited by the AAALAC international. PXB mouse experiments were performed in accordance with ethical approval of the PhoenixBio Ethics Board (approval no. 1701).

Patient consent for publication

Not applicable.

Competing interests

All authors are employees of the company Kyowa Kirin Co., Ltd., who were responsible for manufacturing the novel tetravalent bispecific antibody, PSMA/TRAIL-R2 REGULGENT™, as investigated in the present study.

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