Hexavalent TRAIL fusion protein eftozanermin alfa optimally clusters apoptosis-inducing TRAIL receptors to induce on-target antitumor activity in solid tumors

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Running Title: Eftozanermin alfa induces antitumor activity in solid tumors

Disclosure of Conflicts of Interest: DCP, GB, DC, YX, JX, SKT, MLS HZ, DW, VCA, NX, ZL, LZ, ED, XL NR, BT and SML are employees of AbbVie. LRS is a former employee of AbbVie and was employed during the duration of this study. DCP, GB, LRS, MLS, XL, BT and SML are stockholders of AbbVie Inc. The design study conduct, and financial support for this research were provided by AbbVie. AbbVie Inc. participated in the interpretation of data, review, and approval of the publication. Venetoclax was developed in collaboration between AbbVie and Genentech.

Keywords: TRAIL, Apoptosis, ABBV-621, Solid Tumors, BCL-XL
Abstract

TNF Receptor Apoptosis-Inducing Ligand (TRAIL) can activate cell surface death receptors resulting in potent tumor cell death via induction of the extrinsic apoptosis pathway. Eftozanermin alfa (ABBV-621) is a second-generation TRAIL receptor agonist engineered as an IgG1-Fc mutant backbone linked to two sets of trimeric native single chain TRAIL receptor binding domain monomers. This hexavalent agonistic fusion protein binds to the death-inducing DR4 and DR5 receptors with nanomolar affinity to drive on-target biological activity with enhanced caspase-8 aggregation and DISC formation independent of FcγR-mediated cross-linking, and without clinical signs or pathological evidence of toxicity in non-rodent species. ABBV-621 induced cell death in approximately 36% (45/126) of solid cancer cell lines in vitro at sub-nanomolar concentrations. An in vivo patient-derived xenograft (PDX) screen of ABBV-621 activity across 15 different tumor indications resulted in an overall response (OR) of 29% (47/162). Although DR4 (TNFSFR10A) and/or DR5 (TNFSFR10B) expression levels did not predict the level of response to ABBV-621 activity in vivo, KRAS mutations were associated with elevated TNFSFR10A and TNFSFR10B and were enriched in ABBV-621 responsive colorectal carcinoma (CRC) PDX models. To build upon the OR of ABBV-621 monotherapy in CRC (45%; 10/22) and pancreatic cancer (35%; 7/20), we subsequently demonstrated that inherent resistance to ABBV-621 treatment could be overcome in combination with chemotherapeutics or with selective inhibitors of BCL-X\textsubscript{L}. In summary, these data provide a pre-clinical rationale for the ongoing Phase-1 clinical trial (NCT03082209) evaluating the activity of ABBV-621 in cancer patients.

Statement of significance

This study describes the activity of a hexavalent TRAIL-receptor agonistic fusion protein in pre-clinical models of solid tumors that mechanistically distinguishes this molecular entity from other TRAIL-based therapeutics.
Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, Apo2L or TNFSF10) is a member of the TNF superfamily of cytokines that play diverse roles in immune cell modulation and the activation of intracellular signaling pathways that control cell proliferation, survival and apoptosis. TRAIL is a type-II transmembrane protein that can bind as a trimer to membrane-bound or soluble receptors: TRAIL-R1 (DR4, TNFRSF10A), TRAIL-R2 (DR5, TNFRSF10B), TRAIL-R3 (DcR1, TNFRSF10C), TRAIL-R4 (DcR2, TNFRSF10D) and osteoprotegerin (OPG, TNFRSF11B). Whereas binding of TRAIL to DcR1 and DcR2 has a limited impact on intracellular cell signaling, binding to DR4 and/or DR5 initiates their trimerization that can trigger the formation of the death-inducing signaling complex (DISC) to drive caspase-dependent apoptotic cell death (the extrinsic apoptosis signaling pathway). In type-I cells, caspase-8/-10 activation is robust enough to directly activate effector caspase-3/-7 signaling that results in apoptosis. However, in type-II cells engagement of the intrinsic apoptosis signaling pathway is required to amplify the apoptosis signal. This process requires caspase-8 to induce cleavage of the BH3-only protein BID. The truncated form, tBID, subsequently initiates MOMP following BAX and BAK dimerization to release cytochrome c, which in turn leads to apoptosome formation, effector caspase activation and ultimately apoptosis (1) (2) (3).

The intrinsic apoptosis signaling pathway is regulated by the B-cell lymphoma protein-2 (BCL-2) family of proteins that can be divided into three groups each containing at least one BCL-2 homology (BH) motif (BH1-4). The pro-apoptotic BH3-only proteins BIM, BID, PUMA, NOXA, BAD, BIK, BMF and HRK, and the multidomain effector proteins BAX and BAK, are activated or induced by various cell death stimuli that drive mitochondrial outer membrane permeabilization (MOMP) and subsequently apoptosis. The anti-apoptotic members (BCL-2, BCL-XL, MCL-1, BCL-W, and BCL2-A1) possess BH3-binding grooves that function to counter the activity of the BH3-only and multidomain effectors via direct protein-protein interactions. Aberrant expression and/or function of BCL-2 family proteins are integral to tumorigenesis.
and resistance to cancer therapy by enabling malignant cells to evade apoptosis (4) (5), including that induced by TRAIL (6) (7) (8). A number of small molecule inhibitors of the BCL-2 family members are now in clinical development in various oncology indications including venetoclax, which received FDA approval in the US for use in relapsed/refractory (R/R) CLL patients harboring 17p-deletions (3) (9). However, the development of anti-cancer therapeutics that target the extrinsic apoptosis signaling pathway have faced significant challenges in the clinic (1) (2).

Compelling pre-clinical data demonstrated that TRAIL was capable of selectively inducing the cell death of tumor cells without the adverse effects associated with TNF or CD95 (Fas ligand) (1) (2). Subsequently, a first generation of TRAIL-based therapeutics entered into clinical trials and mainly centered upon the recombinant form of human soluble TRAIL (sTRAIL; exemplified by Apo2L/AMG-951/Dulanermin, (10)) or agonistic antibodies that selectively target DR4 (mapatumumab, (11)) or DR5 (drozitumab, (12); conatumumab/AMG-655, (13); lexatumumab, (14); tigatuzumab, (15)). However, despite being well tolerated in human cancer patients, these TRAIL-based therapeutics failed to develop compelling objective responses as a monotherapy or in combination with other therapeutic agents (1) (2). Much of these clinical failures have been attributed to the biologic properties of these first generation TRAIL-based molecules; Apo2L possesses a short plasma half-life, is rapidly cleared from the circulation (16) (17) and is inherently weak at inducing higher order receptor clustering (18) (19), whereas bi-valent DR5 agonistic mAbs require additional Fc-FcγR-mediated cross-linking to induce optimal clustering of their respective receptors (20). The requirement of Fc-FcγR interactions has been viewed as a critical activity-limiting step in cancer patients in part due to the dependence of FcγR-expressing immune cells in the tumor microenvironment, or competition with high circulating levels of endogenous immunoglobulin G (21). To address these liabilities and improve upon this first-generation agents, eftozanermin alfa (ABBV-621) was engineered as a hexavalent TRAIL receptor agonistic fusion protein derived from its proto-type APG-350 (21). ABBV-621 selectively binds to TRAIL receptors with
nanomolar affinity to induce optimal receptor clustering in human solid tumor cancer cells that drives on-target apoptosis and robust anti-tumorigenic activity that is independent of Fc-FcyR interactions. In addition, ABBV-621 activity is significantly enhanced when combined with sensitizing agents such as taxanes, topoisomerase inhibitors or BCL-X\textsubscript{L} small molecule inhibitors. Together, these data provide a pre-clinical rationale for evaluating ABBV-621 in multiple different cancer types (NCT03082209).
Materials and Methods

Cell culture and reagents

Cell lines were purchased from DSMZ (Braunschweig, Germany), ATCC (Manassas, VA), ECACC (Porton Down, UK), JCRB Cell Bank (Ibaraki city, Japan) or from the KCLB (Seoul, South Korea) and cultivated for 1-15 passages in the recommended culture media supplemented with 20mM HEPES (Gibco), penicillin/streptomycin and 10% fetal bovine serum (FBS; Invitrogen; Supplemental Tables S1 and S2). Cells were grown at 37°C in a humidified atmosphere with 5% CO2. Cell lines were tested for authenticity by short tandem repeat (STR) profiling and mycoplasma by the AbbVie Core Cell Line Facility. ABBV-621, anti-DR5 agonistic mAb with or without mutant Fc (DANA or LALA), anti-ABBV-621 mAb, venetoclax, navitoclax, A-1331852, paclitaxel, docetaxel, erlotinib and 5-FU were all synthesized by AbbVie unless otherwise stated. SN38 was purchased from Sigma-Aldrich (Saint Louis, MO).

Surface Plasmon Resonance (SPR)

Binding kinetics of ABBV-621 for recombinant soluble TRAIL receptor extracellular domains (ECDs) were determined by SPR as described (22). Briefly, recombinant ECDs of human and cynomolgus monkey TRAIL receptors (fused with 6His tag at C-terminal end) were purified from HEK-293 cells. Each assay cycle consisted of the following steps: 1) capture of ABBV-621 on anti-human Fc surface only; 2) TRAIL receptor injection over both reference and test surface, 240 µL at 80 µL/min, after which the dissociation was monitored for 300 seconds at 80 µL/min. All measurements were double referenced against blank surface and running buffer (HBS-EP+). Data were fitted globally to a 1:1 binding model using Biacore T200 Evaluation software to determine the binding kinetic rate constants, $k_a$ (M$^{-1}$s$^{-1}$) and $k_d$ (s$^{-1}$), and the equilibrium dissociation constant $K_D$ (M).

Cell viability and apoptosis determination
Human tumor cell lines were seeded into 96 or 384 well plates at 5000 cells or 1500 cells per well respectively and treated with ABBV-621 alone or in combination with venetoclax, navitoclax, A-1331852 paclitaxel, docetaxel, SN38, gemcitabine, 5FU or erlotinib for the indicated incubation times. Cell viability was subsequently measured using CellTiter-Glo® (Promega, Madison WI). Responses were determined as a percentage of the control treated cells and EC\textsubscript{50}s calculated from the resulting sigmoidal dose-response curves using GraphPad Prism (GraphPad Software, La Jolla, CA USA). The Bliss independence model (23) was used to evaluate combinatorial activity, positive integers indicating synergy. Bliss scores were calculated for each combination within the dose matrix and totaled to give a “Bliss Sum” value. For determination of apoptosis, DLD-1 cells were treated with ABBV-621 and/or SN38 for 24 hours and the sub-G0–G1 content of DNA cell-cycle histograms determined by flow cytometry as described in detail elsewhere (24).

**IncuCyte mediated determination of caspase-3/-7 activation and cell death**

SW620 cells were seeded in 96-well clear-bottom black polystyrene microplates (Corning Inc., Corning, NY) at 5000 cells/well prior to treatment and caspase-3/-7 activity (Cat # 4440, Sartorius, Göttingen, Germany) and cell viability (DRAQ7, Cat. # 7406, Cell Signaling Technology, Danvers, MA) was determined using an IncuCyte Zoom (Sartorius) programmed to take four images per well at a 1-hour interval for 24 hours. Data were analyzed using the IncuCyte S3 2019 software and plotted as the count of green objects (activated caspase-3/-7) and red objects (DRAQ+) divided by area (mm\textsuperscript{2}) per well.

**Western blot analysis**

Cells were harvested, washed with ice cold-PBS and lysed by RIPA buffer (Sigma, St Louis, MO) containing protease inhibitor cocktail (Roche, Indianapolis, IN). The purified lysates were electrophoresed by SDS–PAGE (Invitrogen, Waltham, MA) and transferred to nitrocellulose membranes utilizing an iBlot 2 (Invitrogen) device. The following antibodies were used; mouse anti-β-Actin (Cat. #
ab6276, Abcam, Cambridge, MA). Rabbit anti-BCL-X\(_i\) (Cat. # 2764, Cell Signaling Technologies), anti-PARP (Cat. # 551025, BD Biosciences, San Jose, CA), anti-Caspase-8 (Cat. # M032-3, MBL, International, Woburn, MA), anti-Caspase-3 (Cat. # ab13585, Abcam), anti-Caspase-9 (Cat. # 9502, Cell Signaling Technologies) or GAPDH (Cat. # ab8245, Abcam). Blots were incubated with primary antibodies at 4°C overnight followed by Alexa Fluor 680/800 goat anti-mouse IgG (Cat. # A21057/A32730, Invitrogen) or Alexa Fluor 680/800 goat anti-rabbit IgG (Cat. # A21109/A32735, Invitrogen) secondary antibodies for 60 min at room temperature. Proteins were visualized using the Odyssey® infrared imaging system (LI-COR Biosciences, Lincoln, NB) and were not further manipulated with imaging software.

**Determination of ABBV-621-induced receptor clustering by high content analysis**

For the determination of caspase-8 aggregation by high content analysis, NCI-H460 cells were cultured overnight in black collagen-I coated 96-well plates (Corning). Treated cells were fixed in formaldehyde, washed with PBS, permeabilized with 0.1 % Triton and blocked with 1% BSA (Invitrogen) before incubating with mouse anti-Caspase-8 Ab (1:800, Cat. # M-0323, MBL). Following overnight incubation at 4°C, cells were washed with PBS and incubated with goat anti-mouse secondary Alexa 488 conjugated antibody (1:400, Cat. # A-11029, Thermo Fisher Scientific, Waltham, MA) + Hoechst 33342 (1 µg/mL, Cat. # H-3570, Life Technologies, Carlsbad CA) for 1 hour. Cells were washed again with PBS and the microplate scanned on an ArrayScan (Thermo Fisher Scientific) at 20 x using the Spot Detector algorithm.

To determine the colocalization of ABBV-621 binding with activated caspase-8, NCI-H460 cells were treated with ABBV-621 (2µg/ml) and mouse anti-human mAb (1 µg/ml) (Cat. # ab99757, Abcam) fixed and permeabilized as described above and incubated with a rabbit anti-cleaved caspase-8 Ab (1:100, Cat. # 9496, Cell Signaling Technology), followed by an anti-mouse Alexa 488 secondary Ab (1:400, Cat. # A-11029, Thermo Fisher Scientific) and an anti-rabbit Alexa 594 secondary Ab (1:400, Cat. # A-32740,
Thermo Fisher Scientific). ArrayScan images were analyzed using the colocalization algorithm to measure the overlapping area between independently identified puncta containing ABBV-621 and activated caspase-8.

**Cell line engineering**

Briefly, Colo205 cells were transfected with pCMV-Cas9-RFP containing the CRISPR sequence 5’-GTACATGTAGGTGCCTCTGG targeting TNFRSF10A (Target ID HS0000284207, DR4, Sigma-Aldrich) or sequence 5’-AGAACGCCCCGGCGCTTCGG targeting TNFRSF10B (Target ID HS0000284184, DR5, Sigma-Aldrich). SW620 cells were transfected with pCMV-CAS9-GFP containing the CRISPR sequence 5’AGGAACACCAGCGCAGCGG targeting BID (Target ID HS0000187506, Sigma-Aldrich). H460 cells were transfected with CASP8 CRISPR/Cas9 KO plasmid and CASP8 HDR plasmid (sc-400147 and sc-400147-HDR, Santa Cruz Biotechnology, Dallas, Texas,). Transfection was performed using Lipofectamine® 3000 according to manufacturer’s recommendation (Life Technologies). Cells were assessed for loss in DR5 and/or DR4 expression by flow cytometry, loss of BID or caspase-8 expression by western blot and sensitivity to ABBV-621-induced cell death.

Stable overexpression of DcR1 and DcR2 was conducted by lentiviral transduction using the lentiviral expression vector PLVX-IRES-PURO containing either full-length human DCR1 or DCR2 cDNA, respectively. Overexpression of BCL-Xₐ was conducted by transduction of the expression vector pLOC-BCL-Xₐ (Thermo Fisher Scientific). Colo205 cells expressing PLVX or pLOC vectors were selected for using 1 µg/ml Puromycin (Gibco, Waltham, MA) or 5 µg/mL blasticidin (Gibco), respectively. Expression of DcR1 and DcR2 was confirmed by flow cytometry and BCL-Xₐ by western blot.

**Determination of TRAIL receptor expression by flow cytometry**

Harvested cells were washed twice with ice-cold staining wash buffer (Dulbecco’s PBS without calcium and magnesium containing 0.5 % BSA and 0.1 % sodium azide). Cell samples were subsequently treated
with anti-DR4-PE (Cat. # 12-6644-42, eBiosciences, San Diego, CA), anti-DR5-PE (Cat. # 12-4714-42, eBiosciences), anti-DcR1-PE (Cat. # FAB6302P, R&D Systems), anti-DcR2-Pe (Cat. # FAB633P, R&D Systems) or mouse IgG1-PE isotype control (Cat. # 12-4714-42, eBiosciences) and incubated for 45 minutes on ice in the dark. Cells were washed with staining wash buffer and resuspended in 350 µl of staining wash buffer containing DRAQ7 (Cat. # 7406S, Cell Signaling Technologies) prior to analysis by flow cytometry. TRAIL receptor number was quantified using Quantum Simply Cellular ABC beads (Bang Labs, Fisher, IN) per the manufacturer’s instructions. The isotype control results are subtracted from the resulting TRAIL receptor number, weighted by the percent positive population of cells.

**Assessment of DR4 and DR5 protein expression in vivo**

To determine the DR4 and DR5 protein expression in vivo, ABBV-621 was used as a probe to immunoprecipitate DR4 and DR5. Briefly, ABBV-621 naïve tumors derived from xenograft models were homogenized in PBS using a Precellys (Bertin Instruments, Rockville, MD) and ABBV-621 was added to a final concentration of 200 nM. Tumor samples were incubated on ice for 4 hours and then centrifuged at 15,000 x g for 10 minutes at 4°C. Supernatants were discarded, the remaining cell pellet washed in ice-cold PBS and then resuspended in IP lysis buffer (Pierce Biotechnology Inc.) containing protease inhibitors (Sigma-Aldrich) and phosphatase inhibitors (Roche, Basel, Switzerland). Streptavidin multiarray 96-well plates (Meso Scale Discovery (MSD); Rockville, MD) with immobilized biotin labeled-anti–ABBV-621 were incubated overnight at 4°C with 5 µg of protein sample/well in duplicate. After 3 washes with PBS-Tween, sulfo-tagged mouse anti-human DR4 (2 nM) and sulfo-tagged mouse anti-human DR5 antibody (2 nM) were added to each well and incubated for 1 hour at room temperature with rotation. Samples were then washed three times with PBS-Tween and 150 µL of read buffer (MSD) was added per well. Fluorescence was subsequently measured with an MSD Sector Imager 6000.

**Caspase-3/-7 activation in vivo**
Tumors harvested from xenograft models treated with ABBV-621 were homogenized in IP lysis buffer (Thermo-Fisher, Cat # 87787) using a Precellys (Bertin Instruments, Rockville, MD) under dry ice for 45 seconds twice. Cell supernatants were prepared and 20 µg of protein per sample were used to determine caspase-3/-7 activation (Caspase-3/-7Glo; Promega, Cat. # G8090) according to manufacturer’s instructions.

**Determination of ABBV-621 binding to cellular DR4 and DR5, and ABBV-621 DISC formation in vivo.**

Tumors derived from xenograft models treated with ABBV-621 were homogenized in IP lysis buffer using a Precellys (Bertin Instruments). The protein concentration from whole cell or tumor lysates was subsequently determined (Pierce Biotechnology Inc.). Pre-blocked streptavidin coated multiarray 96-well plates (MSD) were then incubated with 10 nM biotinylated anti-ABBV-621 mAb for 2 hours at room temperature with shaking. Plates were then washed three times with PBS-Tween, 5 µg of protein lysate added in duplicate to each well and samples incubated overnight at 4°C with shaking. Sulfo-tagged anti-human DR4, anti-human DR5 or anti-human caspase-8 antibodies were then added to each sample for 1 hour with rotation to detect ABBV-621 binding to DR4 or DR5, or formation of the ABBV-621-induced DISC, respectively. Finally, plates were washed three times with PBS-tween, 150 µl of MSD reading buffer added and the signal determined using an MSD Sector Imager 6000.

**In vivo pharmacology**

Female SCID and SCID-Beige mice were obtained from Charles River Laboratories (Wilmington, MA), NSG mice were from The Jackson Laboratory (Bar Harbor, Maine). All experiments were approved by AbbVie’s Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health Guide for Care and Use of Laboratory Animals Guidelines in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Cell line-derived xenograft (CDX; Supplemental Table S2) and patient-derived xenograft (PDX) mouse models were weighed at the onset...
of therapy. Each experimental group included 8-10 animals. Tumors were measured two to three times weekly. Measurements of the length (L) and width (W) of the tumor were obtained via electronic calipers and the volume was calculated according to the following equation: tumor volume (TV) = L x W²/2. Mice were euthanized when tumor volume reached a maximum of 2,000 mm³ or upon presentation of skin ulcerations or other morbidities, whichever occurred first.

**In vivo PDX screen**

Studies were conducted at Champions Oncology (Rockville, MD) and approved by the IACUC of Champions Oncology. Athymic nude female mice obtained from Envigo (Indianapolis, IN) between 6-8 weeks of age were used in the studies. Tumor fragments (approximately 5 x 5 x 5 mm³) from Champions TumorGraft® model were implanted SC in the left flank of the female pre-study mice. When the TV reached approximately 150-300 mm³, animals were matched by tumor size and assigned into control (n = 1) and treatment groups (n = 3) and dosing was initiated on Day 0. TV was subsequently measured twice per week and on the final day of study or on the day moribund animals were euthanized. The study was terminated when the mean tumor volume in the vehicle control reached 1500 mm³ or up to Day 60, whichever occurred first. Analysis was based on a modified RECIST 1.1 response criteria (25).

Each tumor response was determined by comparing tumor volume change at time t to its baseline (% TV change = 100 % x ((Vt-Vi) / Vi). The criteria for response were defined as follows: OR, Overall Response = CR + PR; CR, Complete Response < -90 %; PR, Partial Response between 0 % and -90 %; SD, Stable Disease > 0 % and ≤ 100 %; PD, Progressive Disease > 100 %. PDX models in which the control tumor did not reach 3 x initial TV were removed from the data set.

**Pharmacokinetic (PK) studies**

Whole blood exposure of ABBV-621, DRS Mab (conatumumab/AMG-655) or DR5 mAb possessing a LALA or a DANA mutation (20) was measured in Colo205 xenograft or non-tumor bearing CD-1 mice treated at
the indicated concentrations and route of administration. Whole blood was collected at various time points and measured for test article concentrations (N=12 samples/group) by electrochemiluminescence (ECL) immunoassay (QuickPlex 120 Reader, MSD). The pharmacokinetic parameters assessed included: Terminal half-life (t₁/₂), maximum observed serum concentrations (Cmax), Cmax divided by dose (Cmax/D), serum concentration at first time point post dose (Ct), Ct divided by dose (Ct/D) volume of distribution at steady state (Vss), clearance (CL), area under the curve from zero to infinity, (AUC₀–ₚᵢₙ), AUC up to the last measurable concentration (AUC₀–t) and AUC divided by dose (AUC/D). All PK studies were approved by AbbVie’s IACUC.

Toxicokinetic (TK) studies

The TK properties of ABBV-621 were assessed in a Good Laboratory Practice (GLP) repeat dose toxicology study and approved by the IACUC of MPI Research (Mattawan, MI). Cynomolgus monkeys were treated with weekly intravenous doses of 4, 20 and 100 mg/kg for a total of five doses (4-6 animals per sex per dose level) followed by a four-week recovery period (high dose only). TK profiles for all animals were analyzed after the first and fourth doses by ECL immunoassay (QuickPlex 120 Reader, MSD). All animals were euthanized 24 hours after last dose for histological evaluation.

Statistical analysis

In all cases, n refers to the number of independent experiments. Linear regression and Spearman’s correlation were used to determine the correlation between two variables, and the Mann-Whitney U-test or one-way ANOVA with Dunnett’s multiple comparison test was used to determine statistical significance between data sets, all using Prism 7.03 (GraphPad Software).
Results

ABBV-621 selectively binds to TRAIL receptors to drive on-target apoptosis in solid tumor cell lines

ABBV-621 is a hexavalent TRAIL receptor agonistic Fc fusion protein derived from APG-350 (21) that contains a single IgG1-Fc point mutation (asparagine to serine 297; Figure 1A) that effectively removes a glycosylation site to eliminate binding to all Fcy receptors and complement component C1q. ABBV-621 binds to human recombinant DR4 receptor or DR5 receptor with affinities of approximately 780 nM and 635 nM respectively as measured by SPR (Supplemental Table S3). ABBV-621 is highly active across a panel of solid tumor cell lines in vitro, inducing cell death with an IC_{50} of less than 1 nM in approximately 36% (45/126) of cell lines assessed (Figure 1B; Supplemental Table S1), and possesses similar activity to APG-350 in Colo205 cells in vitro and in vivo (Figure 1C). ABBV-621 cellular activity is mechanism-based as demonstrated by the rapid dose-dependent activation of downstream apoptotic signaling in vitro (cleavage of caspase-8, caspase-3, and PARP) and in vivo (caspase-3/-7 activation; Figure 1D). DR4 plasma membrane expression was consistently lower than DR5 across all solid tumor cell lines in vitro (Supplemental Figure S1A). Reflecting this disparity, DR5 protein or gene (TNFRSF10B) expression correlated with ABBV-621 EC_{50} in vitro across solid tumor cell lines, whereas DR4 or TNFRSF10A did not (Figure 1E). However, the tumor type may influence these associations (Supplemental Figure S1B-D). ABBV-621 activity requires DR4 and DR5 tumor cell expression since Colo205 cells deficient in the genes encoding these proteins completely abrogated ABBV-621-induced cell death in vitro and in vivo (Figure 1F). Reflecting the lower plasma membrane expression of DR4 compared to DR5 in Colo205 cells, deletion of TNFRSF10B reduced ABBV-621 activity to a greater extent than TNFRSF10A deletion in vitro (Supplemental Figure S2). Although ABBV-621 binds to human recombinant DcR1 and DcR2 (Supplemental Table S3), endogenous expression of DcR1 or DcR2 across human tumor cell lines did not impact the cell killing activity of ABBV-621 (Supplemental Figure S3A). Similarly, overexpression of DcR1
or DcR2 in Colo205 cells to levels greater than two-fold of DR4 and DR5 combined did not dramatically influence ABBV-621-induced cell death (Figure 1G and Supplemental Figure S3B).

**ABBV-621 is well-tolerated in cynomolgus toxicity studies**

ABBV-621 binds to human and cynomolgus DR4/DR5 receptors with similar affinities (Supplemental Table S3), exhibiting a greater affinity for cynomolgus monkey DcR2 than human DcR2 (approximately 25-fold increase by SPR). No binding of ABBV-621 to cynomolgus DcR1 could be detected (Supplemental Table S3). Considering these binding parameters, and published data generated across species (rat, mouse, monkey; (16) (26)), cynomolgus monkey was therefore considered the only relevant species for which to assess the toxicology profile of ABBV-621. ABBV-621 was well tolerated when administered to cynomolgus monkeys by weekly IV infusion (30 minutes) at doses of 0, 4, 20 or 100 mg/kg/week for four consecutive weeks (five total doses). No ABBV-621-related clinical signs of toxicity, changes in body weight, clinical pathology parameters, gross necropsy or microscopic findings were observed during the study with no injection site reactions. Importantly, in this cynomolgus monkey study ABBV-621 demonstrated approximately dose-proportional increases in maximum observed serum concentrations post dosing with Cmax/D as 21.8 - 25.4 µg/ml/mg/kg and AUC/D as 501 - 567 µg*hr/ml/mg/kg across 4-100 mg/kg IV doses tested. No accumulation was noted in Cmax or AUC between the first and fourth dose of ABBV-621. Serum concentrations and TK parameters for ABBV-621 did not exhibit any sex-specific differences. Approximate dose proportionality was also observed in non-tumor bearing CD1 mice treated with a single dose of ABBV-621 with a Ct/D of 6.6 – 9.06 µg/ml/mg/kg and a terminal half-life of between 43.8 and 51.9 hours (Supplemental Table S4).

**ABBV-621 induces optimal receptor clustering and DISC formation to drive potent anti-tumor activity**

Several TRAIL-related therapeutics have been advanced into clinical trials in cancer patients (10) (11) (12) (13) (14) (15). One of these, conatumumab (anti-DR5 mAb) (13) possesses an extended systemic
exposure profile when compared to ABBV-621 in tumor-bearing SCID beige mice (Figure 2A; IP routes of administration). Despite this, ABBV-621 induced superior anti-tumorigenic activity than the anti-DR5 agonistic mAb in the Colo205 CDX model, the latter requiring Fcγ-Rs to drive DR5 receptor clustering since Fc mutant anti-DR5 mAbs (DANA/LALA mutants) (20) do not impact tumor growth (Figure 2B). Unlike anti-DR5-agonistic Abs, cross-linking does not significantly enhance the activity of ABBV-621 (Figure 2C). Utilizing a fluorescent based approach (12) (18), exposure of the ABBV-621-sensitive human tumor cell line H460 in vitro to ABBV-621 causes active caspase-8 to be rapidly redistributed to regions of ABBV-621 binding (Figure 2D; Supplemental Figure 4A). Pro-caspase-8 was also redistributed from a diffuse staining pattern in untreated H460 cells to defined puncta in ABBV-621 treated cells. This effect was absent with agonistic anti-DR5 mAb treatment without cross-linking (Figure 2E). To evaluate binding of ABBV-621 to DR4 and DR5, and the formation of the DISC in vivo, we developed an ELISA-based assay to measure ABBV-621 binding to DR4, DR5 or caspase-8 (a component of the TRAIL-induced DISC) (1) (2) that is required for ABBV-621 activity (Supplemental Figure 4B). DR5 (and/or DR4) engagement and subsequent DISC formation could be observed within 3 hours of ABBV-621 administration to mice bearing Colo205 tumors and occurred in a dose-dependent fashion (Figure 2F). Collectively, these data demonstrate a requirement for enhanced receptor clustering to induce potent tumor cell death in vitro and in vivo, providing further mechanistic insights as to why other death receptor agonists may have failed in the clinic (18) (20) (21) and thereby distinguishing ABBV-621 from first generation agents. By capturing tumor heterogeneity, human patient-derived xenograft (PDX) models can be more reflective of human disease (27). To further assess the spectrum of ABBV-621 activity, a screen of 162 different human PDX models covering fifteen solid tumor types was performed applying modified RESIST 1.1 response criteria to define progressive disease (PD), stable disease (SD) and overall response (OR; complete response and partial response, CR and PR respectively) in individual PDXs. Across all PDXs, an OR rate of 29% (47/162) was observed in PDX models treated with ABBV-621 monotherapy, with SD and
PD observed in an additional 11% (18/162) and 60% (97/162) of PDX models tested, respectively (Table 1). Importantly this activity was independent of treatment history or tumor sample harvest site; naïve versus pre-treated and primary versus metastatic site (Supplemental Tables S5A and S5B). Of note, ABBV-621 demonstrated better single agent activity within specific tumor types that include CRC, pancreatic cancer (PaCa), gastric cancer and Ewing’s sarcoma, with OR rates ranging from 35% to 60% (Table 1). To understand the impact of DR4 and DR5 protein expression on the response of PDX models to ABBV-621 monotherapy, we developed a quantitative ELISA-based assay that leveraged ABBV-621 as a probe to immunoprecipitate DR4 or DR5 from tumors derived from CRC, gastric and PaCa PDX models naïve to ABBV-621 treatment. Although higher DR5 plasma membrane expression positively correlated with ABBV-621 potency in solid tumor cell lines in vitro (Figure 1E), the measured levels of DR5 protein or gene expression (or DR4/TNFRSF10B expression) did not predict the level of response to ABBV-621 monotherapy in PDX models (Figure 3A; Supplemental Figures S5A and S5B). Of note however, elevated DR4 (TNFRSF4A) and DR5 (TNFRSF5B) gene expression was associated with gain of function mutations in KRAS (Figure 3B). Further, CRC PDX models with strong responses to ABBV-621 treatment (CR and PR) were enriched with KRAS mutations (Supplemental Table S6). These data indicate that while DR4 and DR5 are required for ABBV-621 binding and subsequent anti-tumorigenic activity in vitro and in vivo, predictive response can be multi-factorial and regulated by additional genetic or downstream cell signaling resistance mechanisms.

Since this in vivo PDX screen is limited by the number of mice within each treatment group (n=3), we evaluated the impact of dose on the growth of two CRC PDX tumors with an increased number of mice in each treatment arm (n = 8). ABBV-621 dose-dependently inhibited growth of CRC tumors CTG-0064 and CTG-0069, the 3 mg/kg response group reflecting those obtained under screening conditions (Figure 3C). To validate the high monotherapy response rate observed in some tumor types, we further assessed activity of ABBV-621 in an independent set of PaCa PDX models. ABBV-621 treatment induced
a dose-dependent reduction in tumor growth characterized by robust tumor regression that was maintained for more than 30 days at the maximum dose tested (Figure 3D).

**Anti-tumor agents enhance the activity of ABBV-621 in pre-clinical models of PaCa and CRC.**

Although single agent ABBV-621 activity was demonstrated in 36% of human tumor cell lines *in vitro* and an OR of 29% of solid tumor PDXs across tumor indications, these data also illustrate tumors that are resistant to ABBV-621 monotherapy. To address tumor resistance, we initially evaluated the combinatorial activity of ABBV-621 with therapeutic agents utilized in the management of pancreatic cancer patients (28) in a panel of PaCa cell lines *in vitro*. Broad and robust synergy was observed between ABBV-621 and the microtubule inhibitor paclitaxel or the topoisomerase inhibitor SN38, the active metabolite of irinotecan (Figure 4A, 4B, Supplemental Figure S6A, S6B, and Supplemental Table S7). To further interrogate these combinations *in vivo*, we evaluated their activity in the PaCa CDX model BxPC3. Co-treatment of mice bearing BxPC3 tumors with ABBV-621 and either docetaxel or irinotecan each enhanced the ABBV-621 monotherapy response (Figure 4C and Supplemental Table S8). We subsequently evaluated the docetaxel/ABBV-621 combination in several PaCa PDX models characterized by differential sensitivities to ABBV-621 monotherapy. In all models tested, the combination of ABBV-621 with docetaxel exhibited more pronounced anti-tumorigenic activity than either agent alone (Figure 4D, 4E and Supplemental Table S8). Expanding the combination activity analyses to CRC, an indication where irinotecan is an established treatment option (29), we also observed robust synergistic cell death between ABBV-621 and SN38 in CRC cell lines (Figure 5A, Supplemental Figure S7 and Supplemental Table S9), with SN38 enhancing the sub-G0/G1 DNA content of DLD-1 cells treated with ABBV-621 (Figure 5B). *In vivo*, the combinatorial activity between ABBV-621 and irinotecan was superior to either agent alone in CRC CDX or PDX models that exhibit differential ABBV-621 monotherapy response profiles (Figure 5C, 5D and Supplemental Table S10).
**BCL-X<sub>I</sub> inhibition sensitizes pre-clinical models of CRC and PaCa cancer to ABBV-621**

*BCL2L1* (gene encoding BCL-X<sub>I</sub> protein) is amplified in CRC (30) and is associated with resistance to standard of care (SoC) chemotherapeutics in this and other tumor indications (31) (32) thereby restricting apoptosis mediated by other TRAIL-related therapeutics (7) (33) (34) (35). In agreement with these observations, overexpression of BCL-X<sub>I</sub> in Colo205 cells inhibited ABBV-621 activity (Figure 6A). Subsequently, we evaluated the combinatorial activity between ABBV-621 and the BCL-X<sub>I</sub> selective small molecule inhibitor A-1331852, the BCL-2 selective inhibitor venetoclax, or the BCL-2/BCL-X<sub>I</sub> dual inhibitor navitoclax (31) (36) (37) in numerous pre-clinical CRC models. ABBV-621 in combination with A-1331852 induced broad synergistic cell death in a panel of CRC cell lines *in vitro* that was superior to that observed between ABBV-621 and venetoclax or navitoclax (Figure 6B, Supplemental Figure 8A & Supplemental Table S11). To further probe the tumor sensitization mechanism of BCL-X<sub>I</sub> inhibition combined with TRAIL receptor agonism, we utilized the CRC cell line SW620, which is resistant to both ABBV-621 or A-1331852 monotherapy. Co-treating SW620 cells with these two apoptosis-targeting agents induced robust caspase-dependent apoptosis *in vitro* (Figure 6C, Supplemental Figure S8B) and robust tumor regression *in vivo* (Figure 6D and Supplemental Table S12). As expected, ABBV-621 was shown to bind to DR4 and DR5 and induce DISC derived from treated SW620 tumors (Figure 6E), indicating that resistance mechanisms to ABBV-621 monotherapy can be regulated by downstream cell signaling. Of note, *BID* deletion completely abrogated ABBV-621/A-1331852 synergy (Figure 6F) and caspase-9 activation (Figure 6G) indicating that the BH3-only protein BID is essentially required to link the extrinsic and intrinsic apoptosis signaling pathways to drive combinatorial activity. The anti-tumorigenic activity of ABBV-621 in combination with A-1331852 was also superior to either agent alone in PaCa PDX models (PA117 and PA20; Supplemental Figure S9 and Supplemental Table S12) emphasizing the breadth of activity that can potentially be achieved by combining this TRAIL agonistic fusion protein with BCL-X<sub>I</sub> inhibitors in solid tumors.
Discussion

Herein we characterize the anti-tumorigenic activity of ABBV-621, a hexavalent TRAIL agonistic fusion protein that induces apoptotic cell death in pre-clinical models of human tumors through optimal clustering of the apoptosis-inducing TRAIL receptors DR4 and DR5. In pre-clinical toxicology studies in monkeys, ABBV-621 is well tolerated, with no-test-item related effects recorded at doses of up to 100 mg/kg IV weekly for four weeks. When activation of the extrinsic apoptosis signaling pathway is insufficient to drive robust cell death despite binding to DR4/DR5 and subsequent DISC formation, deeper anti-tumor activity is observed by combining with chemotherapeutics (taxanes or topoisomerase-1 inhibitors) or a tool selective inhibitor of BCL-XL. A Phase-1 clinical trial to assess the safety, tolerability, and activity of ABBV-621 in patients with previously treated solid tumors and hematologic malignancies is underway (NCT03082209).

ABBV-621 binds with high affinity to all four human TRAIL receptors to rapidly drive caspase-dependent apoptosis that is dependent upon DR5 and DR4 expression. In vivo, binding to DR4 or DR5 can be observed within 3 hours of ABBV-621 treatment and is dose proportionally associated with the concomitant formation of the DISC. Agonistic anti-DR5 mAb requires binding to Fcγ-receptors (Fcγ-Rs) since Fc mutations ablated anti-DR5 Ab activity in vivo from our studies and consistent with previous observations (20). Moreover, cellular imaging data indicate that caspase-8 (a component of the DR4/DR5 DISC) (1) (2) redistributes into defined cellular regions post-ABBV-621 treatment, a phenotype that is absent from agonistic anti-DR5 Ab treated cancer cells unless cross-linked artificially. This ability of ABBV-621 to induce enhanced receptor clustering translates into superior activity in vivo despite having a lower AUC and shorter plasma half-life in mice than an agonistic anti-DR5 Ab. ABBV-621 exhibits a dose proportional pharmacokinetic (PK) profile in cynomolgus monkeys and a serum exposure that is superior to that observed with Apo2L (16). A mutation in the Fc domain (Asn297Ser) of ABBV-621 removes a glycosylation site to eliminate Fcγ-R binding and prevents additional cross linking that may
mediate against hyper-clustering of the therapeutic; a process hypothesized to drive the hepatotoxicity (26) and observed following excessive multimerization with other TRAIL-receptor-based therapeutics (38). Collectively, these studies substantiate the ability of ABBV-621 to induce optimized receptor clustering independent of Fcγ-R-mediated receptor cross-linking that translates into on-target antitumor efficacy that is distinguishable from other TRAIL-based therapeutics that have entered human clinical trials.

ABBV-621 is active as a single agent in vitro ($EC_{50} < 1\text{nM}$) across a panel of human cell lines representative of different solid tumor indications; minimal activity observed in SCLC cell lines is attributed to the previously reported low levels of caspase-8 expression (39), and we have demonstrated that caspase-8 is required for ABBV-621 activity. Screening of ABBV-621 against 162 patient-derived xenograft (PDX) models covering 15 different tumor types revealed an OR (CR + PR) of 29% with SD observed in a further 11% of all models tested. ABBV-621 activity was independent of patient treatment history (naïve vs previously treated; Supplemental Table 5A) or tumor sample site (i.e., primary versus metastatic origins; Supplemental Table 5B). Of note, OR or SD was observed in 77% of colon cancer PDX models, 60% of gastric cancer PDX models and 45% of PaCa cancer PDX models following ABBV-621 monotherapy. These high single agent response rates under screening conditions were also reflected in PDX studies with an increased number of test subjects in each treatment arm either in models identified as sensitive (OR or SD) under screening conditions (CTG-0064 and CTG-0069) or from an independent PDX model source (PA20, PA55 and PA-117). Although ABBV-621 activity in vitro significantly correlated with DR5 plasma membrane or gene ($TNFRSF10B$) expression across all solid tumor cell lines ($TNFRSF10A$ expression also correlated, but not the plasma membrane expression of the corresponding protein), this correlation was insufficient to predict ABBV-621 response in PDXs in vivo. However, elevated $DR4$ ($TNFRSF6$) and $DR5$ ($TNFRSF8$) gene expression was associated with PDX models with $KRAS$ mutations, aligning with previously reported data (40). $KRAS$ mutations are frequently
observed in CRC (41) and PaCa patients (28), and these mutations are enriched in CRC PDX models responsive to ABBV-621 monotherapy.

While the breadth of ABBV-621 monotherapy activity observed in pre-clinical models of solid tumors in vitro and in vivo is encouraging, resistance to ABBV-621 is evident to varying degrees across solid tumor indications. To address this, we demonstrated that ABBV-621 synergized with taxanes or SN38, the active metabolite of irinotecan, to induce cell death across a broad panel of PaCa and CRC cancer cell lines in vitro. Subsequent assessment of these combinations in CDX or PDX models of CRC or PaCa demonstrated superior anti-tumorigenic responses to either agent alone; capturing models with differential sensitivities to ABBV-621 monotherapy including those identified in the PDX screen as progressing under ABBV-621 single agent treatment.

Genetic experiments in cell lines indicate that while DR4 and DR5 are required for ABBV-621 activity, additional genomic aberrations within the apoptosis signaling pathway downstream of the TRAIL receptor may regulate apoptosis induction. CASP8 for example is a well-established gene that regulates the activity of various TRAIL-based therapeutics at the DISC (1) (2) and is required for ABBV-621 activity. Further downstream, mitochondria are required to amplify apoptosis signaling originating from death receptors in type-II cells and is a process regulated by the BCL-2 family of proteins (1) (2). BCL-X₅ is highly expressed in CRC (30) and PaCa (33) (34) (35); its elevated expression limiting the activity of ABBV-621 and other TRAIL-related therapeutics (7) (33) (34) (35). Small molecule inhibition of BCL-X₅ with A-1331852 but not BCL-2 with venetoclax induced substantial synergy with ABBV-621 across a breadth of CRC cell lines via caspase-dependent apoptosis. This strong combinatorial activity between ABBV-621 and A-1331852 observed in vitro was mirrored in CRC and PaCa in vivo models, even in the SW620 model of CRC, which minimally responds to either agent alone. Of note, BID was absolutely required for combinatorial activity between ABBV-621 and A-1331852, reflecting the role of this BH3-only protein in linking the extrinsic to the intrinsic apoptosis signaling pathway. BID is variably expressed in human
neoplasms (42) and this differential expression may dictate the combinatorial activity of ABBV-621 with BCL-X<sub>L</sub> inhibitors.

In summary, we demonstrate a well-tolerated safety profile coupled with the ability of ABBV-621 to optimally induce TRAIL receptor clustering independent of Fcγ-R-mediated receptor cross-linking that translates into on-target anti-tumor activity in pre-clinical models of solid tumors. These properties distinguish ABBV-621 from other TRAIL-based therapeutics that have entered human clinical trials. Moreover, the depth and breadth of ABBV-621 activity is enhanced by combining with small molecule inhibitors of BCL-X<sub>L</sub> or SoC therapeutics such as taxanes or topoisomerase-I inhibitors. Collectively, these data support the ongoing Phase-1 clinical trial evaluating the activity of ABBV-621 in patients of multiple different cancer types (NCT03082209).

Acknowledgements

Keith Hickman of AbbVie and Wei Lian of AbbVie for establishing ABBV-621 production protocols, Leyu Wang of AbbVie for plasmid and recombinant TRAIL receptor generation.
References


### Table 1: Solid tumor PDX screen for ABBV-621 monotherapy

162 different PDX models representing the specified solid tumor indications were treated with 5 mg/kg ABBV-621 (q2dx5, IP, n = 3 animals per individual tumor type) under screening conditions and the impact on tumor volume determined as described in the materials and methods. The response of each tumor was defined using a modified RESIST 1.1 criteria; overall response (OS; CR and PR combined), stable disease (SD) and progressive disease (PD). The total number (n) of different PDX models evaluated for response to ABBV-621 activity is shown, along with the percentage (%) of responses.

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**Figure legends**

**Figure 1:** Characterization of ABBV-621 pre-clinical activity in human solid tumor cell lines. Schematic representation of ABBV-621 (A). Human tumor cell lines representing the specified solid tumor indications were treated with ABBV-621 for 24 hours. The effect on cell viability was determined using CellTiter-Glo and \( EC_{50} \) calculated from the resulting dose-response curves. Representative dose response curves from CRC, PaCa and gastric cancer cell lines are shown (B). Colo205 cells were treated with ABBV-621 or APG-350 *in vitro* and the impact on cell viability determined using CellTiter-Glo (left panel). Alternatively, mice bearing Colo205 tumors were treated with ABBV-621 or APG-350 at 0.3 mg/kg and 3 mg/kg (both q2dx5) and the impact on tumor volume determined (n = 8 mice per treatment group, right panel) (C). Colo205 cells were treated with ABBV-621 *in vitro* and the expression of caspase-8, caspase-3, PARP and GAPDH determined by western blot analysis (left panel). Mice bearing Colo205 tumors were treated with a single dose of ABBV-621 at the indicated dose and tumors excised at regular time points post-treatment for determination of caspase-3/-7 activation (4 independent tumors per data point, right panel) (D). The plasma membrane expression of DR4 and DR5 was determined in a panel of solid tumor cell lines and the correlation with ABBV-621 \( EC_{50} \) determined (E). Parental and \( DR4/DR5 \) CRISPR double knock-down (DKD) Colo205 cells were treated with ABBV-621 for 24 hours and the impact on cell viability determined by CellTiter-Glo (center panel). Mice bearing Colo205 tumors deficient in \( TNFRSF10A \) (DR4) and \( TNFRSF10A \) (DR5) were treated with ABBV-621 and the impact on tumor volume was determined (n=8 mice per treatment group, right panel). Flow cytometry histograms depicting DR4 and DR5 expression in the parental and \( DR4/DR5 \) DKD Colo205 cells are shown (left panel) (F). Parental, DcR1 overexpressing and DcR2 overexpressing Colo205 cells were treated with ABBV-621 for 24 hours and the impact on cell viability determined by CellTiter-Glo. Flow cytometry histograms depict the plasma membrane expression of DcR1 and DcR2 in the parental and DCR1 and DCR2 overexpressing cell lines (G).
Figure 2: ABBV-621 induces optimal death receptor clustering and DISC formation. Colo205 CDX models were treated with a single dose of ABBV-621, anti-DR5 agonistic mAb with wild type Fc (DR5\textsuperscript{WT} mAb) or anti-DR5 agonistic mAb with mutant Fc (DANA or LALA, DR5\textsuperscript{DANA} mAb or DR5\textsuperscript{LALA} mAb respectively), all at 5 mg/kg, IP. Serum concentrations were subsequently determined, and AUC\textsubscript{0-t} calculated (A) along with the effect on tumor volume. Data represent the mean ± s.e.m. of 8 mice per treatment arm (B). Colo205 cells were treated ABBV-621 or anti-DR5 mAb with or without cross-linking (goat anti-human IgG Fc-specific; x-ABBV-621 or x-DR5 mAb, respectively) for 24 hours and the impact on cell viability was determined using CellTiter-Glo. Data represent the mean ± s.e.m. of three independent experiments. (C). NCI-H460 cells were treated with ABBV-621 (12 nM) for the indicated times and colocalization (overlap) between ABBV-621 and activated caspase-8 determined using high content analysis. Data are presented as the mean ± Std. Dev. of three replicate wells per condition. UT, untreated. Representative images are shown for illustrative purposes (D). NCI-H460 cells were treated with ABBV-621 (5.96 nM) or anti-DR5 agonistic mAb (5.96 nM with or without cross-linking; x-DR5) for 0 or 30 minutes and the redistribution of caspase-8 into punctate foci quantified using high content analysis (ArrayScan VTI HCS Reader; 20 x magnification) and Spot Detector image analysis algorithm (Thermo Fisher Scientific). Data are presented as the mean ± Std. Dev. of three replicates per condition (E). Colo205 CDXs were treated with a single dose of ABBV-621, IP, at the indicated concentrations and tumor harvested at regular time points post-treatment for determination of DR4 binding, DR5 binding or DISC formation as described in the materials and methods. Each data point is representative of a single tumor where red bars represent the median. Statistical difference was determined using a One-Way ANOVA with Dunnett’s multiple comparison test where p < 0.05 (*), p < 0.01 (**) and p < 0.001 (***), were considered significant (F).

Figure 3: Characterization of ABBV-621 monotherapy in solid tumor PDX models. The plasma membrane expression of DR4 and DR5 was determined by ELISA from ABBV-621 naïve tumors derived
from CRC, PaCa and gastric cancer PDX models described in Table 1. Each data point represents the expression of DR4 or DR5 from different PDX models. Black bar represents the median (A). **DR4 (TNFRSF10A)** and **DR5 (TNFRSF10B)** gene expression was determined from RNAseq data obtained from ABBV-621 naïve tumors described in Table 1 and separated according to their **KRAS** mutation status. WT, wild type; MT, mutant. Statistical difference was determined using the Mann-Whitney U-test, where p < 0.0001 (*** was considered significant. Red bar represents the median (B). ABBV-621-sensitive CRC PDXs (CTG-0064 and CTG-0069) identified from the PDX screen as described in Table 1 (C) or an independent set of PaCa PDXs (PA20, PA55 and PA117) (D) were treated with ABBV-621 at the indicated dose and schedule, and the impact on tumor volume determined. Data are presented as the mean ± s.e.m. of 8 mice per treatment group (C and D).

**Figure 4: Chemotherapeutic agents enhance the activity of ABBV-621 in PaCa.** PaCa cell lines (n = 14) were treated with ABBV-621 in combination with erlotinib, paclitaxel, SN38, gemcitabine or 5FU and the impact on cell viability determined by CellTiter-Glo. Synergy was subsequently assessed using the Bliss independence model, where Bliss Sums represent the cumulative Bliss scores across the combination matrix. Red bar represents the median (A). Examples of the ABBV-621 dose-response curves generated are shown for PA-TU-8977t and BxPC3 PaCa cell lines capturing 4 of the 10 concentrations of SN38 or paclitaxel utilized to determined Bliss Sums in panel A. See Supplemental Figure S6 for full dose-response curves (B). Mice bearing BxPC3 tumors were treated with ABBV-621, docetaxel, irinotecan alone or in combination at the indicated doses and schedules, and the impact on tumor volume determined (C). PaCa PDX sensitive (PA55 and PA117; D) or resistant (CTG-0289, CTG-0314 and CTG-0492) (E) to ABBV-621 monotherapy (as determined in Table 1 and Figure 3) were treated with ABBV-621 or docetaxel alone and in combination with one another at the indicated doses and schedules and the impact on tumor volume determined (D and E). Data in panels C, D and E are presented as the mean ± s.e.m. of 8-10 mice per treatment arm.
Figure 5: **ABBV-621 activity is enhanced by irinotecan in pre-clinical models of CRC.** The indicated CRC cell lines (n = 21) were treated with ABBV-621 in combination with SN38 for 72 hours and the impact on cell viability determined by CellTiter-Glo. Synergy was subsequently assessed using the Bliss independence model, where Bliss Sums represent the cumulative Bliss scores across the combination matrix and are presented as the mean ± s.e.m. of 2-4 independent experiments (A). DLD-1 cells were treated with ABBV-621 (596 pM, 100 ng/ml) and SN38 (500 nM) alone or in combination for 24hrs and the percentage of sub-G0/G1 DNA content (% apoptosis) determined from DNA cell cycle histograms. Data are presented as the mean ± s.e.m. of 3 independent experiments (B). CRC PDX (CTG-0064 and CTG-0069) and CDX (Colo205) models sensitive to ABBV-621 monotherapy were treated with the indicated doses and schedules of ABBV-621 or irinotecan alone and in combination with one another, and the impact on tumor volume determined (C). Mice bearing DLD-1 or SW48 tumors were treated with ABBV-621 (3 mg/kg, q2dx5, IP) or irinotecan (50 mg/kg, q4dx3, IP) alone or in combination with one another and the impact on tumor volume determined (D). Data in panels C and D are presented as the mean ± s.e.m. of 8-10 mice per treatment arm.

Figure 6: **Selective BCL-X\textsubscript{L} inhibition sensitizes pre-clinical models of CRC to ABBV-621.** Colo205 cells overexpressing BCL-X\textsubscript{L} or expressing the vector control (Vct Ctrl) were treated with ABBV-621 and the impact on viability determined using CellTiter-Glo. Inset depicts BCL-X\textsubscript{L} and β-actin expression in the Vct Ctrl and BCL-X\textsubscript{L} expressing Colo205 cells by western blot (A). CRC cell lines (n = 23) were treated with ABBV-621 in combination with venetoclax, navitoclax or A-1331852 for 24hrs. The impact on cell viability was subsequently determined by CellTiter-Glo and the synergy assessed using the Bliss independence model, where Bliss Sums represent the cumulative Bliss scores across the combination matrix. Red bar represents the median (B). SW620 cells were treated with ABBV-621 (80 pM), A-1331852 (20 nM) alone or in combination and the impact on caspase-3/-7 activation or cell viability (DRAQ7 positive cells) determined as a function of time. Alternatively, SW620 cells were pre-treated
with z-VAD-fmk (50 µM) for 1 hour prior to treatment and the impact of ABBV-621 dose at a fixed concentration of A-1331852 (20 nM) determined on caspase-3/-7 activation and DRAQ7 uptake (C). Mice bearing subcutaneous SW620 tumors were treated with ABBV-621 or A-1331852 alone or in combination and the impact on tumor volume determined. Data are presented as the mean ± s.e.m. of 8 mice per treatment arm (D). SW620 CDXs were treated with a single dose of ABBV-621 (3 mg/kg, IP) and tumor harvest at the indicated time points post-treatment. ABBV-621 binding to DR4 and DR5 and formation of the DISC was subsequently determined by ELISA. Data are presented as the mean ± s.e.m. of tumors harvested from four individual mice per time point (E). Parental and BID deficient (BID<sup>−/−</sup>) SW620 cells were co-treated with ABBV-621 and A-1331852 at the indicated concentrations for 24 hours and the impact on cell viability determined using CellTiter-Glo. Inset depicts the expression of BID, caspase-8, and β-actin expression the parental and BID<sup>−/−</sup> SW620 cells by western blot (F). Alternatively, parental and BID deficient (BID<sup>−/−</sup>) SW620 cells were treated with ABBV-621 (80 pM) or A-1331852 (20 nM) alone or in combination for 8 hours, and the impact on caspase-9 was determined by western blot. BID and β-actin expression were also assessed (G).
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Hexavalent TRAIL fusion protein eftozanermin alfa optimally clusters apoptosis-inducing TRAIL receptors to induce on-target antitumor activity in solid tumors


Cancer Res Published OnlineFirst March 9, 2021.