LGR5-targeted antibody-drug conjugate eradicates gastrointestinal tumors and prevents recurrence

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Abstract

Gastrointestinal cancer is one of the leading causes of cancer-related mortality in men and women worldwide. The adult stem cell marker LGR5 (leucine-rich repeat containing, G protein-coupled receptor 5) is highly expressed in a significant fraction of gastrointestinal tumors of the colon, liver, pancreas, and stomach, relative to normal tissues. LGR5 is located on the cell surface and undergoes rapid, constitutive internalization independent of ligand. Furthermore, LGR5-high cancer cells have been shown to exhibit the properties of tumor-initiating cells or cancer stem cells (CSCs). Based on these attributes, we generated two LGR5-targeting antibody-drug conjugates (ADCs) by tethering the tubulin-inhibiting cytotoxic drug monomethyl auristatin E to a highly specific anti-LGR5 monoclonal antibody via a protease cleavable or non-cleavable chemical linker and compared them in receptor binding, cell internalization, and cytotoxic efficacy in cancer cells. Here we show that both ADCs bind LGR5 with high specificity and equivalent nanomolar affinity, and rapidly internalize to the lysosomes of LGR5-expressing gastrointestinal cancer cells. The anti-LGR5 ADCs effectively induced cytotoxicity in LGR5-high gastrointestinal cancer cells, but not in LGR5-negative or -knockdown cancer cell lines. Overall, we demonstrate that the cleavable ADC exhibited higher potency in vitro and was able to eradicate tumors and prevent recurrence in a xenograft model of colon cancer. These findings provide preclinical evidence for the potential of LGR5-targeting ADCs as effective new therapeutics for the treatment and eradication of gastrointestinal tumors and CSCs with high LGR5 expression.
Introduction

Gastrointestinal cancer is a leading cause of cancer-related death worldwide with an estimated 2.3 million new cases and 1.4 million mortalities occurring in 2012 from colorectal and stomach cancers alone (1). LGR5 (leucine-rich repeat containing, G protein-coupled receptor 5) expression is highly elevated in gastrointestinal cancers of the colon, liver, pancreas and stomach (2-5). The LGR5 receptor was initially identified as a marker of intestinal crypt stem cells and has since been established as a bona fide marker of adult stem cells in several major epithelial tissues (6-10). In normal tissues, LGR5 is exclusively expressed at low levels on the cell-surface of the adult stem cells (11, 12). Our group and others showed that LGR5 and its related receptors LGR4 and LGR6 bind the R-spondin growth factors (RSPO1-4) and function to potentiate Wnt signaling (13, 14). The RSPO-LGR system has since emerged as a major ligand-receptor system in the control of stem cell survival and tumorigenesis. A series of studies using mouse models with loss of the tumor suppressor APC (adenomatous polyposis coli) gene have established that LGR5-positive stem cells are the cells-of-origin that drive intestinal and gastric tumor development (6, 15). LGR5-high cancer cells have been shown to exhibit the properties of tumor-initiating cells or cancer stem cells (CSCs) which retain the capacity for self renewal (2, 15-20). CSCs can fuel tumor growth, confer resistance to standard chemotherapy and radiation, and are likely to contribute to recurrent disease (21). Correspondingly, high LGR5 expression in gastrointestinal tumors of different tissue types correlate with higher incidence of metastasis, drug resistance, and poor patient survival (16, 22-24). Thus, LGR5 may serve as an effective therapeutic target for the treatment of gastrointestinal cancers and CSC-based therapy.

Antibody-drug conjugates (ADCs) are unique therapeutics that utilizes highly specific monoclonal antibodies (mAbs) to deliver extremely potent cytotoxic drugs to the tumor site through antigen targets enriched on the cell surface (25, 26). The innovation of ADC design resides within the chemical linker that tethers the cytotoxic drug to the mAb, preventing non-specific drug release to normal tissues during systemic circulation by conferring a therapeutic index to the cytotoxic payload (26). In general, cleavable
chemical linkers release drug through enzymatic cleavage (e.g., cathepsins) following internalization, whereas non-cleavable linkers must undergo lysosomal degradation of the ADC to release active drug. Regardless of the nature of the linker, ADC efficacy is dependent upon its ability to bind tumor-specific antigen with high affinity, robustly internalize within the tumor cell, and traffic to the lysosome for processing and payload release (26). Our previous work showed that LGR5 is rapidly and constitutively internalized through clathrin-mediated endocytosis independent of ligand (27). LGR5 localizes with Rab7/9 (28), which is essential for proper trafficking to the lysosome. As LGR5+ adult stem cells in the mouse have proven to be dispensable (11, 29), its high expression in tumors and rapid internalization make LGR5 a promising candidate for ADC development.

We generated two LGR5-targeting ADCs by conjugating an anti-LGR5 monoclonal antibody (anti-LGR5 mAb) to the antimitotic, tubulin-inhibiting agent monomethyl auristatin E (MMAE) via a cleavable or non-cleavable chemical linker. We compared binding, internalization and cytotoxicity using gastrointestinal cancer cell lines. Here we show that the anti-LGR5 ADC with the cleavable linker was more potent at eradicating gastrointestinal cancer cells in vitro and effectively inhibited tumor growth and recurrence in vivo.

Materials and Methods

Plasmids and cloning

Human RSPO3 (Origene, Rockville, MD; SC128235) and the Fc of mouse IgG2a from mRSPO1-Fc (Calvin Kuo, Stanford) were subcloned into pIRESpuro3 (Clontech, Mountain View, CA) to generate the hRSPO3-Fc plasmid. Super8×TOPflash and pRL-SV40 (Renilla) were purchased from Addgene (Cambridge, MA) and Promega (Madison, WA), respectively. For the anti-LGR5 monoclonal antibody (anti-LGR5 mAb) rat-mouse chimera constructs, codon optimized sequences encoding the light and heavy chain variable regions for the rat 8F2 antibody (14, 30) were synthesized (Epoch Life Sciences, Missouri City, TX) and subcloned into expression vectors containing the constant region for kappa light
chain and the mouse IgG2a heavy chain, respectively (Zhiqiang An, UT Health Science Center at Houston) (31).

**Recombinant proteins, chemicals, and commercial antibodies**

Wnt3a conditioned media (Wnt3aCM) was produced as previously described (13). hRSPO1 was purchased from R&D Systems (Minneapolis, MN). hRSPO3-Fc CM was produced as described (13) and protein was purified using protein A agarose column, verified by SDS-PAGE and quantified (OD280 nm). For organoid cultures: Noggin (Peptrotech, Rocky Hill, NJ), N-Acetylcysteine (Sigma, St. Louis, MO), N2, B27, and mEGF (Life Technologies, Carlsbad, CA). Monomethyl auristatin E (MMAE) was purchased from ALB Technology (Mongkok Kowloon, Hong Kong). All commercial antibodies were used in accordance to manufacturer’s guidelines. For western blot analysis: anti-LGR5 (Abcam, Cambridge, MA; ab75732), and anti-β-actin (Cell Signaling, Danvers, MA; 4970). For immunocytochemistry (ICC): anti-Lamp-1 (Cell Signaling, Danvers, MA; 9091), anti-LGR5 (BD Biosciences, San Jose, CA; 562731), anti-HA-Alexa-594, TO-PRO-3, and secondary antibodies goat anti-rabbit-Alexa-488, goat anti-mouse-Alexa-594, goat anti-mouse-Alexa-488, and goat anti-rat-Alexa-555 (Life Technologies, Carlsbad, CA).

**Cell culture, stable cell line generation**

HEK293T, AGS, and HCT15 cells were authenticated and purchased from ATCC (Manassas, VA). LoVo cells were obtained from the laboratory of Dr. Shao-Cong Sun at M.D. Anderson Cancer Center. Cell lines were authenticated utilizing short tandem repeat profiling and routinely tested for mycoplasma. HEK293T cells were cultured in DMEM, AGS in F-12K, and LoVo and HCT15 cells were cultured in RPMI medium. All medium was supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C with 95% humidity and 5% CO2. To generate stable LGR5 knockdown cells, LoVo cells were infected with lentivirus particles produced by co-transfecting HEK293T cells with a pLKO.1 vector incorporating the LGR5-targeted shRNA and packaging plasmids, psPAX2 and
pMD2.G, using Fugene 6 (Roche, Indianapolis, IN). Virus infected cells were selected with puromycin followed by clonal selection. The shRNA for human LGR5, TRCN0000011589 (shLGR5) was from GE Dharmacon (Lafayette, CO). Stable HEK293T cell lines over-expressing hLGR5 or hLGR4 and vector cells were generated as previously reported (13).

**Synthesis of maleimido-functionalized MMAE**

MMAE was modified with a short linker to permit conjugation to partially reduced thiols. A solution of MMAE in anhydrous DMSO was treated with N, N-diisopropylethylamine and 3-(maleimido)propionic acid N-hydroxysuccinimide ester (Thermo Fisher Scientific, Waltham, MA) and the mixture was stirred 2 days at room temperature. The reaction mixture was evaporated and purified by silica gel chromatography using dichloromethane:methanol (100:3) as an eluant (~67% yield). The expected molecular weight of the purified product was confirmed by mass spectroscopy: ESI-MS (m/z): 869.8(M+H)+; 891.0(M+Na)+.

**Anti-LGR5 mAb production and ADC generation**

The anti-LGR5 mAb was transiently expressed in 293F suspension cells by co-transfecting the light and heavy chain constructs using polyethylenimine (PEI) reagent. Medium was harvested approximately 7 days post transfection. Antibodies were purified using protein A agarose (Life Technologies, Carlsbad, CA) affinity chromatography, eluted in PBS (pH7.4), analyzed for purity and homogeneity (SDS-PAGE and HPLC), and quantified (OD280 nm). The cleavable and non-cleavable ADCs were both generated using cysteine-based conjugation methods. Anti-LGR5-mc-vc-PAB-MMAE was generated by The Chemistry Research Solution, LLC (Bristol, PA) by conjugating mc-vc-PAB-MMAE to the mAb through accessible disulfide bonds using similar methodology as has been previously described (32). Anti-LGR5-mp-MMAE was generated in house as follows: Purified mAb was diluted with PBS (pH 7.4) to a final concentration of 3 mg/mL and warmed to 37°C. A stock solution of DTT (50 mM) was prepared and a 2.5 molar equivalent was added. After 3 hours, the reaction was cooled to room temperature. The mAb was purified with a Zeba desalting spin column (Thermo Fisher Scientific, Waltham, MA) and collected.
in 0.1 M Na₂HPO₄, pH 8.3. A 5 molar equivalent of maleimido-functionalized MMAE payload (2 mM in DMSO) was added to the mAb and payload conjugation was performed for 2 hours at 25°C followed by 4°C overnight. The ADC was Zeba purified and collected in PBS (pH 7.4). ADCs were analyzed by HPLC to identify possible aggregate formation and by UV-VIS spectroscopy to assess drug-antibody ratio (DAR) using previously published methodology (33). An average DAR of 4 was determined for both anti-LGR5 ADCs.

**Western blot and SuperTopflash luciferase assays**

SuperTopFlash assays were performed as previously described (13). Cell were treated with serial dilutions of anti-LGR5 mAb, hRSPO1 (30 ng/ml), or Wnt3aCM (1:5 dilution) as indicated and incubated overnight at 37°C. Luciferase measurements were carried out using the Dual-Glo® luciferase assay kit (Promega, Madison, WI) according to the manufacturer’s protocol and EnVision multilabel plate reader (PerkinElmer, Waltham, MA). Each conditioned was tested in quadruplicate for 3 experiments. For western blot, protein extraction was performed using RIPA buffer (Sigma, St. Louis, MO) supplemented with protease/phosphatase inhibitors. Cell lysates and tumor samples were incubated at 37°C for 1 hour in 2xSDS buffer prior to equal loading on SDS-PAGE. HRP-labeled secondary antibodies were utilized for detection with the standard ECL protocol. Quantification was performed using ImageJ.

**Internalization and binding assays**

For ICC internalization assays, HEK293T, LoVo or AGS cells were seeded into 8-well chamber slides (BD Biosciences, San Jose, CA) and allowed to adhere overnight. The next day, cells were incubated with anti-LGR5 mAb, anti-LGR5 ADCs, or co-incubated with hRSPO3-Fc and commercial anti-LGR5 antibody at 37°C for 1 hr. Cells were washed, fixed, and permeabilized. For lysosome co-localization studies, cells were incubated with anti-Lamp-1 for 45 min a followed by anti-rabbit-Alexa-488 and anti-mouse-Alexa-594 for 1 hour at room temperature, respectively. For hRSPO3-Fc studies, cells were incubated with anti-mouse-Alexa-488 and anti-rat-Alexa-555 antibodies. Nuclei were counterstained with
TO-PRO®-3. Images were acquired using confocal microscopy (Leica TCS SP5 microscope) with the LAS AF Lite software (Leica Microsystems, Inc., Buffalo Grove, IL). For binding assays, stable HEK293T cells over-expressing vector, hLGR5, or hLGR4 were seeded onto poly-D-lysine coated 96-well plates and allowed to attach overnight. Serial dilutions of anti-LGR5 mAb or anti-LGR5 ADCs were added for 3 hours at 4°C. Plates were washed, fixed and incubated with anti-mouse Alexa-594 for 1 hour at room temperature. Fluorescence intensity was quantified using Tecan Infinite M1000 plate reader (Tecan, Männedorf, Switzerland). Data were analyzed with GraphPad Prism software using the logistic nonlinear regression model.

In vitro cytotoxicity

The cytotoxicity of the various antibodies was tested on 293T-hLGR5, 293T-hLGR4, AGS, LoVo, LoVo-shLGR5, and HCT15 cell lines. 293T cells were plated at 500 cells/well and the cancer cell lines were plated at 1000 cells/well in 96 half-well plates. Serial dilutions of unconjugated anti-LGR5 antibody, anti-LGR5 ADCs, or free MMAE were added to the plate and allowed to incubate for 4 days at 37°C. Each condition was tested in triplicate in 2-4 separate experiments. The resulting cell viability was measured using CellTiter-Glo (Promega, Madison, WI) according to manufacturer’s protocol and the EnVision multilabel plate reader (PerkinElmer, Waltham, MA). Data were analyzed with GraphPad Prism software using the logistic nonlinear regression model.

Evaluation of in vivo efficacy in xenografts

Animal studies were carried out in strict accordance with the recommendations of the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at Houston. Female 6-8 week old nu/nu mice (Charles River Laboratories, Houston, TX) were subcutaneously inoculated with 3.5 x 10^6 LoVo cells in 1:1 mixture with matrigel (BD Biosciences, San Jose, CA). After 2 weeks, when tumor size reached ~ 100 mm^3, mice were randomized into 3 groups of 6-7 mice and intravenously treated with either PBS vehicle, unconjugated anti-LGR5 mAb (8 mg mAb/kg), or anti-LGR5-mc-vc-PAB-MMAE (8
mg ADC/kg) weekly for 3 weeks. Dosing regimen of 8 mg/kg Q7Dx3 was selected to evaluate ADC efficacy and any potential toxicity until a complete response was observed. Tumor volumes were measured bi-weekly and estimated by the formula: Tumor volume= length x width\(^2\)/2. Mice were euthanized when tumor volume reached 1000 mm\(^3\). Tumors and intestines were harvested for western blot and immunohistochemical analysis, respectively. Intestinal tissue was fixed then paraffin embedded, sectioned and H&E stained by the Tissue Histopathology Center (UT Health, Houston, TX). Five mice from the anti-LGR5-mc-vc-PAB-MMAE treatment group were further monitored for tumor recurrence 35 days post-treatment or until the size of first tumor re-growth reached ≥ 200 mm\(^3\).

**Ex vivo intestinal organoid culture**

Mouse intestinal organoid cultures were established as previously published (34). Briefly, small intestines were harvested from wild-type B6.129P2-Lgr5tm1(cre/ERT2)Cle/J (Jackson Labs, Bar Harbor, ME) and washed to remove contaminants and villi. Intestinal fragments were incubated in EDTA for 30 min, strained, and centrifuged. Crypts were resuspended in matrigel with DMEM/F12 media containing 10mM HEPES, Glutamax, 1x B27, 1x N2, 1mM N-acetylcysteine, 50 ng/ml mEGF, 100 ng/ml mNoggin, 20 ng/ml RSPO1 and Pen/Strep. The media was replenished every 2-3 days and the organoids were mechanically broken down by glass pipette and passaged every 5-6 days. Organoids were treated with PBS vehicle, anit-LGR5-mc-vc-PAB-MMAE (1 or 10 \(\mu\)g/ml), or 7 ng/ml MMAE to test toxicity. Organoid viability (~11-20 organoids/treatment) was quantified using bright field microscopy and image analysis.

**Statistical analysis**

Data are expressed as mean +/- SEM or SD as indicated in results section. CCLE and TCGA datasets were partitioned into low, medium, and high expression values based on overall distribution range of each cohort. For TCGA data, normal and tumor samples were compared and analyzed using paired t-test. For in vivo studies, differences between groups were analyzed by unpaired t-test. Multiple comparisons used
one-way ANOVA and Tukey’s post-hoc analysis. P-values ≤ 0.05 were considered statistically significant.

Results

Increased expression of LGR5 in gastrointestinal tumors

To evaluate the level of LGR5 gene expression in gastrointestinal tumors, we examined whole transcriptome sequencing datasets of different tumor types from The Cancer Genome Atlas (TCGA) (35). Based on the distribution of Log2 RSEM (RNA-Seq by Expectation-Maximization) or RPKM (Reads Per Kilobase of transcript per Million) of the transcripts, LGR5 was shown to be expressed at moderate to high levels in a significant fraction of cases (Fig 1A). Discretization of the data into low, medium, and high partitioned values demonstrated high level expression of LGR5 in 67%, 28%, 20%, and 22% of cases in the colorectal, liver, pancreatic, and stomach cohorts, respectively (Fig. 1A). Similarly, mining of publically accessible microarray datasets from the Cancer Cell Line Encyclopedia (CCLE) showed moderate to high LGR5 expression (Log2 Robust Multi-array Average (RMA)-normalized) in 49%, 28%, 10%, and 29% of colon, liver, pancreatic, and stomach cancer cell lines, respectively (Fig. 1B) (36).

Utilizing individual patient data extracted from the TCGA data matrix, we investigated the difference in LGR5 expression between tumors and matched normal samples from the colon adenocarcinoma (COAD) (Fig. 1C) and stomach adenocarcinoma (STAD) (Fig. 1D) cohorts (p< 0.0001). By implementing a fold change cut-off of 2, we found that 70% of the COAD and 78% of the STAD patient population exhibited increased LGR5 expression in primary tumor compared to normal adjacent tissue (Fig. 1C and 1D). To assess LGR5 protein expression in gastrointestinal cancer cell lines, we conducted western blot analysis (Fig. 1E). As we reported previously, HEK293T cells express LGR4 with an insignificant level of LGR5 (13). Therefore, we used established stable cell lines over-expressing recombinant myc-tagged human LGR5 (293T-hLGR5) or vector control (293T-vector) as positive and negative controls for LGR5 expression (Fig. 1E) (13). AGS gastric cancer and LoVo colon cancer cells were found to express high
levels of LGR5, whereas no LGR5 was detected in HCT15 colon cancer cells or in Lovo cells with lentivirus-mediated shRNA knockdown of LGR5 (LoVo-shLGR5; Fig. 1E). These western blot findings were consistent with LGR5 gene expression data extracted from the CCLE for each of the aforementioned cancer cell lines (Fig. 1F). Collectively, these data show that LGR5 expression is significantly elevated in gastrointestinal tumors and cancer cell lines of diverse tissue types.

Characterization of the anti-LGR5 mAb

We produced and purified the 8F2 anti-LGR5 mAb as a rat-mouse chimera (Supplementary Fig. S1A-B), which recognizes the N-terminus of LGR5 as shown through epitope mapping (14). Using a fluorescence-based binding assay we showed that anti-LGR5 mAb binds LGR5 expressed on the surface of 293T-hLGR5 cells with high affinity (Kd = 0.92 μg/ml or 6 nM; Fig. 2A). Anti-LGR5 mAb does not bind 293T-vector cells or 293T cells over-expressing related receptor hLGR4 (293T-hLGR4 cells; Supplementary Fig. S2), indicating antigen specificity (Fig. 2A). Next, we tested whether the anti-LGR5 mAb could block binding of RSPO ligand to the LGR5 receptor. 293T-vector, -hLGR4, and –hLGR5 cells lines were incubated with 1.5 μg/ml RSPO3-Fc and an approximate 10-fold molar access of anti-LGR5 mAb and incubated for 1 hour at 37°C. Of note, a commercial rat anti-LGR5 mAb with the identical variable region as our mAb was used since both our mAb and the RSPO3-Fc fusion protein incorporate the mouse IgG2a Fc. Using ICC and confocal analysis we found that both RSPO3-Fc and anti-LGR5 mAb simultaneously bound and co-internalized in 293T-hLGR5 cells (Fig. 2B). As expected RSPO3-Fc binding was detected in 293T-hLGR4, but not 293T-vector cells and no binding of anti-LGR5 mAb was detected in either 293T-vector or -hLGR4 cells (Fig. 2B). These data suggest that the anti-LGR5 mAb does not compete with RSPO ligand for binding to the LGR5 receptor and that the mAb epitope is separate from the ligand binding site of the LGR5 extracellular domain. Since RSPO binding to LGR5 potentiates activation of the Wnt/β-catenin signaling pathway, we examined the effect of anti-LGR5 mAb on LGR5 function using the SuperTopFlash assay. This Wnt/β-catenin-responsive luciferase
reporter assay measures the level of active or stabilized nuclear β-catenin/TCF (T-cell factor) complexes binding to 8 tandem repeats of the Wnt/TCF transcriptional response element. 293T-vector and -hLGR5 cells were treated with serial dilutions of anti-LGR5 mAb in the presence of vehicle, Wnt3a CM, or Wnt3aCM+RSPO1 (30 ng/ml). As shown in Fig. 2C and 2D, anti-LGR5 mAb does not inhibit RSPO1-mediated potentiation of the Wnt pathway. Interestingly, at higher concentrations (> 0.4 μg/ml or 2.5 nM), anti-LGR5 mAb essentially enhances Wnt/β-catenin signaling in 293T-hLGR5 cells treated with Wnt3aCM and to a greater extent in the presence of exogenous RSPO1 (Fig. 2D). We can speculate that this synergy between the anti-LGR5 mAb and LGR5 ligands is due to changes in receptor conformation, yet the molecular mechanism remains unknown. These studies establish that anti-LGR5 mAb-8F2 is highly specific, binds LGR5 at the cell surface with nanomolar affinity and can co-internalize with receptor in the presence or absence of ligand without neutralizing RSPO-LGR5 signaling function.

**Generation, binding, and internalization of anti-LGR5 ADCs with cleavable and non-cleavable chemical linkers**

The anti-LGR5 mAb was conjugated to MMAE toxin via the cathepsin B protease-sensitive linker maleimidocaproyl-valine-citrulline-p-aminobenzylxycarbonyl (mc-vc-PAB) as has been described (32, 37) and via a non-cleavable maleimidopropionyl linker resulting in the generation of two separate ADCs, anti-LGR5-mc-vc-PAB-MMAE and anti-LGR5-mp-MMAE, respectively (Fig. 3A). For the latter conjugation method, MMAE was successfully functionalized with a maleimidopropionyl linker and then conjugated to free thiols of the anti-LGR5 mAb under partial reduction conditions. The ADCs were tested for purity and homogeneity using HPLC (not shown) and no aggregates were detected. Coomassie staining of SDS-PAGE run showed a gel shift of the ADCs compared to unconjugated anti-LGR5 mAb, indicating an increased molecular weight due to the addition of MMAE (Fig. S1B). Both ADCs were determined to have an equivalent average drug-to-antibody ratio (DAR=4) making them suitable for direct comparison of binding and functional efficacy.
Using a fluorescence-based binding assay, we found that anti-LGR5-mp-MMAE and anti-LGR5-mc-vc-PAB-MMAE were comparable to unconjugated anti-LGR5 mAb in binding to 293T-hLGR5 cells and lacked interaction with LGR5-negative 293T-vector cells (Fig. 2A and 3B). Furthermore, no measurable binding of ADCs to 293T-hLGR4 cells was detected (Supplementary Fig. S3A). These results indicate that neither conjugation approach affected mAb binding affinity or specificity. To evaluate the trafficking and internalization of the anti-LGR5 ADCs, AGS (gastric cancer) and LoVo (colon cancer) cells were incubated with anti-LGR5-mp-MMAE or LGR5-mc-vc-PAB-MMAE or for 1 hour at 37°C. ICC and confocal microscopy showed that both ADCs were rapidly internalized within the cancer cells (Fig 3C, top panels). Since ADC efficacy is dependent upon the extent of trafficking to the lysosome for either peptide linker- or total degradation-mediated release of the toxin (26), we investigated intracellular co-localization of ADCs to the acidic lysosomal compartment. Lysosomes were visualized using an antibody specific to the lysosome-associated membrane protein 1 (Lamp-1; Fig. 3C, middle panels). Both ADCs co-localized with LAMP1 in a distinct and punctate pattern in AGS and LoVo cells (Fig. 3C, bottom panels). No ADC binding or lysosome co-localization was detected in LGR5-negative LoVo-shLGR5 cells (Fig. 3C, right-hand panels). Collectively, these results suggest that conjugation of MMAE to anti-LGR5 mAb via a cleavable or non-cleavable linker does not affect its binding efficacy and that both anti-LGR5 ADCs are trafficked to the lysosome in a manner consistent with the ADC mechanism of action (26).

**Anti-LGR5 ADC with the cleavable linker is more effective at inducing gastrointestinal cancer cell cytotoxicity**

To evaluate target-dependent in vitro cytotoxicity, AGS and LoVo cancer cells which express high levels of LGR5 (Fig. 1E and 1F) were incubated with increasing concentrations of unconjugated anti-LGR5 mAb, anti-LGR5-mp-MMAE, anti-LGR5-mc-vc-PAB-MMAE, or free MMAE and cell viability was measured after 4 days (Fig. 4A, 4B, and Supplementary Fig. S3B). Both AGS and LoVo cells demonstrated MMAE sensitivity, with exposure to free drug mediating cell cytotoxicity at EC₅₀ = 0.08
nM and 0.46 nM, respectively (Supplementary Fig. S3B). In AGS cells, anti-LGR5-mc-vc-PAB-MMAE exhibited 11-fold greater potency than anti-LGR5-mp-MMAE at cell killing (EC₅₀ = 0.69 μg/ml or 4.5 nM and 7.9 μg/ml or 51 nM, respectively; Fig. 4A). Furthermore, anti-LGR5-mc-vc-PAB-MMAE was more cytotoxic to LoVo cells compared to AGS cells and showed a 20-fold greater potency compared to anti-LGR5-mp-MMAE (EC₅₀ = 0.36 μg/ml or 2.3 nM and 7.3 μg/ml or 47 nM, respectively; Fig. 4B). Unconjugated anti-LGR5 mAb had no significant effect on cell viability in either cell line (Fig. 4A and 4B). Since, anti-LGR5-mc-vc-PAB-MMAE was more effective at killing cancer cells; it was selected as our lead ADC for the remainder of this study. To confirm target antigen specificity, we tested cytotoxicity using LGR5 negative cell lines LoVo-shLGR5 and HCT15 (Fig. 1E-F and 4C-D). Anti-LGR5-mc-vc-PAB-MMAE and anti-LGR5 mAb had no significant effect on either cell line up to 10 μg/ml (Fig. 4C and 4D). Cytotoxicity was also measured in the receptor over-expression cell lines, 293T-LGR4 and -hLGR5. As shown in Fig. 4E, anti-LGR5-mc-vc-PAB-MMAE killed 293T-hLGR5 cells with high potency (EC₅₀ = 3.6 x 10⁻³ μg/ml or 2.3 pM) and had no effect on 293T-hLGR4 cells up to 3 μg/ml, further validating target specificity. As expected, anti-LGR5 mAb had no effect on either cell line (Fig. 4E). These data demonstrate that although both cleavable and non-cleavable linker anti-LGR5 ADCs can mediate cytotoxicity in LGR5-expressing cancer cell lines in a concentration-dependent manner, the cleavable anti-LGR5-mc-vc-PAB-MMAE exhibits the highest overall potency.

**Anti-LGR5 ADC inhibits tumor growth and recurrence in vivo**

Since LGR5 is normally expressed at low levels throughout the body with its most notable expression in the stem cells of the intestinal crypts (8, 11), we presumed that if anti-LGR5 ADC treatment were to result in any toxicity in the mouse, it would likely occur within the intestine. Using a fluorescence-based binding assay we showed that anti-LGR5 mAb binds mouse LGR5 expressed on the surface of 293T-mLGR5 cells, however with much lower affinity and maximum binding compared to human LGR5 (Supplementary Fig. S4A and S4B). Prior to in vivo testing, we established an ex vivo intestinal crypt
organoid model and investigated whether anti-LGR5-mc-vc-PAB-MMAE had any effect on organoid viability (Fig. 5A and Supplementary Fig. S4C). Organoids were treated with anti-LGR5-mc-vc-PAB-MMAE (1 or 10 μg/ml) for 1 week and subsequently passaged with no observable cytotoxicity (Fig. 5A and Supplementary Fig. S4). In contrast, exposure to free MMAE resulted in complete cell death (Fig. 5A and Supplementary Fig. S4). This suggests that anti-LGR5-mc-vc-PAB-MMAE should not induce significant target-mediated toxicity in the intestinal system vivo.

To assess the in vivo efficacy of anti-LGR5-mc-vc-PAB-MMAE, athymic nu/nu mice were implanted with LoVo colon cancer cells and randomized into groups of 6-7 mice once tumors reached ~100 mm³. Each group was administered intravenously via tail vein with vehicle (PBS), unconjugated anti-LGR5 mAb (8 mg/kg), or anti-LGR5-mc-vc-PAB-MMAE (8 mg/kg) one dose every week for 3 weeks (Q7Dx3; Fig. 5B). All 7 mice treated with anti-LGR5-mc-vc-PAB-MMAE had a significant reduction in tumor size compared to the other treatment cohorts, with 4 mice exhibiting a complete response (i.e. no detectable tumor; Fig. 5B and Supplementary Fig. S5A). There was a significant reduction in the rate of tumor growth of the anti-LGR5 mAb-treated cohort compared to the vehicle cohort (Fig. 5B). Since exposure to anti-LGR5 mAb had no effect on cancer cell viability in vitro (Fig. 4), this suggests an anti-tumor immune response mechanism such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (38, 39). No indications of overt toxicity were observed in any group. There were no significant effects in body weight (Supplementary Fig. S5B) and H&E staining of intestinal tissue sections from each treatment group did not show any morphological changes or signs of cell death (Supplementary Fig. S6). Five mice from the anti-LGR5-mc-vc-PAB-MMAE cohort (2 with residual tumor and 3 with complete regression) were monitored for tumor recurrence subsequent to last treatment dose and until tumor volume reached >200 mm³. Three mice exhibited re-growth, whereas two of the mice with complete regression after treatment had no tumor recurrence (Fig. 5C and Supplementary Fig. S7). We then measured the LGR5 expression levels in vehicle, anti-LGR5 mAb, and recurrent tumors from the anti-LGR5-mc-vc-PAB-MMAE group using western blot analysis (Fig 5D). On average,
the recurrent tumors expressed significantly lower levels of LGR5 compared to vehicle and anti-LGR5 mAb groups. These data provide evidence that anti-LGR5-mc-vc-PAB-MMAE can effectively target and destroy LGR5-expressing tumor and suppress tumor recurrence.

Discussion

A major dilemma in cancer therapeutics has been the need to target and destroy tumors while minimizing undesired toxicity to normal tissue. First, many cytotoxic drugs fail to have a therapeutic index since they are equally damaging to both cancer and normal cells. Secondly, several mAb-based therapies that target tumor-specific antigens and limit toxicity to normal tissues do not always destroy cancer cells. ADC technology remedies this conflict by coupling a nonspecific cytotoxic drug to a highly specific mAb (which need not be functionally effective against the tumor on its own) through a chemical linker with high systemic stability. The clinical success of ADCs, brentuximab-vedotin and trastuzumab-emtansine, has validated this approach and many more ADCs are at various stages of clinical development (25, 26). The selection of target is critical in that it must be highly expressed in tumor compared to normal tissue and be effectively internalized and trafficked to the lysosome for the release of the cytotoxin. We selected LGR5 as a target for ADC development due to its high expression in gastrointestinal cancers of different tissue types relative to its low cell-surface expression in normal tissues which is restricted to the adult stem cells (2-5, 11). LGR5 is also rapidly and constitutively internalized in a ligand-independent manner, making it an ideal transit for ADC uptake into cancer cells (27).

We generated two LGR5-targeting ADCs, one with the enzyme cleavable valine-citrulline peptide linker (anti-LGR5-mc-vc-PAB-MMAE) and the other with a non-cleavable maleimidopropionyl linker (anti-LGR5-mp-MMAE). We found that both ADCs bind LGR5 with equal nanomolar affinity and rapidly internalize to the lysosome of gastrointestinal cancer cells. However, cleavable anti-LGR5-mc-vc-PAB-MMAE exhibited 10-20 fold greater potency at killing gastrointestinal cancer cells in vitro. The difference in potency may be a result of enzyme-mediated proteolytic cleavage (e.g. lysosomal
cathepsins) being more efficient for drug release than lysosome-mediated degradation of the non-cleavable ADC. In fact, cleavable ADCs against poorly internalized targets have shown to be effective in vivo (37), suggesting a role for extracellular proteases in ADC activation. Yet, another mechanism that could explain why the cleavable ADC is more cytotoxic is the bystander effect (38, 40). This phenomenon is attributed to the efflux of hydrophobic cytotoxic drugs, like MMAE, which can diffuse from target cells and subsequently permeate adjacent cells that need not be positive for the target antigen. This bystander effect is not observed with non-cleavable ADCs since its degradation results in charged metabolites (e.g., amino acid-linker-cytotoxin) that are impermeable to the membrane. While bystander cytotoxicity may reduce ADC specificity, the extent of the effect depends largely on conjugation chemistry and may ultimately be advantageous when treating solid tumors that are composed of heterogeneous cell populations (40).

Using a xenograft model of colon cancer, we demonstrated that treatment with anti-LGR5-mc-vc-PAB-MMAE can effectively eliminate tumors with high LGR5 expression and prevent recurrence. Interestingly, of the tumors that were not completely ablated and eventually grew back, LGR5 expression was significantly lower compared to vehicle and nearly all tumors treated with anti-LGR5 mAb. This suggests that either (1) a subpopulation of LGR5-low/null LoVo cells exists in which the ADC was ineffective at targeting or (2) ADC treatment destroyed the majority of LGR5-high LoVo cells, yet the residual LGR5-high cells that were not eliminated essentially gave rise to a LGR5-low/null subpopulation of cells to drive tumor re-growth. However, since two mice showed no recurrent tumors subsequent to ADC treatment, it seems more feasible that the latter is true. In fact, it has been reported that both LGR5-positive intestinal crypt stem cells and colon CSCs can interconvert with LGR5-negative cells (19, 41). The conversion of LGR5-positive CSCs was dependent upon exposure to chemotherapy resulting in an LGR5-negative drug-resistant phenotype, though both cell types retained tumor-initiating activity (19). We also observed that anti-LGR5 mAb-treated tumors grew more slowly compared to vehicle and two of them expressed low levels of LGR5. This suggests a potential immune response effect that may have lead
to an interconversion at some time during the course of treatment. LGR5 mAb-based therapy mediated by complement-dependent cytotoxicity has been reported (39). Future studies involving adjustments in ADC dosing conditions and comprehensive testing in LoVo and other cancer models will be required to address these issues.

Several lines of evidence have identified LGR5 as a marker of CSCs making it an important target for development of CSC-directed therapeutics. APC deletion in LGR5+ stem cells of the intestinal crypt and stomach leads to rapid transformation and growth of macroscopic adenomas, suggesting LGR5-positive stem cells are tumor-initiating cells (6, 15). LGR5 antibody enrichment of CSCs in colorectal cancer demonstrated that LGR5 enhances clonogenicity (18). In a number of gastrointestinal cancer cell lines, LGR5 was shown to increase drug resistance and in colon primary tumors LGR5-high expression correlated with chemoresistance and lower disease-free and overall survival (16, 17, 20, 23, 42, 43). Thus, an LGR5-targeting ADC may not only effectively treat gastrointestinal tumors, but also function to eliminate CSCs. In fact, while this manuscript was in preparation a report was published further validating the importance and effectiveness of targeting LGR5 for the treatment of colon cancer using different ADCs (44). In summary, our anti-LGR5-mc-vc-PAB-MMAE has demonstrated significant efficacy in gastrointestinal cancer cell lines and in a colon cancer xenograft model, suggesting its potential as a promising new therapeutic to target CSCs, eradicate a LGR5-positive tumors of different tissue types, and prevent recurrence.
References


Figure Legends

Fig 1. Aberrant expression of LGR5 in gastrointestinal cancer. (A) LGR5 RNA-seq data from TCGA cohorts, COADREAD/colorectal (RSEM, 382-samples), LIHC/liver (RSEM, 373-samples), PAAD/pancreatic (RSEM, 179-samples), and STAD/stomach (RPKM, 265-samples). (B) CCLE data distribution partitioned by cancer type. Horizontal bars represent the median. (C-D) LGR5 RSEM values for matched tumor and adjacent normal from the TCGA cohorts (C) COAD/colon and (D) STAD/stomach. Statistical analysis was performed using paired t-test. (E) Western blot of LGR5 protein expression and (F) normalized LGR5 gene expression (CCLE) for cell lines used in this study. Baseline indicates no detectable expression.

Fig 2. Characterization of anti-LGR5 mAb binding specificity and its effect on Wnt signaling activity. (A) Anti-LGR5 mAb specifically binds LGR5 and not LGR4 expressed on the surface of 293T cells. (B) Anti-LGR5 mAb is non-competitive with R-spondin (RSPO) ligands for binding to the receptor as demonstrated by ICC and confocal imaging. (C-D) Anti-LGR5 mAb does not neutralize RSPO-induced SuperTopflash activity in the presence of vehicle or Wnt3aCM (1:5) ± RSPO1 (30 ng/ml) (3a ± R) in 293T (C) vector or (D) LGR5 cells.

Fig 3. Binding and internalization of anti-LGR5 ADCs. (A) Schematic of anti-LGR5 ADCs; anti-LGR5-mp-MMAE (non-cleavable linker) and anti-LGR5-mc-vc-PAB-MMAE (cleavable linker). mp, maleimidopropionyl; mc, maleimidoacaproyl; vc, valine-citrulline; PAB, para-aminobenzyloxy carbonyl; MMAE, monomethyl auristatin E. (B) Anti-LGR5 ADCs bind the surface of 293T-LGR5 cells, but not 293T-vector cells. (C) Confocal images showing anti-LGR5 ADCs in complex with the LGR5 receptor and co-localization with the lysosome marker LAMP1 in AGS and LoVo cancer cells after 1 hour incubation at 37°C. No binding was observed in LoVo cells with LGR5-shRNA knockdown (LoVo-shLGR5).
Fig. 4. Anti-LGR5 ADC-mediated in vitro cytotoxicity. Cell viability was measured after 4 days of treatment with anti-LGR5 mAb or ADCs using CellTiter-Glo assay. Anti-LGR5-mc-vc-PAB-MMAE induced a stronger cytotoxic effect on cell viability compared to anti-LGR5-mp-MMAE and anti-LGR5 mAb in (A) AGS and (B) LoVo cells. No significant cytotoxic effect was observed in (C) LoVo-shLGR5 cells or (D) the LGR5-null colon cancer cell line, HCT15. (E) Anti-LGR5-mc-vc-PAB-MMAE induces cytotoxicity in 293T cells over-expressing recombinant hLGR5, but not related receptor hLGR4.

Fig 5. Efficacy of anti-LGR5-mc-vc-PAB-MMAE on xenograft tumors and recurrence in vivo. (A) Quantification of intestinal organoid viability 6 days after treatment with vehicle (PBS), anti-LGR5 ADC, or 7ng/ml MMAE. Error bars indicate SD. (B) Established LoVo xenograft tumors in nu/nu mice were treated weekly with vehicle (n=7), anti-LGR5 mAb (8 mg/kg, n=6), or anti-LGR5 ADC (8 mg/kg, n=7) for 3 weeks (arrows indicate time of therapeutic i.v. injection); **p<0.01 versus vehicle group; ***p<0.0001 versus vehicle and anti-LGR5 group. Error bars indicate SEM. (C) Tumor recurrence examined in five mice after anti-LGR5 ADC treatment was terminated. (D) Western blot and quantification of LGR5 in tumors from mice treated with vehicle or anti-LGR5 mAb and recurrent tumors that developed subsequent to anti-LGR5 ADC treatment. Average LGR5 expression and SEM for each group are indicated in parenthesis; *p<0.02 versus vehicle and anti-LGR5 mAb groups.
Figure 1.

A

Expression of LGR5 Log2 [RSEM]

Normal

Tumor

0

3

6

9

12

p < 0.0001

TCGA STAD Samples

Expression of LGR5 Log2 [RSEM]

B

Expression of LGR5 Log2 [RPKM]

Colon

Liver

Pancreatic

Stomach

-5

0

5

10

15

-4

-2

0

2

4

6

Tumor Type

LGR5 Expression Log2 [RSEM]

LGR5 Expression Log2 [RPKM]

C

Expression of LGR5 Log2 [RSEM]

Normal

Tumor

TCGA COAD Samples

p < 0.0001

D

Expression of LGR5 Log2 [RSEM]

Normal

Tumor

TCGA STAD Samples

p < 0.0001

E

1: LoVo

2: LoVo-shLGR5

3: AGS

4: HCT15

5: 293T-hLGR5

6: 293T-vector

F

Log2 RMA-norm. LGR5 Expression

Baseline

Cancer Cell Type

Log2 RMA-norm. LGR5 Expression

Cancer Cell Line
Figure 2.

A

B

anti-LGR5  RSPO3-Fc  Merge

LGR5  LGR4

C

D

Vector  LGR5

Normalized TopFlash Activity

Normalized TopFlash Activity
Figure 3.

A

(1) LGR5 mAb mp MMAE Toxin

(2) LGR5 mAb mc vc PAB MMAE Toxin

B

-2 -1 0 1 2

Log [LGR5 antibody, $\mu$g/ml ]

Norm. Fluorescence

-0.5 0 0.5 1 1.5 2 2.5

-2 -1 0 1 2

anti-LGR5-mp-MMAE/ Vector cells

anti-LGR5-mp-MMAE/LGR5 cells

anti-LGR5-mc-vc-PAB-MMAE/Vector cells

anti-LGR5-mc-vc-PAB-MMAE/ LGR5 cells

C

<table>
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<tbody>
<tr>
<td>AGS</td>
<td>LoVo</td>
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LGR5

LAMP1

Merge

0 µm 25 µm
Figure 4.

A) AGS

B) LoVo

C) LoVo-shLGR5

D) HCT15

E) 293T-hLGR5

- anti-LGR5 mAb
- anti-LGR5-mp-MMAE
- anti-LGR5-mc-vc-PAB-MMAE

Log [LGR5 antibody, \(\mu\)g/ml]

% Cell Viability
Figure 5.

A) % Organoid Viability

B) Average Tumor Volume (mm³)

C) Tumor Volume (mm³)

D) Relative LGR5 Expression
Molecular Cancer Therapeutics

LGR5-targeted antibody-drug conjugate eradicates gastrointestinal tumors and prevents recurrence


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