A Novel GUCY2C-CD3 T-Cell Engaging Bispecific Construct (PF-07062119) for the Treatment of Gastrointestinal Cancers


**ABSTRACT**

**Purpose:** Gastrointestinal cancers remain areas of high unmet need despite advances in targeted and immunotherapies. Here, we demonstrate potent, tumor-selective efficacy with PF-07062119, a T-cell engaging CD3 bispecific targeting tumors expressing Guanylyl Cyclase C (GUCY2C), which is expressed widely across colorectal cancer and other gastrointestinal malignancies. In addition, to address immune evasion mechanisms, we explore combinations with immune checkpoint blockade agents and with anti-angiogenesis therapy.

**Experimental Design:** PF-07062119 activity was evaluated in vitro in multiple tumor cell lines, and in vivo in established subcutaneous and orthotopic human colorectal cancer xenograft tumors with adoptive transfer of human T cells. Efficacy was also evaluated in mouse syngeneic tumors using human CD3ε transgenic mice. IHC and mass cytometry were performed to demonstrate drug biodistribution, recruitment of activated T cells, and to identify markers of immune evasion. Combination studies were performed with anti–PD-1/PD-L1 and anti-VEGF antibodies. Toxicity and pharmacokinetic studies were done in cynomolgus macaque.

**Results:** We demonstrate that GUCY2C-positive tumors can be targeted with an anti-GUCY2C/anti-CD3ε bispecific, with selective drug biodistribution to tumors. PF-07062119 showed potent T-cell–mediated in vitro activity and in vivo efficacy in multiple colorectal cancer human xenograft tumor models, including KRAS- and BRAF-mutant tumors, as well as in the immunocompetent mouse syngeneic tumor model. PF-07062119 activity was further enhanced when combined with anti–PD-1/PD-L1 treatment or in combination with antiangiogenic therapy. Toxicity studies in cynomolgus indicated a monitorable and manageable toxicity profile.

**Conclusions:** These data highlight the potential for PF-07062119 to demonstrate efficacy and improve patient outcomes in colorectal cancer and other gastrointestinal malignancies.

**Introduction**

Gastrointestinal malignancies, including colorectal cancer, gastric cancer, and esophageal cancer, continue to be areas of high unmet medical need despite advances in targeted therapies (1, 2). Colorectal cancer alone is a worldwide issue affecting both men and women, and responsible for 9.2% of all cancer deaths. The lack of response to targeted therapy, such as anti-EGFR anti-

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Colorectal cancer is an area of high unmet need and a leading cause of cancer-related deaths worldwide. The majority of colorectal cancers is microsatellite stable, frequently with mutated KRAS or BRAF oncogenes, resulting in poor prognoses, and shows a lack of response to currently approved immunotherapy. T-cell engaging CD3-bispecific antibodies hold potential as potent therapeutics against solid tumors. Here, we demonstrate T-cell-mediated antitumor activity with PF-07062119, a novel CD3 bispecific against tumors expressing Guanylyl Cyclase C (GUCY2C), a target expressed widely across colorectal cancer and other gastrointestinal tumors. PF-07062119 shows efficacy in multiple colorectal cancer models independent of their KRAS or BRAF mutational status, because bispecific activity is dependent on GUCY2C expression. We also show significant combination benefits with checkpoint blockade and antiangiogenesis therapy. PF-07062119 has a well-tolerated toxicity profile in cynomolgus monkeys. Accordingly, PF-07062119 warrants further clinical investigation for the treatment of colorectal cancer and other gastrointestinal cancers.

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saline to remove systemic therapeutic, which might interfere with IHC detection. To immunohistochemically detect the bispecifc molecule, sections underwent heat-induced epitope retrieval in Borq Decloaker (Biocare Medical) for 30 minutes, followed by peroxidase block in Peroxidized 1 (Biocare Medical) for 10 minutes, and protein block with Background Punisher (Biocare Medical) for 10 minutes. Primary antibody rabbit mAb anti-Human IgG (EPR4421, Abcam ab109498) was applied at 1:200 (0.41 μg/mL) for 1 hour followed by MACH2 Rabbit HRP-Polymer (Biocare Medical) for 30 minutes. Color was developed using Betazoid DAB (Biocare Medical) chromogen for 5 minutes. After a rinse in water, the sections were counterstained for 30 seconds in Tacha’s Hematoxylin (Biocare Medical), dehydrated in 100% reagent alcohol, and cleared in xylene. Slides were coverslipped using Permount medium.

For GUCY2C staining, sections underwent heat-induced epitope retrieval in Borq Decloaker for 30 minutes, followed by peroxidase block in Peroxidized 1 for 10 minutes, and protein block with Background Punisher for 10 minutes. The primary antibody anti-GUCY2C (9H3-rabbit IgG) was applied at 2 μg/mL for 60 minutes followed by the labeled polymer MACH2 Rabbit HRP-Polymer for 30 minutes. Color was developed using Betazoid DAB chromogen for 5 minutes. After a rinse in water, the sections were counterstained for 10 seconds in Tacha’s hematoxylin, dehydrated in 100% reagent alcohol, and cleared in xylene. Slides were coverslipped using Permount medium and examined under a microscope for Histo score (H-score) and cleared in xylene. Slides were coverslipped using Permount medium and examined under a microscope for Histo score (H-score) and cleared in xylene. Slides were coverslipped using Permount medium.

For CD3, Granzyme B, and PD-L1 expression in the LS0134 colorectal orthotopic tumor model

Female NOD-SCID IL2R-null (NSG) animals (Jackson Laboratory) were used for experiments under approval by the Institutional Animal Care and Use Committee, and all applicable animal care and use regulations, guidelines, and policies were followed. For xenograft studies, NSG mice were inoculated with LS1034, HT55, LS174T, or HCT116 cells in the flank in a total injection volume of 0.2 mL. For the LS1034, HT55, and HCT116 subcutaneous CLX models, 5 × 10^6 cells per mouse were administered in 50% Matrigel Basement Membrane Matrix. For the LS174T CLX model, 2 × 10^6 cells per mouse were administered in 50% Matrigel Basement Membrane Matrix. PDX-CRX-11201 (Asterand) tumor fragments (4 mm × 4 mm) were generated from 500 to 800 mm^3 tumors, expanded, and then implanted subcutaneously in the flank of NSG mice.

Tumor measurements were collected using Vernier caliper, and volumes were calculated by use of the modified ellipsoid formula: \( \frac{4}{3} \pi \times \text{length} \times \text{width} \). Mice were randomized and staged at tumor size of 150 to 200 mm^3. An initial dose of PF-07062119, a nontargeted CD3-bispecific control, or PBS (vehicle) was administered to animals on day 0, and 2 × 10^6 cultured activated pan human T-cells (containing CD8 and CD4 T cells) were inoculated the following day. Mice were dosed in 0.2 mL bolus injections weekly for 3 doses in all studies except in the single-agent efficacy study in PDX-CRX-11201, which was dosed weekly for 4 doses. In combination studies, combination agents were administered starting on day 0. Anti-human PD-L1 was administered at 10 mg/kg every 3 days for 6 doses, anti-human PD-1 was dosed at 5 mg/kg weekly for 3 doses, and anti–VEGF-A mAb (G6-31) that blocks both human and mouse VEGF (26) was dosed at 5 mg/kg every 3 days for 4 doses. All compound and T-cell administrations were intravenous via the lateral tail vein of each animal. Tumor measurements were collected twice weekly along with continuous monitoring for signs of a graft versus host response.

Tumor volume data were log-transformed after an adjustment was made for zero tumor size. A separate ANOVA analysis using all groups was performed for each day. From each analysis, a one-sided \( P \) value from a Student t pairwise comparison of each group with the control was reported. For observed differences between the combination agent, treatments and their respective single-agent treatments, statistical analysis was performed to determine statistical significance (\( P \) values) for each difference. In the studies using anti–PD-L1 and anti–PD-1, the tumor size was log-transformed, and a one-sided \( t \) test of the log-transformed tumor sizes was used to assess significance. Because the difference observed varied with day, this assessment was performed separately for each day. For the study using anti–VEGF-A, many tumors had zero size, so a nonparametric Wilcoxon ranked sum test (one-sided with t-approximation) of tumor sizes was used in place of a parametric t-test. The \( P \) values reported in sections 7.4 and 7.5 are those for the last day of measurements because that \( P \) value is similar (in most case identical) across the last 5 days of measurements. A separate \( P \) value is computed for each single agent (when compared with the combination agent), and the more conservative (higher) of the two \( P \) values is reported for each combination being evaluated.

IHC analysis of CD3-positive T cells in the anti–VEGF-A and PF-07062119 combination study in PDX-CRX-11201 was done on tumors harvested on day 7 after the first bispecifc dose, using the CD3 staining method described above.
weeks after cell inoculation, the subcutaneous tumors (250 to 300 mm³) were harvested, and tumor fragments (4 × 4 mm) were prepared for orthotopic implants. Laparotomy was performed through a midline incision, and the cecum was exposed. Then LS1034-luc tumor fragment was sutured to the cecum adjacent to the ascending colon. The mice with orthotopic tumor implants were monitored weekly with the tumor growth assessed by In Vivo Imaging System (Perkin Elmer) scans of luciferin bioluminescence using Living Image version 4.3.1 software. Animals were staged to an average bioluminescence level of 24 to 26 × 10⁶ photons/s and a dose of 50 as intravenously for 5 doses via tail vein injection with PF-07062119 at 0.3, 0.1, and 0.03 mg/kg or nontargeted CD3 bispecific at 0.3 mg/kg. Activated/expanded T cells were administered as described earlier for adoptive T-cell transfer, 24 hours after dosing with bispecifics.

**CT26-mGUCY2C efficacy study in human CD3+ transgenic mice**

To generate CT26-mGUCY2C cells, mouse Gucy2c nucleotide sequence (NM_001127318) encoding the mature extracellular domain, transmembrane domain, and the first three amino acids of the cytoplasmic domain (nucleotide sequence 194-1507 from GenBank accession # NM_001127318, corresponding to amino acid sequence V20-Y457 of GenBank accession # NP_001120790) was inserted into a mammalian expression vector downstream of the synthetic CAGGS promoter. The vector also contained the neomycin resistance gene for growth selection. CT26 cells (ATCC) were transfected with this expression construct, and a G418-resistant, single-cell clonal population was generated using limiting dilution.

CT26-mGUCY2C tumor cells were cultured in vitro with RPMI medium supplemented with 10% FBS in a humidified chamber at 37°C under 5% CO₂ atmosphere. Cells were harvested during the exponential growth phase, and 1 × 10⁶ cells were inoculated in each human CD3e transgenic mouse (CrownBio) subcutaneously in the right rear flank region in 0.1 mL of PBS mixed with Matrigel (1:1) for tumor development. Mice were randomized (n = 5 mice/group) when mean tumor size reached approximately 68 mm³. On the day of randomization, mice were dosed with PF-07062119 at 3, 1, or 0.3 mg/kg, or with the nontargeted CD3-bispecific control at 3 mg/kg every 3 days for 6 doses. Tumor volumes were measured twice a week using calipers, and animals were monitored for changes in bodyweight, behavior, or signs of toxicity.

**Pharmacokinetic measurements of GUCY2C(M)-CD3 in LS1034 adoptive transfer model**

A qualitative ligand-binding assay was used to measure GUCY2C(M)-CD3 in NSG mouse-diluted whole blood, using the Meso-Scale Discovery (MSD) assay platform. The assay used biotinylated goat anti-human IgG (Southern Biotech, 2049-08) coated on the surface of the MSD plate to capture GUCY2C(M)-CD3. After washing, the bound drug was detected with a mouse anti-human IgG Fc (Clone JDC10 Southern Biotech, 9040-01), which was labeled with ruthenium. After a final set of washes, tripropylamine was added and plates read on the MSD SECTOR Imager 6000. The electrochemiluminescent signal generated was proportional to the amount of bound drug. Sample concentrations were determined by interpolation from a standard curve that was fit using a 4-parameter logistic equation with 1/y² weighting. The range of quantitation in 100% diluted whole blood was 80.0 ng/mL to 5,120 ng/mL. The pharmacokinetic parameters were determined from individual animal data using noncompartmental analysis in Watson LIMS (Version 7.5, Thermo, Inc.). Concentrations below the limit of quantitation were not used in the calculations. In addition, pharmacokinetic (PK) data were also analyzed using a two-compartment PK model with linear elimination from the central compartment using Phoenix 64 Win Non Lin (Certara L.P.).

**Cell culture**

T84, LS1034, LS174T, and HCT116 cells were obtained from the ATCC. HT55 cells were obtained from the European Collection of Authenticated Cell Cultures (Sigma-Aldrich). T84 cells were cultured in a 1:1 mixture of Ham’s F12 medium and DMEM with Glutamax and 10% FBS. LS1034 cells were cultured in RPMI-1640 with 10% FBS. HT55 cells were cultured in modified Eagle’s medium with 20% FBS. LS174T cells were cultured in modified Eagle’s medium with Glutamax and 10% FBS. HCT116 cells were grown in McCoy’s 5a medium with 10% FBS. HCT116-hGUCY2C2 cells were generated by transducing HCT116 cells with human GUCY2C lentiviral particles (OriGene, RC21390113V) and selecting puromycin-resistant clonal populations.

**Characterization of PF-07062119 binding to human T cells and GUCY2C-expressing tumor cells**

Freshly isolated human T cells and tumor cells (HCT116, HCT116-human GUCY2C, CT26, CT-26-mouse GUCY2C) dissociated with CDB (1 × 10⁶ cells per sample) were blocked and washed in cold FACs buffer containing PBS and 3% BSA. Cells were stained in flow buffer containing 0.25 μmol/L DAPI and acquired using BD LSRII Fortessa with FACs Diva software version 8.0.1. QuantiBRITE PE-labeled beads were used in the same acquisition PE voltage settings to calculate the number of PE-labeled antibodies per cell (ABC) based on the background-corrected PE geometric median fluorescence intensity.

**CTL assay**

To test PF-07062119 sensitivity on a panel of colorectal tumor cell lines endogenously expressing GUCY2C, monolayer cultures of firefly luciferase–expressing tumor cell lines were isolated from cell culture...
flasks using TrypLE dissociation reagent. Tumor cells were resuspended in R10 medium (RPMI, 10% FBS, 3 mL of 45% glucose). Five thousand tumor cells per well were plated into clear-bottom white 96-well plates (Costar). Human T cells were also resuspended in R10 media and added to tumor cells at an effector to target ratio (E:T ratio) of 5:1. The cells were treated with serial dilutions of PF-07062119 or non–targeted-CD3 control bispecific and incubated at 37°C under 5% CO₂ for 48 hours. The luciferase signal in viable cells was measured in relative lights units using the neocitrate reagent read on a Victor plate reader (Perkin Elmer) at 0.1 seconds/well. EC₅₀ values were calculated in Graphpad PRISM v7.04 using variable slope four parameter non-linear regression analysis of percent cytotoxicity versus Log₁₀ concentration of bispecifics. For each tumor cell line, 3 separate donor CD₃⁺ lymphocytes were used in cytotoxicity assays, and the average EC₅₀ for each cell line was calculated. PF-07062119 activity was also measured in GUCY2C-expressing tumor cells in the absence of T cells.

**IFNγ induced in vitro by PF-07062119**

To test PF-07062119–induced cytokine release, supernatants were collected at the end of the CTL assays described above and analyzed using a Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel according to the manufacturer’s guidelines, read on a Luminex 200 (Luminex Xmap Technology) with Luminex xPONENT software 3.1, and analyzed using MILLIPEX ANALYST v5.1.0.0 to measure human IFNγ levels.

**Mass cytometry deep phenotyping**

The mass cytometry panel included the following metal-conjugated antibodies from Fluidigm: anti–CD45-89Y (3089003B), anti–CD3-154Sm (Tonbo Biosciences, 70-0038-U100), anti–CD4-200 (Luminex Xmap Technology) with Luminex xPONENT software 3.1, and analyzed using MILLIPEX ANALYST v5.1.0.0 to measure human IFNγ levels.

### Results

**GUCY2C expression in gastrointestinal cancer and normal tissues**

Although GUCY2C expression in colorectal, gastric, esophageal, and pancreatic cancers has been evaluated previously (11, 12), both membrane and cytoplasmic expressions of the target have been observed across these indications. Because our bispecific targeting modality relies on binding to cell surface GUCY2C, we sought to specifically characterize membrane expression of GUCY2C in primary gastrointestinal tumors and in normal tissues (Indivudum, Protagenix, Avaden, University of Michigan, and Cornell University) using IHC with an anti-GUCY2C mAb (Fig. 1; Supplementary Table S1). GUCY2C was expressed in the majority of colorectal adenocarcinomas across all stages, including liver metastases (Fig. 1A; Supplementary Table S1). It was also expressed in gastric (Fig. 1A and G; Supplementary Table S1) and esophageal adenocarcinomas (Fig. 1A and H; Supplementary Table S1). Moderately to well differentiated tumors tended to be associated with higher membrane expression of GUCY2C compared with those that were poorly differentiated (Supplementary Table S1). Relatively heterogeneous and low incidence of membrane expression was observed in pancreatic adenocarcinomas (Fig. 1A; Supplementary Table S1). In normal tissues, GUCY2C expression was primarily observed on the apical side of the colon (Fig. II) and small intestinal epithelium (Fig. IJ). A lower level of expression was present in prostatic apical epithelium, and all other tissues evaluated were negative (Supplementary Fig. S1).

### Tumor selective targeting with an anti-GUCY2C/anti-CD3e bispecific

To test the ability to target GUCY2C-expressing tumors with an anti-GUCY2C/anti-CD3e bispecific, we developed a heterodimeric diabody Fc-fusion protein comprised of two single-chain Fv (scFv) domains, one targeting GUCY2C and the other targeting CD3e. The
Figure 1.
GUCY2C expression in gastrointestinal tumors and normal tissues. A, H-scores depicting GUCY2C cell surface expression across colorectal, pancreatic, gastric, and esophageal tumors (red line, median H-score). IHC showing GUCY2C expression (brown) in (B) colorectal adenocarcinoma stage I; (C) colorectal adenocarcinoma stage II; (D) colorectal adenocarcinoma stage III; (E) colorectal adenocarcinoma stage IV; (F) liver metastasis of colorectal adenocarcinoma (H, hepatocytes; T, tumor; dashed line, tumor/normal boundary); (G) gastric adenocarcinoma; (H) esophageal adenocarcinoma; (I) normal colon; and (J) normal small intestine. Scale bar, 200 μm.
VH of one binding domain pair is joined with the VL of the other binding domain pair such that when the two constructs are coexpressed, the result is the formation of a diabody. These scFv domains are fused to the Fc domain of human IgG1 (Fig. 2A). Mutations (L234A, L235A, and G237A EU numbering) were introduced in the CH2 region of the Fc to reduce binding to Fc gamma receptor (FcγR; ref. 27). In addition, knob-in-hole mutations in the CH3 region of the Fc domain were used to facilitate correct heterodimeric pairing and bispecific purification (28, 29). The Fc domain fusion was also designed to extend the bispecific half-life and allow for less frequent dosing than antibody fragment–based bispecifics such as BiTEs (e.g., blinatumomab), which require constant i.v. infusion into patients via a pump. BiTEs contain only two tandem antibody variable domains (scFv-scFv) and no Fc domain. The diabody-Fc format is similar to the BiTE format in that it contains two scFv domains. However, the variable domains in the diabody are more tightly packed together than those of the BiTE, allowing for more potent killing activity (30), and introduction of the Fc further extends half-life from 1 hour to approximately 1 week (31).

To illustrate both tumor selective targeting and efficacy, we generated an anti-GUCY2C/anti-CD3ε bispecific (GUCY2C(M)-CD3), which had equivalent binding affinity to human and mouse GUCY2C (Supplementary Table S2). NOD-scid IL2Rγnull (NSG) mice bearing LS1034 colorectal cancer CLX tumors were treated with GUCY2C(M)-CD3 at 1 and 0.3 mg/kg, along with adoptive human T-cell transfer. Pharmacokinetic analyses demonstrated that the diabody-Fc fusion design of GUCY2C(M)-CD3 led to a systemic estimated half-life of 11 days in these tumor-bearing mice, which enabled weekly dosing with the bispecific (Fig. 2B). Even though GUCY2C expression was observed in both the LS1034 tumors and the normal intestinal tract epithelium of the same mice (Fig. 2C, left plot), only tumors showed uptake of GUCY2C(M)-CD3 (Fig. 2C, middle plot and Fig. 2D).

**Figure 2.** An anti-GUCY2C/anti-CD3ε bispecific selectively targets tumors versus normal tissue. A, Schematic of an anti-GUCY2C/anti-CD3ε bispecific human IgG1 FcγR-diabody. B, Pharmacokinetics of a murine GUCY2C cross-reactive bispecific GUCY2C(M)-CD3 at 1 and 0.3 mg/kg showing an estimated half-life of 11 days in LS1034 tumor–bearing female NSG mice with human adoptive T-cell transfer. C, IHC showing GUCY2C expression (left plot, brown staining) in LS1034 CLX tumor (top) and in the colon from an LS1034 tumor–bearing mouse (bottom). Middle plot shows biodistribution of the bispecific GUCY2C(M)-CD3 (brown) in the same LS1034 tumor (top) and lack of bispecific uptake in the colon (bottom); 7 days after dosing in an adoptive transfer study. Right plot shows infiltration of human CD3-positive T cells (brown) in the bispecific-treated tumors (top) but not in colonies from the same mice (bottom). Scale bar, 200 μm. D, GUCY2C(M)-CD3 showed antitumor activity in the LS1034 human T-cell adoptive transfer model at 1 and 0.3 mg/kg (n = 10 mice/group, Q3DX3 dosing, P value < 0.0001).
plot) and infiltration of human CD3–positive T cells (Fig. 2C, right plot), whereas no drug or human CD3 T-cell infiltration could be detected in the intestinal tract. Importantly, antitumor efficacy was observed with GUCY2C(M)-CD3 at 1 and 0.3 mg/kg but not with a nontargeted CD3 bispecific (Fig. 2D). No changes in body weight were noted in response to bispecific treatment (Supplementary Fig. S2). These observations support the hypothesis that GUCY2C can be selectively targeted on tumors with an anti–GUCY2C-targeting agent.

PF-07062119 elicits target-dependent CTL activity in vitro

After establishing the ability to preferentially target GUCY2C-expressing tumors, we developed a fully humanized and lead-optimized anti-GUCY2C/anti-CD3 bispecific (described herein as PF-07062119). PF-07062119 was generated in the diabody Fc fusion format shown in Fig. 2A and characterized in vitro for target binding and T-cell–mediated cytotoxicity. PF-07062119 showed cell surface binding to human T cells, as well as HCT116 tumor cells overexpressing GUCY2C, but not to GUCY2C-negative HCT116 parental cells (Fig. 3A; Supplementary Fig. S3). To understand the range of GUCY2C expression capable of eliciting T-cell–mediated cytotoxicity, cell surface receptor density was quantitatively measured across a panel of colorectal tumor cell lines. These cells showed receptor densities ranging from 875 to 8,067 receptors per cell (Fig. 3B). Treatment of these cells with PF-07062119 and human T cells showed dose-dependent and GUCY2C expression–dependent cytotoxicity, as measured by tumor cell survival (Fig. 3C). Consistent with cytotoxicity measurement, supernatants harvested from these cytotoxicity assays showed release of IFNγ in a PF-07062119 dose-dependent manner (Fig. 3D). No change in tumor viability or IFNγ release was observed in GUCY2C-negative HCT116 cells, confirming the need for GUCY2C expression for induction of PF-07062119–mediated T-cell effector function.

Because the GUCY2C pathway regulates intestinal fluidity, PF-07062119 was also characterized for its potential to modulate GUCY2C signaling by measuring production of the downstream effector, cyclic guanosine monophosphate (cGMP). Although the GUCY2C pathway agonist bacterial enterotoxin STp increased cGMP in a dose-dependent manner in GUCY2C-expressing T84 tumor cells, cGMP production was not enhanced by the addition of increasing concentrations of PF-07062119 (Supplementary Fig. S4). A nontargeted CD3-bispecific control also did not show cGMP production. In addition, PF-07062119 did not affect cGMP production induced by STp. These findings indicate the PF-07062119 is not a GUCY2C pathway agonist and does not neutralize GUCY2C pathway function in the presence of ligand.

Table 1.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>H-Score (GUCY2C)</th>
<th>KRAS mutation</th>
<th>BRAF mutation</th>
<th>Differentiation status</th>
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</thead>
<tbody>
<tr>
<td>LS1034</td>
<td>210</td>
<td>A146T</td>
<td>WT</td>
<td>Moderately differentiated</td>
</tr>
<tr>
<td>HT55</td>
<td>180</td>
<td>WT</td>
<td>N581Y</td>
<td>Moderately differentiated</td>
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<tr>
<td>LS174T</td>
<td>105</td>
<td>G12D</td>
<td>WT</td>
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<tr>
<td>HCT116</td>
<td>0</td>
<td>G13D</td>
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<td>Poorly differentiated</td>
</tr>
<tr>
<td>PDX-CRX-II1201</td>
<td>185</td>
<td>G12V</td>
<td>WT</td>
<td>Moderately differentiated</td>
</tr>
</tbody>
</table>

Figure 3.

Figure 4.
PF-07062119 mediates antitumor efficacy in multiple in vivo models. A, GUCY2C expression in LS1034 (CLX), HT55 (CLX), PDX-CRX-11201 (PDX), LS174T (CLX), and HCT116 (CLX) tumor models (scale bar, 200 μm). B, PF-07062119 induced antitumor efficacy in LS1034, HT55, PDX-CRX-11201, and LS174T, and lack of efficacy in GUCY2C-negative HCT116 subcutaneous established models using adoptive human T-cell transfer. Mice with established tumors of 150 to 200 mm³ were dosed with PF-07062119, a nontargeted CD3-bispecific control or PBS (vehicle) weekly up to 3 times for all models, and up to 4 times for PDX-CRX-11201. Human T cells were administered intravenously 24 hours after the first bispecific or vehicle dose. C, LS1034 colon orthotopic established tumor model using adoptive human T-cell transfer described in (B) with bispecific dosed weekly up to five doses. GUCY2C expression (brown) in LS1034 orthotopic tumor and normal adjacent tissue. H&E staining (purple) shows tumor and normal tissue boundary. Bioluminescence imaging showing complete tumor regression with PF-07062119 at 0.3 mg/kg. D, Flow cytometry assay showing PF-07062119 binding to CT26-mGUCY2C and lack of binding to CT26 cells (110 nmol/L Ab 9H3-hIgG used for binding). E, CT26-mGUCY2C tumors show membrane expression of GUCY2C (brown) by IHC (scale bar, 200 μm). F, PF-07062119 mediated efficacy in CT26-mGUCY2C tumors in hCD3e mice. Tumors staged to 50 to 80 mm³ were treated with PF-07062119 or nontargeted CD3 every 3 days up to 6 doses.
PF-07062119 demonstrates antitumor efficacy in GUCY2C-positive human xenograft and mouse syngeneic tumors

After characterizing the in vitro range of activity of PF-07062119, we next evaluated in vivo efficacy with this bispecific in CLX and PDX models of colorectal cancer. GUCY2C expression was characterized in four CLX models and one PDX model by IHC to generate an H-score reflective of cell surface expression of GUCY2C (Table 1, Fig. 4A). The tumors were also characterized for their mutational status of KRAS and BRAF oncoproteins, as well as their differentiation status (Table 1). Among the models tested, LS1034 and HT55 CLX models showed relatively high GUCY2C expression, reflective of human colorectal tumors with high GUCY2C expression (Fig. 1A; Supplementary Table S1). LS1034 and HT35 were mutant for KRAS and BRAF, respectively (Table 1). PDX-CRX-11201 also had relatively high GUCY2C expression and was mutant for KRAS. The LS174T CLX model was mutant for KRAS and showed lower GUCY2C expression, representative of human tumors expressing GUCY2C below the median H-score level in colorectal cancer (Table 1, Fig. 1A). The HCT116 model was negative for GUCY2C. Both LS174T and HCT116 were poorly differentiated tumors. Apically enhanced expression of GUCY2C was noted in all tumors with high GUCY2C expression, and higher differentiation state.

To assess PF-07062119 activity in vivo, NSG mice were implanted subcutaneously with the characterized colorectal cancer cell lines and PDX-CRX-11201 fragments. An initial dose of PF-07062119, nontargeted CD3-negative control bispecific, or vehicle was administered to animals with established tumors, along with adoptive transfer of human T cells, followed by weekly dosing with PF-07062119 or control agents. In the LS1034 model, which had the highest expression of GUCY2C, PF-07062119 treatment at 0.1 mg/kg or higher doses led to complete tumor regressions (P value < 0.0001; Fig. 4B). The antitumor activity was dose dependent, because partial reduction in tumor volumes was observed at 0.03 mg/kg of PF-07062119. The HT55 CLX model and PDX-CRX-11201 (Fig. 4B), which both had similar H-scores, showed complete tumor regressions at doses as low as 0.15 mg/kg of PF-07062119 (P value < 0.0001). The LS174T CLX model, which had low GUCY2C expression, showed a small but significant reduction in tumor burden only at the high dose of PF-07062119 treatment (1 mg/kg, P value < 0.0001; Fig. 4B). The GUCY2C-negative HCT116 colorectal cancer model was not responsive to PF-07062119 at any dose tested (Fig. 4B). In summary, tumors with the highest H-scores showed complete tumor regressions with 0.1 to 0.15 mg/kg or higher doses of PF-07062119, whereas tumors with lower GUCY2C tumors had a moderate antitumor response with a higher dose of 1 mg/kg. In all models, treatment with the PBS vehicle control, or with a nontargeted CD3 bispecific at the same or higher doses that were efficacious with PF-07062119, did not result in any antitumor activity. These data demonstrate GUCY2C expression–dependent antitumor efficacy with PF-07062119, independent of the KRAS or BRAF mutational status of the tumor.

We also examined PF-07062119 activity in a colon orthotopic model to evaluate the ability of the drug to target tumors located within the gastrointestinal tract. LS1034 cells expressing luciferase were generated to monitor tumor growth in vivo using bioluminescence. GUCY2C expression in this model was confirmed by IHC analyses and found to be relatively comparable between LS1034 tumors and the adjacent mouse colon epithelium (Fig. 4C, top right). Similar to subcutaneous models, treatment of these established orthotopic tumors with PF-07062119 following adoptive human T-cell transfer led to complete tumor regressions at 0.3 mg/kg, as measured by bioluminescence imaging scans (P value < 0.0001; Fig. 4C, top left). In addition, necropsy findings at the end of the study indicated that no tumors were observed at secondary sites in mice treated with PF-07062119 at doses above 0.1 mg/kg. However, in animals treated with PF-07062119 at 0.03 mg/kg, 2 of 9 mice had tumors spread to the liver and 2 other mice had secondary spread at the abdominal wall. In the nontargeted CD3 treatment group, 2 of 9 animals had tumor spread to the liver. No body weight loss was observed in the bispecific-treated animals (Supplementary Fig. S5). These data indicate that PF-07062119 has the ability to target primary tumors located in the intestinal tract and could potentially prevent tumor spreading to secondary sites.

Although the above experiments demonstrate the ability of PF-07062119 to target tumors in immunodeficient mice using adoptive human T-cell transfer, we also sought to examine PF-07062119–driven antitumor activity in an immunocompetent mouse model. Although PF-07062119 binds to mouse GUCY2C albeit with lower affinity than to human GUCY2C (Supplementary Table S2), it does not bind to mouse CD3. Therefore, CT26 mouse syngeneic tumor cells were engineered to express mouse GUCY2C (CT26-mGUCY2C), and a transgenic mouse model expressing human CD3ε on mouse T cells was used for efficacy studies. PF-07062119 showed specific binding to CT26-mGUCY2C cells but not to GUCY2C-negative CT26 cells (Fig. 4D). GUCY2C expression on the tumor cell surface was confirmed by IHC in CT26-mGUCY2C subcutaneous tumors established in the human CD3ε transgenic model (Fig. 4E). Similar to NSG models with CLX and PDX tumors, PF-07062119 treatment showed reduction of tumor burden at all doses tested and resulted in complete regressions at doses above 1 mg/kg (Fig. 4F). No body weight loss was observed with PF-07062119 treatment (Supplementary Fig. S6), suggesting that PF-07062119 has tumor selective activity in an immunocompetent model, thereby showing the ability to harness T-cell effector function in the setting of cancer immune escape.

PF-07062119 treatment increases tumor-infiltrating lymphocyte infiltration and activation, and enhanced efficacy with checkpoint blockade

To better understand the mechanism of action of PF-07062119–mediated human T-cell activity, the LS1034 subcutaneous adoptive transfer model was used to evaluate changes in tumors and tumor-infiltrating lymphocytes (TIL) in vivo. Tumors treated at 1 mg/kg of PF-07062119 showed increased T-cell infiltration compared with tumors treated at the minimally efficacious dose of 0.03 mg/kg, whereas tumors in the vehicle treatment group did not show any T-cell infiltration (Fig. 5A, top row). TILs in PF-07062119–treated tumors showed expression of granzyme B that was polarized toward tumor cells, suggesting that these TILs were poised to form immune synapses and kill tumor cells (Fig. 5A, second row). Tumors treated with PF-07062119 also showed upregulation of PD-L1 at both 1 mg/kg and 0.03 mg/kg doses (Fig. 5A, third row). The upregulation of PD-L1 was specific to PF-07062119–treated tumors, and was not observed in vehicle treated, indicating that checkpoint mechanisms that could dampen T-cell activity were being induced with bispecific treatment. These PF-07062119–induced changes in LS1034 tumors were concomitant with tumor necrosis (Fig. 5A, bottom row). In addition, tSNE analyses on CYTOF data from TILs from these tumors identified two T-cell clusters that were unique to the PF-07062119 treatment groups. These clusters were CD4+ and CD8+–positive T cells that were PD-1+ 41-BB “Tim-3” Lag-3+, indicating that these TILs were activated, but also expressed activation-induced checkpoint markers, which if chronically expressed could negatively regulate T-cell effector function (Fig. 5B and C). Although these data imply that PF-07062119

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treatment induced T-cell infiltration into tumors in a dose-dependent manner, the upregulation of PD-L1 on tumors and PD-1 on TILs suggests that a PD-1/PD-L1 axis that could dampen T-cell activity is induced with PF-07062119 treatment.

Based on the rationale that PF-07062119 treatment induced expression of PD-1/PD-L1 on TILs and tumors, we tested if checkpoint blockade with anti-PD-1 or anti-PD-L1 antibodies could enhance antitumor efficacy of PF-07062119. LS1034 subcutaneous tumors...
A GUCY2C-CD3 Bispecific Targets Gastrointestinal Cancers

Figure 6. Combination study of PF-07062119 with anti–VEGF-A mAb. A, Combination of a minimally efficacious dose of PF-07062119 in PDX-CRX-11201 tumor, which is partially responsive to anti–VEGF-A mAb treatment, results in a significant combination benefit compared with single agents. B, Increased CD3+ T cells (brown) were observed in the PF-07062119 + Anti–VEGF-A combination treatment group compared with single agents or nontargeted CD3 treatment.

Discussion

Despite the benefit and approval of checkpoint blockade agents for the treatment of microsatellite instability–high colorectal tumors, immunotherapy has had limited success in the majority of gastrointestinal cancers. Attempts to enhance T-cell activity against these cancers with CD3 bispecifics have shown early signs of activity in the clinic but have been limited by target expression on normal tissues (32, 33). Here, we have described the expression of GUCY2C across gastrointestinal cancers, particularly in moderately to well-differentiated colorectal, gastric, and esophageal adenocarcinomas, and provided support for the hypothesis that GUCY2C-expressing tumors can be selectively targeted with an anti-GUCY2C/anti-CD3e bispecific, owing to the restricted expression of the target in the apical

treated with a suboptimally efficacious PF-07062119 of 0.03 mg/kg showed significant improvement in efficacy in combination with either an anti–PD-L1 or an anti–PD-1 antibody (P value < 0.0001 and 0.0004, respectively), whereas these agents were not efficacious as single agents in this model (Fig. 5D). These findings indicate that although checkpoint mechanisms may limit efficacy of PF-07062119, the bispecific activity can be further enhanced in combination with checkpoint blockade agents.

Anti-VEGF blockade enhances PF-07062119 antitumor activity

Antiangiogenesis treatment is currently used for the treatment of colorectal cancer and is also reported to improve responses to T-cell–mediated therapy by enhancing T-cell infiltration into the tumor. Because suboptimal doses of PF-07062119 showed reduced T-cell infiltration, we tested the effects of combining anti–VEGF-A blockade with PF-07062119 treatment in vivo. PDX-CRX-11201 was used for this evaluation because this tumor model is only partially responsive to G6-31, an anti–VEGF-A mAb that blocks murine VEGF-A. A combination of anti–VEGF-A treatment with PF-07062119 administered at a suboptimal dose of 0.05 mg/kg led to a significant combination benefit leading to complete tumor regressions (P value < 0.0001; Fig. 6A). In addition, IHC analyses showed that tumors that received the combination treatment had notably increased T-cell infiltration compared with tumors treated with either PF-07062119 or anti–VEGF-A alone (Fig. 6B). Therefore, these data suggest that apart from the combination benefit observed with checkpoint blockade agents, antiangiogenesis treatment could further enhance the efficacy observed with PF-07062119.

Toxicology and pharmacokinetics of PF-07062119 in cynomolgus monkey

Cynomolgus monkeys were selected as relevant species for toxicity studies after confirming binding of PF-07062119 to cynomolgus T cells and GUCY2C protein (Supplementary Table S2; Supplementary Fig. S7). Nontoxic clinical and pharmacokinetics of PF-07062119 were evaluated in cynomolgus monkeys in an exploratory intra-animal dose escalation (2 doses, once weekly) toxicity study at 30 mg/kg followed a week later by 100 mg/kg, and 60 mg/kg followed a week later by 180 mg/kg (Supplementary Fig. S8A). The main in-life effects observed with PF-07062119 treatment included emesis, a slight increase in body temperature, decreased activity, hunched posture, reduced appetite, dehydration, body weight loss (≤ 7%), and soft or liquid feces. However, animals in both cohorts tolerated the treatment with administration of oral and/or subcutaneous fluids. Importantly, histopathologic evaluations of the intestinal tract, which is the main site of GUCY2C expression in normal tissues, showed only minimal to mild infiltrates and crypt hyperplasia in multiple segments of the large and small intestine, along with typically minimal to mild villous atrophy in the small intestine (Supplementary Fig. S8B). However, there were no erosions, ulcerations, or other evidence of overt necrosis or loss of overlying epithelium. PF-07062119 treatment induced pharmacodynamic effects in the monkeys, as measured by increases in the percentage of activated (CD69+) CD8+ T cells (Supplementary Fig. S7C), and in peripheral cytokines, including IFNγ, IL2, IL10, and IL6 (Supplementary Fig. S7D). Cytokine induction observed after the first dose in each cohort was dampened after the second dose administration of PF-07062119, indicative of a priming effect. Toxicokinetic analysis of PF-07062119 in both treatment cohorts, assessed by maximum observed concentration (Cmax) and area under the concentration–time curve, showed dose-proportional systemic exposure, as well as a linear PK profile (Supplementary Fig. S7E). No sex-related differences in systemic exposure were noted in this study.
intestinal epithelium. For this evaluation, a bispecific with equivalent affinity to mouse and human GUCY2C was used to show selective biodistribution to xenograft tumors expressing human GUCY2C versus the intestinal tract of the host mice expressing mouse GUCY2C. In further support of this hypothesis, antitumor efficacy was observed in this study in the absence of any tissue damage to the intestinal tract (as evaluated by histopathology), suggesting that GUCY2C-positive tumors can be preferentially targeted with an anti-GUCY2C/anti-CD3ε bispecific T-cell redirection approach.

Based on these data, PF-07062119, a fully humanized and lead-optimized anti-GUCY2C/anti-CD3ε bispecific, was developed and characterized in vitro and in several in vivo tumor models for pharmacologic activity. Both immunodeficient mice with human T-cell adoptive transfer and immunocompetent mice were used in a complementary manner to characterize in vivo efficacy with PF-07062119. Notably, PF-07062119-mediated antitumor activity was observed in all models in a dose-dependent manner and was associated with the level of GUCY2C expressed in human xenograft models, suggesting a potential to obtain activity through dose modulation in the clinic. The human xenograft tumors evaluated for in vivo efficacy included those with mutations in the KRAS or BRAF oncogenes, which are clinically associated with a lack of response to currently approved EGFR-targeted therapies, suggesting that PF-07062119 can target tumors regardless of the mutational status of these genes.

Furthermore, a colon orthotopic xenograft model showed that PF-07062119 was effective at selectively eliminating primary tumors in the intestinal tract without damaging the normal adjacent tissue (as assessed by histopathology) and had the potential to prevent tumor spreading to secondary sites, such as the liver. Although these T-cell adoptive transfer studies highlighted PF-07062119-mediated redirected T-cell-induced killing, we also demonstrated that dose-dependent efficacy could be achieved in a mouse syngeneic tumor model in the presence of a functional immune system. Even with this caveat that both mouse GUCY2C and human CD3ε proteins are overexpressed in the syngeneic system, this model allows for long-term evaluation of antitumor responses, whereas the duration of adoptive transfer tumor models is limited by the eventual onset of graft versus host disease.

We further explored the mechanisms of PF-07062119 activity using the T-cell adoptive transfer model and showed that activity was mediated through recruitment of TILs into GUCY2C-expressing tumors. These T cells had granzyme B expression, which was polarized toward target-expressing tumor cells, suggesting that they were poised to form immune synapses with the tumor cells and initiate cell killing. Such analyses of T-cell infiltration and activity could be informative in the clinic as pharmacodynamic biomarkers of PF-07062119-mediated activity. Further evaluations showed that tumors treated with PF-07062119 also upregulated PD-L1, and TILs from these tumors had expression of activation markers including PD-1, indicating initiation of checkpoint mechanisms that could dampen T-cell-mediated cytotoxicity. Based on these observations, PF-07062119 was combined with checkpoint blockade via anti–PD-1 and anti–PD-L1 antibodies. Combinations of anti–PD-1 or anti–PD-L1 blocking antibodies with a suboptimal dose of PF-07062119 showed improved efficacy compared with single agents, indicating that checkpoint blockade combinations will be important to consider during clinical evaluation of PF-07062119. A limitation of murine models is that due to their lack of sensitivity to systemic cytokine release, the sequencing of combinations and their effects on cytokine release syndrome will need to be evaluated in the clinic.

Recent preclinical and clinical studies with antiangiogenesis agents have been reported to improve responses to immunotherapy through various mechanisms, including enhancing T-cell infiltration into tumors (34–36). For example, the FDA recently approved combinations with axitinib (VEGFR inhibitor) and avadumab (anti–PD-L1 mAb) in renal cell carcinoma (37), and with bevacizumab (anti-VEGF mAb) and atezolizumab (anti–PD-L1 mAb) in non–small cell lung cancer (38), due to significant improvements in progression-free survival and overall response rate. Based on these observations, we tested a combination of PF-07062119 with an anti-VEGF mAb, which induced complete tumor regressions, whereas single agents were only minimally active in the PDX model tested. Although increased T-cell infiltration was observed in the combination treatment group, further mechanistic studies will elucidate whether this increase in TILs was due to changes in the tumor vasculature or was a consequence of increased PF-07062119 uptake following anti-VEGF blockade. A limitation of the adoptive transfer model is that T-cell engraftment in the tumor is dependent on bispecific-mediated recruitment of T cells, thereby resulting in minimal T-cell engraftment with antiangiogenesis treatment alone. Therefore, additional combination studies in immunocompetent mice will be pursued to further elucidate the mechanisms by which VEGF blockade improves T-cell infiltration in PF-07062119–treated tumors.

Finally, the exploratory toxicity study with PF-07062119 in cynomolgus monkeys suggests a clinically monitorable and manageable toxicity profile. PF-07062119 treatment did not induce any erosions, ulcerations, or evidence of overt necrosis or loss of overlying epithelium, and the overall in-life findings were tolerated with administration of oral and/or subcutaneous fluids. Furthermore, the gastrointestinal findings of minimal to mild immune infiltrates, crypt hyperplasia, and villous atrophy would all be expected to resolve following cessation of treatment. Pharmacologic activity in the monkeys was shown by CD69 upregulation on CD8⁺ T cells and increases of systemic cytokines (Supplementary Fig. S7); although there are polymorphisms in macaque CD3ε that abolish activity of a specific anti-CD3ε antibody (FN18) in a subset of animals (39–41), PF-07062119 has shown binding and pharmacologic activity in all monkeys tested (Supplementary Figs. S7 and S8; Supplementary Table S2). In addition, pharmacokinetic analyses in cynomolgus monkeys estimated the terminal half-life of PF-07062119 to be similar to a regular mAb. A longer time-course PK study would be needed to calculate the precise terminal half-life.

Collectively, these studies demonstrate that GUCY2C-positive tumors can be preferentially targeted with an anti-GUCY2C/anti-CD3ε bispecific. Our lead clinical candidate, PF-07062119, has shown potent single-agent antitumor efficacy with PF-07062119 in multiple in vitro and in vivo models. This activity can be further enhanced with immune checkpoint blockade agents, which prevent T-cell exhaustion, as well as with antiangiogenesis agents, which could increase T-cell infiltration (Supplementary Fig. S9). Further evaluation of PF-07062119 along with these combination agents will be pursued in clinical studies to examine their ability to reduce tumor burden in patients with gastrointestinal cancers.

Disclosure of Potential Conflicts of Interest

D. Mathur, A.R. Root, B. Bugaj-Gaweda, W. Fang, J.C. Kearney, C.M. Rohde, C. Stevens, C. Kamperschroer, K. Kelleher, E. Upeslacis, E.R. LaVallie, D.R. Fernandez, B.S. Buettow, E. Rosford, L. Bloom, and L. Chistiakova are employees/paid consultants for Pfizer. P. Sapra is an employee/paid consultant for and holds ownership interest (including patents) in Pfizer. No potential conflicts of interest were disclosed by the other authors.
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References


A Novel GUCY2C-CD3 T-Cell Engaging Bispecific Construct (PF-07062119) for the Treatment of Gastrointestinal Cancers

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