Title:

Preclinical Antitumor Activity and Biodistribution of a Novel Anti–GCC Antibody–Drug Conjugate in Patient-Derived Xenografts.

Authors: Adnan O. Abu-Yousif¹*, Donna Cvet¹, Melissa Gallery¹, Bret M. Bannerman¹, Michelle L. Ganno¹, Michael D. Smith¹, Katharine C. Lai², Thomas A. Keating², Bradley Stringer¹, Afrand Kamali¹,†, Kurt Eng¹, Secil Koseoglu¹,‡, Andy Zhu, Cindy Q. Xia¹, Melissa Saylor Landen¹,§, Maria Borland¹,§, Robbie Robertson³, Jayaprakasam Bolledula¹, Mark G. Qian¹, Jennifer Fretland¶, O. Petter Veiby¹

Affiliations:
¹ Millennium Pharmaceuticals, Inc., Cambridge, MA, USA, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited.
² ImmunoGen, Inc. Waltham, MA, USA.
³ Quanta Imaging, Boston, MA, USA.

* To whom correspondence should be addressed: Adnan Abu-Yousif, Takeda Pharmaceuticals International Co., 40 Landsdowne Street, Cambridge, MA, 02139, USA. Phone: (617) 444-1667. Fax: (617) 551-8906. Email: Adnan.Abu-Yousif@Takeda.com
† Current affiliation: Kintai Therapeutics, Cambridge, MA, USA.
‡ Current affiliation: Surface Oncology, Cambridge, MA, USA
§ Employed by Millennium Pharmaceuticals, Inc. at the time of this work
¶ Current affiliation: Sanofi, Cambridge, MA, USA.

Conflict of Interest:
The following authors are (or were) employees of Millennium Pharmaceuticals, Inc., Cambridge, MA, USA, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited at the time of the study.

Adnan O. Abu-Yousif, Donna Cvet, Melissa Gallery, Bret M. Bannerman, Michelle L. Ganno, Michael D. Smith, Bradley Stringer, Afrand Kamali, Kurt Eng, Secil Koseoglu, Andy Zhu, Cindy Q. Xia, Melissa Saylor Landen, Maria Borland, Robbie Robertson, Jayaprakasam Bolledula, Mark G. Qian, Jennifer Fretland, O. Petter Veiby

Current affiliation: Afrand Kamali: Kintai Therapeutics, Cambridge, MA, USA.
Current affiliation: Secil Koseoglu: Surface Oncology, Cambridge, MA, USA
Current affiliation: for Jennifer Fretland: Sanofi, Cambridge, MA, USA.
Current affiliation: for Robbie Robertson: Quanta Imaging, Boston, MA, USA.

During preparation of the manuscript, the following were employed by ImmunoGen, Inc. Waltham, MA, USA.

Katharine C. Lai, Thomas A. Keating

In addition, Thomas A. Keating claims the following potential conflicts: Ownership of stock: ImmunoGen, AstraZeneca, and Merck
No other conflicts are reported.
Guanylyl cyclase C (GCC) is a unique therapeutic target with expression restricted to the apical side of epithelial cell tight junctions thought to be only accessible by intravenously administered agents on malignant tissues where GCC expression is aberrant. In the present study, we sought to evaluate the therapeutic potential of a second-generation investigational ADC, TAK-164, comprised of a human anti-GCC monoclonal antibody conjugated via a peptide linker to the highly cytotoxic DNA alkylator, DGN549. The in vitro binding, payload release, and in vitro activity of TAK-164 was characterized motivating in vivo evaluation. The efficacy of TAK-164 and the relationship to exposure, pharmacodynamic marker activation, and biodistribution was evaluated in xenograft models and primary human tumor xenograft (PHTX) models. We demonstrate TAK-164 selectively binds to, is internalized by, and has potent cytotoxic effects against GCC-expressing cells in vitro. A single intravenous administration of TAK-164 (0.76 mg/kg) resulted in significant growth rate inhibition in PHTX models of mCRC. Furthermore, imaging studies characterized TAK-164 uptake and activity and showed positive relationships between GCC expression and tumor uptake which correlated with antitumor activity.

Collectively, our data suggest that TAK-164 is highly active in multiple GCC positive tumors including those refractory to TAK-264, a GCC-targeted auristatin ADC. A strong relationship between uptake of 89Zr-labeled TAK-164, levels of GCC expression and, most notably, response to TAK-164 therapy in GCC expressing xenografts and PHTX models. These data
supported the clinical development of TAK-164 as part of a first-in-human clinical trial (NCT03449030).
**Introduction**

Gastrointestinal cancers, particularly colorectal cancer (CRC), are among the most common cancers worldwide (1). CRC is the third most diagnosed cancer in the United States, with an estimated 145,600 new cases and 51,020 deaths in 2018 (2). Current treatment options consist of a chemotherapy backbone combined with targeted antibody therapies (3,4). Despite advances in the treatment of CRC, prognosis is generally poor. Approximately 75% of patients either have metastases at diagnosis or develop metastases as the disease progresses (4). Although the 5-year survival rate in CRC is nearly 65%, the 5-year survival rate is only 14.2% in patients with distant metastases (5,6). In pancreatic, esophageal, and gastric cancers, 5-year survival rates are also low at 9.3%, 19.9%, and 31.5%, respectively (6). Thus, new treatment options that can improve these poor survival rates are needed. Recently, a subset of these patients, classified as microsatellite instability-high (MSI-high), are now eligible for treatment with immunotherapy offering some hope, however, they represent a small subset of patients with GI malignancies (7,8).

Our group and others have demonstrated that the cell surface receptor, guanylyl cyclase C (GCC), is expressed in >95% of primary CRC and metastatic CRC (mCRC), and in approximately 65% of esophageal, gastric, and pancreatic tumors (9–13). GCC is an attractive therapeutic target, because it is only accessible by intravenously administered agents on malignant tissues, where GCC expression is aberrant and accessible through the vascular compartment (13).

In normal gastrointestinal tissue, GCC is expressed exclusively on the apical side of epithelial cell tight junctions, and thus isolated to the luminal environment (14,15) and internalizes through receptor-mediated endocytosis upon ligand binding (16). In mice, GCC
ligands have been shown to selectively target colon tumor cells without accumulation in normal intestinal tissue (17,18) forming the basis for clinical testing of $[^{68}\text{Ga}]$DOTA-MLN6907, a GCC-targeted peptide, in patients with CRC (NCT02056015). Collectively, these data motivated the design of TAK-264, an MMAE-conjugated monoclonal antibody (mAb) targeting GCC.

Preclinical investigation of TAK-264 showed cytotoxicity in GCC-expressing cells both in vitro and in vivo; however, some GCC-expressing tumors were refractory to high concentrations of TAK-264 (13). A subsequent phase 1 study showed promising preliminary efficacy in patients with advanced gastric and pancreatic malignancies (19), but only limited activity was observed in phase 2 studies in patients with metastatic or recurrent pancreatic or gastroesophageal adenocarcinomas (20,21). Clinical experience with TAK-264 and other antibody-drug conjugates (ADCs) suggest that clinical efficacy depends on 3 key variables: antibody (or other targeting moiety), conjugation/linker strategy, and payload (mechanism and potency) (21–24).

Given the high specificity and favorable pharmacokinetics of the mAb in TAK-264, we have utilized an alternative payload mechanistically aligned with currently approved agents in CRC and other gastrointestinal-expressing tumors (3), where GCC is most frequently expressed. Recent preclinical studies of ADCs containing a new class of DNA alkylating agents, indolinobenzodiazepine pseudodimers (termed IGNS), have demonstrated favorable in vivo tolerability and impressive therapeutic indices in preclinical models (25–27). The first clinical studies to validate these observations were first tested in patients with acute myeloid leukemia where IMGN779, a CD33-targeted ADC (28) was tested and resulted in a follow-on approach with IMGN632, a CD123-targeted ADC (29). None of these ADCs are being investigated for the treatment of gastrointestinal malignancies; however, preliminary results from a phase 1 trial of
fam-trastuzumab deruxtecan-nxki (DS-8201a), a HER-2 targeted ADC bearing a camptothecin-class DNA topoisomerase I inhibitor, showed an overall response rate of 39% in HER-2 expressing CRC (30,31) and was granted accelerated approval by the FDA for patients with unresectable or metastatic HER2-positive breast cancer who have received two or more prior anti-HER2-based regimens in the metastatic setting. This suggests that ADCs could reach targets in mCRC and deliver a cytotoxic payload that can drive clinically meaningful antitumor activity.

In the present study, we describe preclinical observations with the second-generation anti-GCC ADC, TAK-164, which is comprised of a fully human IgG1 monoclonal antibody (mAb) targeting the extracellular domain of GCC as described in Supplemental Figure 1 and in issued patents (32,33). The payload of TAK-164 is DGN549, a highly potent indolinobenzodiazepine DNA alkylator, conjugated to the anti-GCC antibody via an intracellularly cleavable alanine-alanine dipeptide linked via mAb lysine residues (25) and the intact ADC is described as CDA-3 in (33). We demonstrate that TAK-164 binds to, is internalized by, and leads to dose-dependent, highly selective cytotoxicity and antitumor activity in GCC-expressing cells and xenograft mouse models, including those previously refractory to TAK-264. Most notably, tumor uptake of an $^{89}$Zr-labeled TAK-164 molecule in mouse CRC models was concordant with levels of GCC expression; immuno-positron emission tomography (PET) yielded rank-order correlation for response to TAK-164 therapy in various GCC-positive cell lines and patient-derived xenograft models.

**Materials and Methods**

*Generation of anti-GCC antibodies and conjugation to DGN549*
Recombinant anti-GCC mAb was generated and produced in large-scale as previously described (13,32). Conjugation to DGN549 was achieved by concentration of the antibody to 10 mg/ml using Amicon Ultra-15, 10 kDa MWCO centrifugal filters (EMD Millipore) and exchanged into 75 mM EPPS, pH 8.0 buffer using Illustra NAP Sephadex G25 desalting columns (GE Healthcare). Approximately four-fold molar excess of sulfonated DGN549-NHS was added to antibody with a final concentration of 2 mg/ml and 10% total dimethylacetamide. The reaction was incubated in a water bath at 25 °C for 4 hours before purification into 20 mM histidine, 50 mM sodium chloride, 8.5% sucrose, 0.01% Tween-20, 50 µM sodium bisulfite, pH 6.2, via Sephadex G25. The TAK-164 conjugates were analyzed and found to have approximately 2.6 moles of conjugated DGN549 per mole of antibody, 99% monomer and less than 2% unconjugated DGN549 (33). Conjugation of the non-targeting isotype control antibody, chKTI, was performed under similar conditions. Representative SDS-PAGE and LC-MS Results are shown in supplemental figure 2 for TAK-164 and chKTI ADCs.

Cell lines

HEK293 cells (ATCC, Manassas, VA, USA) were maintained in 90% Dulbecco's Modified Eagle's Medium (DMEM, 11995-040, Invitrogen), 10% fetal bovine serum (FBS, SH 30071.02, HyClone), 1% L-glutamine (25030-081, Invitrogen), and (HEK-293 GCC cells) 10 µg/ml of blasticidin (R21001, Invitrogen). Introduction of GCC into the HEK-293 cells was performed at Millennium Pharmaceuticals, Inc., Cambridge, MA, USA, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited, as previously described (13). All cells were grown at 37 °C in a humidified atmosphere with 5% CO₂.

Flow cytometry
Binding of anti-GCC antibody and TAK-164 to HEK-293 GCC cells was assessed with flow cytometry. Cells were incubated on ice for 1 hour in a V-bottom 96-well plate (1×10^6 cells/well) with serial antibody dilutions of 0.02 nM to 26.7 nM. Cells were washed twice with 3% FBS in ice-cold phosphate-buffered saline (PBS) and incubated with 1:200 goat anti-human phycoerythrin (PE) IgG1 (Southern Biotech, Birmingham, AL, USA) for 1 hour on ice. Cells were washed again and analyzed by flow cytometry on a BD FACS CANTO II (Becton Dickinson, Franklin Lanes, NJ, USA) flow cytometer. Data was analyzed using BD FACS Diva Software Version 8 (Becton Dickinson) and the PE-H geometric mean fluorescence intensity height was determined. The concentration producing a half-maximal response (EC_{50}) was calculated for TAK-164 and antibody by plotting the Log concentration of each molecule versus the PE-H geometric mean fluorescence intensity height value; results were analyzed by nonlinear regression using GraphPad Prism Version 5.

**Internalization assay**

HEK-293 GCC cells were grown in 6-well plates in FBS medium to a density of 600,000/well. FBS media was aspirated out of each well using suction, and immediately replaced with 1.5 ml/well of DMEM media containing 1 μg/ml of [³H]TAK-164 (tritiation on the payload). Cells were incubated at 37 °C for up to 24 h. Harvesting was carried out at 0, 4, and 24 hours by gently pipetting up-and-down the media in each well and transferring into a 15 ml conical tube per each 2 wells. A 0.5 ml aliquot of PBS was subsequently used to wash the same 2 wells, and added to the conical tube. For separation of cells from the media, the tubes were centrifuged for 10 min at 1000 rpm at 4 °C. The cell pellet was washed with 1 ml PBS and extracted with 1 ml of acetone. Similarly, media was extracted with 3 volumes of acetone. The acetone extracts from cells and media were dried, reconstituted, and radioactivity was measured by adding 5 ml of Ultima Gold.
scintillation cocktail (PerkinElmer, Waltham, MA, USA) followed by liquid scintillation counting (LS 6500, Beckman Coulter, Fullerton, CA). The resulting pellets from cells and media were solubilized with Solvable reagent (Perkin-Elmer), processed, and radioactivity was measured. For DNA isolation, tubes containing the cell pellet were vortexed for resuspension of cells in the residual supernatant. DNA isolation was performed using the DNA Puregene Gentra Kit (Qiagen, Germantown, MD) according to the manufacturer’s instructions. DNA pellets were re-suspended in 300 µl water (Optima LC/MS grade®, Fisher Scientific, Waltham, MA). All the solution was added to 5 ml scintillation cocktail and radioactivity was measured by liquid scintillation counting.

In vitro cytotoxicity assay

HEK-293 vector and HEK-293 GCC cells were plated in 96-well plates at a cell density of 4000 cells/well and incubated at 37 ºC with a titration of 100 nM to 5.12 fM of the unconjugated anti-GCC mAb or TAK-164 for 96 h. Cell viability was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. Triplicate wells were used for each treatment, and viability was normalized to untreated cells. Error was calculated as the standard error of the mean (SEM) of 3 independent experiments.

Human xenograft tumor studies in mice

All animal research and veterinary care was performed at Takeda Boston, and the study was conducted using a protocol approved by the Takeda Boston Institutional Animal Care and Use Committee (IACUC) in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Immunocompromised mice
were housed in a controlled environment and received food and water ad libitum. Seven-week old CB17 severe combined immunodeficient (SCID) or nude female mice were inoculated subcutaneously with $5 \times 10^6$ of HEK-293 GCC cells or human primary tumors in DMEM without 10% FBS medium. Human colorectal cancer tissue was obtained from the National Disease Research Interchange, Philadelphia, PA and used to develop primary human tumor xenograft models annotated with PHTX at Takeda. For PHTX studies (PHTX-09C, PHTX-11C, PHTX-17C, PHTXM-46C), mice were implanted subcutaneously in the right flank with tumor fragments from primary human colon tumor tissue propagated in mice. Tumor growth was monitored using Vernier calipers, and the mean tumor volume was calculated using the formula $(0.5 \times \text{length} \times \text{width}^2)$.

For in vivo pharmacokinetic and pharmacodynamic studies, when the mean tumor volume reached approximately $500 \text{ mm}^3$, animals were randomized into treatment groups ($n = 3$, TAK-164 treatment group; $n = 4$, vehicle group). Mice were treated with a single IV dose of vehicle (0.9% saline) or 0.38, 0.76, and 1.53 mg/kg TAK-164 (containing 7.5, 15, or 30 µg/kg of payload). The animals were sacrificed at defined time points (1, 24, 48, 96, 168, 336, or 504 h) following treatment administration, and tumor and whole blood samples were harvested. For plasma collection, approximately 0.5 ml of whole blood was collected via cardiac-puncture using a 25½ G, 1-cc syringe (Becton Dickinson) and transferred into labeled microcentrifuge tubes containing dipotassium ethylenediaminetetraacetic acid (Becton Dickenson). Samples were kept on wet ice until processing. All blood samples were processed into plasma by centrifugation (10,000 rpm for 10 min) at 4 °C. Tumors were dissected into 2 pieces; one piece was placed into labeled microcentrifuge tubes, snap-frozen on dry ice, and stored frozen at approximately −80 °C until time of analysis; the other piece was placed in 10% neutral buffered formalin (NBF). For in
vivo activity studies, mice were randomized into treatment groups of 7 animals for HEK-293 GCC and 8 animals for all PHTX studies, when tumors reached approximately 200 mm$^3$. Mice received a single IV dose of vehicle, or 0.19, 0.38, 0.76, or 1.53 mg/kg of TAK-164 or non-binding control chKTI-DGN549. Tumor GRI was calculated from treatment to termination of the experiment, using the formula: $100\% \times \frac{\text{tumor growth rate with vehicle} - \text{tumor growth rate with treatment}}{\text{tumor growth rate with vehicle}}$.

Mice were anesthetized using an isoflurane/oxygen mix and kept under anesthesia while tumor fragments were implanted, and no analgesics were used or required per IACUC guidelines. This method of anesthesia was used for its short induction and recovery time and the reliability of its effects (34). There were no unexpected animal deaths due to experimental procedures in the above-mentioned in vivo studies. Mice were euthanized using inhaled CO$_2$ when the following humane endpoints were reached: tumor volumes reaching ≥10% of the mouse's body weight, tumor length ≥2 cm, or tumors of any size interfering with eating, drinking, urinating, defecating, or walking. No mice showed any signs of illness due to tumor formation. Animal health observations were performed twice daily at 10 AM and 3 PM by husbandry staff. Temperature and relative humidity within the barrier facility rooms were checked (VWR thermometers) and recorded every morning by husbandry staff. The temperature of the rooms was maintained at 70ºF, and temperature fluctuations of ± 2 ºF were reported to the Animal Facility Supervisor. The relative humidity for animal holding rooms was set to 50%. Any reading more than ± 20% was also immediately reported to the Animal Facility Supervisor. Animals were housed on Alpha Dri1 or Alpha Dri+1 bedding with two forms of enrichment (one of which had to be for sheltering or nesting) in Thoren cage setups that had been autoclaved. Water bottles were filled with
chlorinated RO water, capped with a neoprene stopper, and then autoclaved in a covered container before placing them in the wire lid.

**Pharmacokinetics analysis**

Total and conjugated antibody levels in plasma samples were determined using qualified sandwich immunoassay methods with electrochemiluminescence (ECL) detection. The duration between the collection and analysis was within the documented 30 day storage stability timeframe. The lower limit of quantitation (LLOQ) was 0.065 μg/ml in plasma for assays measuring total and conjugated antibody from TAK-164. Briefly, 96 well plates were coated with the extra-cellular domain of GCC grafted onto the mouse Fc region to capture free and conjugated antibody present in plasma samples. Ruthenylated donkey anti-human Fcγ was used as the detection antibody for the total antibody assay, while ruthenylated anti-IGN antibody was used for the conjugated antibody assay. In the presence of tripropylamine-containing buffer, ruthenium tag produces an ECL signal that is triggered by a voltage. The resulting signal was measured on the MESCO QuickPlex SQ 120 instrument (Meso Scale Discovery [MSD]).

**Immunohistochemistry**

For γ-H2AX expression measurements, xenograft tumors were fixed in 10% NBF for 24 h as detailed above. Tissues were embedded in paraffin and then cut in 5-micron sections. The primary antibody was the Phospho-Histone H2A.X (Ser139) (20E3) Rabbit mAb (Cell Signaling Technologies, Danvers, MA, USA), diluted 1:500. Detection was performed using the Bond polymer refine 3,3’-diaminobenzidine (DAB) kit in combination with a Bond RX automated stainer (Leica Biosystems, Buffalo Grove, IL, USA), according to the manufacturer’s instructions. Stained images were captured using an Aperio Scanscope XT (Aperio ePathology
Solutions, Vista, CA, USA). Data analysis was performed with a Definiens Tissue Studio algorithm (Definiens, Cambridge, MA, USA). GCC protein levels in formalin-fixed paraffin-embedded tissues were assessed as previously described (13).

$^{89}$Zr-immuno PET imaging

TAK-164 was chemically modified with a chelator and radiolabeled with $^{89}$Zr to generate a non-site-selectively modified radio-immunoconjugate ($^{89}$Zr-DFO-TAK-164). $[^{89}\text{Zr}]$TAK-164 was provided as ready-to-use, isotonic, clear and colorless solutions for preclinical use (Invicro, Boston, MA, USA). Animals received ~60 µCi $[^{89}\text{Zr}]$TAK-164 (approximately 0.3 mg/kg) via tail vein by a 27 gauge x ½” insulin syringe (Terumo Medical, Somerset, NJ, USA). Injected animals were maintained in the animal facility and placed on a warmed water blanket when brought into the PET lab for imaging. Animals were anesthetized by isoflurane, placed in a 4-bed holder, and 1–3% isoflurane was maintained via nosecone during the imaging procedure. The mice were imaged using an Inveon preclinical PET/computed tomography (CT) scanner (Siemens, Knoxville, TN USA). A 30–60 min static PET image was acquired for each imaging session, followed by a 5–8 min attenuation correction CT scan. After imaging, animals were allowed to recover from the anesthesia in a regular cage containing bedding, food, and water. The recovery cage was placed on a heated water-jacketed blanket at least until mice were mobile.

$[^{18}\text{F}]$FDG PET imaging

Mice were fasted for 6–8 hours prior to the time of the scan. Animals received 300 µCi (+/-10%) FDG via tail vein by a 27 gauge x ½” insulin syringe and 1 hour was allowed for conscious distribution of the tracer. Animals were anesthetized as described above. The mice were imaged using an Inveon preclinical PET/CT scanner (Siemens). A 5–10 min static PET image, followed
by a 5–8 min attenuation correction CT scan, was acquired 1 hour after the FDG injection. After imaging, animals were allowed to recover as described above.

Reconstructed images from the Inveon PET/CT were generated in units of activity per volume. Namely, the values assigned to the voxels (volume elements) comprising the 3D reconstructed PET images were in units of nCi/cc. Reconstructed images were co-registered to one another, resampled to 0.20 mm$^3$ voxels, and cropped to a uniform size prior to analysis.

Tumor regions of interest (ROIs) were manually segmented using CT as an anatomical reference. A master spreadsheet was generated which included the volume, activity, and concentration (Activity/Volume) at each time point for each ROI generated. Results were presented in units of SUV$^{\text{max}}$. All images for the study were presented in units of percent injected dose per gram (%ID/g) scaled to 0 to 25 %ID/g.

**Statistical analysis**

The differences in the tumor growth trends over time between pairs of treatment groups were assessed by fitting each animal’s data to a simple exponential growth model and comparing the mean growth rates of the 2 groups. The difference in the growth rates was summarized by the GRI, which is the reduction in growth rate experienced by the treatment group relative to that of the reference group, expressed as a percent of the vehicle growth rate. A positive GRI indicates that the tumors in the treatment group grew at a reduced rate relative to the reference group. A statistically significant p value suggests that the trends over time for the 2 groups (i.e. vehicle and treatment) were different. A p value < 0.05 was considered statistically significant. For correlation analysis between tumor uptake of radiolabeled TAK-164 in tumor bearing mice vs
IHC or GRI, a one-tailed t-test with the nonparametric Spearman correlation (GraphPad Prism 7.03 Software) was utilized.

**Results**

*TAK-164 selectively binds, is internalized by, and is cytotoxic to GCC-expressing cells.*

Conjugation to a payload via lysine residues may affect the affinity of an antibody to its target. Using flow cytometry, we compared the relative binding of unconjugated anti-GCC mAb and TAK-164 to HEK-293 GCC, a cell line engineered to express GCC that allows for in vitro testing. (Fig. 1A). TAK-164 and unconjugated anti-GCC mAb bound to HEK-293 GCC cells in a dose-dependent manner with comparable affinities (EC$_{50}$: 0.33 nM and 0.14 nM, respectively), indicating that conjugation of DGN549 (approximately three molecules/antibody) to the anti-GCC mAb has modest impact on antigen-binding properties.

Target-dependent internalization and DNA binding of TAK-164 was demonstrated in HEK-293 GCC cells using tritium-labeled TAK-164 (tritiation on the DGN549 payload). Cells were incubated in medium containing $[^{3}]$H]TAK-164; samples were collected after 0, 4, and 24 hours of incubation. Total radioactivity decreased with time in the media pellet sample and increased in the cell pellet sample, suggesting cellular uptake of $[^{3}]$H]TAK-164. Evidence of cellular uptake is supported by increased radioactivity observed in DNA samples extracted from HEK-293 GCC cells exposed to $[^{3}]$H]TAK-164 also showed increased radioactivity with time, demonstrating binding of $[^{3}]$H]TAK-164 to DNA.

To evaluate TAK-164 in vitro cytotoxicity, HEK-293 GCC or HEK-293-vector (i.e., nonGCC-expressing) cells were incubated with TAK-164 (Fig. 1C). TAK-164 reduced HEK-293 GCC cell viability, with a 50% lethal dose (LD$_{50}$) of 2.8 pM. This is approximately 33-fold more
potent than the TAK-164 LD_{50} on HEK-293-vector cells (93.5 pM), indicating that the cytotoxic activity of TAK-164 is GCC target-dependent. The shift in potency observed warranted testing in vivo where the anatomical compartmentalization of GCC is preserved and linker stability can more readily be assessed.

**TAK-164 in vivo pharmacokinetics and pharmacodynamics**

To assess the pharmacokinetics of TAK-164 in plasma, mice carrying HEK-293 GCC xenograft tumors were injected once with 0.38, 0.76, or 1.53 mg/kg of TAK-164 (containing 7.5, 15, or 30 μg/kg of payload; Fig. 2A). Total mAb (conjugated and unconjugated) and conjugated mAb were detected in plasma for up to 504, 336 or 168 h after a single injection with 1.53, 0.76 or 0.38 mg/kg of TAK-164, respectively. The total mAb and conjugated mAb concentrations in plasma were similar, suggesting a slow rate of spontaneous cleavage of the payload over time, and high stability of the unbound ADC in plasma.

Mice carrying HEK-293 GCC xenograft tumors were evaluated by immunofluorescence for expression levels of γ-H2AX, a DNA-damage marker (35) following a single intravenous (IV) dose of TAK-164 at 0.38, 0.76, and 1.53 mg/kg (containing 7.5, 15, or 30 μg/kg of payload) or vehicle (Fig. 2B). The proportion of γ-H2AX–positive cells increased at a dose-dependent fashion, peaking at 168 hours and remaining relatively high throughout the experiment up to 504 hours after a single injection. For the 1.53 mg/kg TAK-164 dose (containing 30 μg/kg of payload), approximately 80% of the cells were γ-H2AX–positive at 168 hours after the injection and decreased to approximately 63% through 504 hours post-injection. The highest proportion of γ-H2AX–positive cells observed with 0.38 and 0.76 mg/kg TAK-164 was 57.7% and 72%, respectively.
TAK-164 has strong antitumor activity in GCC-expressing xenografts and primary human tumor xenograft mouse models of mCRC.

To characterize TAK-164, we followed up our PK assessment in HEK-293 GCC tumor bearing mice. In addition, we selected primary human tumor xenograft (PHTX) mouse models of mCRC previously shown to express different levels of GCC closely resembling the GCC expression pattern in human disease and where antitumor activity of TAK-264, a GCC-targeted auristatin ADC had been evaluated (13). Importantly, the dose regimen for TAK-164 administration was guided by prior work reported by Miller et al. (25) and resulted in a regimen where body weight loss for all animals included in the present study are less than 5%. Tumor-bearing mice were treated once with vehicle or TAK-164 at various doses. In HEK-293 GCC tumors, treatment with TAK-164 at 0.19, 0.38, or 0.76 mg/kg resulted in tumor growth inhibition in a dose-dependent fashion (Fig. 3A). In the PHTX-09C model, dose-dependent antitumor activity was also observed up to 65 days after a single injection with 0.38, 0.76, and 1.53 mg/kg TAK-164 compared with vehicle (Fig 3B). In the PHTX-17C and PHTX-11C tumor models, both previously refractory to treatment with TAK-264 (13), antitumor activity was achieved with 0.76 and 1.53 mg/kg TAK-164 compared with vehicle (Fig. 3C and D). In the HEK-293 GCC and PHTX-09C models, tumor regressions were observed; in the PHTX-17C and 11C models, tumor stabilization was observed for approximately 40 days post-injection at the highest dose level. Interestingly, antitumor activity to a chKTI nontargeting isotype control ADC varied in these models (Supplemental Table 1)

In vivo tumor uptake of TAK-164 correlates with GCC expression and antitumor activity
We used $^{89}$Zr-immuno PET to visualize uptake of radiolabeled TAK-164 by HEK-293 GCC and PHTX tumor tissues in mice (Fig. 4). After a single injection with ~60 $\mu$Ci $[^{89}$Zr]TAK-164 (average 0.3 mg/kg), uptake was visualized in tumor tissue, with a stronger signal in tumors with higher H-scores for GCC expression (Fig. 4A). Tumor uptake was first detected after approximately 24 hours and increased steadily up to 144–192 hours after a single injection with $[^{89}$Zr]TAK-164 in GCC-positive tumors (Fig. 4B). Maximum standard uptake values ($SUV_{\text{max}}$) were two- to three-fold higher in high GCC-expressing tumors (HEK-293 GCC, PHTX-09C and PHTX-11C) compared with low-expressing tumors (HEK-293, PHTX-17C, PHTXM-46C).

To further explore the relationship between GCC expression and uptake of TAK-164 by tumor tissue in vivo, we plotted the $SUV_{\text{max}}$ measured by $^{89}$Zr-immuno PET against GCC expression H scores in each tumor tissue (Fig. 4C). A significant correlation was observed between GCC-expression H-scores and $SUV_{\text{max}}$ ($P = 0.0290$). In high-expressing tumors (GCC expression H-score of 300: HEK-293 GCC, PHTX-09C, and PHTX-11C), the $SUV_{\text{max}}$ was between 4.5 and 7.4; in low-expressing tumors (H-score $\leq$150: HEK-293, PHTXM-46C and PHTX-17C), the $SUV_{\text{max}}$ was between 2.3 and 2.7.

To determine whether the antitumor activity displayed by TAK-164 in mouse models correlated with efficacy, the GRI at a TAK-164 dose of 0.38 mg/kg determined from previous pharmacology studies was plotted against the $SUV_{\text{max}}$ extrapolated from the PET imaging. A correlation was observed between GRI values and $SUV_{\text{max}}$ ($P = 0.0313$), indicating that the antitumor activity of TAK-164 is dependent on tumor uptake (Fig. 4D).
The growth rate inhibition (GRI) was determined for each TAK-164 dose versus vehicle in each tumor model. A non-GCC-binding ADC (chKTI-DGN549) was used as a control. A statistically significant GRI was demonstrated in HEK293-GCC tumors for 0.19, 0.38, and 0.76 mg/kg TAK-164. In PHTX-09C and PHTX-17C, significant GRI was observed for TAK-164 doses of 0.38, 0.76, and 1.53 mg/kg. In PHTX-11C tumors, a significant GRI was shown at 0.76, and 1.53 mg/kg TAK-164.

**TAK-164 antitumor activity can be monitored by \(^{18}\)F-fluorodeoxyglucose (FDG)-PET**

We used \(^{18}\)F-FDG-PET to monitor tumor viability following treatment with a single injection of TAK-164 or vehicle in HEK-293 GCC tumor-bearing mice (Fig. 5). Tumor size was increased in mice treated with vehicle, while tumor size was decreased in mice treated with TAK-164. Correspondingly, tumor volume values and \(^{18}\)FFDG uptake significantly increased following treatment with vehicle (Fig. 5A). In mice treated with TAK-164, a significant decrease in \(SUV_{\text{max}}\) was observed by day 12 after treatment, which correlated with decreased tumor size. Tumor growth and \(^{18}\)FFDG uptake could be detected again at 28 days following treatment (Fig. 5B).

**Discussion**

We here describe preclinical characterization of a second-generation ADC, TAK-164, comprising a fully human IgG\(_1\) conjugated to DGN549, a highly cytotoxic indolinobenzodiazepine DNA alkylator via an intracellularly cleavable linker with a demonstrated high level of bystander killing (36) that could be advantageous in solid tumors with heterogenous target expression. Further, the mechanism of action of DGN549 is aligned with approved treatments for GI malignancies (3,4), and while preserving similar potency to PBDs,
indolinobenzodiazapine pseudodimers appear to be better tolerated, particularly in repeat-dose scenarios practiced in the clinic (27,36). TAK-164 is being assessed for potential therapeutic application in GCC-positive gastrointestinal malignancies (NCT03449030) following limited clinical efficacy for the first-in-class MMAE-conjugated anti-GCC ADC, TAK-264 (19–21). GCC remains an attractive therapeutic target due to its restricted expression pattern in mCRC and other gastrointestinal cancers (9,14,15,20).

TAK-164 effectively binds to, and is internalized by, GCC-expressing cells, and the payload associates with DNA in these cells resulting in picomolar LD_{50} values in HEK293-GCC expressing cells, but not HEK293-vector cells (Fig. 1). Following a single administration, TAK-164 was detectable in the plasma of mice bearing HEK-293 GCC xenografts for up to 500 hours, with evidence of minimal to no release of the payload in plasma, which is thought to limit potential off-target toxicity due to early release of potent payloads (37). Furthermore, a single dose of TAK-164 induced dose-dependent expression of phosphorylated histone γ-H2AX, a sensitive biomarker of DNA double-strand breaks (35), consistent with DGN549 alkylating activity. These observations motivated exploration of TAK-164 activity in PHTX models that more closely resemble the architecture and heterogeneity observed in clinical patient samples. Following a single IV injection, we found that TAK-164 had antitumor activity against several GCC-expressing PHTX tumors, including those refractory to a weekly administration for 3 weeks of TAK-264 at much higher concentrations (13). Building on these observations, we looked to evaluate TAK-164 single-agent activity more broadly in 68 Subcutaneous HuPrime® Colorectal Cancer PDX Models, in which generated data suggest that higher GCC+ IHC score increases time to rise and that TAK-164 might be active across various subtypes of CRC (38). Furthermore, TAK-164 uptake by human GCC-positive xenograft tumors was visualized in vivo.
by $^{89}$Zr-immuno PET and, most importantly, correlated with GCC expression, as assessed by immunohistochemistry, demonstrating tumor-selective accumulation. TAK-164 GRI also correlated with tumor uptake assessed by $^{89}$Zr-immuno PET, suggesting that GRI achieved with TAK-164 may be associated with GCC expression. While the correlations observed are encouraging, the present study is limited by the number of preclinical models that could be evaluated for tumor uptake thus broader testing including evaluation of clinical biodistribution may help understand accessibility of TAK-164 in GCC expressing tumors particularly if such work can be performed along with conventional IHC methods. Such techniques, combined with early response assessments, have been shown to predict response with the FDA-approved ado-trastuzumab, emtansine (39). A recent review by Coats et al. calls attention to additional studies that underscore the need to more fully understand clinical biodistribution of ADCs, and refinement of translational strategies while highlighting the promise of next generation ADCs such as trastuzumab deruxtecan (DS-8201a) and sacituzumab govitecan in solid tumor settings (22).

Treatment paradigms for solid tumors including GI malignancies continue to evolve, most recently to include immunomodulatory agents in microsatellite instability-high solid tumors (40). Observations in the present study are limited by the lack of a competent immune system, however, others have demonstrated that various ADCs bearing DNA-directed agents, such as PBD and a topoisomerase I inhibitor, elicit immune responses in preclinical models (41,42). These findings suggest ADCs may be successfully combined with immune checkpoint inhibition (ICI) and have motivated clinical testing of ADCs in combination with ICI. Future work aims to better understand the potential of TAK-164 in preclinical models of other GCC-expressing tumor models as single agents and in combination with relevant therapeutics.
In conclusion, TAK-164 is a novel GCC-targeting ADC that employs a unique cytotoxic payload to achieve potent antitumor activity in preclinical models of CRC that can be monitored using clinically relevant approaches (pharmacodynamic assays and FDG-PET). Our findings identify TAK-164 as a promising new therapeutic candidate, warranting further clinical evaluation for the treatment of CRC and other gastrointestinal malignancies.

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Fig. 1. Characterization of TAK-164 binding, internalization, and in vitro cytotoxicity. (A) Unconjugated anti-GCC mAb and TAK-164 bind to GCC-expressing cells. Flow cytometry was performed with unconjugated anti-GCC mAb and TAK-164 on HEK-293 GCC cells. Mean fluorescence intensity is plotted versus mAb concentration (log) nM. (B) $[^3]$H TAK-164 is internalized by HEK-293 GCC cells and the intracellularly deconjugated payload binds to DNA. Cells were incubated in medium containing $[^3]$H TAK-164 (tritiation on the DGN549 payload), and harvested at different timepoints for measuring of radioactivity. (C) In vitro cytotoxicity of TAK-164. Cell viability was assessed 96 hours after incubation of HEK-293 GCC cells or HEK293-vector cells with increasing concentrations of TAK-164. The average of three independent experiments is plotted with error represented as standard error of the mean (SEM).

Fig. 2. TAK-164 in vivo pharmacokinetics and pharmacodynamics in HEK-293 GCC tumor bearing mice. (A) Plasma concentration of total anti-GCC antibody (conjugated and unconjugated) or conjugated antibody after a single injection of 0.38, 0.76, and 1.53 mg/kg TAK-164. (B) Levels of the DNA-damage biomarker γ-H2AX in tumors were quantified by immunofluorescence following a single injection with 0.38, 0.76, and 1.53 mg/kg of TAK-164, and corrected for baseline levels detected in vehicle-treated xenografts.

Fig. 3. TAK-164 induces tumor regression and long-term tumor growth delay of GCC-expressing tumors. Female severe combined immunodeficient (SCID) mice bearing (A) GCC-expressing HEK293 xenograft tumors or female nude mice bearing (B) PHTX-09C, (C) PHTX-17C, or (D) PHTX-11C tumors, patient-derived xenograft models of mCRC, were treated with a single injection of either vehicle, TAK-164, or nontargeting control ADC at various doses, when the tumor reached approximately 200 mm$^3$. Average tumor volume was determined at multiple time points following the injection and is shown ± SEM.

Fig. 4. Tumor uptake of radiolabeled TAK-164 in xenograft mice models and correlation with GCC expression. (A) $^{89}$Zr-immuno PET was used to quantitate in vivo uptake of TAK-164 by tumor tissue in mice after 144 hours of an average 0.3 mg/kg injection with $[^{89}Zr]$ TAK-164. (B) SUV$_{max}$ versus time at 144h post injection of $[^{89}Zr]$ TAK-164 in tumor-bearing mice (C) GCC expression H scores versus SUV
for $[^{89}\text{Zr}]$TAK-164 in each xenograft tumor line. (D) GRI (%) versus standard uptake values for $[^{89}\text{Zr}]$TAK-164. Growth-rate inhibition (%) was determined for tumor-bearing mice after 21 days of a single injection with 0.38 mg/kg TAK-164; SUV$_{\text{max}}$ were determined by PET. H scores were determined by immunohistochemistry.

**Fig. 5.** $[^{18}\text{F}]$FDG-PET is effective for monitoring tumor viability following treatment with TAK-164. (A) PET images and (B) tumor volume (dotted line) and SUV$_{\text{max}}$ of $[^{18}\text{F}]$FDG (solid lines) determined by PET in HEK-293 GCC tumor-bearing mice following a single injection with vehicle (V, blue triangles) or TAK-164 0.38 mg/kg (T, red squares). Data until day 21 were included for linear mixed-effects modeling with post hoc general linear multiple comparisons. Testing with Tukey’s all-pair comparisons asterisks indicate statistically significant changes in values from the time point before the treatment. *$P < 0.05$, ***$P < 0.001$. 
Figure 1

A) Mean Fluorescence Intensity vs. Log₁₀ (nM) for Anti-GCC Antibody and TAK-164.

B) Total Radioactivity (%): Cell extract, Media extract, Media pellet, Cell pellet, DNA. Comparison at 0 hours, 4 hours, and 24 hours.

C) Viability (%) vs. Log₁₀ TAK-164 concentration [M]: HEK-293 Vector and HEK-293 GCC.

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<th>Table: Anti-GCC Antibody</th>
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Figure 2
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Preclinical Antitumor Activity and Biodistribution of a Novel Anti-GCC Antibody-Drug Conjugate in Patient-Derived Xenografts.

Adnan O Abu-Yousif, Donna Cvet, Melissa Gallery, et al.

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