**Scribner et al., MGC018, a duocarmycin-based ADC targeting B7-H3 for cancer**

**Supplemental Materials and Methods**

**Generation of Hs700T/B7‑H3 knockout tumor cell line**

Briefly, 6 x 105 cells were seeded in 6‑well plates and transfected the following day with 2.5 µg of vector using Lipofectamine 3000, according to the manufacturer’s protocol (Invitrogen). Transfectants were selected with puromycin, sorted by flow cytometry, and individual clones were expanded for subsequent analysis.

**Bystander killing time lapse microscopy**

Hs700T/B7-H3 KO cells stably expressing red fluorescent protein (Hs700T/B7-H3 KO/RFP) were generated using the IncuCyte NucLight Red Lentivirus (Sartorius) according to manufacturer’s protocol. Cells were harvested by trypsinization and plated into 96-well flat bottom plates. Parental Hs700T cells were plated at densities ranging from 0 – 5,000 cells/well. Hs700T/B7-H3 KO/RFP cells were plated at 5,000 cells/well. MGC018 was added to plates at the indicated concentrations, then plates were incubated at 37oC and monitored in an IncuCyte Live-Cell Analysis System (Sartorius) over 5 days.

**Flow cytometry/quantitative flow cytometry**

For flow cytometry, cells were removed from tissue culture flasks with 0.05% trypsin-EDTA, then washed in Hank’s Balanced Salt Solution (HBSS) containing 1.0% bovine serum albumin (BSA; fraction V). 2 x 105 cells were stained with an anti-B7‑H3 antibody (BRCA69D) at 1 µg/mL for 15 minutes at 4°C, then washed and counterstained with 2 µg/mL r-phycoerythrin-conjugated goat anti-mouse antibody for 15 minutes at 4°C. Cells were washed, resuspended in HBSS containing 1.0% BSA, and 7500 live cells acquired on a Guava-PCA-96 cytometer (MilliporeSigma).

For quantitative flow cytometry (QFACS), cells were removed with 0.05% trypsin-EDTA, washed with HBSS containing 1.0% BSA; fraction V), then labeled with the anti-B7‑H3 mAb PRCA157 at a saturating concentration of 2 µg/mL. Cells, set-up beads and calibration beads were then labeled with fluorescein-conjugated anti-mouse secondary antibody at saturating concentration. Antibody Binding Capacity (ABC) was calculated based on the interpolation of the calibration curve equation.

**Immunohistochemistry**

FFPE tissues were sectioned at 4 µm, mounted on Superfrost Plus Micro charged glass slides, then baked for 1 hour at 60ºC. Tissue was deparaffinized with 100% xylene. Slides were hydrated using graded ethanol and rinsed with distilled water. Antigen retrieval was performed in a Biocare Medical Decloaking Chamber using Agilent antigen retrieval solution, pH 6, heated to 125°C for 30 seconds and then cooled to 90°C over approximately 20 minutes. Sections were further cooled to 650C for an additional 10 minutes. The sections were rinsed with deionized water and equilibrated in wash buffer prior to loading them into the autostainer to preform Immunohistochemistry (IHC) staining.

IHC staining was conducted on a Thermo Scientific Lab Vision 480s Autostainer (Thermo Fisher Scientific). Sections were treated with 3% hydrogen peroxide for 10 minutes, avidin and biotin for 15 minutes each, then 5% normal donkey serum for 10 minutes prior to staining. Sections were incubated with goat anti-human B7-H3 affinity purified polyclonal antibody (R&D Systems Cat# AF1027) at 1 µg/mL for 40 minutes. Primary antibody was detected using Biotin-SP-AffiniPure Donkey Anti-Goat IgG (H+L) at 5 µg/mL for 30 minutes, Streptavidin-HRP for 30 minutes, followed by 3, 3’ diaminobenzimide (DAB) for 6 minutes. A goat IgG antibody (R&D Systems Cat# AB-108-C) was used as a negative control in the staining runs. The sections were counterstained with hematoxylin for 6 minutes. Upon completion of staining, the slides were dehydrated in graded ethanol, cleared in 100% xylene, and cover slipped.

To evaluate the IHC staining, staining intensity was scored on a semi-quantitative integer scale from 0 (negative) to 3 (the highest intensity). The percentage of cells staining positively for membrane with or without cytoplasm staining was recorded. An H-score was then calculated for each sample based on the following formulation: [1 × (% tumor cell (membrane) at 1+) + 2 × (% tumor cell (membrane) at 2+) + 3 × (% tumor cell (membrane) at 3+)].

**Surface plasmon resonance**

Anti-penta-His tag mAb was immobilized on the CM5 sensor chip according to the procedure recommended by the manufacturer (GE Healthcare). The His tagged human or cynomolgus monkey B7-H3(4Ig) was captured at the level of approximately 30 resonance units (RU). MGC018 or MGA017 was injected for 120 seconds at a flow rate of 30 µL/min (in duplicates) in HBS-EP buffer at concentrations of 0, 6.25, 12.5, 25, 50, 100 and 200 nM. Regeneration of the immobilized anti-penta-His mAb surface was performed by pulse injection of 10 mM glycine, pH 1.5. Reference curves were obtained by injection of each dilution of MGC018 or MGA017 over the treated surface with no immobilized protein. Binding curves at zero concentration were subtracted as a blank. Kinetic constants, ka and kd, for one arm affinity interaction were estimated by global analysis of the association/dissociation curves to the Bivalent analyte interaction model.  (BIAevaluation software v4.1). The dissociation equilibrium constant (KD) was calculated as KD=kd/ka.

**In vivo efficacy**

Statistical analyses were carried out between treated and vehicle control groups comparing tumor volumes. Percent Tumor/Control (% T/C) was calculated based on the mean tumor volume of the treatment group divided by the mean tumor volume of the vehicle group on, or near, the last day when all vehicle group individuals were still on study and prior to any tumor ulcerations. Complete regression (CR) was determined at the end of the study and was defined as the lack of a palpable tumor (tumor volume ≤ 5 mm3) during the study. All analyses were performed using Study Director software (v3.1.399.17, Studylog Systems), GraphPad Prism software (v7.02, GraphPad), or calculated from the raw data.

**In vitro serum stability**

MGC018 was diluted to 100 or 200 µg/mL in human, cynomolgus monkey, or mouse serum, and individual aliquots were incubated at 37°C for 0, 1, 6 hours, and 1, 2, 4, 6, 8, and 10 days. Samples were snap frozen into dry ice and stored at ‑80°C until assayed. For detection of total antibody, 96‑well nickel‑coated plates were coated with 2 µg/mL rhB7‑H3-His protein in phosphate-buffered saline (PBS). To detect conjugated antibody(containing ≥ 1 linker-payload), 96‑well ELISA plates were coated with 2 µg/mL anti‑Hapten B-10-2-6 IgG, which binds specifically to the DUBA payload. Following overnight incubation at 4°C, plates were blocked with HBSS containing 1% BSA, then washed with PBS/0.05% Tween‑20. A standard curve of MGC018 was diluted into HBSS/1% BSA solution containing human, cynomolgus monkey, or mouse serum to match the highest concentration of serum in samples to be tested for each plate. Test serum samples containing preincubated MGC018 were diluted in HBSS/1% BSA containing human, cynomolgus monkey, or mouse serum. Following a 1-hour incubation, plates were washed with PBS/0.05% Tween‑20, incubated with a peroxidase-conjugated affinity pure goat anti-human IgG (1: 30,000 into HBSS/1% BSA), and detected with Sure Blue TMB peroxidase substrate (Seracare). The reactions were stopped by the addition of 1 M phosphoric acid and plates were read at 450 nm on an EMax plate reader (Molecular Devices) and standard curves were generated with GraphPad Prism software (v7.02, GraphPad) using a 4‑paramater curve fit. The concentration of total antibody and conjugated antibody in serum stability samples was interpolated using the standard curves generated.

Stability analysis was performed on the data with Phoenix WinNonlin software (v8.1, Certara), uniform weighting, and linear up log down method to obtain the relevant parameters Co (serum concentration at time 0), Clast (last measurable serum concentration) and AUC (area under the concentration curve).

**Pharmacokinetics – mouse**

Female CD1 Nude (homozygous; Crl:CD1-*Foxn1nu*) or SCID/CES1c knockout mice (Charles Rivers Lab), weighing 20-30 grams and 5‑7 weeks of age were used in the pharmacokinetic (PK) studies. 3-5 mice were used per time point.

MGC018 was administered to mice by intravenous (IV) injection at 5 mg/kg in a volume of 100 µL. Mouse blood samples were collected by retro-orbital bleeds or by terminal cardiac puncture at 0, 0.5, 1, 4, 8 hours and 1, 2, 4, 7, and 10 days following administration of MGC018. Samples were allowed to clot at room temperature, centrifuged for serum separation, then snap frozen in dry ice and stored at ‑80°C.

96-well ELISA plates were coated with anti-human IgG (total antibody assay) or anti-Hapten B-10-2-6 IgG (conjugated antibody assay; detects antibody with ≥ 1 linker-payload), both at 2 µg/mL in PBS. Following overnight incubation at 4°C, plates were washed and blocked with a HBSS containing 1% BSA then washed with PBS/0.05% Tween-20. Plates were incubated with MGC018 standard calibration samples, quality control samples, test samples, and/or dilution control samples. Plates were washed with PBS/0.05% Tween-20, incubated with a peroxidase-conjugated affinity pure goat anti-human IgG (1:30,000 in HBSS/1% BSA) and bound peroxidase-conjugated IgG was detected with Sure Blue TMB peroxidase substrate. 1 M phosphoric acid was added to stop the reaction. Plates were read at 450 nm on an EMax plate reader (Molecular Devices) and standard curves were generated using GraphPad Prism software (v7.02, GraphPad), using a 4-paramater curve fit. Total antibody and conjugated antibody concentrations were interpolated using the standard curve generated. Noncompartmental analysis (NCA) was performed on the data with Phoenix WinNonlin software (V8.1, Certara), Model 200 and 201 (sparse, plasma data, extravascular administration or IV bolus), uniform weighting, and linear trapezoidal linear/log interpolation to obtain the PK parameters.

**Pharmacokinetics – cynomolgus monkey**

An electrochemiluminescence method was used to quantify total antibody. Briefly, Meso Scale Discovery (MSD®) standard plates were coated with 0.125 µg/mL goat anti-human IgG (H+L) antibody and incubated overnight at 2-8°C. After blocking the nonspecific sites with 5% BSA in PBS, the plates were incubated with MGC018 standard calibration samples, quality control samples, test samples, and/or dilution control samples. The immobilized goat anti-human IgG (H+L) antibody captures the total human antibody (conjugated antibody and unconjugated antibody). The captured total human antibody was detected by the sequential addition of 0.125 µg/mL biotinylated goat anti-human IgG(Fc) monkey adsorbed antibody, followed by SULFO-TAG Streptavidin at 0.125 µg/mL. A quantitative measure of the enhanced chemiluminescence (ECL) signal emitted by each well was recorded, and the ECL counts emitted by the standard calibrators were used to generate a standard curve using a SoftMax Pro GxP (v5.4.1, Molecular Devices) 4-parameter logistic (4PL) fit. The unknown concentrations of total human antibody in test samples were then determined from the interpolation of the samples’ ECL counts with the MGC018 standard curve.

A sandwich ELISA was used to quantify conjugated antibody containing ≥ 1 linker-payload. Assay plates were coated overnight with 1 µg/mL goat anti-human IgG (H+L) antibody. After blocking the non-specific sites with 0.5% BSA in PBS with 0.1% Tween-20, plates were incubated with MGC018 standard calibration samples, quality control samples, test samples, and/or dilution control samples. The captured conjugated antibody was specifically detected by the sequential addition of 0.13 µg/mL biotinylated mouse anti-Hapten B 10-2-6 monoclonal antibody, followed by 1:20,000 dilution of Streptavidin-horseradish peroxidase (HRP). The bound HRP activity was quantified by luminescence light generation using Pico substrate, and measured by the Victor X4 plate reader (Perkin Elmer). The standard curve was generated by fitting the relative light unit signal from the MGC018 standards with a 4PL model using SoftMax Pro GxP (v5.4.1, Molecular Devices). The concentration of total and conjugated antibody in the serum samples was determined by interpolation from a standard curve using a four-parameter curve fit with 1/Y2 weighting relating the light intensity to the concentration of MGC018.