Supplementary Materials and Methods

Title: MRTX1719 is an MTA-cooperative PRMT5 inhibitor that exhibits synthetic lethality in preclinical models and patients with *MTAP* deleted cancer

Running Title: MRTX1719 is a PRMT5/MTA inhibitor for *MTAP* deleted cancers

Authors: Lars D. Engstrom[1], Ruth Aranda[1], Laura Waters[1], Krystal Moya[1], Vickie Bowcut[1], Laura Vegar[1], David Trinh[1], Allan Hebbert[1], Christopher R. Smith[1], Svitlana Kulyk[1], J. David Lawson[1], Leo He[2], Laura D. Hover[2], Julio Fernandez-Banet[2], Jill Hallin[1], Darin Vanderpool[1], David M. Briere[1], Alice Blaj[1], Matthew A. Marx[1], Jordi Rodon[3], Michael Offin[4], Kathryn C. Arbour[4], Melissa Johnson[5], David J. Kwiatkowski[6, 7], Pasi A. Janne[6, 7], Candace L. Haddox[6], Kyriakos P. Papadopoulos[8], Jason T. Henry[9], Konstantinos Leventakos[10], James G. Christensen[1], Ronald Shazer[1], Peter Olson[1*]

Cell Lines and Culture Reagents

Parental PK-1 cells were obtained from Riken BioResource Research Center (RCB1972, RRID: CVCL_4717). MRC-5 cells were obtained from ATCC (CCL-171, RRID: CVCL_0440) and 293FT cells were obtained from Thermo Fisher Scientific (R70007, RRID: CVCL_6911).

McCoy's 5A medium (#16600082), RPMI 1640 (#11875085), Minimum Essential Medium (#11095080), DMEM (#10566-016), Dulbecco's phosphate-buffered saline (DPBS, #14190136), Trypsin-EDTA (#25200056), Sodium Pyruvate (#11360070), puromycin (# A1113803) and Trypan blue solution, 0.4% (#15250061) were obtained from Gibco/Thermo Fisher Scientific. Fetal Bovine Serum (FBS) was obtained from Corning (#35-011-CV). Antibiotic-Antimycotic (Anti-Anti) Solution was obtained from Caisson Labs (#ABL02).

MTAP del Cell Line Engineering

HCT116 and PK-1 *MTAP* del cell clones were generated from the parental cell lines using CRISPR. Single guide RNA (sgRNA) lentiviral constructs targeting the human *MTAP* gene were ordered from Cellecta in a single vector system (Item # - CVPRC-PX; vector - pRSGC2-U6-(XX)-UbiC-Cas9-2A-Puro; MTAP sgRNA-1: R# 92879-1P, lot # 16081602; *MTAP* sgRNA-2: R# 92879-2P, lot # 16081603; MTAP sgRNA-3: R# 92879-3P, lot # 16081604). Lentivirus was produced in 293FT cells and sterile filtered. Parental HCT116 and PK-1 cells were transduced with fresh virus mixed with 8 µg/mL polybrene. Following selection with 2 µg/mL puromycin, ~20-30% of the cells were puromycin resistant suggesting the majority of cells harbored single integration events. Single cell clones were expanded and screened for *MTAP* deletion by western blot (1:500, Abcam #ab55517, RRID: AB_944282). Multiple single clones for each model were

confirmed to have *MTAP* knock out and increased sensitivity to MRTX1719 compared to the *MTAP* wild-type (WT) parental cell lines.

Tissue Microarray Immunohistochemistry

Immunohistochemistry was performed for MTAP expression on formalin fixed paraffin embedded (FFPE) tissue microarrays (TMA) of human bladder, head and neck, lung adenocarcinoma, lung squamous cell carcinoma, mesothelioma and pancreatic tumor samples. TMAs were sectioned at 4 micron thickness onto positively charged glass slides and stained for MTAP with an anti-MTAP antibody (Abcam #ab55517, RRID: AB_944282) and automated detection on a Dako Link 48 autostainer at Mosaic Laboratories. Immunohistochemistry was performed on slides from a serial TMA section for SDMA using a rabbit MultiMab SDMA antibody (Cell Signaling Technology #13222; RRID: AB_2714013).

MTAP staining in neoplastic cells was evaluated by a pathologist and MTAP H-scores were calculated. For each of the listed cancer types, 30 core biopsies, with roughly half given an MTAP H-score of zero, were selected for pathologist review to determine SDMA H-scores. The SDMA H-scores of neoplastic cells were graphed as a dot plot grouped by samples with no MTAP expression (MTAP H-score of ≥ 1) and MTAP expressing samples using GraphPad Prism annotated with the group mean and 95% confidence interval. Statistical significance was determined using a two-tailed Student's t-test comparing SDMA H-scores of *MTAP* WT and *MTAP* del tumors from each tumor type or combined analysis assuming equal variance.

PRMT5 Substrate Competitive Biochemical Assay

To determine substrate competitive nature of PRMT5 inhibitors MRTX1719 and GSK3326595, an AlphaLISA biochemical assay was performed at BPS biosciences. The serial dilution of the compounds was first performed in 100% DMSO with the highest concentration at 2 mM or 1 mM. Each intermediate compound dilution (in 100% DMSO) was diluted 30x fold into assay buffer for 3.3x conc (DMSO). Enzyme only and blank only wells have a final DMSO concentration of 1%. From the intermediates, 3 µl of compound is added to 2 µl of diluted PRMT5 enzyme and incubated for 30 minutes at room temperature. PRMT5 reactions were conducted in duplicate 120 minutes at RT in a 10 µl mixture containing assay buffer, various PRMT5 substrates - biotinylated histone H4 peptide at selected concentrations, 2 µM SAM concentrations, enzyme, and test compound. MTA at 0.25 µM, an approximate IC50 concentration in this assay, was added to reactions containing MRTX1719. These 10 µl reactions were carried out in 384-well Optiplates (Perkin Elmer). After enzymatic reactions, 10 µl of anti-Rabbit acceptor beads (Perkin Elmer, diluted 1:500 with 1x detection buffer) and Primary antibody 4-3 (BPS, diluted 1:60 with 1x detection buffer) were added to the reaction mix, briefly mixed on a plate shaker and incubated for 30 minutes. Finally, 10 µl of AlphaScreen Streptavidin-conjugated donor beads (Perkin, diluted 1:125 with 1x detection buffer) were added, incubated for 30 minutes and the samples were measured using an AlphaScreen

microplate reader (EnSpire Alpha 2390 Multilabel Reader, PerkinElmer). Enzyme activity assays were performed in duplicate at each concentration. The A-screen intensity data or luminescence data were analyzed and compared. In the absence of the compound, the intensity (Ce) in each data set was defined as 100% activity. In the absence of enzyme, the intensity (C0) in each data set was defined as 0% activity. The percent activity in the presence of each compound was calculated according to the following equation: % activity = (C-C0)/(Ce-C0), where C = the A-screen intensity or luminescence in the presence of the compound. The values of % activity versus a series of compound concentrations were then plotted using non-linear regression analysis of Sigmoidal dose-response curve generated with the equation Y = B+(T-B)/1+10((LogEC50-X)×Hill Slope), where Y = percent activity, B = minimum percent activity, T = maximum percent activity, X = logarithm of compound and Hill Slope = slope factor or Hill coefficient. The IC_{50} value was determined by the concentration causing a half-maximal percent activity.

Thermal Shift Assay

Solutions of 4 nM 4X SyproOrange Dye, 80 μM 8X MTA (10 μM final concentration) with 10 μM final concentration of inhibitor, 80 μM 8X SAM (10 μM final concentration) with 10 μM final concentration of inhibitor, and buffer controls with 10 μM inhibitor were prepared, as well as a 2 μM 2X PRMT5/MEP50 (1 μM final concentration) solution. 5 μL of Buffer/SAM/MTA-inhibitor, 10 μL of PRMT5, and 5 μL of SyproOrange dye were added to the plate in stated order. The prepared plate was sealed and centrifuged at 1000 RPM for one minute. Using a Quantstudio Flex 7 (Applied Biosystems), the plate was heated to 25°C, then increased 0.5°C per second to 99°C, over the course of 25 minutes. The thermal shift assay measures the melting temperature (Tm) of PRMT5, which is the temperature at which PRMT5 has denatured by 50%. The denaturation is monitored via the increase in fluorescence of the SyproOrange Dye which binds to the hydrophobic residues of PRMT5 that are exposed as the protein unfolds. The first derivative of the Tm (Tm D) and ΔTm D were calculated from the acquired data using Protein Thermal Shift™ Software (Applied Biosystems).

Immunoblotting

Reagents and antibodies not listed here are included in the main materials and methods section. The following antibodies were used at the indicated dilution: cleaved caspase 3 (1:500, Cell Signaling Technology #9661; RRID: AB_2341188), cleaved caspase 7 (1:500; Cell Signaling Technology #9491; AB_2068144), PARP1 (1:500; Cell Signaling Technology #9532; RRID: AB_659884).

For the LU99 in vivo 21-day time course study, approximately 20 μg of total protein was added to the previously referenced sample loading buffer and reducing agent and boiled for 5 minutes. Processed samples were then loaded onto NuPage 10% Bis-Tris Midi Protein 26-well gels (Invitrogen #WG1203BOX) using MES SDS 20X running buffer (ThermoFisher #J62138.K2),

with proteins transferred from gels to a nitrocellulose membrane using the previously referenced iBlot protocol. The membranes containing SDMA, pRb, p21, and p27 were blocked, incubated, washed, and imaged with the same methods as previous blots. Membranes probed for full/cleaved PARP1, cleaved caspase 3 and cleaved caspase 7 were blocked with 3% Milk-TBST (Bio-Rad, Hercules #1706404) for 1 hour at room temperature on a rocking platform. Primary antibodies were also diluted in 3% Milk-TBST and incubated overnight at 4°C on a rocking platform. Membranes were washed with TBST three times for 10 minutes, then incubated with anti-rabbit IgG-HRP conjugate for 1 hour at room temperature, then washed three times for 10 minutes. Membranes were then incubated with SuperSignal West Dura Extended Duration Substrate (ThermoFisher #34076) for 2-3 minutes on a rocking platform at room temperature and immediately imaged for chemiluminescent signal using the LI-COR Odyssey Fc Imaging system set to 2-minute image integration time.

Co-culture Viability Assay

LU99/Luc cells were engineered by transducing parental LU99 cells with a firefly luciferase lentivirus (Cellomics Technology # PLV-1017) at an MOI of 6. A stable firefly luciferase expressing cell pool was generated with puromycin (2 µg/mL) selection for 2 weeks prior to co-culture experiments. For co-culture assays, LU99/Luc and MRC-5 cells were plated in 96-well plates at 500 cells per well alone or as a co-culture where indicated, in 90 µl of RPMI 1640 media containing 10% fetal bovine serum, 10 mM HEPES and 1 mM Sodium Pyruvate. The following day baseline reads were taken after addition of 100 µl CTG or Bright-Glo Luciferase Assay (Promega #E2610) reagents to designated wells. The same day, assay plates were dosed with DMSO or a dose response of MRTX1719. After 5 days of drug treatment, CTG and Bright-Glo luminescent measurements were measured on a plate reader. Raw data was Day 0 subtracted and % viability was calculated as percent of DMSO controls, data curves were fit using GraphPad and IC50s of each culture condition were calculated for MRTX1719.

In Vivo Studies

The NCI-H2228 cell line derived xenograft (CDX) model and the PA0372, LU6408, GA2254, ES0218 and CC1470 patient derived xenograft (PDX) models were conducted at Crown Bioscience with similar study designs and procedures as HCT116 and LU99 studies. Briefly, 6-8-week-old female BALB/c nude mice were inoculated subcutaneously with the indicated cell line or a 2-3 mm^3 PDX fragment and randomized for treatment initiation when the mean tumor volumes reached ~120 to 170 mm^3. Mice were treated with vehicle or test compounds formulated as described in main manuscript methods section (n=3-5 per group). GA2254 and CC1470 were dosed with MRTX1719 at 50 mg/kg while all other Crown Bioscience models were dosed at 100 mg/kg.