Supplementary Materials

Translational Studies Using the MALT1 Inhibitor (*S*)-Mepazine to Induce Treg Fragility and Potentiate Immune Checkpoint Therapy in Cancer

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**Table of Contents**

[1. In Vivo Studies: Tumor Cell Preparation and Animal Care 2](#_Toc108472476)

[2. Generation of the anti-neoCYLDct monoclonal antibody 3](#_Toc108472477)

[3. Organotypic Tumor Spheroid Preparation 3](#_Toc108472478)

[4. Supplementary Tables 5](#_Toc108472479)

[Table S1. Patient Cohort from Massachusetts General Hospital for PDOTS Profiling 5](#_Toc108472480)

[Table S2. Selected Properties of (*S*)-Mepazine and Its Salts 6](#_Toc108472481)

[Table S3. Plasma Pharmacokinetic Parameters of MPT-0118 and MPT-0308 in Male C57BL/6J Mice After a Single Dosea 7](#_Toc108472482)

[Table S4. Mean Plasma and Tissue Pharmacokinetics in Fasted Female ICR Mice and Female Wistar Rats Following a Single Oral 16 mg/kg Dose of MPT-0308a 8](#_Toc108472483)

[5. Supplementary Figures 9](#_Toc108472484)

[Figure S1. Comparison of the efficacy of MPT-0118 and MPT-0308 in the D4M.3A syngeneic tumor model and assessment of lead-in therapy with MPT-0118. 9](#_Toc108472485)

[Figure S2. Dose-response of MPT-0308 in immune-proficient MDOTS and immune-deficient spheroids. 9](#_Toc108472486)

# 1. In Vivo Studies: Tumor Cell Preparation and Animal Care

*Cell Preparation*

MC38 cells*: C*ells were provided by Dr. Andrew Luster at Massachusetts General Hospital (Boston, MA, USA), who in turn had received them from Dr. Mark Smyth at the QIMR Berghofer Medical Research Institute (Brisbane, Australia). Cells were cultured in DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin, in a humidified incubator at 37°C with 5% CO2. Normocin was also added according to the manufacturer’s recommendation to prevent mycoplasma growth.

D4M.3A cells: Cells were provided by Dr. David Fisher, Massachusetts General Hospital (Boston, MA, USA). Cells were cultured in RPMI + L-glutamine, supplemented with 10% FBS, 1% penicillin/streptomycin solution, and 0.2% normocin, in a humidified incubator at 37°C with 5% CO2. Cells were split at 80% confluency and harvested for implantation at 70% confluency.

B16-F10 cells: Cells were purchased through ATCC (Manassas, VA, USA), and early aliquots were frozen for future use. Cells were cultured in RPMI + L-glutamine, supplemented with 10% FBS, 1% penicillin/streptomycin solution, and 0.2% normocin, in a humidified incubator at 37°C with 5% CO2. Cells were split at 80% confluency and harvested for implantation at 70% confluency.

*Animal Care*

In vivo mouse studies with C57BL/6J mice: Studies were performed by Rincon Bio (Salt Lake City, UT, USA). Mice were fed Teklad irradiated (sterilized) mouse diet and bedded with Teklad irradiated (sterilized) corncob bedding (Envigo; Indianapolis, IN, USA). Mice were housed in Optimice carousel sterile quarters with filtered air supply in disposable cages (Animal Care Systems, Inc.; Centennial, CO, USA). Animals also received hydrogel during dosing period.

In vivo rat studies with female Harlan Sprague Dawley rats: Study was performed by Rincon Bio (Salt Lake City, UT, USA). Rats were fed Teklad rodent diet and bedded with Teklad corncob bedding (Envigo). Rats were individually housed in an Optirat carousel with a filtered air supply (Animal Care Systems, Inc.).

# 2. Generation of the anti-neoCYLDct monoclonal antibody

Experiments were performed in compliance with the German Animal Welfare Law and were approved by the institutional committee on animal experimentation and the government of Upper Bavaria (approved No. ROB-55.2-2532.Vet\_03-17-68). Mouse monoclonal antibodies were generated by immunization of BALB/c mice with a peptide comprising MALT1-cleaved CYLD neoepitope NH2-GVGDKGSS (CYLD[325-332]) coupled to ovalbumin (Peps4LS; Heidelberg, Germany). Animals were injected subcutaneously and intraperitoneally with a mixture of 40 μg peptide, 5 nmol CpG (Tib Molbiol; Berlin, Germany) and an equal volume of incomplete Freund’s adjuvant. A booster injection was given 10 weeks later without Freund’s adjuvant, and spleen cells were fused three days later with P3X63Ag8.653 myeloma cells using standard procedures. Hybridoma supernatants were screened in a flow cytometry assay (iQue® [Sartorius; Goettingen, Germany]) on biotinylated CYLD neoepitope peptide captured on streptavidin beads (PolyAN; Berlin, Germany) and incubated for 90 minutes with hybridoma supernatant and Atto-488-coupled isotype-specific monoclonal rat-anti-mouse IgG secondary antibodies. CYLD peptide with N-terminal His tag served as the control to exclude antibodies binding to uncleaved CYLD. Antibody binding was analyzed using ForeCyt software (Version 9.0 [Sartorius; Goettingen, Germany]). Neoepitope-specific supernatants were further validated in Western blot and flow cytometry analyses on Jurkat wild type and MALT1 knock-out cells. Hybridoma cells from selected clones were subcloned by limiting dilution to obtain stable monoclonal cell lines. The hybridoma supernatant producing anti-neoCYLDct (clone 25F10; IgG2a/ƙ) was selected for use in CYLD cleavage cell assays.

*Intracellular CYLD neo-antibody staining in Jurkat T cells.* Jurkat T cells were treated with the indicated concentrations of MPT-0308 for 1h and stimulated for 2h with PMA (200 ng/mL) and CD28 (300 ng/mL [BD Pharmingen]). Cells were centrifuged (400 x g, 5 min, 4°C) and washed twice with fluorescence-activated cell sorting (FACS) buffer, fixed in 2% paraformaldehyde for 15 min at room temperature (RT), and permeabilized in 70% ice-cold methanol for 20 minutes at 4°C. Fc block was performed using anti-human CD16/CD32 (eBioscience, 9161-73; 1:50 in FACS buffer). Cells were stained with anti-neoCYLDct antibody (clone 25F10; 1:100 in FACS buffer) for 30 minutes at 4°C. After washing, cells were treated with rat anti-mouse IgG2a Alexa Fluor 488-conjugated secondary antibody (Biozol Diagnostica; 1:200 in FACS buffer) for 30 minutes at 4°C. To wash out unbound antibodies, cells were washed twice with PBS (second wash was incubated >15 min at RT). Cells were resuspended in FACS buffer, and acquired using an Attune Acoustic Focusing Cytometer (Thermo Fisher Scientific). For analysis, differences in median fluorescence intensity (MFI) were calculated (stimulated with primary/secondary staining minus stimulated with secondary only staining), and the percentage of MALT1 activity was determined relative to that of DMSO treated cells.

*Intracellular CYLD neo-antibody staining in primary murine splenocytes***.** Primary murine splenocytes from C57B16 mice were treated with Red Blood Cell Lysis Solution (Miltenyi Biotec; 1:10 in MilliQ H2O), and 1 x 106 cells were resuspended in T cell medium (2 mL; RPMI 1640, penicillin [100 U/mL], streptomycin [100 μg/mL], fetal calf serum [10%], and β-mercaptoethanol [50 nM] [all Gibco Thermo Fisher Scientific]). MPT-0308 was added, and cells were incubated for 3 hours prior to stimulation with ionomycin (300 ng/mL) and PMA (200 ng/mL) for an additional 2 hours. Cells were centrifuged (400 x g, 5 min, 4°C) and washed twice with phosphate-buffered saline (PBS) followed by live/dead staining with Fixable Viability Dye eFluor™ 780 (eBioscience; 1:1000 in PBS) for 30 minutes at 4°C. Afterwards, cells were washed with FACS buffer (3% fetal calf serum in PBS), fixed in 2% paraformaldehyde for 15 min at RT, and permeabilized in 70% ice-cold methanol for 20 minutes at 4°C. Unspecific antibody binding was blocked by treatment with anti-mouse CD16/CD32 (Fc block [eBioscience, 14-0161-85]; 1:50 in FACS buffer) for 15 minutes at 4°C. Cells were stained with anti-neoCYLDct antibody (CYLD 25F10; 1:100 in FACS buffer) for 30 minutes at 4°C. After washing, cells were treated with rat anti-mouse IgG2a Alexa Fluor 488-conjugated secondary antibody (1:200 in FACS buffer) for 30 minutes at 4°C. To wash out unbound antibodies, cells were washed twice with FACS buffer (second wash was incubated >15 min at RT). For surface staining, cells were incubated with anti-CD4-PerCP (eBioscience, 45-0042-82; 1:300 in FACS buffer) for 30 minutes at RT. Cells were washed, resuspended in FACS buffer, and acquired and analyzed as above. Animal care was in accordance with institutional guidelines.

# 3. Organotypic Tumor Spheroid Preparation

Fresh tumor specimens were minced in a standard 10-cm dish using sterile forceps and scalpel and were resuspended in high-glucose DMEM (supplemented with 10% FBS plus 1% pen-strep), 100 U/mL type IV collagenase, and 15 mM HEPES (Life Technologies) for 15 to 30 minutes. Equal volumes of media were then added, and cell suspensions were pelleted (5 min, 1,000 x g, 4 °C). Pellets were resuspended in fresh pre-warmed media and passed over 100-μm and 40-μm filters sequentially to obtain spheroid fractions S1 (>100 μm), S2 (40-100 μm), and S3 (<40 μm). Spheroid fractions were subsequently transferred to ultra-low attachment tissue culture plates. The S2 fractions were pelleted and resuspended in type I rat tail collagen (Corning) at a concentration of 2.5 mg/mL following the addition of 10× PBS with phenol red with pH adjusted using NaOH (final pH 7.0–7.5).

# 4. Supplementary Tables

## Table S1. Patient Cohort from Massachusetts General Hospital for PDOTS Profiling

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Sample ID | Tumor type | Age | Gender | Mutational status | MMR status | TMB (absolute count) | Stage |
| 10011 | Colon cancer | 76 | F | MLH1, PMS2 | MMR-D | N/A | II |
| 10018 | Colon cancer | 85 | F | N/A | MSI-H | N/A | II |
| 10019 | Cutaneous melanoma | 65 | M | TERT | N/A | N/A | IV |
| 10020 | Colon cancer | 72 | F | N/A | MSI-H | N/A | II |
| 10021 | Cutaneous melanoma | 73 | M | TP63, DDX3X, PIK3CA, BRAF, TSC2, MYC, TERT, SMARCA4 | N/A | N/A | IV |
| 10022 | Ocular melanoma | 70 | F | N/A | N/A | N/A | IV |
| 10137 | Colon cancer | 51 | F | N/A | N/A | N/A | I |
| 10140 | Colon cancer | 70 | M | KRAS, CDH1, TP53, STK11, PIK3CA, APC, MEN1 | N/A | LOW | IV |
| 10142 | Colon cancer | 29 | M | PIK3CA, TP53 | N/A | LOW | IV |
| 10162 | Colon cancer | 62 | F | ERBB2, APC, TP53 | MSS | LOW | IV |
| 10165 | Cutaneous melanoma | 73 | M | BRCA1, MAP3K1, TERT, NRAS | N/A | N/A | IV |

## Table S2. Selected Properties of (*S*)-Mepazine and Its Salts

|  |  |  |
| --- | --- | --- |
| Property | Result | Test Compound |
| pKa | 9.2 | MPT-0118c |
| logP | 5.29 | MPT-0118c |
| Caco-2 permeability | 13.73 ± 0.15 (10-6 cm/s)a | MPT-0308d |
| MDCK-MDR1 permeability | Efflux ratio [BA/AB]: 0.38b | MPT-0308d |
| Aqueous solubility | 35-50 mg/mL | MPT-0118c |
| Melting point | 165.6°C | MPT-0118c |

Abbreviations: logP, octanol–water partition coefficient.

 aMean apparent AB permeability coefficient. bNot a PGP substrate. c(*S*)-mepazine succinate. d(*S*)-mepazine hydrochloride.

## Table S3. Plasma Pharmacokinetic Parameters of MPT-0118 and MPT-0308 in Male C57BL/6J Mice After a Single Dosea

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Parameter | Unit | MPT-030832 mg/kg IP | MPT-0308 64 mg/kg PO | MPT-0118 64 mg/kg PO |
| Doseb | mg/kg | 32 | 64 | 64 |
| t1/2 | h | 2.82 | 4.37 | 3.36 |
| Tmax | h | 1 | 2 | 2 |
| Cmax | ng/mL | 1563 | 1177 | 1543 |
| AUC 0-t | ng/mL\*h | 6921 | 6012 | 8746 |

Abbreviations: AUC0-t, area under the concentration-time curve from time 0 to time ‘t’; Cmax, maximum concentration; IP, intraperitoneal, PO, oral; t1/2, half-life; Tmax, time to reach maximum concentration.

aPlasma samples obtained at 1, 2, 4, and 12 hours post-dose. MPT-0118 and MPT-0308 are the succinate and hydrochloride salts of (*S*)-mepazine, respectively. bDoses expressed as (*S*)-mepazine free-base.

## Table S4. Mean Plasma and Tissue Pharmacokinetics in Fasted Female ICR Mice and Female Wistar Rats Following a Single Oral 16 mg/kg Dose of MPT-0308a

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Plasma or tissues | Cmax(ng/g) | Tmax(h) | AUC0-last(ng•h/mL) | Tissue AUC:plasma AUC ratio |
|  | Mice | Rats | Mice | Rats | Mice | Rats | Mice | Rats |
| Plasma | 339b | 421b | 1.0 | 6 | 1345 | 4093 | -- | -- |
| Lung | 10153 | 19067 | 1.0 | 6 | 51862 | 242987 | 39/1 | 59/1 |
| Liver | 9860 | 25500 | 0.25 | 0.5 | 33459 | 137765 | 25/1 | 34/1 |
| Kidney | 3810 | 5620 | 1.0 | 6 | 11979 | 55126 | 9/1 | 13/1 |
| Brain | 4343 | 6597 | 1.0 | 6 | 19167 | 71456 | 14/1 | 17/1 |
| Lymph node | 1373 | 3867 | 1.0 | 8 | 5701 | 50476 | 4/1 | 12/1 |
| Heart | 752 | 2800 | 0.5 | 6 | 2210 | 13099 | 2/1 | 3/1 |
| Skin | 1007 | 1567 | 2 | 8 | 5422 | 19629 | 4/1 | 5/1 |
| Eye | 239 | 961 | 2 | 6 | 1323 | 10370 | 1/1 | 3/1 |

Abbreviations: AUC, area under the curve; Cmax, peak (or maximum) concentration; Tmax, time to reach maximum concentration.

aPlasma samples obtained at 0.25, 0.5, 1, 2, 4, 6, 8, 24 hours post-dose (doses expressed as (*S*)-mepazine free base). MPT-0308 is (*S*)-mepazine hydrochloride. bConcentration in ng/mL

# 5. Supplementary Figures

Figure S1. Comparison of the efficacy of MPT-0118 and MPT-0308 in the D4M.3A syngeneic tumor model and assessment of lead-in therapy with MPT-0118. **A**) MPT-0308 and MPT-0118 monotherapy. **B**) MPT-0308 and MPT-0118 in combination with anti-PD-1. **A** and **B**) MPT dosing: 32 mg/kg IP, QD; anti-PD-1 dosing: 3 x 200 μg, IP, QOD. Doses expressed as (*S*)-mepazine free base (MW: 310.46). Results are expressed as means ± SEM (*n* = 10 per cohort). No statistical differences were found between (*S*)-mepazine salt forms using two-way ANOVA. Statistical differences for the monotherapy and anti-PD-1 groups vs vehicle (A) and for the combination groups vs anti-PD-1 (B) are not shown. Peritoneally infiltrated tumors are included; mice with early mortality are excluded (*n* = 4). **C**) Comparison of tumor volumes between anti-PD-1/MPT-0118 combination therapy starting on day 9 (orange) versus MPT-0118 lead-in treatment (days 6-8) followed by anti-PD-1/MPT-0118 combination therapy starting on day 9 (green). MPT-0118 dosing: 64 mg/kg, PO, QD; anti-PD-1 dosing: 3 x 200 μg, IP, QOD. Doses are expressed as (*S*)-mepazine free base (MW: 310.46). Results are expressed as means ± SEM (*n* = 10 per cohort). No significant difference was found between lead-in dosing with MPT-0118 followed by combination therapy vs simultaneous initiation of combination therapy using two-way ANOVA. Statistical differences for combination treatments vs anti-PD-1 are not shown. Peritoneally infiltrated tumors are included; no mice are excluded for early mortality. Abbreviations: anti-PD-1, anti-programmed cell death protein 1 antibody; ns, not significant; TU, tumor.

Figure S2. Dose-response of MPT-0308 in immune-proficient MDOTS and immune-deficient spheroids. **A**) Effect of MPT-0308 or anti-mouse PD-1 (10 μg/mL) on growth of cell-line-derived D4M.3A murine melanoma tumor spheroids (immune deficient) in 3D microfluidic culture (day 4). **B**) Effect of MPT-0308 on growth of D4M.3A MDOTS (immune proficient) in 3D microfluidic culture (day 4). **C**) Effect of anti-IFNγ (10 μg/mL) on cell viability of MC38 MDOTS (immune proficient) from the MC38 murine tumor model in 3D microfluidic culture with indicated treatments (day 6). Statistical analysis: one-way ANOVA with Dunn’s multiple comparisons test, *n* = 3 biological replicates; \* *p* < 0.05; \*\* *p* < 0.01; \*\*\* *p* < 0.001; \*\*\*\* *p* < 0.0001). Abbreviations: anti-IFN-γ, anti-interferon-γ antibody; anti-PD-1, anti-programmed cell death protein 1 antibody; MDOTS, murine-derived organotypic tumor spheroids; TME, tumor microenvironment.