Pre-clinical characterization of signal transducer and activator of transcription 3 small molecule inhibitors for primary and metastatic brain cancer therapy

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Supplementary Materials and Methods

Synthesis, Administration, and Validation of STAT3 small molecule inhibitors.

CPA-7 (fac-[$Pt(NH_3)_2Cl_3NO_2$]; M.W. 381.5) was synthesized according to a previously published report (Littlefield et al., 2008). The reaction product was purified of KCl contaminants by acetone purification. Briefly, the pale yellow solution was dried using a rotary evaporator at 25°C. The solid sample was then transferred to a 250 mL Erlenmeyer flask and 50 mL of reagent grade acetone was added to dissolve the crude CPA-7 product. KCl, which remained undissolved, was removed by filtering through a medium-porosity filter into a single-neck round-bottom flask. The acetone was evaporated under vacuum and the residue was then dried under pressure then vacuum for 2 hours at 45°C.

The purity of the recrystallized product was assayed by HPLC (using an ICS-5000 ThermoFisher system) and determined to be 97.48% pure. Analysis of the product by ^{1}H-NMR (in acetone-d6) shows a mulitplet (septet) centered at 5.760 ppm, consistent with cis-diammines bonded to a Pt(Mora, #7) center in this asymmetric Pt(Mora, #7) complex.

Sensitivity to light and stability in darkness was also assessed for CPA-7 (**Supplementary Figure 1**). The UV spectrum of CPA-7 features an absorption maximum at a wavelength of 264 nm. As a measure of the stability of CPA-7, the absorbance of a solution of CPA-7 (6.0 mg/100 mL H_2O) was monitored at 264 nm over a period of 7 days. (After the solution was prepared, it was divided in half: 50 mL exposed to light & 50 mL kept in the dark). The data illustrate the changes in the absorbance as a function of time. It is clear that CPA-7 is sensitive to light and thus stock solutions must be protected from light to minimize changes.

Aliquots of 20 mM CPA-7 were prepared at in a 50:50 mixture of H_20 and DMSO, wrapped in foil (CPA-7 is light sensitive) and stored at -80°C long-term. Prior to *in vivo* administration of CPA-7, 20 mM (7.63 mg/mL) aliquots prepared in a 50:50 mixture of DMSO and H_20 were thawed and diluted with serum-free DMEM to the desired concentration (3.28 mM or 1.25 mg/mL). For intracranial and flank tumor survival studies, mice were administered 100 µL (5 mg/kg or 0.125 mg) of the diluted CPA-7 solution via tail vein injection. Six injections of CPA-7 were administered 3 days apart and were initiated 4 days post-implantation of intracranial tumors, and 25 days post-implantation of flank tumors.

Synthesis of WP1066 ($C_{17}H_{14}BrN_3O$; M.W. 356.2) was carried out by the Vahlteich Medicinal Chemistry Core at the University of Michigan according to the synthetic route outlined in **Supplementary Figure 2a** (Hussain et al., 2007).

Analytical HPLC was performed on an Agilent 1100 Series system with an Agilent Eclipse plus C18 (4.6 + 7.5 mm, 3.5 mm particle size) column (**Supplementary Figure 2b**). The mobile phase was a binary gradient of acetonitrile (containing 0.1 % TFA) and water (10–90%). Mass spectra were recorded on a Micromass LCT time-of-flight instrument utilizing the

electrospray ionization mode (**Supplementary Figure 2c**). ^{1}H NMR spectra were recorded on a Varian 400 instrument at 400 MHz (**Supplementary Figure 2d**).

Aliquots of 10 mM WP1066 were prepared in DMSO and stored at -80°C long-term. Prior to *in vivo* administration of the drug, WP1066 aliquots were thawed and diluted to 10 mg/mL with PEG300 to make a final solution that is 80% PEG300 and 20% DMSO. For intracranial and flank tumor survival studies, 100 µL of 10 mg/mL WP1066 solution (40 mg/kg) was administered daily via oral gavage for 5 days, followed by 2 days of rest. Treatment of intracranial tumor-bearing mice began 4 days after tumor implantation, while treatment for flank tumors commenced 25 days after tumor implantation and continued until the animals showed signs of morbidity.

ML116 ($C_{18}H_{19}N_3S$; M.W. 309.4) was purchased from Ryan Scientific, Inc. and solubilized in DMSO as per the original synthetic route (Madoux et al., 2010). 10 mM aliquots were prepared for long-term storage at -80°C. For *in vivo* delivery of ML116, 16 mg/mL aliquots prepared in pure DMSO then diluted 1:4 in a carrier solution consisting of 30% hydroxypropyl-β-cyclodextrin and 25% PEG300 (final concentration of ML116 in the solution was 4 mg/mL). Mice bearing intracranial tumors were treated with ML116 (15 mg/kg) daily by intraperitoneal injection. Animals were treated with ML116 until they began to display symptoms of morbidity.

Tumor models

Female C57BL/6J mice (6-12 weeks) were anesthetized by IP injection of Ketamine (75 mg/kg) and Medetomidine (0.5 mg/kg). Once fully anesthetized, the mice were mounted onto a stereotactic frame and a small burr hole was drilled in the skull using a hand dremel. 20,000 GL26, 3,000 B16-f10, or 1,000 B16-f0 cells in 1 µl of PBS were injected unilaterally into the right striatum (+0.5 mm AP, +2.2 mm ML, -3.0 mm DV from bregma) using a 5µl Hamilton

syringe fitted with a 33-gauge needle. The needle was left in place for 3 minutes following the injection before being withdrawn slowly. The injection site was washed with sterile saline and the incision was closed using nylon sutures. Mice were resuscitated using atipamezole (Alonzi, #15) and administered buprinex (SQ) as an analgesic. To generate peripheral flank tumors $1x10^6$ GL26, $2x10^5$ B16-f10, or $5x10^4$ B16-f0 were prepared in 100 µl of PBS and injected in the hind flanks of C57BL/6J mice. Using a hand caliper, tumor volume was estimated by measuring the width and length of the tumor mass and applying the formula $v = \frac{1}{2}(l \times w^2)$ where w is the smaller of two measurements. All animals were housed in specific pathogen free environment, and monitored closely. Animal tissues requiring histopathology were perfused using oxygenated, heparinized Tyrode's solution (132 mM NaCl, 1.8 mM $CaCl_2$, 0.32 mM NaH_2PO_4, 5.56 mM glucose, 11.6 mM $NaHCO_3$, and 2.68 mM KCl) and perfused-fixed with 4% paraformaldehyde, respectively.

Luciferase assay for transcription factor activity.

U251 human glioma cells were transfected with the following plasmids to generate stable clones: pGL4.32[luc2P/NF-κβ-RE/Hygro], pGL4.52[luc2P/STAT5-RE/Hygro]. After selection with antibiotic, clones were screened and selected based on their luciferase activity. MG-132 (Sigma, St. Louis, MO, USA), Pimozide (Sigma, St. Louis, MO, USA), and nifuroxazide were used as inhibitors of NF-κβ, STAT5, and STAT1 respectively. LPS (100 ng/mL) and IFNγ (100 pg/mL) were used to stimulate NF- κβ and STAT1 activity, respectively. Firefly luciferase activity was measured using Promega's dual luciferase assay kit. 30,000 U251 cells were plated in 12 well plates (Fisher, Waltham, MA, USA) in Complete DMEM. On day 2, CPA-7, ML116, and WP1066 were added to the appropriate wells at the indicated final concentrations. NF-κβ or STAT1 activity was stimulated with their appropriate cytokines 1.5 hours after addition of

inhibitor. 6 hours after LPS addition or incubation with STAT3 inhibitor, the wells were rinsed with PBS and lysed in 150 μL of Promega Passive Lysis Buffer (Promega), shaken for 20 minutes. Seventy-five μL of each well was then transferred to an opaque 96-well plate for measurement with 100 μL of Luciferase Assay Reagent (Promega) for 10 seconds in a Veritas Microplate Luminometer (Turner Biosystems). In a separate treatment plate, effects on cell viability were monitored by measuring ATP levels and described earlier.

Western Blot

Whole cell extracts were prepared by lysing the cells with RIPA buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1 mM each NaF, NaVO4, and EGTA; 1% Igepol; 0.25% sodium deoxycholate; 1x protease/phosphotase inhibitors (Thermo Scientific, Waltham, MA) for 5 min on ice, then centrifuging at 12,000g for 10 minutes at 4°C to remove cell debris. Protein concentration was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific) and 15-30 μg of protein was denatured and reduced before loading onto a 4-12% SDS-Polyacrylamide gradient gel. Samples were transferred onto PVDF membranes for immunoblotting. Membranes were blocked with 5% milk in TBS+0.05% Tween20 and incubated with primary antibodies at the manufactures suggested dilution overnight at 4°C. Membranes were washed 3 times in TBS with 0.05% Tween20 and incubated with the appropriate HRP-conjugated secondary (Dako, Carpinteria, CA). Immunoreactivity was visualized using the SuperSignal West Femto substrate solution (Thermo Scientific) and imaged on a ChemiDoc MP imaging system (BioRad, Hercules, CA). Band intensity was digitally quantified using the imaging software which accompanies the ChemiDoc MP imaging system.

Paraffin IHC

Tumor specimens were fixed by perfusing with a 4% solution of paraformaldehyde, processed and embedded in paraffin for sectioning. 5 μM microtome sections of flank tumors, brain tumors, liver, kidney, and spleen were cut, mounted on a slide, deparaffinized and rehydrated for IHC or H&E. Tissue slides for IHC were placed in an antigen retrieval solution (1 mM EDTA, 10 mM Tris pH 9.0) and heated inside a pressure cooker to expose antigens. Sections were washed and permeabilized with TBS-TritonX100 (TBS-Tx 0.05%) solution. To inactivate endogenous peroxidases, a solution of 0.3% H_2O_2 /PBS was added to the section and allowed to sit for 20 minutes at room temperature before proceeding with a blocking step (TBS-Tx with 10% normal goat serum for 1 hour at room temperature). After blocking, primary antibody was added to the sections at the manufacturer's suggested dilution and allowed to bind at 4°C overnight. The following day, sections were washed five times with TBS-Tx before adding the appropriate biotinylated secondary antibody. After secondary incubation, sections were washed five times with TBS-Tx before the addition of Vectastain ABC solution (Vector Labs, Burlingame, CA), which contains the avidin/biotin complex. Chromogenic detection of peroxidase was detected using DAB as an enzyme substrate. Sections were then dehydrated and coverslipped for imaging on a Zeiss Axioplan 2 microscope.

CBC and Serum Chemistry

CBC was performed on an IDEXX Procyte Dx Hematology Analyzer within 15 minutes of blood draw according to the manufacturer's instructions. Serum chemistry was performed on a VetTest 8008 Chemistry Analyzer according to the manufacturer's instructions.

Hematoxylin & Eosin Histology

The livers, kidneys, and spleens were post-fixed for 2 days in 4 % paraformaldehyde prior to dehydration on a Leica ASP 300 processor, embedded on a Tissue-Tek embedding station, and sectioned on a Leica RM 2135 microtome at 5 µm. Liver, kidney, and spleen sections were stained with Hematoxylin and Eosin. Slides were analyzed at the Department of Pathology at the University of Michigan and imaged on a Zeiss Axioplan 2 microscope.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Quality control for CPA-7 synthesis. (**a**) Chromatogram of HPLC performed on the purified synthesized product indicates the presence of a single compound with trace contaminants (97.5% purity). (**b**) Stability of CPA-7 over time when kept in the dark. (**c**) Light sensitivity of CPA-7.

Supplementary Figure 3. Synthesis and quality control measurements for WP1066. (a) Proposed synthetic route for WP0166. **(b)** HPLC data indicates high purity of a single compound (>99.7%). **(c)** Positive ion mass spectrometry analysis showing the ions produced from the fragmentation of the synthesized product. Peaks at 356 m/z indicate presence of a compound with a molecular mass equivalent to that of WP1066. **(d)** ^{1}H-NMR spectrum conforms to the predicted molecular structure of WP1066.

Supplementary Figure 3. Immunoblot for pSTAT3 against glioma and melanoma cells. Expression of phosphorylated STAT3 is variable amongst glioma cell lines. Whole cell lysates were obtained from cultured tumor cells using RIPA buffer. Equal amounts of total clarified protein were separated on an SDS polyacrylamide gel and immunoblotted with antibodies against pSTAT3, total STAT3, and actin.

Supplementary Figure 4. Inhibition of STAT3 phosphorylation in response to ML116 treatment. U251 human glioma cells were treated with increasing doses of ML116 before undergoing lysis. Equivalent amounts of clarified whole cell lysate were analyzed by Western blot using antibodies against p_{Tyr705}STAT3, total STAT3, and actin. Inhibition of STAT3 was achieved at a high dose of ML116 (100 μM).

Supplementary Figure 5. Therapeutic efficacy of WP1066 and ML116 in mice bearing GL26 tumors. (**a**) Schematic showing the experimental approach to intracranial GL26 tumor implantation and treatment strategies. Mean survival time of C57BL/6J mice implanted with 20,000 GL26 tumor cells and treated 4 days after tumor implantation with (**b**) WP1066 (40 mg/kg; oral gavage, 5 days "on," and 2 days "off") or (**c**) ML116 (15 mg/kg; I.P., daily) was similar to vehicle treated controls. (**d**) Schematic showing the experimental approach to GL26 flank tumor implantation and treatment strategies. Similarly, C57BL/6J mice implanted with $1x10^6$ GL26 cells in the hind flank and treated 25 days after tumor implantation with (**e**) WP1066 (40 mg/kg; oral gavage, 5 days "on," and 2 days "off") or (**f**) ML116 (15 mg/kg; I.P., daily) showed no statistically significant difference in the growth of peripheral GL26 tumors.

Supplementary Figure 6. Passive permeability of STAT3 inhibitors using Parallel Artificial Membrane Permeability Assay (PAMPA). PAMPA was used to measure the permeability of drugs across a lipid membrane. Briefly, compounds were added to the bottom chamber of donor plates at a concentration of 50 μM. A membrane was coated with a lipid solution and sandwiched between the donor and acceptor plates. The plates were incubated for 4 hours before measuring the passive diffusion across chambers with UV absorption. The effective permeability (cm/s) or P_{eff} was derived with the software provided by Pion, Inc.

Supplementary Figure 7. Treatment of intracranial melanoma tumors with WP1066 and ML116. 1,000 B16-f0 or 1,000 B16-f10 melanoma cells were injected in the striatum of C57BL/6J mice. Treatment was initiated with CPA-7 (n=5; 5 mg/kg, IV, every three days),

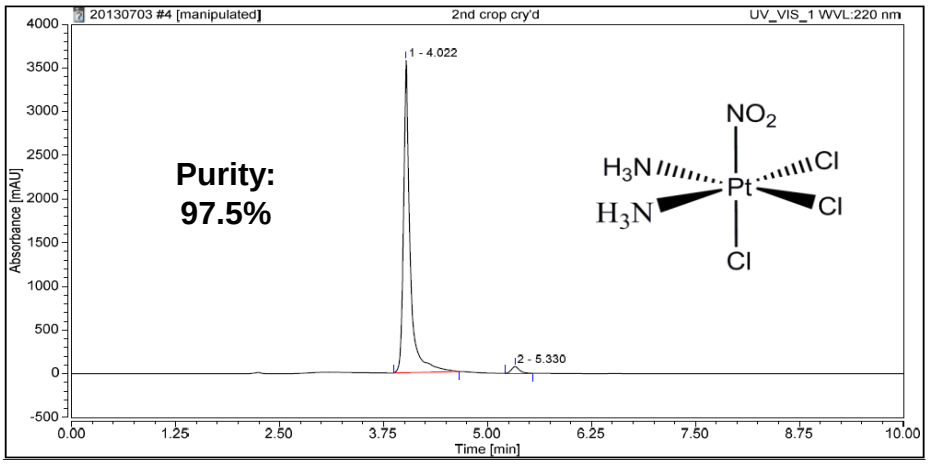
ML116 (n=5; 20 mg/kg, IP, every day), or WP1066 (n=5; 40 mg/kg, oral gavage, 5 days "on," and 2 days "off") on day 4 and continued until animals became moribund.

Supplementary Figure 8. Toxicity in the kidneys of B16-f0 melanoma flank tumor-bearing mice treated with STAT3 small molecule inhibitors. Kidneys featured normal tissue for both the vehicle- and WP1066-treated animals in the central cortexes and subcapsular tubules. In the CPA-7-treated animals (n=3), however, protein was noted in many of the tubules of the central cortex (**a**) accompanied by flattened epithelium surrounding these tubules (**b**), characteristic of tissue regeneration as a result of an injury. Within the subcapsular tubules, CPA-7-treated mice revealed relatively smaller cells with darker cytoplasm (**c**), also indicating injury.

Supplementary Figure 9. Treatment of flank B16-f10 melanoma tumors with ML116. 200,000 B16-f10 melanoma cells were injected subcutaneously in the flanks of C57BL/6J mice. Treatment with ML116 began 25 days after tumor implantation (20 mg/kg, IP, every day) showed no significant difference in growth kinetics when compared to the vehicle.

Supplementary Figure 1

A



B

CPA-7 Sample Kept in the Dark

Elapsed Time	As at 264 nm	% Change
0	1.197	0
2 hours	1.191	0.5
4 hours	1.184	1.1
4 days	1.125	6.0

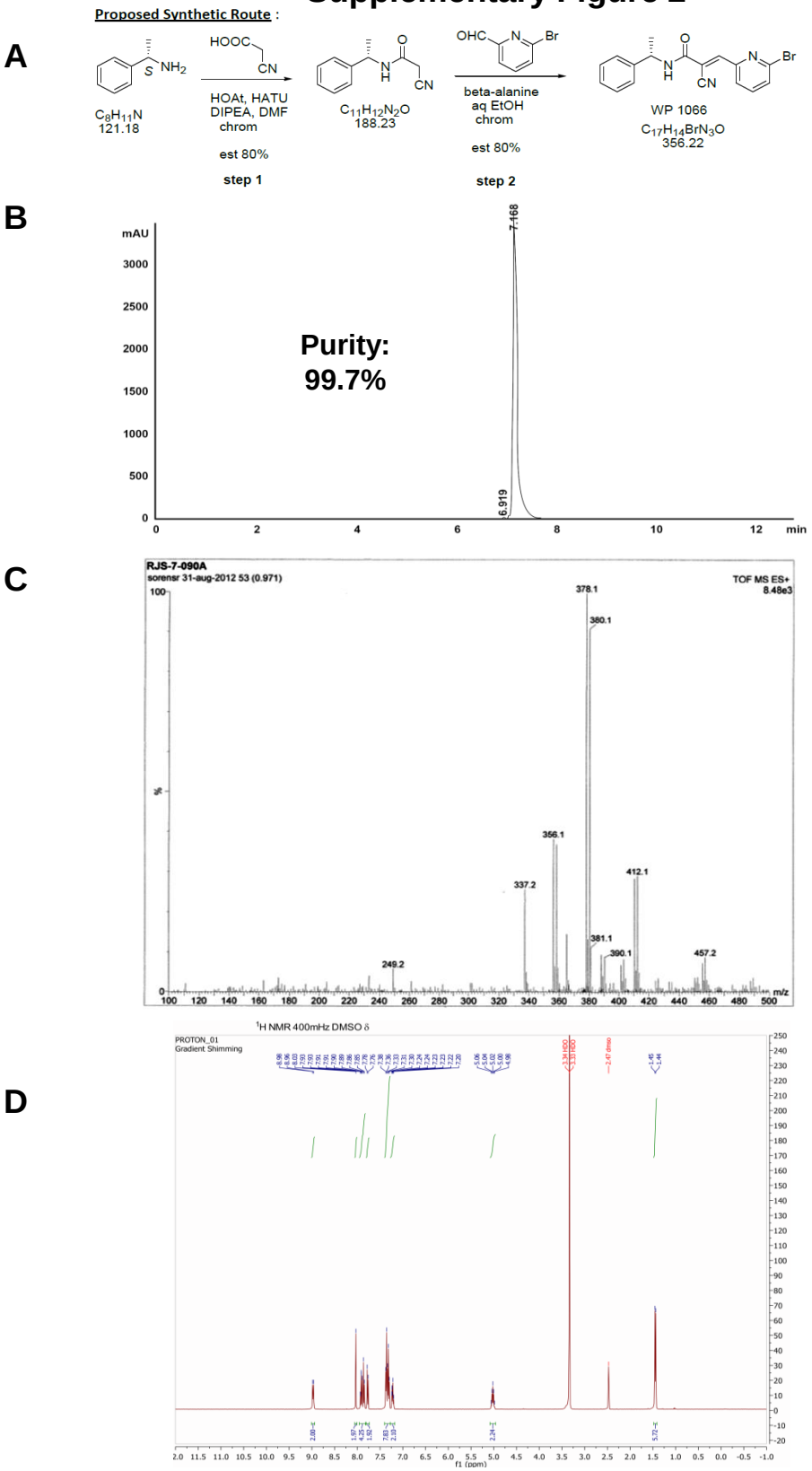
C

CPA-7 Sample Exposed to Light (~8-10 h/day)

Elapsed Time	As at 264 nm	% Change
0	1.197	0
2 hours	1.163	2.8
4 hours	1.129	5.7
4 days	0.583	51.3

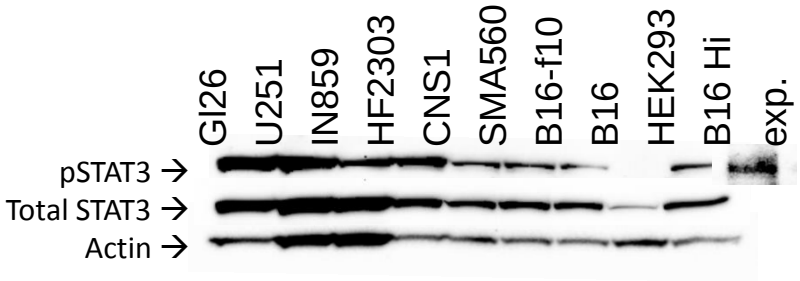
Supplementary Figure 1. Quality control for CPA-7 synthesis. **A.** Chromatogram of HPLC performed on the purified synthesized product indicates the presence of a single compound with trace contaminants (97.5% purity). **B.** Stability of CPA-7 over time when kept in the dark. **C.** Light sensitivity of CPA-7.

Supplementary Figure 2



Supplementary Figure 2. Synthesis and quality control measurements for WP1066. **A.** Proposed synthetic route for WP0166. **B.** HPLC data indicates high purity of a single compound (>99.7%). **C.** Positive ion mass spectrometry analysis showing the ions produced from the fragmentation of the synthesized product. Peaks at 356 m/z indicate presence of a compound with a molecular mass equivalent to that of WP1066. **D.** ^{1}H-NMR spectrum conforms to the predicted molecular structure of WP1066.

Supplementary Figure 3



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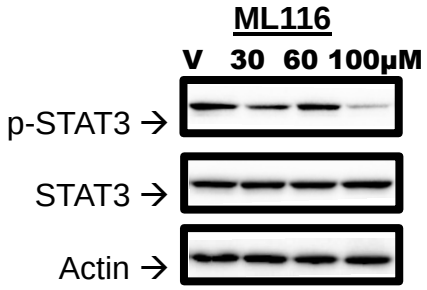

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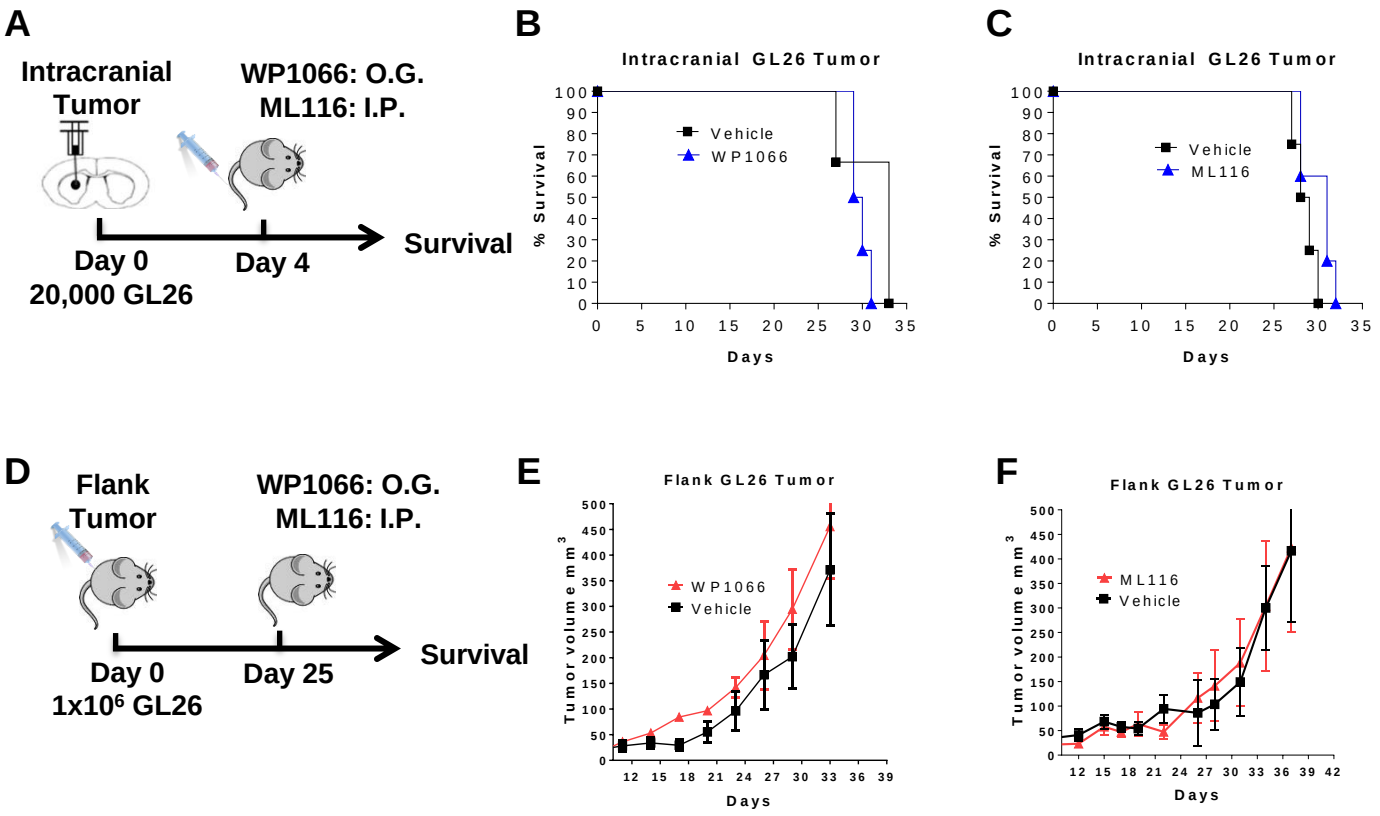
Supplementary Figure 5



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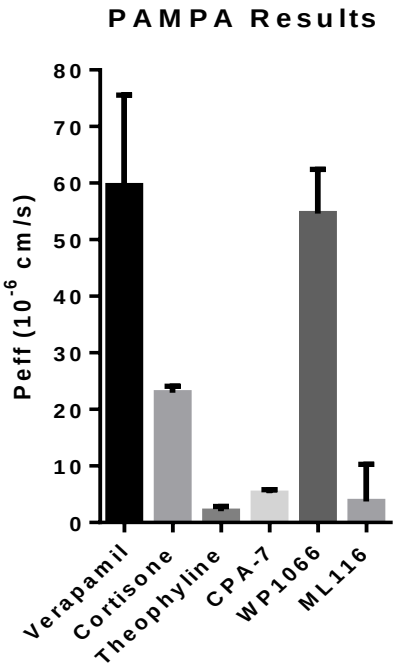
Supplementary Figure 6



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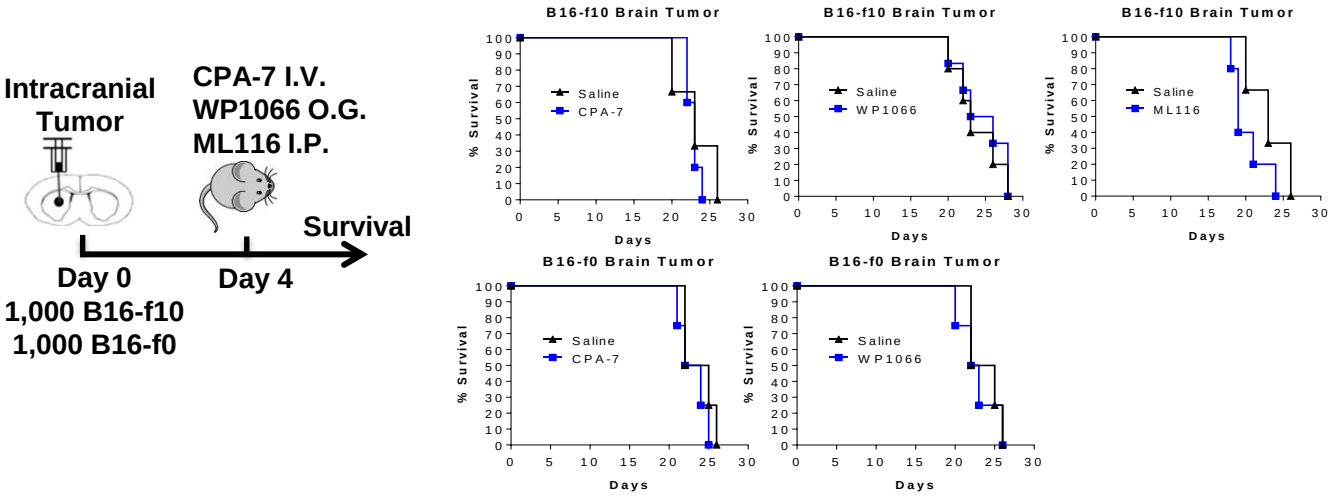
Supplementary Figure 7



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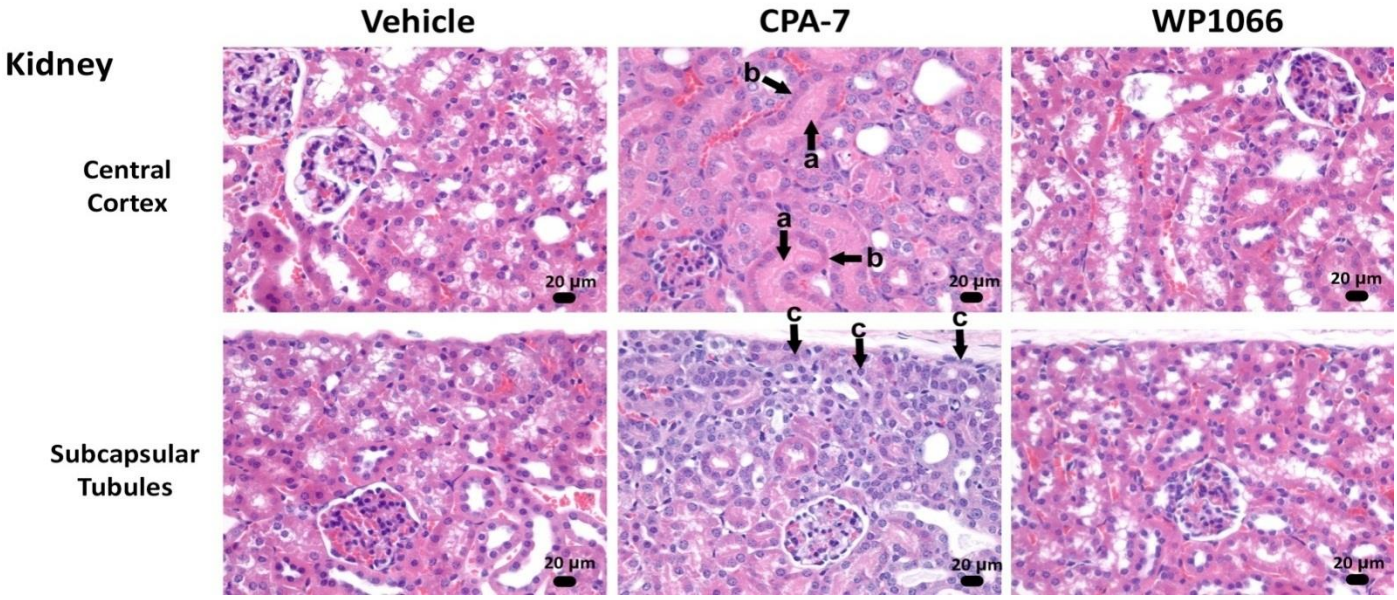
Supplementary Figure 8



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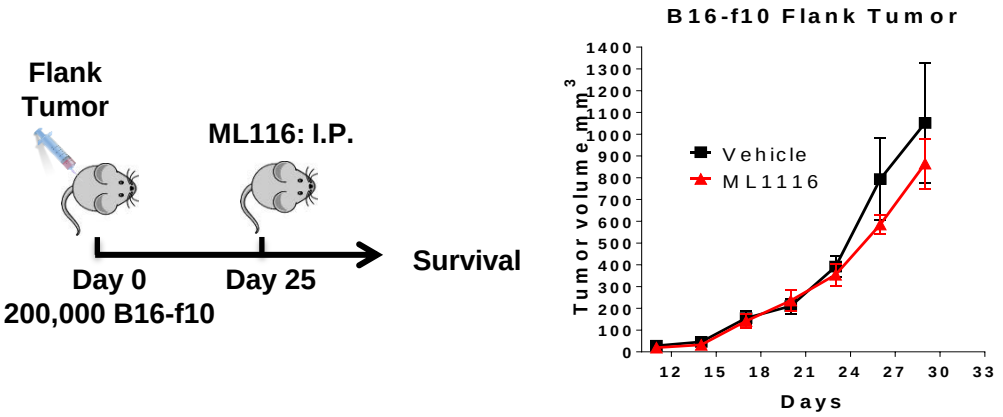

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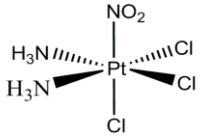
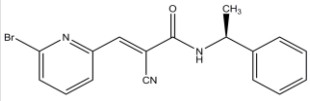
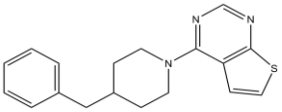
Supplementary Table 1

Compound	Characteristics	References
 **CPA-7 M.W.: 381**	**-Good Solubility -Selective for STAT3 -High doses of CPA-7 can block STAT1 and STAT5 -Low IC50; 1-10μM -Blocks STAT3 DNA binding**	**18, 19, 20, 21**
 **WP1066 M.W.: 356**	**- Inhibits upstream kinase JAK2 -Low IC50; 0.5-10μM -Nonselective for STAT3**	**12, 23, 24, 26, 38**
 **ML116 MW: 309**	**-Newly identified compound -Inhibits STAT3 DNA binding -Poor Solubility: 1.6μM in PBS -High IC_{50}**	**25**

Supplementary Table 1. STAT3 small molecule inhibitor characterization. The structure, physical properties, and specificity within the JAK-STAT pathway are shown.

Supplementary Table 2

Parameters	Reference Range	Vehicle		WP1066		CPA-7	
		Mean	SEM	Mean	SEM	Mean	SEM
RBC (Red Blood Cells; M/µL)	7.4 -1 2	5.85	1.55	6.71	1.97	3.57	0.37
HGB (Hemoglobin; g/dL)	10.8 - 19.2	8.58	2.77	9.58	2.72	5.13	0.45
HCT % (Percent Hematocrit)	37.3 - 62	29.40	7.59	34.03	9.06	15.27	1.76
RET (Reticulocytes; K/µL)	2.4 - 10.6	542.35	76.04	686.83	270.37	10.62	0.72
RET % (Percent Reticulocytes)	2.2 - 6.4	16.15	9.30	17.53	8.41	0.17	0.04
Plt (Platelet; K/µL)	840 - 2200	646.00	144.01	649.25	59.39	638.67	173.98
WBC (White Blood Cells; K/µL)	3.9 - 14	2.81	0.69	2.29	0.31	0.78	0.28
Neutrophils (K/µL)	0.4 - 3	0.36	0.07	0.41	0.16	0.31	0.15
Neutrophil %	7.3 - 28.6	14.83	4.03	16.10	4.13	33.27	11.51
Lymphocytes (K/µL)	2.8 - 11.2	2.12	0.51	1.75	0.13	0.40	0.14
Lymphocyte %	61 - 87	75.63	3.77	78.45	5.45	59.10	16.46
Monocytes (K/µ/L)	0.15 - 0.94	0.31	0.21	0.10	0.04	0.03	0.02
Monocyte %	2 - 11	8.20	3.86	4.03	1.44	3.80	2.69
Eosinophils (K/µL)	0.01 - 0.5	0.03	0.01	0.03	0.01	0.03	0.03
Eosinophil %	0.013 - 4.5	1.05	0.50	1.18	0.39	3.83	3.83
Basophils (K/µL)	0 - 0.14	0.01	0.00	0.01	0.00	0.00	0.00
Basophils %	0.01 - 1.24	0.15	0.10	0.25	0.15	0.00	0.00
ALB (Albumin; g/dL)	2.6 - 3.8	1.40	0.17	1.75	0.15	1.97	0.26
ALKP (Alkaline Phosphatase; U/L)	35 - 96	51.50	10.10	55.50	19.50	73.33	6.36
ALT (Alanine Aminotransferase; U/L)	28 - 129	45.25	11.26	67.00	8.00	41.00	6.51
Ca (Calcium; mg/ml)	110 - 129	9.63	0.34	-	-	-	-
CREA (Creatinine; mg/dL)	0.2 - 0.5	0.23	0.08	0.15	0.05	0.47	0.03
PHOS (Phosphorus; mg/dL)	7.9 - 14.5	12.83	1.33	-	-	-	-
TBIL (Total Bilirubin; mg/dL)	0.2 - 0.6	0.18	0.05	0.20	0.00	0.17	0.07
TP (Total Protein; g/dL)	4.8 - 7	3.60	0.27	4.25	0.35	4.40	0.42
BUN (Blood Urea Nitrogen; mg/dL)	7 - 28	30.50	4.19	19.50	2.50	84.33	5.78
GLOB (Globulin; g/dL)		2.18	0.09	2.40	0.20	2.43	0.18

Supplementary Table 2. Systemic toxicity in flank tumor-bearing mice following treatment with STAT3 inhibitors, i.e., WP1066 or CPA-7. Hematological and biochemical parameters are shown for mice bearing flank tumors with mean values and SEM (Standard Error of the Mean).