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Pro-905, a novel purine antimetabolite, combines with glutamine amidotransferase inhibition to suppress growth of malignant peripheral nerve sheath tumor

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Supplementary Materials: The following supporting information is available: Supporting Methods: Detailed Pro-905 Synthesis and Characterization, Compound dose response and colony area formation, FGAR quantification, Bioanalysis of purine metabolites from NPc1s tumors, In vivo Pro-905 tolerability, Western blotting; Supplemental Figure 1: Additional data on effect of JHU395 in NPc1s mouse model; Supplemental Figure 2: Clinically used antimetabolite 6-mercaptopurine has partial antitumor activity in MPNST models with evidence of formation of toxic metabolites; Supplemental Figure 3: Pro-905 has antitumor activity in a human MPNST patient-derived xenograft model; Supplemental Figure 4: Additional data on effect of JHU395 and Pro-905 in MPNST cells; Supplemental Figure 5: Full scan images for western blots; Supplemental Figure 6: Additional data on effect of combination JHU395 and Pro-905 on metabolites in murine tumors; Supplemental Figure 7: Model of purine nucleotide inhibition by JHU395 and Pro-905 in tumor cells.

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Abstract

Malignant peripheral nerve sheath tumors (MPNST) are highly aggressive soft tissue sarcomas that arise from neural tissues and carry a poor prognosis. Previously, we found that the glutamine amidotransferase (GA) inhibitor JHU395 partially impeded tumor growth in preclinical models of MPNST. JHU395 inhibits de novo purine synthesis in human MPNST cells and murine tumors with partial decreases in purine monophosphates. Based on prior studies showing enhanced efficacy when GA inhibition was combined with the antimetabolite 6-mercaptopurine (6-MP), we hypothesized that such a combination would be efficacious in MPNST. Given the known toxicity associated with 6-MP, we set out to develop a more efficient and well-tolerated drug that targets the purine salvage pathway. Here, we report the discovery of Pro-905, a phosphoramidate protide that delivered the active nucleotide antimetabolite thioguanosine monophosphate (TGMP) to tumors over 2.5 times better than equimolar 6-MP. Pro-905 effectively prevented the incorporation of purine salvage substrates into nucleic acids and inhibited colony formation of human MPNST cells in a dose-dependent manner. Additionally, Pro-905 inhibited MPNST growth and was well-tolerated in both human patient-derived xenograft and murine flank MPNST models. When combined with JHU395, Pro-905 enhanced the colony formation inhibitory potency of JHU395 in human MPNST cells and augmented the antitumor efficacy of JHU395 in mice. In summary, the dual inhibition of the de novo and purine salvage pathways in preclinical models may safely be used to enhance therapeutic efficacy against MPNST.

Keywords

tumor metabolism; malignant peripheral nerve sheath tumor; purine synthesis; protide; 6-mercaptopurine; glutamine amidotransferase

Introduction

Purine nucleotides are foundational building blocks for cellular macromolecules (e.g. DNA, RNA) and play important roles in cellular bioenergetics, intracellular signaling, and production of immune modulating substrates in the extracellular environment [1, 2]. Purines may be made in cells by a de novo pathway starting from amino acids, sugars, bicarbonate, and energy substrates or recycled from previously synthesized nucleosides or bases via a salvage pathway. Due to the importance of purines in cell growth and survival, multiple efforts have been made to investigate purine synthesis regulation in cancer.

Enhanced activation of RAS-ERK signaling in tumors, often through oncogenic *RAS* pathway mutations, affects tumor metabolism including nucleotide synthesis [3–5]. The role of inhibition of purine nucleotide synthesis has been less well-characterized in tumors with loss of the RAS GTPase activating protein neurofibromin 1 (NF1), including the NF1-associated aggressive sarcoma malignant peripheral nerve sheath tumor (MPNST). We

recently reported that the glutamine amidotransferase (GA) inhibitor JHU395 slows growth of MPNST in mice with prominent perturbations of purine metabolites [6]. While promising, the antitumor effect was partial and its mechanism incompletely understood. Herein, we sought to better understand the mechanism of JHU395 in MPNST and to rationally develop an improved combination treatment.

In addition to de novo purine synthesis, tumor cells also recycle purine bases into nucleotides via the salvage pathway. Clinically used purine antimetabolites including 6-mercaptopurine (6-MP), used in the treatment of hematologic malignancies, require metabolism by enzymes in the purine salvage pathway to form active phosphorylated nucleosides which are incorporated to nucleic acids and lead to anti-cancer activity [7]. While 6-MP is an effective agent, its metabolism is complex, subject to multiple side reactions and pharmacogenomic variations, yielding non-therapeutic and GI/hepatotoxic methylated metabolites [8].

Based on the prior observed antitumor effects of the GA inhibitor JHU395 in MPNST and observed perturbation of purine metabolites, we sought to develop a combination strategy that would strategically exploit the dependence of MPNST on purine metabolism by disrupting additional points in purine nucleotide synthesis. Prior clinical studies demonstrated that the GA inhibitor 6-diazo-5-oxo-norleucine (DON) when combined with 6-MP led to improved efficacy in pediatric cancer patients. Unfortunately, the combination of DON with 6-MP also resulted in increased toxicity, including GI toxicity and mucositis [9, 10]. Although 6-MP is not currently clinically used in pediatric solid tumors [11], we hypothesized that the partial effects of JHU395 on MPNST might be enhanced by combination therapy with 6-MP's active metabolite, thioguanine monophosphate (TGMP).

Nucleoside monophosphates are not suitable as drugs given the negative charge on the phosphate, affecting drug distribution and pharmacokinetics as well as the ability to cross the plasma membrane of tumor cells. Phosphoramidate protides are one class of prodrugs designed to evade these challenges that are already used clinically as antivirals [12–15] and are in clinical development for use as anticancer agents [16]. Protides release monophosphorylated nucleosides upon metabolism, enabling efficient transformation to nucleoside triphosphates in cells. In principle, this strategy should permit lower equivalent doses of the protide antimetabolite compared to the parent inhibitor and minimize toxic side effects, particularly those arising from methylated metabolites commonly generated during 6-MP metabolism [17, 18].

Here, we describe the therapeutic potential of a novel combination strategy targeting purine metabolism in the RAS pathway-active sarcoma MPNST. The GA inhibitor JHU395 blocks multiple points in de novo purine synthesis in MPNST, with partial decreases in purine monophosphates. We hypothesized that combining JHU395 with an efficient and well-tolerated purine antimetabolite, designed to incorporate into nucleic acids without generating toxic methylated metabolites, would further enhance anti-tumor efficacy. We demonstrate success of this therapeutic strategy, using the newly designed Protide-TGMP-905 (Pro-905); a novel purine nucleotide antimetabolite designed to deliver active TGMP to tumors. We find that Pro-905 has robust single agent activity in multiple MPNST models including

human MPNST cell lines and a patient derived xenograft (PDX) murine model [19, 20]. Pro-905 also enhances the inhibitory potency of JHU395 when co-administered in both human MPNST cell lines and in mice bearing murine flank MPNST. In combination with GA inhibitor, Pro-905 provides a novel effective therapeutic strategy for these aggressive, therapy-resistant sarcomas.

Materials and Methods

Cell culture:

The sNF96.2 cells (RRID:CVCL_K281) were received from American Type Culture Collection and grown in DMEM (Life Technologies #11965), 10% FBS (Hyclone), 2mM L-glutamine and 1% penicillin/streptomycin. JH-2-002 and JH-2-031 MPNST cells were received from the JH NF1 Biospecimen Repository (<http://bit.ly/nf1bank>) and grown in DMEM/F12 (Life Technologies 11320-033), 10% FBS (Gemini), 2 mM L-glutamine, and 1% penicillin/streptomycin [19, 21]. Cell lines were authenticated at the Johns Hopkins Genetic Resources DNA Services Core Facility by STR profiling using Promega GenePrint10 in comparison to available data [19, 21] and tested negative for Mycoplasma using a Captivate Bio EZ-PCR kit. For establishment of murine flank MPNST, cells harvested from a tumor in an NPcis (*NF1*^{+/-}; *p53*^{+/-}) mouse and frozen in 5% DMSO were cultured as previously described [6]. All cells were incubated at 37°C, in a humidified atmosphere with 5% CO₂. Confluency was monitored using an Axiovert 25 optical microscope. JHU395 was synthesized as previously described [22]. 6-mercaptopurine (Tocris #4103) was purchased commercially. Pro-905 was synthesized as described below and in the Supplemental Methods. For details of compound dose response and colony area assays [23] in cell culture please see Supplemental Methods.

Cell-based targeted metabolomics:

sNF96.2 and JH-2-002 cells were plated in equal numbers and grown to 70% confluence per 10 cm² plate in media. Cells were treated in quadruplicate with JHU395 (10 μM) or DMSO for 6 hours, then media was aspirated and cells were washed with cold PBS. Polar metabolites were extracted in 2 mL ice cold 80% methanol/20% water following the published protocol of Yuan et. al. [24]. Dried metabolite pellets were resuspended in 20 uL LC/MS grade water and 5 uL was injected onto an AB/SCIEX 6500 QTRAP hybrid triple quadrupole mass spectrometer targeting >300 polar metabolites via selected reaction monitoring (SRM) with polarity switching. Metabolites were separated using amide XBridge HILIC (Waters Corp.) chromatography with a Shimadzu Prominence UFLC instrument. Data were integrated using MultiQuant 3.0 software to generate peak area intensities. For details of FGAR quantification please see Supplemental Methods.

Radiolabel incorporation to DNA/RNA synthesis:

Radiolabeled glycine, hypoxanthine, and guanine incorporation to DNA and RNA were measured in sNF96.2 cells and JH-2-002 cells respectively by a protocol similar to described in [4]. Briefly, cells (~85% confluent) were incubated in dialyzed FBS media for 15 hours, then treated with vehicle (DMSO), JHU395 (10μM), Pro-905 (10μM), or combination for 6 hours. At the same time of drug treatment, cells were also labeled with the given

radionuclide (2- μ Ci of either U-¹⁴C-glycine, ³H-hypoxanthine or 8-³H-guanine) for 6 hours. After 6 hours, cells were harvested and RNA or DNA was isolated using Allprep DNA/RNA kits according to the manufacturer's instructions and quantified using a spectrophotometer. 30 μ l of eluted RNA or 70 μ l of eluted DNA were added to scintillation vials and radioactivity was measured by liquid scintillation counter and normalized to the total RNA or DNA concentrations, respectively. All conditions were analyzed with biological triplicates and representative of at least two independent experiments.

Animal studies:

Studies in laboratory mice were conducted under a protocol approved by the Johns Hopkins Animal Care and Use Committee. C57BL6/NHsd (B6) male mice, at ~12 weeks of age between 25 and 30 g were obtained from Envigo. NSG mice (#005557) were obtained from Jackson Labs and bred in house. NPcis (B6;129S2-*Trp53^{tm1Tyj} Nf1^{tm1Tyj}/J*, RRID:IMSR_JAX:008191) transgenic mice [25] were obtained from Jackson Labs, Inc, and bred in house. Animals were genotyped using previously validated primers on ear punches (Transnetyx).

Evaluation of JHU395 antitumor efficacy in the NPcis transgenic mouse model:

To explore the anti-tumor efficacy of JHU395 in a genetically-engineered mouse model of MPNST, NPcis mice were bred to generate double heterozygote (*NF1^{+/-};p53^{+/-}*) mice. Animals were monitored three times weekly for spontaneous tumor development. Once a palpable tumor reached a volume of 100 mm³ as measured by calipers and calculated by the formula: volume = ($L \times (W^2)$)/2, the animal was enrolled onto the treatment study and began treatment with either vehicle (PBS + 1% Tween-80 and 2.5% ethanol TIW p.o., $n = 10$) or JHU395 (2.4 mg/kg TIW p.o., $n = 11$). Animal weights, body condition scores (BCS), and tumor volumes were measured three times weekly during the study.

Reasons for animal euthanasia were defined by guidelines set forth by the Johns Hopkins University Animal Care and Use Committee including tumor size exceeding 2 cm in either dimension, poor body condition scoring (BCS), and other clinical signs of pain and distress. On the day of euthanasia, each animal received a final timed dose of vehicle or JHU395 and was euthanized two hours later by CO₂ inhalation. Whole body perfusion (10% neutral buffered formalin) was performed on a subset of animals ($n = 3-4$ /treatment group) and specimens saved for necropsy and histopathology with H&E staining at IDEXX, Inc. Tumor and plasma were collected from the rest of the animals. Tumor was flash frozen in liquid nitrogen and all samples were stored at -80°C until further bioanalysis. For bioanalysis details, please see Supplemental Methods.

Pro-905 synthesis and characterization:

Pro-905 was synthesized in a four-step synthesis starting from 2',3'-O-isopropylidene-guanosine. 2',3'-O-isopropylidene-guanosine was prepared based on previously described methods [26] and reacted with isopropyl [chloro(phenoxy)phosphoryl]-L-alaninate. The product (Cpd 902) was transformed to its 6-thioguanosine analog (Cpd 904) by reaction with anhydrous sodium hydrogen sulfide, followed by deprotection of the isopropylidene group to give Cpd 905 (Pro-905). Products were characterized by ¹H-NMR, ¹³C-NMR and

high-resolution mass spectrometry. The final product Pro-905 was achieved with 99.5% purity. For detailed methods regarding synthetic techniques and molecular characterization please refer to Supplemental Methods, Detailed Pro-905 Synthesis and Characterization. Subsequent scale up to achieve gram quantities was performed with Redicuis, Inc.

6-MMP and TGMP Bioanalysis:

Mice (B6, ~12 weeks of age, bearing murine flank MPNST at ~500 mm³) were dosed with 6-MP, Pro-905, or vehicle at doses and routes indicated. The mice were euthanized at specified time points post-drug administration and blood samples (~0.8 mL) were collected in heparinized microtubes by cardiac puncture. Tumors and liver were removed and flash frozen on dry ice. Blood samples were centrifuged at a temperature of 4 °C at 3000 × *g* for 10 min. Plasma (~300 µL) was collected in polypropylene tubes, and all plasma and tissue samples were stored at –80 °C until bioanalysis.

For quantifying 6-MMP/6-TGMP levels, plasma samples (25 µL), were extracted using a protein precipitation method by addition of 125 µL of methanol containing internal standard (IS; 2-Chloroadenine, 1 µM), followed by vortex-mixing and then centrifugation at 16000 × *g* for 5 min at 4 °C. The tumor tissues were diluted 1:5 w/v with methanol containing the IS (1 µM), homogenized, then vortex-mixed and centrifuged at 16000 × *g* for 5 min at 4 °C. Supernatants were analyzed by LC-MS/MS using an UltiMate 3000 UHPLC coupled to Q Exactive Focus Orbitrap Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The mobile phase used for chromatographic separation consisted of water + 0.01% formic acid (A), and acetonitrile + 0.01% formic acid (B), delivered at a flow rate of 0.4 mL/min.

For 6-MMP analysis, a gradient LC method (time (min)/%B: 0–0.25/2.5, 2.25–3.25/95, 3.25–4.25/2.5) was used for the analyses. Separation was achieved using an Atlantis dC18 2.1 × 150 mm, 3 µm particle size column (Waters, Milford, MA, USA). The mass spectrometer was operated with the capillary temperature setting at 350°C and a spray voltage of 3 kV. Nitrogen was used as the sheath and auxiliary gas set to 30 and 3 arbitrary units, respectively. The mass spectrometer operating in positive PRM mode dissociated the parent ion of 6-MMP at *m/z* 167.0386 with a collision energy (CE) setting of 23 quantifying product ions of 126.0119, 134.0585 and 152.0152. The parent ion of IS at *m/z* 170.0228 was dissociated with a CE setting of 23 quantifying product ion of 152.0566.

For TGMP analysis, a gradient LC method (time (min)/%B: 0–1.5/0, 2–3/95, 3–4.5/0) was used for the analyses. Separation was achieved using an SB-Aq 2.1 × 100 mm, 1.3 µm particle size column (Agilent, Santa Clara, CA, USA). The mass spectrometer was operated with the capillary temperature setting at 350°C and a spray voltage of 3.5 kV. Nitrogen was used as the sheath and auxiliary gas set to 25 and 15 arbitrary units, respectively. The mass spectrometer operating in negative PRM mode dissociated the parent ion of TGMP at *m/z* 378.0279 with a CE setting of 21 quantifying product ions of 78.9591 and 211.0015. The parent ion of IS at *m/z* 168.0082 was dissociated with a CE setting of 17 quantifying product ions of 97.0237 and 125.0187.

Patient-derived xenograft (PDX) studies:

JH-2-031 MPNST patient derived xenograft specimens at mouse passage 3 were obtained from the JH NF1 Biospecimen Repository [21]. Female NSG mice at 10 weeks of age were anesthetized with ketamine/xylazine (100 mg/kg ketamine + 10 mg/kg xylazine i.p.) until surgical plane confirmed. ~3 mm³ tumor xenograft pieces were implanted subcutaneously in the right flank in Matrigel (Corning Inc; 354230). When palpable tumors > ~500 mm³ formed, mice were euthanized and tumors harvested under sterile conditions, dissected to ~3 mm³ pieces, and immediately passed to recipient experimental mice. For the experiment shown in Supplemental Figure 3, tumors at mouse passage 7 were used. To evaluate Pro-905 efficacy, mice with tumor volumes > 100 mm³ were randomized to receive vehicle (PBS + 1% Tween + 10% EtOH) or Pro-905 (20 mg/kg) i.p. 5d/week. Mean starting tumor volume per group was ~225 mm³. Mice were weighed and tumors measured with calipers three times per week. Tumor volume was calculated with the formula $V = (L * (W^2)) / 2$. At study endpoint, which was four weeks from treatment initiation, mice were euthanized by CO₂ inhalation and tumors were harvested and weighed. Data shown are representative of two independent experiments.

6-MP, Pro-905, and combination JHU395 + Pro-905 activity in vivo in B6 allograft flank tumored mice:

Male B6 mice (Envigo) approximately 10 weeks of age were injected in the right flank with 3×10^6 cells that were originally harvested from the tumor of an NPCis mouse and grow in culture. Tumors were allowed to form for 3–4 weeks. Mice were randomized to treatment groups to achieve equivalent mean tumor volumes (based on caliper measurements) at start of treatment.

For 6-MP antitumor activity, the mean tumor volume for the groups at start of experiment was 450 mm³. Mice were treated with 6-MP (20 mg/kg i.p. daily 5d/week) or vehicle for two weeks. Animal weights and tumor volumes were measured three times per week. Blood and tumor were harvested at the conclusion of the study and evaluated for tumor weights and clinical chemistry/complete blood counts.

For Pro-905 and combination Pro-905+JHU395 efficacy studies: mice were randomized to 4 groups (n = 7–8 mice/group) for treatment with mean tumor volume ~ 350 mm³ at start of dosing. Mice were administered Pro-905 (10 mg/kg i.p. daily until day 10; dose reduced on days 10–12 to 5 mg/kg i.p. in both combination and single agent Pro-905 arms due to ~15% weight loss occurring in combination therapy arm); JHU395 (1.2 mg/kg p.o. daily 5 days /week); the combination or Vehicle. Animal weights and tumor volumes were measured 3x/week. Tumor volume was calculated with the formula $V = (L \times W^2) / 2$. On the day of tissue harvest (day 12 since start of study), ~two hours following final dosing, mice were euthanized by CO₂ inhalation. Blood and tumor were harvested. Tumor was flash frozen in liquid nitrogen. A subset of mice (n=2–3 per group) were terminally perfused with 10% neutral buffered formalin and submitted for processing and histopathologic review by a veterinary pathologist (IDEXX Bioanalytics, Inc.)

Tumor metabolomics in Pro-905, JHU395, and Combination treated tumors:

For the polar metabolomics studies in single agent JHU395, Pro-905, and Combination treated tumors, the tumors were generated and animals treated similarly to above. Tumors were harvested after five days of treatment and flash frozen in liquid nitrogen. Metabolites were extracted in 80% cold methanol: water and separated from insoluble material by centrifugation. Supernatants were divided to equal aliquots based on tissue mass and dried at room temperature overnight under nitrogen gas. Dried metabolite pellets from 25 mg wet tumor tissue were analyzed by targeted LC-MS/MS via SRM with polarity switching on a 6500 QTRAP. Peak intensities were normalized to vehicle treated samples and analyzed further using Metaboanalyst [24, 27].

Tumor immunohistochemistry:

Tumor sections were stained for Ki67 to assess proliferation. Briefly, slides were deparaffinized and rehydrated through graded alcohols, followed by quenching of endogenous peroxidase activity in 3% H₂O₂ (30m, RT). Heated antigen retrieval was performed in 10 mM citric acid (20m, microwave). Slides were blocked with 2.5% BSA, followed by incubation with 1° anti-Ki67 (Abcam ab16667; 1:200, O/N, 4C) or rabbit IgG (Cell Signaling DA1E; concentration-matched, O/N, 4C) in 2.5% BSA. A polymer-based secondary detection system was employed according to manufacturer's instructions (Vector, ImmPRESS-HRP Anti-Rabbit IgG, MP-7401). All slides were individually developed with Sigmafast™ DAB (Sigma, D4293) for 90 seconds under microscopic control, followed by counterstaining with Hematoxylin QS (Vector, H-3404). Slides were then digitized with an Aperio CS2 slide scanner (Leica Biosystems) and analyzed by a trained blinded investigator using Aperio Imagescope™ software. Representative images of each sample were photographed, and tumors were manually circumscribed to calculate tumor area. A representative area, equivalent in size to the smallest tumor (14 mm²), was then selected within each sample for quantification. Automated total nuclei counts and Ki67 positive nuclei counts were obtained using the Aperio Nuclear v9 algorithm, with thresholds set to detect moderate-to-strong nuclear DAB. Counts were converted to % proliferation ([Ki67 Nuclei Count/Total Nuclei Count]*100), plotted in GraphPad Prism (RRID:SCR_002798) and analyzed by ordinary one-way ANOVA with multiple comparisons (*p < 0.05).

Data availability statement:

The data generated in this study are available upon request from the corresponding author.

Results

JHU395 inhibits glutamine amidotransferases in de novo purine synthesis leading to partial inhibition of tumor cell proliferation

JHU395 is a nervous tissue-penetrant prodrug of the GA inhibitor DON [22, 28] which has been shown to inhibit three enzymes in de novo purine synthesis including phosphoribosyl pyrophosphate amidotransferase (PPAT), phosphoribosyl formylglynamidine amidotransferase (PFAS), and guanosine monophosphate synthetase (GMPS) [29–31] (Fig 1A). Using quantitative liquid chromatography-mass spectrometry

(LC-MS) methods recently described [32], we observed that 24h incubation of JHU395 (10 μ M) in both sNF96.2 and JH-2-002 human MPNST cells dramatically increased the PFAS substrate formylglycinamide ribonucleotide (FGAR) over 100-fold (Fig 1B). Using polar metabolomics, we further demonstrated that GA substrates phosphoribosyl pyrophosphate (PRPP) and xanthosine monophosphate (XMP) were also significantly increased by JHU395 (10 μ M) in sNF96.2 cells (Fig 1C). Radiolabeled glycine was used to monitor de novo purine synthesis and incorporation of glycine-containing metabolites into newly synthesized nucleic acids of DNA and RNA. JHU395 treatment of MPNST cells significantly reduced de novo purine synthesis, as evidenced by a >25% reduction of glycine label incorporation in DNA and RNA (Fig 1D). Human NF1-MPNST are genomically heterogeneous with now well-established diversity in *NF1* mutations, alterations in tumor suppressors (e.g. *CDKN2A*, *TP53*), epigenetic regulators (PRC2 complex components), and other variations [33, 34] [35]. Thus, we compared JHU395 inhibitory potency in three different MPNST cell lines including commercially available sNF96.2 cells, as well as two patient-derived cell lines (JH-2-002, JH-2-031) [19, 21, 36]. We found that JHU395 partially inhibited growth in all three cell lines with IC₅₀ values in the low micromolar range (Fig 1E).

JHU395 decreases tumor growth, improves survival, and alters tumor purine metabolism in a murine NF1-MPNST model.

JHU395 was evaluated for single agent antitumor activity in the transgenic NPCis spontaneous MPNST model [25]. In brief, male and female mice (Supplemental Fig 1A) were assigned to treatment (vehicle or JHU395 2.4 mg/kg p.o. t.i.w.) as they developed palpable tumors (defined as > 100 mm³). Animals continued treatment until necessitating euthanasia due to tumor volume or other parameter (weight change, body condition score) exceeding institutional standards (Fig 2A). Mice tolerated JHU395 treatment well with no significant weight loss over the treatment period (Supplemental Fig 1B). Similar to prior studies in this model [37–39], tumor volumes were assessed at day 10 relative to day 1 to evaluate drug activity. JHU395 displayed anti-tumor effects as demonstrated by a significant reduction in tumor volume (day 10/day 1) in the JHU395 treated mice relative to vehicle treated mice (JHU395 mean log₂ fold change 0.66 versus vehicle mean log₂ fold change 2.60; $p = 0.045$ by t-test) (Fig 2B). Notably, one JHU395-treated animal showed regression in tumor volume by day 10 and a second JHU395-treated animal had an impalpable tumor by day 10. JHU395 treatment slowed tumor growth compared to vehicle-treatment in mice over the first thirty days of dosing (Supplemental Fig 1C). There was also a significant survival difference between the JHU395- and vehicle-treated animals (22 versus 12 day median survival respectively; log rank p -value = 0.014) (Fig 2C). At the time of euthanasia, tumors were excised, flash frozen, and metabolomic markers were analyzed ($n=5-6$ tumors/treatment). JHU395-treated tumors had elevated FGAR levels indicative of de novo purine synthesis inhibition, with corresponding decreases in the purine nucleotide monophosphates IMP and GMP (Fig 2D). Representative tumor histology from vehicle and JHU395 treated mice is shown in Supplemental Figure 1D.

Pro-905, a novel protide of TGMP, was developed to circumvent the toxicities observed with 6-MP.

Given the promising partial antitumor activity observed for JHU395 in MPNST, we hypothesized that combination treatment could lead to improved efficacy. Prior pediatric cancer clinical studies showed enhanced efficacy when GA inhibition was combined with the purine antimetabolite 6-MP, though the combination was limited by toxicity [9].

We initially investigated single agent 6-MP activity in MPNST cell and mouse models as proof-of-concept. Following administration, 6-MP is transformed to an active monophosphorylated nucleoside antimetabolite 6-thioguanosine monophosphate (TGMP) through multiple steps involving enzymes in the purine salvage pathway [7] (Supplemental Fig 2A). The active metabolites generated have myelosuppressive effects and monitoring of peripheral blood cell counts are used as a surrogate marker of antitumor activity [40]. It is also well-known that this 6-MP metabolic process generates inactive and hepatotoxic methylated metabolites (e.g. 6-methylmercaptopurine, 6-MMP). In the clinic 6-MP treatment requires consistent monitoring, to identify effects of these toxic metabolites, as well as titrate dosing appropriately and provide adequate supportive care [41].

In our studies, 6-MP dose-dependently inhibited proliferation of MPNST cells in culture (Supplemental Fig 2B) and tumor growth in a flank tumor model (Supplemental Figure 2C). Following 10 days of treatment (6-MP 20 mg/kg i.p. daily, the equivalent to 60 mg/m²/day in humans) [42, 43], the mean tumor volume was smaller in the 6-MP group compared to vehicle (1107 ± 654 mm³ vs 2038 ± 468 mm³, respectively; p = 0.0098). Consistent with clinical reports [44], some mice treated with 6-MP had increased alanine liver transaminases (46 U/L ± 13 versus 21 U/L ± 4; p=0.024; Supplemental Fig 2D). In addition, 6-MP's hepatotoxic methylated metabolite 6-MMP was detectable in the liver of these mice up to 6 hours following treatment (Supplemental Fig 2E).

We hypothesized that generating a prodrug to more efficiently deliver TGMP to tumor cells, and bypass the metabolic processing required to activate 6-MP, would lead to a better tolerated antimetabolite devoid of 6-MP's hepatotoxic methylated metabolites (Fig 3A). As TGMP is a nucleotide and negatively charged, a protide approach was taken. We synthesized a phosphoramidate protide of the nucleoside analog 6-thioguanosine termed Pro-905 (Fig 3B). The synthesis was accomplished in a three-step process starting from 2',3'-O-isopropylidene-guanosine and isopropyl [chloro(phenoxy)phosphoryl]-L-alaninate. Pro-905 was purified to >99.5% by HPLC and characterized by ¹H and ¹³C NMR and mass spectrometry (see Supplemental Methods). Pro-905 inhibited purine salvage-dependent ³H-hypoxanthine incorporation to DNA and RNA in tumor cells (Fig 3C). Pro-905 also inhibited sNF96.2 and JH-2-002 human MPNST colony formation in a dose dependent manner (Fig 3D), with a magnitude of effect similar to 6-MP (Fig 3E).

Pro-905 is well-tolerated and active in MPNST models

Based on the promising *in vitro* data with Pro-905, we next tested its pharmacokinetic profile and tumor delivery in mice. Given the more direct metabolic route of Pro-905 to active TGMP, we hypothesized that Pro-905 would be a more efficient purine antimetabolite

versus 6-MP. In support of this, when dosed on an equimolar basis, Pro-905 had >2.5-fold enhanced tumor delivery of TGMP versus 6-MP (Pro-905 TGMP $AUC_{0 \rightarrow t}$ 268 nmol/g \times hr versus 6-MP TGMP $AUC_{0 \rightarrow t}$ 114 nmol/g \times hr; Fig 4A). Moreover, while 6-MP treatment resulted in exposure to the hepatotoxic metabolite MMP, Pro-905 did not (Fig 4B).

Having confirmed increased delivery of TGMP to tumor, we next evaluated Pro-905 for myelosuppression, as assessed by peripheral blood counts, to serve as a biomarker of antitumor activity *in vivo* [40]. White blood cell (WBC) counts and platelets from B6 mice treated with Pro-905 at two dose levels (1 and 10 mg/kg) were compared to those from mice treated with 6-MP (20 mg/kg) to assess activity. Following 12 days of treatment, WBC and platelet values from the 10 mg/kg i.p. Pro-905 were comparable to those from the 20 mg/kg 6-MP treated mice (Fig 4C). When Pro-905 at 10 mg/kg was administered daily to B6 mice bearing flank MPNST for three weeks, tumor growth was slowed compared to vehicle (Day 15 vehicle tumor volume 3049 ± 317 mm³ vs Day 15 Pro-905 tumor volume 1279 ± 777 mm³; $p = 0.0047$) (Fig 4D). Pro-905 treated animal weights remained within 10% of starting weight over this time (Fig 4E) with no elevations in liver transaminases or bilirubin (Fig 4F).

In parallel we evaluated tolerability and efficacy of Pro-905 in immunodeficient *Nod scid gamma* (NSG) mice, bearing patient-derived xenograft (PDX) MPNST. Unlike the B6 mice, NSG mice treated with 10 mg/kg Pro-905 i.p. did not have evidence of myelosuppression as a marker of TGMP activity (white blood cell counts: vehicle 1.002 K/ μ l ± 0.1899 versus Pro-905 0.856 K/ μ l ± 0.178 . Absolute neutrophil counts: vehicle 0.710 K/ μ l ± 0.140 versus Pro-905 0.644 K/ μ l ± 0.198). However in a second tolerability study, NSG mice treated with 20mg/kg i.p. Pro-905 did have evidence of myelosuppression. Compared to vehicle-treated mice, Pro-905-treated animals (20 mg/kg i.p.) exhibited lower white blood counts (vehicle 1.53 ± 0.64 K/ μ L versus Pro-905 0.970 ± 0.33 K/ μ L) and absolute neutrophil counts (vehicle 1.190 K/ μ L ± 0.629 versus Pro-905 0.688 K/ μ L ± 0.269). Given that the 20 mg/kg i.p. Pro-905 dose had signs of activity it was chosen for efficacy studies.

NSG mice bearing the human MPNST PDX JH-2-031 were dosed with Pro-905 (20 mg/kg i.p. 5 days per week for 4 weeks) and tumor growth was monitored. Pro-905 slowed tumor growth over the duration of the study compared to vehicle (Supplemental Fig 3A). At sacrifice the Pro-905-treated mice had significantly smaller mean tumor masses (1322 mg versus 2733 mg respectively; $p = 0.013$ by t-test; Supplemental Fig 3B), Pro-905 as a single agent was well-tolerated in this model; animal body weights remained within 10% of starting weights over two weeks of dosing (Supplemental Fig 3C). Taken together these studies support the antitumor activity of Pro-905 against human MPNST *in vivo*.

Combination of Pro-905 and JHU395 inhibits human MPNST cell proliferation with prominent effects on nucleic acids.

Based on these promising results showing single agent Pro-905 activity against MPNST we hypothesized that more prominent antitumor activity would be observed when Pro-905 is combined with the de novo purine synthesis inhibitor JHU395 (Fig 5A). We tested the combination in cell culture using a colony forming assay in human MPNST cells. Using JH-2-002 cells, single agent Pro-905 (10 μ M) or JHU395 (1 μ M) each decreased colony area ~20% compared to no treatment. When combined at these doses, the two agents reduced

colony formation by ~80% compared to no treatment (Fig 5B). A similar trend was observed in sNF96.2 cells (Supplemental Fig 4A). We next sought to identify the effects Pro-905 and JHU395 on nucleic acids in MPNST cells. Pro-905 and the combination, but not JHU395 alone, inhibited guanine incorporation to newly synthesized DNA and RNA (Fig 5C). We also explored the effects of Pro-905, JHU395, and their combination, on markers of DNA damage (γ H2AX) and apoptotic cell death (cleaved PARP). Single agent Pro-905 (10 μ M), JHU395 (1 μ M), and the combination induced DNA damage (γ H2AX) following 120h of treatment, corresponding to more than one cell doubling time, but not at 72h of treatment (Fig 5D). Cleaved PARP as a marker of apoptosis was not observed at this dose of JHU395 at either time point, but was observed when MPNST cells were treated with JHU395 at higher doses or with the anthracycline doxorubicin as a positive control (Supplemental Fig 4B, Fig 5D, lanes 2 & 10). Full scan western blots are pictured in Supplemental Figure 5. Taken together, these results demonstrate that Pro-905 and JHU395 combine in MPNST cells to decrease cell proliferation with effects on nucleic acid synthesis and DNA damage, but without some markers of classical apoptosis.

Combination of Pro-905 and JHU395 inhibits tumor nucleotide metabolism and abrogates MPNST proliferation in mice.

B6 mice with flank MPNST were treated with JHU395 (1.2 mg/kg p.o. 5d/week), Pro-905 (10 mg/kg i.p. 5d/week), the combination, or vehicle. Following five days of treatment, tumors were harvested, flash frozen, and metabolites extracted for LC-MS analysis. We observed that compared to vehicle treated tumors, combination treated tumors demonstrated forty polar metabolites with $|\log_2 \text{fold change}| > 1$ and (negative $\log_{10} \text{p-value} > 1$; notably this was more than were observed compared to either Pro-905 or JHU395 single agent treatments (Supplemental Fig 6A and 6B). Analysis of these metabolites based on pathways demonstrated that purine and pyrimidine metabolism pathways were the most significantly impacted by combination treatment (Fig 6A). Metabolites in purine pathways were decreased upon combination treatment compared to control (Fig 6B). Of note, guanosine nucleotides dGMP and GDP were among the purine metabolites decreased by the combination treatment.

In a two-week efficacy study, the combination of Pro-905 with JHU395 prevented growth of established tumors to a greater extent than either single agent (Day 12 mean tumor volume: Vehicle 2225 \pm 941 mm³; Pro-905 1018 \pm 287 mm³; JHU395 1423 \pm 1038 mm³; Combo 319 \pm 243 mm³.) (Fig 6C). Tumors from combination treated mice weighed significantly less than those from vehicle treated mice at tissue harvest (Combination 194.9 \pm 162.2 mg versus Vehicle 1356.0 \pm 570.0 mg; $p = 0.004$ by one-way ANOVA) (Fig 6D) and had fewer apparent mitoses (Fig 6E). Ki67 staining as a marker of proliferation was significantly reduced in the combination group compared to vehicle treated tumors or single agent Pro-905, and trended towards decreased when compared to JHU395 (mean percent proliferation Vehicle 1.63%, JHU395 0.82%, Pro-905 1.25%, Combination 0.18%) (Fig 6F).

Discussion

Here we show that preclinical models of the chemotherapy resistant sarcoma MPNST are sensitive to inhibition of multiple points in purine metabolism. Both inhibition of glutamine-dependent de novo purine biosynthesis with a GA inhibitor and purine antimetabolite incorporation to nucleotide synthesis with the novel protide Pro-905, perturb MPNST cell use of purine substrates. Individually JHU395 and Pro-905 each exert antiproliferative effects on MPNST in both cell-based and mouse models, with increased markers of cell death at concentrations of JHU395 IC_{50} . When co-administered, these effects are enhanced with further perturbation of purine metabolites and abrogation of MPNST growth. Here, we have not only developed a novel purine antimetabolite drug, but also provide strong evidence that inhibition of nucleic acid synthesis pathways is a viable therapeutic strategy for the treatment of RAS-active sarcomas.

Broadly active small molecule GA inhibitors, which have recently advanced to clinical trials ([NCT04471415](#)) [45], have preclinical single agent activity in RAS-active tumors [6, 46] including MPNST. JHU395, a lipophilic GA inhibitor with activity in nervous system tumors [6, 47], perturbs multiple glutamine-dependent steps in de novo purine biosynthesis leading to alterations in nucleic acid synthesis and DNA damage. To our knowledge, the work presented here is the first demonstration of GA inhibition of nucleic acid synthesis in any sarcoma model. In both flank tumor and transgenic mouse models of MPNST, JHU395 partially inhibits tumor growth, prolongs survival, and alters tumor nucleotides. Similar inhibitory effects of another GA inhibitor, JHU083 on purine and pyrimidine nucleosides were recently shown in models of renal cell carcinoma [48].

Based on the altered de novo purine synthesis observed upon GA inhibitor treatment of MPNST models, paired with prior data suggesting that GA inhibition in combination with the purine antimetabolite 6-MP increased anti-tumor efficacy [9], we hypothesized that combining JHU395 with a purine antimetabolite would be a novel and effective therapeutic strategy for MPNST. Because of the prior toxicity associated with the combination [9], we further hypothesized that a protide approach could be taken to develop a more efficient and better tolerated purine antimetabolite that could be more safely co-administered. Phosphoramidate protides have been successfully used as a drug delivery approach for nucleoside monophosphates as antiviral and antitumor agents [12, 49, 50]. We developed Pro-905, a novel protide of the active 6-MP metabolite 6-TGMP. Characterizations of Pro-905 in vitro revealed an ability to inhibit purine salvage incorporation to nucleic acids and to prevent cell growth. In vivo Pro-905 was efficient at TGMP tumor delivery. When directly compared to equimolar 6-MP, Pro-905 had >2.5-fold enhanced delivery of TGMP to tumor. As might be expected, Pro-905 did not generate any detectable 6-MMP, a metabolite linked to much of the GI and hepatotoxicity of 6-MP. Importantly in vivo Pro-905 as a single agent, and in combination with JHU395, abrogated MPNST growth. Pro-905 is well-poised for exploration in additional disease models where 6-MP has been utilized including inflammatory bowel disease and other cancer models, such as acute leukemia.

When MPNST cells were treated with JHU395, guanine and hypoxanthine incorporation to RNA and DNA increased compared to vehicle. These substrates are metabolized through

purine salvage pathways. Flux through purine salvage likely increases in response to single agent JHU395 de novo purine synthesis blockade as a compensatory measure. Pro-905 derived TGMP competes with endogenous substrates and blocks guanine and hypoxanthine incorporation to nucleic acids, decreasing availability of purine pool substrates for biosynthesis. In addition, TGMP, when incorporated to nucleic acids, triggers a DNA damage response and replication arrest [51]. Taken together these findings support a model (Supplemental Figure 7) in which JHU395 and Pro-905 affect substrate flux for biosynthesis through de novo purine synthesis and purine salvage respectively. By blocking both purine pathways, the combination triggers effects that tumor cells are unable to evade. Under this model Pro-905-derived TGMP, by competing with GMP nucleotides, decreases total nucleotide flux downstream of GMP from both de novo and salvage pathways to DNA and RNA synthesis. Indeed, when Pro-905 and JHU395 were co-administered in a murine flank MPNST model, the combination decreased multiple purine nucleotides including dGMP and GDP as well as markers of tumor growth such as Ki67.

While some studies have evaluated the effect of mutant NF1 on tumor metabolism [52], there is room for improved understanding of the role that metabolism plays in MPNST development and response to treatment. Our finding that GA inhibition disrupts de novo purine synthesis leading to antitumor effects raises important questions about regulation of tumor metabolism in MPNST. Others have observed that in tumors with signaling modification similar to MPNST such as RAS-ERK driven tumor cells increase phosphorylation of enzymes in purine and pyrimidine synthesis and have increased flux through these pathways [3, 4]. Some MPNST also have mutations in *TP53* [53]; p53 also regulates nucleotide metabolism [54], though this has yet to be thoroughly explored in MPNST. More recently, amplifications of chromosome 8 including the *MYC* oncogene have been reported in MPNST [20, 35]. *MYC* amplification has been linked to metabolic alterations in multiple tumor types and sensitivity to GA [47, 55].

Using existing RNAseq data (C. Pratilas lab/J. Banerjee, unpublished data, NF data portal and [21]) to determine gene expression differences between human plexiform neurofibroma and MPNST we observed differential expression of gene products involved in purine synthesis, suggesting these pathways are affected as part of malignant transformation, and therefore may be a therapeutic target for further investigation. We did not directly assess the response of MPNST cells to GA inhibition or Pro-905 antimetabolite effects in terms of regulation of purine nucleotide metabolic gene products, but this will be an important area for future research, in order to better understand patterns of tumor resistance and sensitivity.

In summary we have characterized purine biosynthesis as a target pathway in MPNST; have developed Pro-905, an entirely novel, efficient, and well tolerated purine antimetabolite ProTide; and described a new combination therapy targeting both de novo and salvage purine metabolism pathways that has exquisite anti-tumor activity in preclinical models of MPNST. Pro-905 is well poised for additional investigation of its activity in other cancer and disease models. In addition our finding that purine biosynthesis is a therapeutic target pathway in MPNST suggests additional research directions to understand the connection between metabolic vulnerabilities and ongoing genomic and transcriptomic characterizations of this complex and difficult-to-treat sarcoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

MPNST	malignant peripheral nerve sheath tumor
GA	glutamine amidotransferase
6-MP	6-mercaptopurine
6-MMP	6-methylmercaptopurine
TGMP	6-thioguanosine monophosphate
FGAR	formylglycinamide ribonucleotide
NF1	neurofibromatosis type 1
PDX	patient derived xenograft
BLQ	below limit of quantitation

References:

1. Leone RD and Emens LA, Targeting adenosine for cancer immunotherapy. *J Immunother Cancer*, 2018. 6(1): p. 57. [PubMed: 29914571]
2. Ali ES and Ben-Sahra I, Regulation of nucleotide metabolism in cancers and immune disorders. *Trends in Cell Biology*, 2023.
3. Graves LM, et al. , Regulation of carbamoyl phosphate synthetase by MAP kinase. *Nature*, 2000. 403(6767): p. 328–32. [PubMed: 10659854]
4. Ali ES, et al. , ERK2 Phosphorylates PFAS to Mediate Posttranslational Control of De Novo Purine Synthesis. *Mol Cell*, 2020. 78(6): p. 1178–1191 e6. [PubMed: 32485148]
5. Santana-Codina N, et al. , Oncogenic KRAS supports pancreatic cancer through regulation of nucleotide synthesis. *Nat Commun*, 2018. 9(1): p. 4945. [PubMed: 30470748]

6. Lemberg KM, et al. , The novel glutamine antagonist prodrug JHU395 has antitumor activity in malignant peripheral nerve sheath tumor. *Mol Cancer Ther*, 2019.
7. Karran P and Attard N, Thiopurines in current medical practice: molecular mechanisms and contributions to therapy-related cancer. *Nat Rev Cancer*, 2008. 8(1): p. 24–36. [PubMed: 18097462]
8. Hartford C, et al. , Differential effects of targeted disruption of thiopurine methyltransferase on mercaptopurine and thioguanine pharmacodynamics. *Cancer Res*, 2007. 67(10): p. 4965–72. [PubMed: 17510427]
9. Sullivan MP, et al. , A comparison of the effectiveness of standard dose 6-mercaptopurine, combination 6-mercaptopurine and DON, and high-loading 6-mercaptopurine therapies in treatment of the acute leukemias of childhood: results of a cooperative study. *Cancer Chemother Rep*, 1962. 18: p. 83–95. [PubMed: 13918321]
10. Sullivan MP, et al. , A comparison of the effectiveness of standard dose 6-mercaptopurine, combination 6-mercaptopurine and DON, and high-loading 6-mercaptopurine therapies in the treatment of acute leukemia in children: results of cooperative study. *Cancer Chemother Rep*, 1962. 16: p. 161–4. [PubMed: 13918322]
11. Adamson PC, et al. , A phase II trial of continuous-infusion 6-mercaptopurine for childhood solid tumors. *Cancer Chemother Pharmacol*, 1990. 26(5): p. 343–4. [PubMed: 2208575]
12. Mehellou Y, Rattan HS, and Balzarini J, The ProTide Prodrug Technology: From the Concept to the Clinic. *J Med Chem*, 2018. 61(6): p. 2211–2226. [PubMed: 28792763]
13. Sofia MJ, et al. , Discovery of a beta-D-2'-Deoxy-2'-alpha-fluoro-2'-beta-C-methyluridine Nucleotide Prodrug (PSI-7977) for the Treatment of Hepatitis C Virus. *Journal of Medicinal Chemistry*, 2010. 53(19): p. 7202–7218. [PubMed: 20845908]
14. Eastman RT, et al. , Remdesivir: A Review of Its Discovery and Development Leading to Emergency Use Authorization for Treatment of COVID-19. *ACS Cent Sci*, 2020. 6(5): p. 672–683. [PubMed: 32483554]
15. Ray AS, Fordyce MW, and Hitchcock MJ, Tenofovir alafenamide: A novel prodrug of tenofovir for the treatment of Human Immunodeficiency Virus. *Antiviral Res*, 2016. 125: p. 63–70. [PubMed: 26640223]
16. Schwenzer H, et al. , The Novel Nucleoside Analogue ProTide NUC-7738 Overcomes Cancer Resistance Mechanisms In Vitro and in a First-In-Human Phase I Clinical Trial. *Clin Cancer Res*, 2021. 27(23): p. 6500–6513. [PubMed: 34497073]
17. Nygaard U, Toft N, and Schmiegelow K, Methylated metabolites of 6-mercaptopurine are associated with hepatotoxicity. *Clin Pharmacol Ther*, 2004. 75(4): p. 274–81. [PubMed: 15060506]
18. Melachuri S, Gandrud L, and Bostrom B, The association between fasting hypoglycemia and methylated mercaptopurine metabolites in children with acute lymphoblastic leukemia. *Pediatr Blood Cancer*, 2014. 61(6): p. 1003–6. [PubMed: 24415675]
19. Wang J, et al. , Activation of receptor tyrosine kinases mediates acquired resistance to MEK inhibition in malignant peripheral nerve sheath tumors. *Cancer Res*, 2020.
20. Dehner C, et al. , Chromosome 8 gain is associated with high-grade transformation in MPNST. *JCI Insight*, 2021. 6(6).
21. Pollard K, et al. , A clinically and genomically annotated nerve sheath tumor biospecimen repository. *Sci Data*, 2020. 7(1): p. 184. [PubMed: 32561749]
22. Nedelcovych MT, et al. , N-(Pivaloyloxy)alkoxy-carbonyl Prodrugs of the Glutamine Antagonist 6-Diazo-5-oxo-1-norleucine (DON) as a Potential Treatment for HIV Associated Neurocognitive Disorders. *J Med Chem*, 2017. 60(16): p. 7186–7198. [PubMed: 28759224]
23. Guzman C, et al. , ColonyArea: an ImageJ plugin to automatically quantify colony formation in clonogenic assays. *PLoS One*, 2014. 9(3): p. e92444. [PubMed: 24647355]
24. Yuan M, et al. , Ex vivo and in vivo stable isotope labelling of central carbon metabolism and related pathways with analysis by LC-MS/MS. *Nat Protoc*, 2019. 14(2): p. 313–330. [PubMed: 30683937]
25. Cichowski K, et al. , Mouse models of tumor development in neurofibromatosis type 1. *Science*, 1999. 286(5447): p. 2172–6. [PubMed: 10591652]
26. Foitzik RC, et al. , Linear and convergent approaches to 2-substituted adenosine-5'-N-alkylcarboxamides. *Tetrahedron*, 2009. 65(43): p. 8851–8857.

27. Xia J and Wishart DS, Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. *Curr Protoc Bioinformatics*, 2016. 55: p. 14 10 1–14 10 91.
28. Lemberg KM, et al. , We're Not "DON" Yet: Optimal Dosing and Prodrug Delivery of 6-Diazo-5-oxo-L-norleucine. *Mol Cancer Ther*, 2018. 17(9): p. 1824–1832. [PubMed: 30181331]
29. Hartman SC, The Interaction of 6-Diazo-5-Oxo-L-Norleucine with Phosphoribosyl Pyrophosphate Amidotransferase. *J Biol Chem*, 1963. 238: p. 3036–47. [PubMed: 14081921]
30. Levenberg B, Melnick I, and Buchanan JM, Biosynthesis of the purines. XV. The effect of aza-L-serine and 6-diazo-5-oxo-L-norleucine on inosinic acid biosynthesis de novo. *J Biol Chem*, 1957. 225(1): p. 163–76. [PubMed: 13416227]
31. Kaufman ER, Isolation and characterization of a mutant Chinese hamster cell line resistant to the glutamine analog 6-diazo-5-oxo-L-norleucine. *Somatic Cell and Molecular Genetics*, 1985. 11(1): p. 1–10. [PubMed: 3856328]
32. Alt J, et al. , Glutamine Antagonist GA-607 Causes a Dramatic Accumulation of FGAR which can be used to Monitor Target Engagement. *Curr Drug Metab*, 2021. 22(9): p. 735–745. [PubMed: 34488583]
33. Pemov A, et al. , Low mutation burden and frequent loss of CDKN2A/B and SMARCA2, but not PRC2, define pre-malignant neurofibromatosis type 1-associated atypical neurofibromas. *Neuro Oncol*, 2019.
34. Lemberg KM, Wang J, and Pratilas CA, From Genes to -Omics: The Evolving Molecular Landscape of Malignant Peripheral Nerve Sheath Tumor. *Genes (Basel)*, 2020. 11(6).
35. Cortes-Ciriano I, et al. , Genomic patterns of malignant peripheral nerve sheath tumor (MPNST) evolution correlate with clinical outcome and are detectable in cell-free DNA. *Cancer Discov*, 2023.
36. Wang J, et al. , Combined inhibition of SHP2 and MEK is effective in models of NF1-deficient malignant peripheral nerve sheath tumors. *Cancer Res*, 2020.
37. Malone CF, et al. , Defining key signaling nodes and therapeutic biomarkers in NF1-mutant cancers. *Cancer Discov*, 2014. 4(9): p. 1062–73. [PubMed: 24913553]
38. De Raedt T, et al. , Exploiting cancer cell vulnerabilities to develop a combination therapy for ras-driven tumors. *Cancer Cell*, 2011. 20(3): p. 400–13. [PubMed: 21907929]
39. De Raedt T, et al. , PRC2 loss amplifies Ras-driven transcription and confers sensitivity to BRD4-based therapies. *Nature*, 2014. 514(7521): p. 247–51. [PubMed: 25119042]
40. Lennard L, et al. , Childhood leukaemia: a relationship between intracellular 6-mercaptopurine metabolites and neutropenia. *Br J Clin Pharmacol*, 1983. 16(4): p. 359–63. [PubMed: 6578834]
41. Conneely SE, Cooper SL, and Rau RE, Use of Allopurinol to Mitigate 6-Mercaptopurine Associated Gastrointestinal Toxicity in Acute Lymphoblastic Leukemia. *Front Oncol*, 2020. 10: p. 1129. [PubMed: 32766146]
42. Reagan-Shaw S, Nihal M, and Ahmad N, Dose translation from animal to human studies revisited. *FASEB J*, 2008. 22(3): p. 659–61. [PubMed: 17942826]
43. Nishii R, et al. , Preclinical evaluation of NUDT15-guided thiopurine therapy and its effects on toxicity and antileukemic efficacy. *Blood*, 2018. 131(22): p. 2466–2474. [PubMed: 29572377]
44. Cohen G, et al. , Allopurinol use during pediatric acute lymphoblastic leukemia maintenance therapy safely corrects skewed 6-mercaptopurine metabolism, improving inadequate myelosuppression and reducing gastrointestinal toxicity. *Pediatr Blood Cancer*, 2020: p. e28360. [PubMed: 32909665]
45. Rais R, et al. , Discovery of DRP-104, a tumor-targeted metabolic inhibitor prodrug. *Sci Adv*, 2022. 8(46): p. eabq5925. [PubMed: 36383674]
46. Sharma NS, et al. , Targeting tumor-intrinsic hexosamine biosynthesis sensitizes pancreatic cancer to anti-PD1 therapy. *J Clin Invest*, 2019.
47. Pham K, et al. , Novel Glutamine Antagonist JHU395 Suppresses MYC-Driven Medulloblastoma Growth and Induces Apoptosis. *J Neuropathol Exp Neurol*, 2021. 80(4): p. 336–344. [PubMed: 33712838]
48. Kaushik AK, et al. , In vivo characterization of glutamine metabolism identifies therapeutic targets in clear cell renal cell carcinoma. *Sci Adv*, 2022. 8(50): p. eabp8293. [PubMed: 36525494]

49. Krecmerova M, et al. , Phosphonates and Phosphonate Prodrugs in Medicinal Chemistry: Past Successes and Future Prospects. *Front Chem*, 2022. 10: p. 889737. [PubMed: 35668826]
50. Slusarczyk M, et al. , Single Diastereomers of the Clinical Anticancer ProTide Agents NUC-1031 and NUC-3373 Preferentially Target Cancer Stem Cells In Vitro. *J Med Chem*, 2021. 64(12): p. 8179–8193. [PubMed: 34085825]
51. Karran P, Thiopurines DNA damage, DNA repair and therapy-related cancer. *British Medical Bulletin*, 2006. 79–80(1): p. 153–170.
52. Masgras I, et al. , Absence of Neurofibromin Induces an Oncogenic Metabolic Switch via Mitochondrial ERK-Mediated Phosphorylation of the Chaperone TRAP1. *Cell Rep*, 2017. 18(3): p. 659–672. [PubMed: 28099845]
53. Lee W, et al. , PRC2 is recurrently inactivated through EED or SUZ12 loss in malignant peripheral nerve sheath tumors. *Nat Genet*, 2014. 46(11): p. 1227–32. [PubMed: 25240281]
54. Tarangelo A, et al. , Nucleotide biosynthesis links glutathione metabolism to ferroptosis sensitivity. *Life Sci Alliance*, 2022. 5(4).
55. Wang SZ, et al. , Unbiased metabolic profiling predicts sensitivity of high MYC-expressing atypical teratoid/rhabdoid tumors to glutamine inhibition with 6-diazo-5-oxo-L-norleucine. *Clin Cancer Res*, 2019.

Statement of Significance:

We discovered a novel protide targeting purine salvage and show that when it is combined with de novo purine synthesis inhibition, robust antitumor efficacy is observed in malignant peripheral nerve sheath tumor.

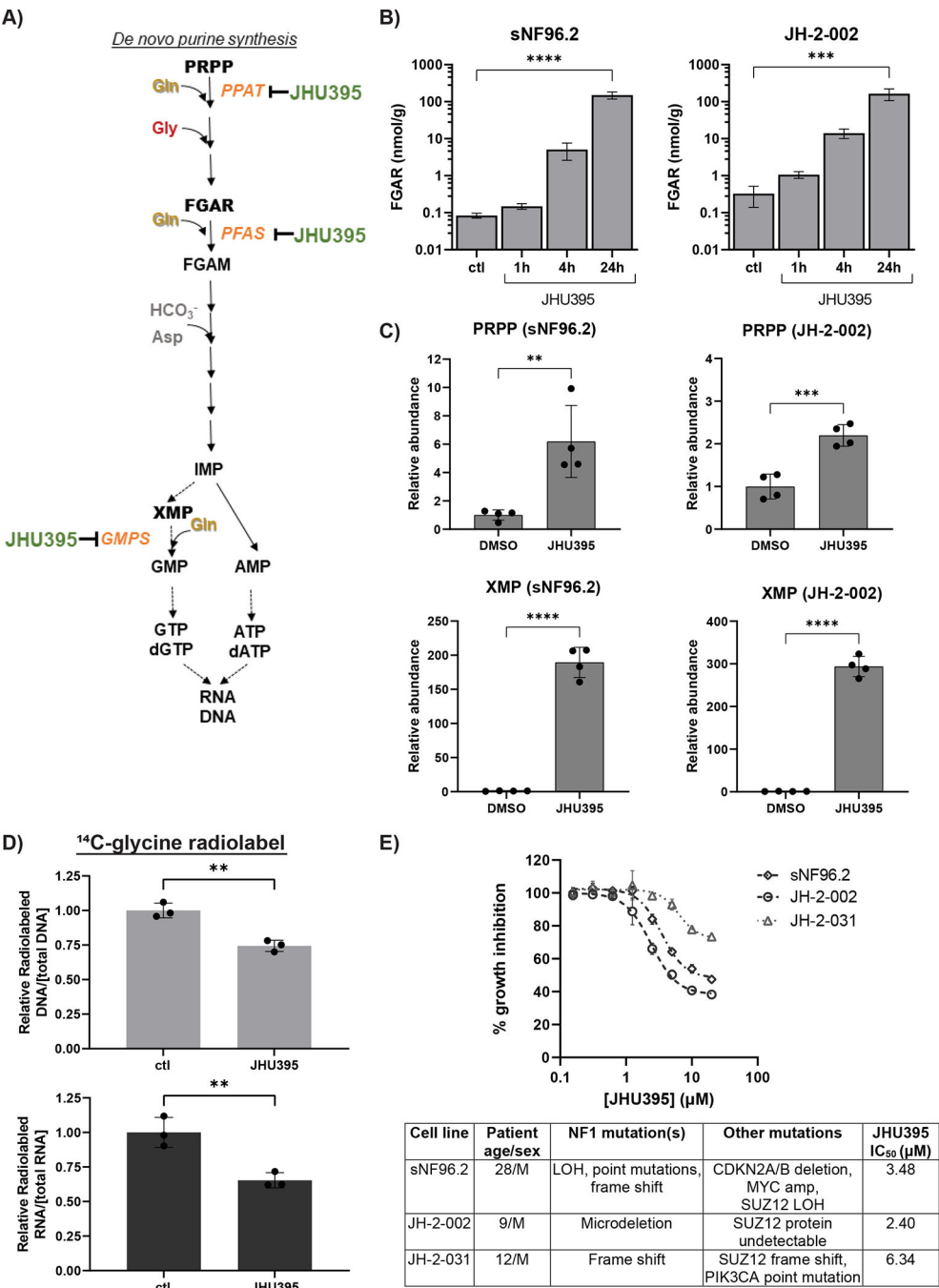


Figure 1. JHU395 inhibits glutamine amidotransferases in de novo purine synthesis leading to partial inhibition of tumor cell proliferation.
A) Schematic of de novo purine synthesis. Glutamine amidotransferases inhibited by JHU395 are shown in orange. B) Quantitative measurement of formylglycinamide ribonucleotide (FGAR) over 24h in sNF96.2 and JH-2-002 cells following treatment with JHU395 (10μM). *** indicates 0.0001 p<0.001; **** indicates p<0.0001 by one-way ANOVA with Dunnett’s multiple comparisons test. C) Relative measurement of glutamine amidotransferase substrates phosphoribosyl pyrophosphate (PRPP) and xanthosine-5-monophosphate (XMP) in sNF96.2 and JH-2-002 cells after 6h treatment with

JHU395 (10 μ M) or DMSO. ** indicates 0.001 $p < 0.01$, *** indicates 0.0001 $p < 0.001$, **** indicates $p < 0.0001$ by t-test. D) Effect of JHU395 (10 μ M for 6h) on 14 C-glycine label incorporation to DNA and RNA of sNF96.2 cells compared to vehicle. Data graphed as mean \pm standard deviation. ** indicates 0.001 $p < 0.01$, *** indicates 0.001 $p < 0.01$ by t-test. E) Dose response of human MPNST cells treated with JHU395 for 72 hours. Percent growth inhibition was calculated by ratio of fluorescence at 590 nm of treated cells compared to no treatment. All samples done in triplicate and shown as mean \pm standard deviation. IC₅₀ value for JHU395 in each cell line calculated from curve fit summarized in table alongside previously characterized genomic alterations (ref. 36).

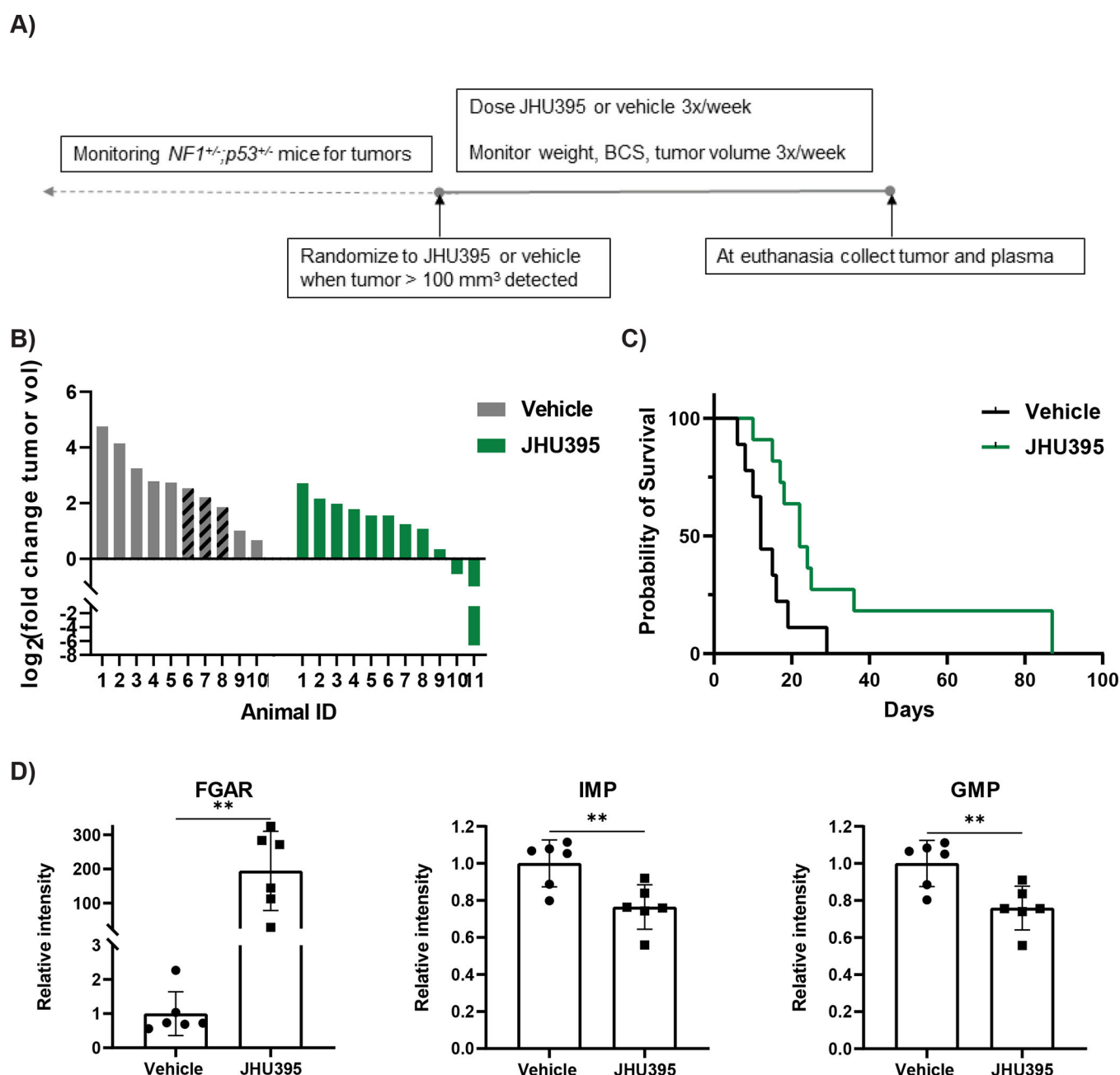


Figure 2. Single agent JHU395 slows growth of MPNST in a transgenic mouse model with effects on purine nucleotides.

A) Schematic of experimental design. B) Waterfall plot comparing log₂ fold change in tumor volume in NPcis mice from day 1 of treatment tumor volumes with day 10 tumor volumes (JHU395 group n = 11 animals; vehicle group n = 10 animals). Shaded bars in vehicle group indicate animals euthanized prior to day 10, in which case fold change is based on the last day of tumor measurement. Treatments were JHU395 2.4 mg/kg p.o. three times per week or vehicle. C) Survival plot for vehicle and JHU395 treated mice enrolled on this study. Animals were the same as in panel B. D) Relative intensities of the metabolites FGAR,

inosine monophosphate (IMP), and guanosine monophosphate (GMP) in JHU395 or vehicle treated tumors at conclusion of treatment. ** indicates 0.001 $p < 0.01$ by t-test.

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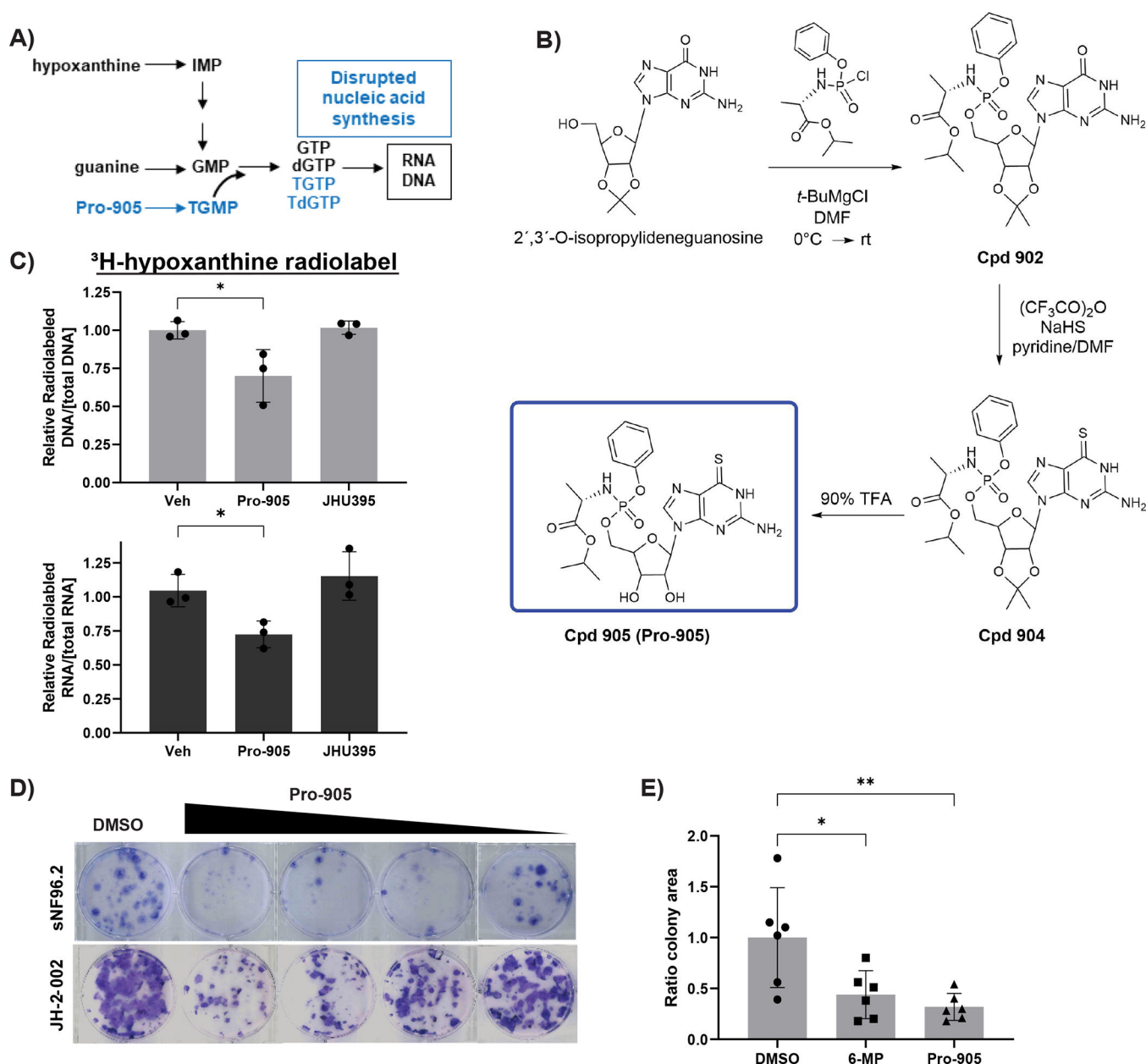


Figure 3. Synthesis and characterization of Pro-905, a novel thioguanine protide with activity in MPNST.

A) Schematic of purine base salvage and antimetabolite TGMP incorporation into this process. Pro-905 is shown in blue. B) Synthetic scheme for Pro-905. C) Effect of Pro-905 (10 μM) or JHU395 (10 μM) on ^3H -hypoxanthine incorporation to DNA and RNA in sNF96.2 cells 6h after treatment. * indicates $0.01 < p < 0.05$ by one-way ANOVA with Dunnett's multiple comparisons test. D) Effect of Pro-905 on colony formation of sNF96.2 cells. Cells stained with crystal violet. Concentrations of Pro-905 shown are 30, 10, 3, and 1 μM , compared to DMSO. E) Comparison of Pro-905 (10 μM), 6-MP (10 μM), and DMSO effect on colony area of sNF96.2 cells. * indicates $0.01 < p < 0.05$, ** indicates $0.001 < p < 0.01$ by one-way ANOVA with Dunnett's multiple comparisons test.

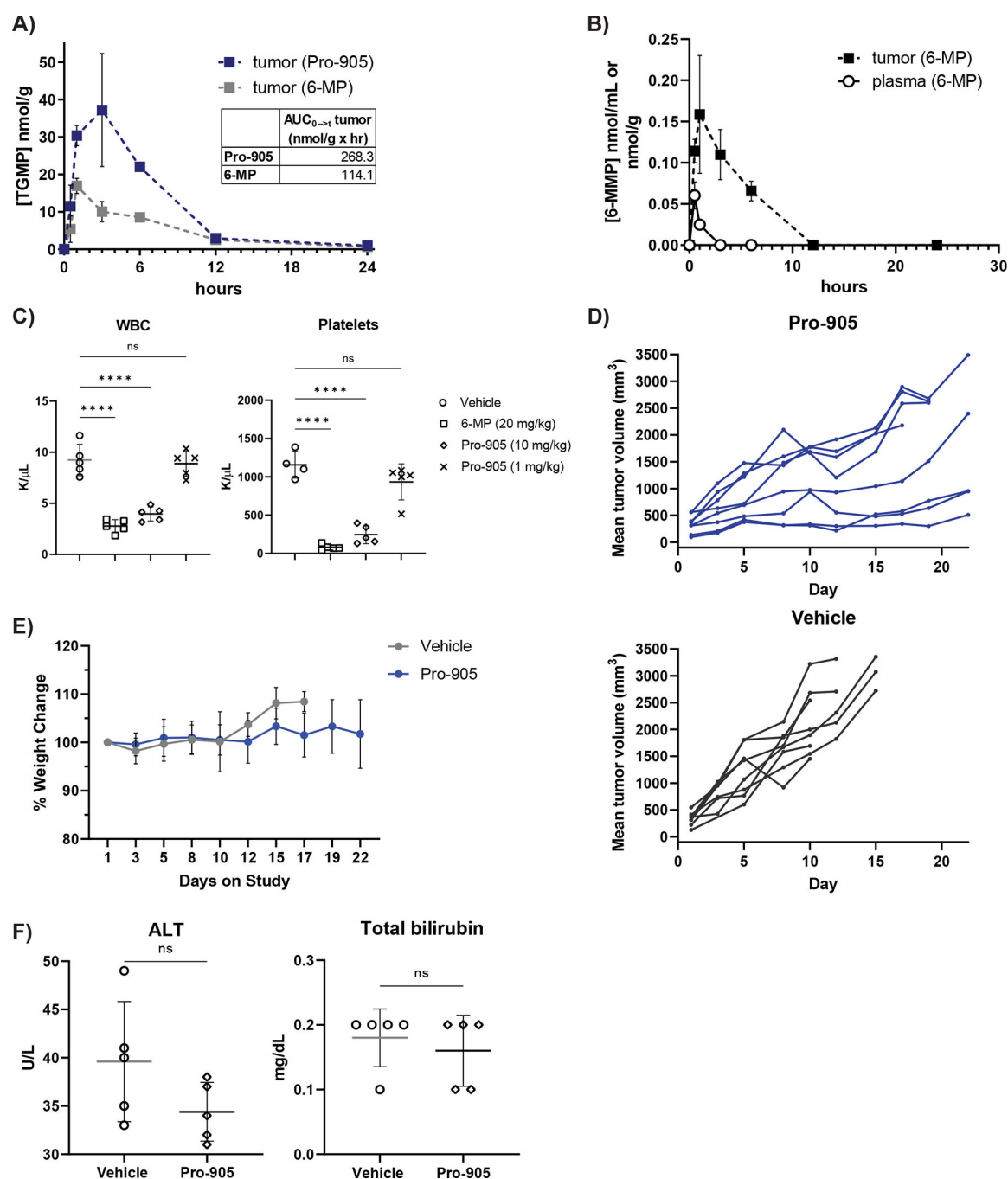


Figure 4. Characterization of Pro-905 pharmacokinetics and effect in vivo.

A) 6-TGMP active metabolite present in tumor over time following dosing of Pro-905 (10 mg/kg i.p.) or equimolar 6-MP (2.9 mg/kg i.p.) in B6 mice bearing murine flank MPNST. Error bars indicate S.D. Three mice were used per time point. B) 6-MMP identified in tumor and plasma of B6 mice with flank MPNST following treatment with 6-MP (2.9 mg/kg i.p.) or Pro-905 (all below limit of quantitation). C) White blood cells and platelets in B6 mice after 12 days dosing with 6-MP (20 mg/kg i.p.) or Pro-905 (10 mg/kg or 1 mg/kg i.p.) **** indicates p < 0.0001 by one-way ANOVA with Dunnett's multiple comparisons test.

D) Spider plot of tumor volumes measured from B6 mice with flank MPNST treated with Pro-905 (10 mg/kg i.p.) or vehicle for three weeks. E) Mean weights compared to day 1 during three week study for mice shown in D. F) Plasma alanine aminotransferase (ALT) and bilirubin measured from B6 mice after 12 days of dosing with Pro-905 (10 mg/kg i.p.).

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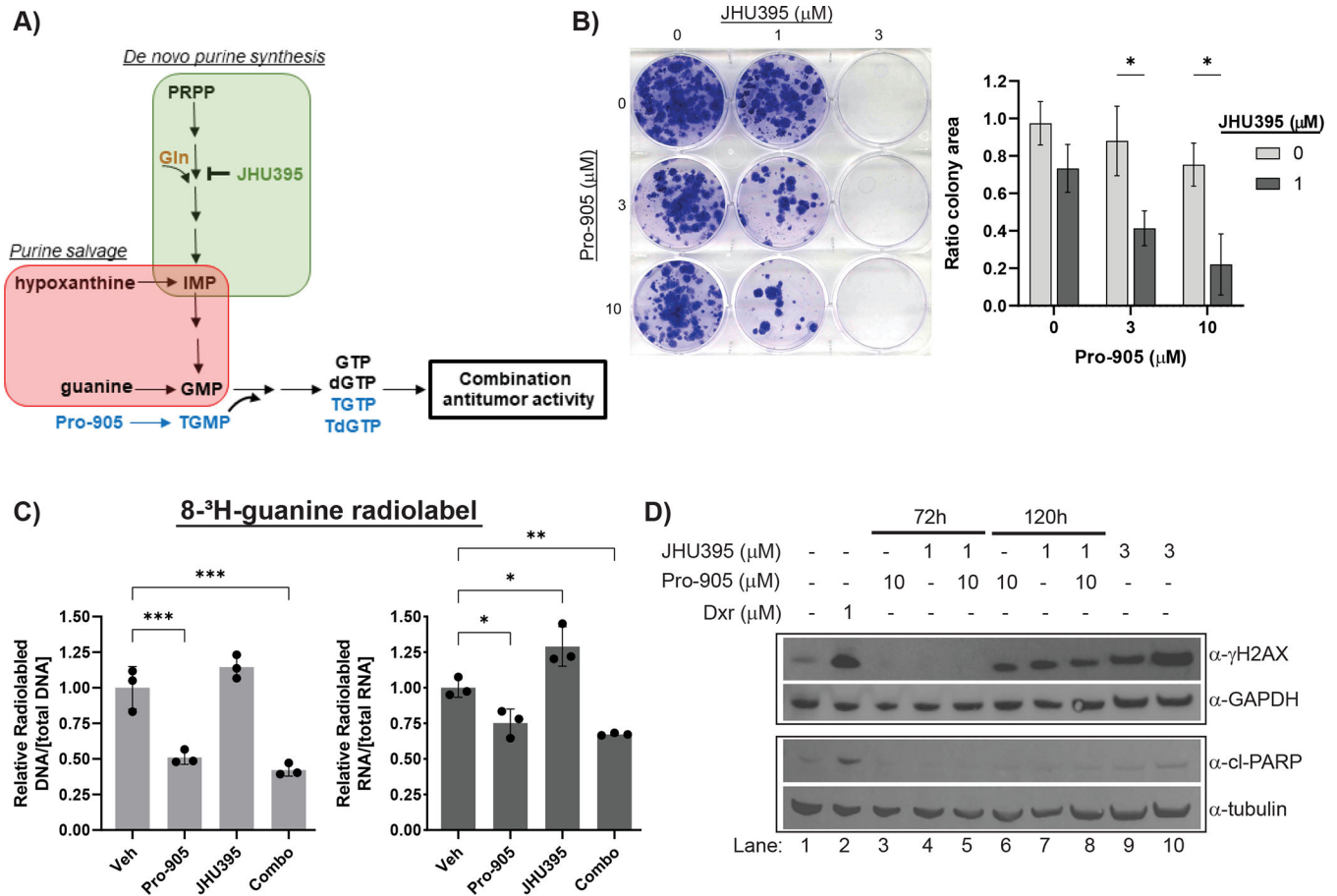


Figure 5. Combination Pro-905 and GA inhibition promotes considerable anti-tumor response in cellular models of MPNST.

A) Schematic of combination of JHU395 and Pro-905 effect on purine nucleotide synthesis and incorporation to nucleic acids. B) Effect of combination JHU395 and Pro-905 at indicated concentrations on colony area formation of JH-2-002 cells. Cells stained with crystal violet. Quantification performed using FIJI Colony Area plugin. Ratio colony area to no treatment shown for JHU395 0 and 1 μM with Pro-905 0, 3, and 10 μM. JHU395 3 μM colony area was BLQ and is not shown. * adjusted $p < 0.05$ by multiple t-tests. C) Effect of Pro-905, JHU395, or Combination on radiolabeled guanine incorporation to DNA and RNA of JH-2-002 cells. Cells were treated with indicated compounds at 10 μM each for 6h. *** indicates $0.0001 < p < 0.001$ by one-way ANOVA with Dunnett's multiple comparisons test. D) Effect of single agent Pro-905, JHU395, or the combination on γH2AX and cleaved PARP in JH-2-002 cells. GAPDH and tubulin used as loading controls. Doxorubicin (Dxr) shown as a positive control for DNA damage. Samples were assessed on two separate blots, denoted by black rectangles.

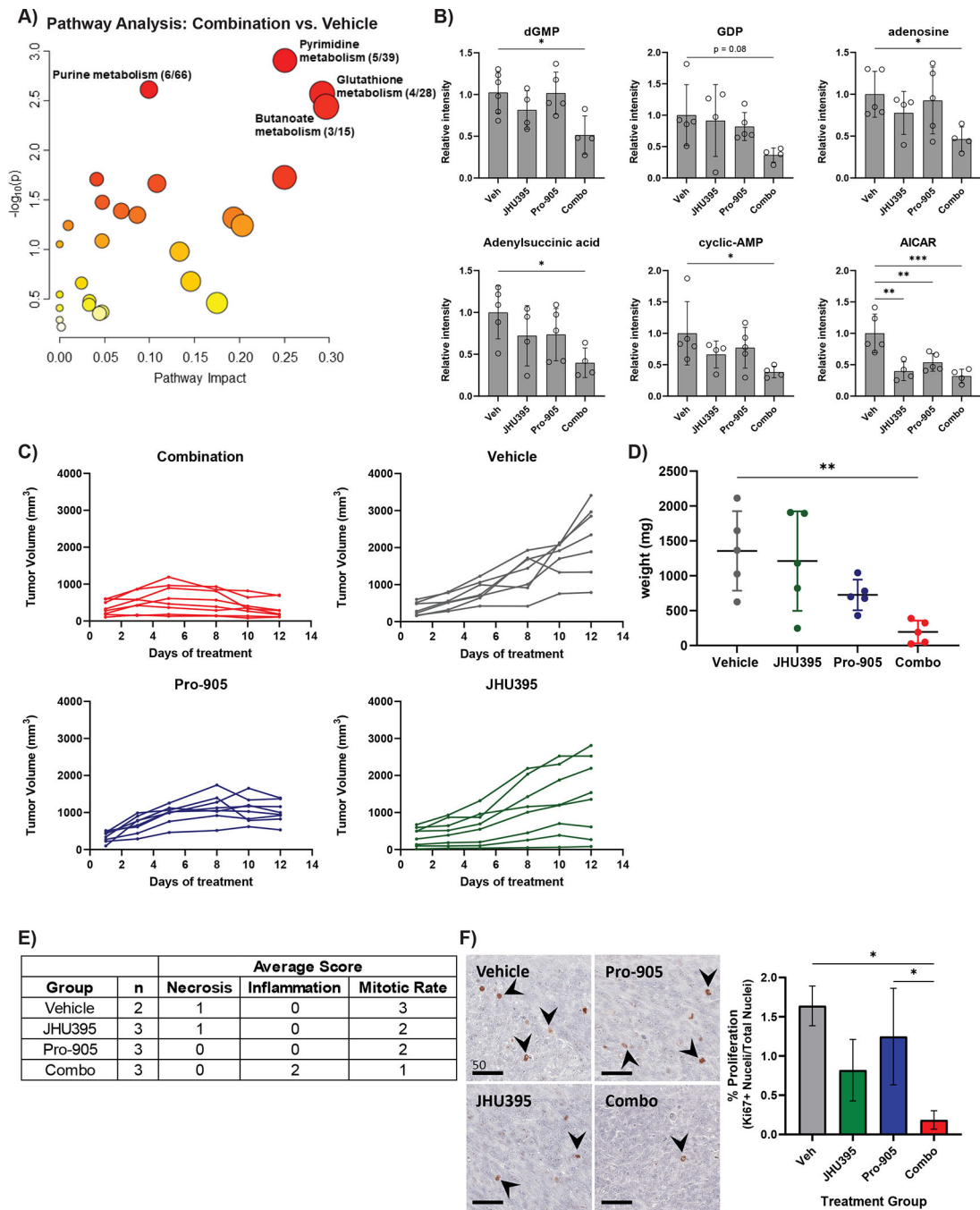


Figure 6. Combination Pro-905 and GA JHU395 affects nucleotide metabolism in murine MPNST and significantly inhibits tumor proliferation.

A) Pathway analysis of tumor metabolites identified as considerably affected by combination treatment compared to vehicle treatment of B6 mice with flank MPNST. Threshold for metabolites used in pathway analysis was: absolute value (\log_2 fold change) > 1 and $-\log_{10}$ pvalue > 1 . Mice with flank MPNST were treated for five days with vehicle, JHU395 (1.2 mg/kg p.o.), Pro-905 (10 mg/kg i.p.) or the combination. Targeted metabolomics performed with LC-MS and pathways analyzed using Metaboanalyst. B) Relative intensities of six purine metabolites identified by pathway analysis compared

across all four treatment groups (vehicle, JHU395, Pro-905, and combination). C) Tumor volumes and D) Tumor masses at tissue harvest of murine flank MPNST in B6 mice during treatment with vehicle, JHU395 (1.2 mg/kg p.o. 5d per week), Pro-905 (10 mg/kg i.p. 5d per week, reduced to 5 mg/kg i.p. for days 10–12 treatment), or the combination. Data points are mean \pm S.D. E) Table of features on H&E stained tumors assessed by veterinary pathologist after 12 days of treatment. F) Evaluation of Ki67 staining in a subset of murine flank MPNST from B6 mice after 12 days treatment with indicated agents. Quantification performed in Aperio ImageScope. * $p < 0.05$ by one-way ANOVA.