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Leonard C Rogers  
*Washington University School of Medicine in St. Louis*

Jeff C Kremer  
*Washington University School of Medicine in St. Louis*

Caitlyn B Brashears  
*Washington University School of Medicine in St. Louis*

Zongtao Lin  
*Washington University School of Medicine in St. Louis*

Alliny C S Bastos  
*Washington University School of Medicine in St. Louis*

See next page for additional authors

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**Recommended Citation**  
Rogers, Leonard C; Kremer, Jeff C; Brashears, Caitlyn B; Lin, Zongtao; Bastos, Alliny C S; Baker, Adriana; Fettig, Nicole; Zhou, Dong; Shoghi, Kooresh I; Dehner, Carina A; Chrisinger, John S A; Garcia, Benjamin A; Oyama, Toshinoa; Van Tine, Brian A; and et al., "Discovery and targeting of a noncanonical mechanism of sarcoma resistance to ADI-PEG20 mediated by the microenvironment." Clinical Cancer Research. 29, 16. 3189 - 3202. (2023).  
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Authors
Discovery and Targeting of a Noncanonical Mechanism of Sarcoma Resistance to ADI-PEG20 Mediated by the Microenvironment

Leonard C. Rogers1, Jeff C. Kremer1, Caitlyn B. Brashears1, Zongtao Lin2, Zhixian Hu3, Alliny C.S. Bastos1, Adriana Baker1, Nicole Fettig4, Dong Zhou4, Kooresh I. Shoghi1,2,5, Carina A. Dehner6, John S.A. Chrisinger6, John S. Bomalaski7, Benjamin A. Garcia2, Toshinaga Ohyama4, Eileen P. White3,8, and Brian A. Van Tine1,9,10

ABSTRACT

Purpose: Many cancers lack argininosuccinate synthetase 1 (ASS1), the rate-limiting enzyme of arginine biosynthesis. This deficiency causes arginine auxotrophy, targetable by extracellular arginineramping enzymes such as ADI-PEG20. Long-term tumor resistance has thus far been attributed solely to ASS1 reexpression. This study examines the role of ASS1 silencing on tumor growth and initiation and identifies a noncanonical mechanism of resistance, aiming to improve clinical responses to ADI-PEG20.

Experimental Design: Tumor initiation and growth rates were measured for a spontaneous Ass1 knockout (KO) murine sarcoma model. Tumor cell lines were generated, and resistance to arginine deprivation therapy was studied in vitro and in vivo.

Results: Conditional Ass1 KO affected neither tumor initiation nor growth rates in a sarcoma model, contradicting the prevalent idea that ASS1 silencing confers a proliferative advantage. Ass1 KO cells grew robustly through arginine starvation in vivo, while ADI-PEG20 remained completely lethal in vitro, evidence that pointed toward a novel mechanism of resistance mediated by the microenvironment. Coculture with Ass1-competent fibroblasts rescued growth through macropinocytosis of vesicles and/or cell fragments, followed by recycling of protein-bound arginine through autophagy/lysosomal degradation. Inhibition of either macropinocytosis or autophagy/lysosomal degradation abrogated this growth support effect in vitro and in vivo.

Conclusions: Noncanonical, ASS1-independent tumor resistance to ADI-PEG20 is driven by the microenvironment. This mechanism can be targeted by either the macropinocytosis inhibitor imipramine or the autophagy inhibitor chloroquine. These safe, widely available drugs should be added to current clinical trials to overcome microenvironmental arginine support of tumors and improve patient outcomes.

Introduction
The enzyme argininosuccinate synthetase 1 (ASS1) is essential for both humans and mice to synthesize arginine, an amino acid that is required for protein synthesis and survival. However, most solid tumors, including many hepatocellular carcinomas, prostate cancers, bladder cancers, small-cell lung cancers, and melanomas, among others, lack a functional level of ASS1, making it one of the most commonly silenced genes in cancers (1–6). This is also true of sarcomas, with more than 85% being deficient in this enzyme (7). Lack of ASS1 causes these tumors to be auxotrophic for arginine, relying on the naturally plentiful extracellular supply for growth and survival.

Studies have shown that ASS1 downregulation can confer proliferation advantages, particularly by reducing aspartate consumption by ASS1 and diverting it toward pyrimidine synthesis (8). However, this has not been established as the reason for frequent silencing of ASS1 in tumors, nor has it been shown to affect initiation or growth in a spontaneous tumor model. In the case of sarcomas, where this phenotype is frequent, the cells of origin should be considered. For example, ASS1 is essentially undetectable in muscle, from which many sarcomas originate, and is also low in adipose tissue (9). This study directly investigates the effects of a conditional knockout of Ass1 in a spontaneous murine sarcoma model.

Despite the advantage that ASS1 deficiency might confer to tumors in a typical environment, this characteristic makes tumors metabolically vulnerable to arginine deprivation. PEGylated arginine deiminase (ADI-PEG20) is an enzymatic drug that degrades extracellular arginine to citrulline, limiting arginine-driven polyamine biology and protein synthesis in cells without ASS1 expression. Treatment with ADI-PEG20 has little effect on most normal cells because they express a functional level of ASS1 to convert citrulline into arginine as part of the urea cycle. However, ASS1-deficient cancer cells, with their high demand for arginine and inability to synthesize it, enter a starvation state and become cytostatic (4, 6, 7, 10–12). Therefore, they must adapt metabolically to survive or die.

Upregulation of autophagy in response to ADI-PEG20 has been shown to compensate in tumor cells without an extracellular source of arginine (4, 6, 7, 13, 14). This adaptation is driven by low levels of
in intracellular arginine inhibiting mTORC1 that in turn causes autophagy to increase. Autophagy can recycle arginine within the cell and provide enough nutrients to sustain the cell in the short term.

To gain long-term resistance to arginine deprivation therapy, studies have shown that these cancers simply increase their expression of ASS1, often through a c-Myc-dependent mechanism, which is possible because the gene is typically epigenetically repressed rather than being mutated or deleted (3, 7, 11, 15). ASS1 upregulation and the development of an autoimmune response are the only published mechanisms of long-term resistance to ADI-PEG20 (16).

Ultimately, cancer cells must undergo a metabolic adaptation to acquire an adequate supply of arginine to grow and proliferate. This may not necessarily be achieved solely by upregulating ASS1 and synthesizing more arginine. There are a multitude of other mechanisms by which cancer cells could theoretically acquire arginine (17). Among others, macropinocytosis, necrocytosis, receptor-mediated endocytosis, phagocytosis, entosis, extracellular vesicle transfer, nutrient sharing through gap junctions, and arginine secretion by nearby cells provide plentiful possibilities (17–29). It is unclear which, if any, of these mechanisms have the capacity to supply a sufficient amount of arginine for tumors to progress in the absence of plasma arginine, but a recent report suggests that macropinocytosis helps to overcome similar aspartate limitations (30). Most import mechanisms would also require the newly acquired materials to be broken down by autophagy/lysosomal degradation to produce free arginine to incorporate into new proteins. This study defines the first alternative mechanism for resistance to arginine starvation whereby the microenvironment supports tumor growth in the absence of ASS1 expression.

Materials and Methods

**Spontaneous murine sarcoma model**

Assf/F mice were bred as previously described (12). Strain #008462 r5/F mice were obtained from The Jackson Laboratory (31). Strain #010529 Myf5/Cre mice were also obtained from The Jackson Laboratory (32). Mice were bred so that all experimental mice were females that were heterozygous for Myf5-Cre and homozygous r5/F. The indicated mice were bred to also be homozygous Assf/F.

**Cell culture**

SKLMS1, SKUT1, and HDFa cell lines were obtained from the ATCC. Atg200/F MEFs were generated from Atg200/F mice, which were made and provided by Dr. M. Komatsu (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; refs. 33, 34). Fip200/F MEFs were generated from Fip200/F mice (35, 36). All MEFs used in experiments were first spontaneously immortalized as previously described (12). All long-term ADI-PEG20-treated (LTAT) cells were developed by passaging cells normally with 1 μg/mL ADI-PEG20 (Polaris Pharmaceuticals) until growth rate stabilized. SKLMS1 and SKUT1 cells were grown in media consisting of minimum essential medium (Thermo Fisher Scientific) with 10% FBS (Bio-Technne), 1% 100 × penicillin/streptomycin (Thermo Fisher Scientific), and 2.5 μg/mL Plasmocin (InvivoGen). All other cell lines were grown in I-20 media, consisting of minimum essential medium (IMDM; Thermo Fisher Scientific) with 20% FBS, 1% 100 × penicillin/streptomycin, 1% 100 × MEM nonessential amino acids (Corning), 0.0007% 2-mercaptoethanol (Millipore Sigma), and 2.5 μg/mL Plasmocin. Phenol red-free versions of these media were used for any experiment requiring quantification of green fluorescence. Arginine-free media was a modified I-20 made with IMDM for SILAC (Thermo Fisher Scientific) and...
dialyzed One Shot FBS (Thermo Fisher Scientific), with l-lysine hydrochloride (Millipore Sigma) added to 146 mg/mL. MEFs were labeled in this same modified I-20 with the addition of 398 μmol/L 13C,15N-L-Arginine hydrochloride (Silantes). Cells were grown in a cell culture incubator with 5% CO2 at 37°C.

Cell line experiments were conducted in 96-well plates and started the day after plating except where indicated. For these plates, cells were plated at a density per well of 3,000 for SKLMS1, HDFa, and MEFs; 5,000 for murine tumor cell lines; and 7,500 for SKUT1. For coculture experiments, cells were plated and allowed to settle and attach together. The growth media of the cell line being counted was used when cell lines in the same well typically used different media. Feeder layers of cells were plated two days before the start of the experiment and treated with mitomycin C (MMC; Millipore Sigma) the next day, immediately before the tumor cells were seeded on top.

MMC treatment was performed by adding 10 μg/mL MMC to cells for 2 hours at 37°C, then washing three times with DPBS (Thermo Fisher Scientific). DiOC18(3) (3,3'-dioctadecyloxacarbocyanine perchlorate; Thermo Fisher Scientific) staining was performed according to the manufacturer’s instructions. All in vitro ADI-PEG20 treatment was done with media that had been pretreated with 1 μg/mL ADI-PEG20 overnight at 37°C to ensure complete degradation of free arginine in the media. EST (aloxistatin; MedChemExpress), imipramine (Millipore Sigma), 5-((N-ethyl-N-isopropyl)-amiloride (EIPA; Millipore Sigma), chloroquine (Millipore Sigma), tonabersat (Cayman Chemical Company), carbenoxolone (Apexbio Technology), PitsStop 2 (Millipore Sigma), docetaxel (Millipore Sigma), and gemicitabine (Millipore Sigma) were administered at the indicated concentrations, and controls were treated with the same volume of vehicle.

**Vesicle uptake assay**

A total of 3 × 10⁴ DiOC18(3)-stained or unstained MEFs were plated in a 10-cm dish, and 10 mL phenol red-free media was added the next day and left on for 24 hours. Media were then taken off MEFs and centrifuged for 5 minutes at 200 × g, and the supernatant was collected. Conditioned media for the supplemental growth support experiment was obtained in the same way with unstained MEFs, plating them at the same density per cm² and adding the same depth of media as in the 96-well plates, and treating with MMC before conditioning media.

MMC-treated BVM01R cells were plated in a 96-well plate at 15,000 cells per well, and media collected from MEFs was pretreated with ADI-PEG20 and added to the BVM01R cells with or without imipramine. This media was collected again after 24 hours, and DiOC18(3) fluorescence was measured on a Tecan Infinite M200 plate reader (Tecan). Data was normalized so that 0% represented the average fluorescence of conditioned media from unstained MEFs and 100% represented the average fluorescence of media from stained MEFs, both without being added to BVM01R cells.

**Heavy arginine transfer assay**

MEFs were cultured in 1-20 with 13C,15N-labeled arginine until all arginine in the cells was labeled as measured by mass spectrometry. A total of 1.33 × 10⁵ 1037 cells expressing a red fluorescent nuclear marker were then plated simultaneously for coculture with 7,95 × 10⁵ MEFs in a 10-cm dish, either labeled or unlabeled, with or without 20 μmol/L imipramine as indicated. Cells were incubated together for 24 hours, then harvested for FACS, keeping imipramine in all solutions for the imipramine-treated sample. 1037 cells were isolated away from MEFs by FACs, using pure MEFs, which were not fluorescent, as a gating control. Mass spectrometry followed by proteomics analysis was then performed on the 1037 cells to determine the extent of heavy arginine labeling, with the unlabeled control being normalized to zero.

**Extracellular vesicle isolation and quantification**

MEFs were plated on five 10-cm dishes of 8 × 10⁶ cells each. The next day, 5 mL of media was added to each plate for 24 hours, then harvested with Total Exosome Isolation Reagent (from cell culture media; Thermo Fisher Scientific) according to the manufacturer’s instructions. Isolated EVs were resuspended in ADI-PEG20 pretreated media and added to 1037 cells in a 96-well plate at the indicated concentrations. For quantification, EVs were suspended in PBS and analyzed by a Nanosight NS300 (Malvern Panalytical).

**Automated cell imaging**

All automated cell imaging was done on cells in 96-well plates with either an Incucyte ZOOM (Sartorius) or Incucyte S3 (Sartorius) automated cell imaging system. Cells that were counted were first transduced with either Incucyte Nuclight Red Lentivirus (EF1a, Puro; Sartorius) or Incucyte Nuclight Green Lentivirus (EF1a, Puro; Sartorius) and selected with puromycin for stable expression. Incucyte software was used to count the red or green nuclei of transduced cells to measure the number of live cells at each timepoint. The cell impermeable DNA-binding dye YOYO-1 Iodide (Thermo Fisher Scientific) was used to stain dead cells and was quantified in the same way. mApple ArgSen expression was measured as the average integrated intensity of the nuclei.

**Immunoblotting**

Cells were plated at 1 × 10⁴ cells per well of a 6-well plate for all immunoblot experiments. Fresh media were added the next day, and the cells were harvested 24 hours later. Cell pellets were lysed in 1 × Cell Lysis Buffer (Cell Signaling Technology) with 1 × Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) added. Mouse tissues were lysed in 2 × Cell Lysis Buffer with 1 × Halt Protease and Phosphatase Inhibitor Cocktail. Tissues were mashed with Bio-masher II Disposable Micro Tissue Homogenizers (DWK Life Sciences). After resuspension or homogenization in lysis buffer, samples were incubated on ice for 20 minutes with periodic vortexing, then sonicated. Samples were centrifuged at 21,130 × g at 4°C for 10 minutes, and the supernatant was collected. Samples were run on a Wes-automated immunoblot machine (Bio-Techne) according to the manufacturer’s instructions, using the primary antibodies indicated in Supplementary Table S2A. Protein amounts were quantified with Compass software for Simple Western (Bio-Techne) and normalized to total protein.

**Cloning**

All primer sequences are listed in Supplementary Table S2B. Primers were ordered from Integrated DNA Technologies. The mApple gene was PCR amplified from plasmid mApple-N1 (Addgene) with SaI BamHI mApple fwd and NotI NLS mApple rev primers to add restriction sites and a C-terminal nuclear localization signal. mApple-N1 was a gift from Michael Davidson (Addgene plasmid #54567; http://n2t.net/addgene:54567; RRID: Addgene_54567; ref. 37). The amplicon was then cloned into the pKLV2-EF1a-GFP ArgSen plasmid (Addgene plasmid #184695; ref. 12) with restriction sites BamHI and NotI, replacing EGFP with mApple. The sensor gene with mApple reporter was then subcloned into the pLV-EF1a-GFP ArgSen plasmid (Addgene plasmid #184704; ref. 12), completely replacing the original insert. This pLV-EF1a-mApple ArgSen plasmid was used for experiments.
The EGFP gene was PCR amplified with BamHI EGFP fwd and BglII EGFP rev primers, then cloned into the MSCV puro plasmid (Plasmid #68469, ref. 38) by digesting BglII on the vector and C terminus of the insert, and BamHI (compatible with BglII) on the N terminus of the insert, followed by ligation. Cloning destroyed the N terminal restriction site and preserved the C terminal BglII site. MSCV Puro was a gift from Tyler Jacks (Addgene plasmid #68469; http://n2t.net/ addgene:68469; RRID: Addgene_68469). ASS1 cDNA was then PCR amplified from a HEK 293T cDNA library with ASS1 fwd and ASS1 rev primers. This PCR product was further amplified while adding restriction sites with primers. This PCR product was then Cloned into the MSCV EGFP puro plasmid with BglII on the N terminus of the insert, linking the ASS1 and EGFP genes, and leaving the C terminus of the insert undigested and ligating with the blunt end left by HpaI digestion of the vector. The resulting GFP-ASS1 fusion sequence was then PCR amplified with AscI EGFP fwd and NotI ASS1 rev primers and cloned into the pLV vector with AscI and NotI restriction sites.

All cloned constructs were integrated into lentiviruses and used to transduce the indicated cells, which were selected with puromycin.

**In vivo arginase sensor**

1.5 million SKLMS1 cells expressing mApple ArgSen were grafted subcutaneously into female NU/J mice (Strain #002019, The Jackson Laboratory) 4–6 weeks of age. All subcutaneous grafts in this study were done by suspending cells in media, mixing 1:1 with Matrigel (Corning), and injecting 100 μl under the skin on the flank. mApple ArgSen mice were fed an alfalfa-free, low fluorescence diet, the Teklad global 18% protein diet (Inovit). Treatment and imaging were started after tumors reached 200 mm³. Images of mApple fluorescence were acquired by a Bruker Multispectral FX Pro system (Bruker), and images were analyzed with Fiji software (fiji.sc).

**Tumor growth experiments**

Five million of the indicated cells were grafted subcutaneously into female C57BL/6j (strain #000664, The Jackson Laboratory) or NU/J mice 4–6 weeks of age, except for the ADI-PEG20, gemcitabine, docetaxel, and imipramine (4-drug) experiment, for which 3 million cells were grafted. Treatments began after tumors reached 200 mm³. ADI-PEG20 treatments were administered by intramuscular injection as 13 μL of 11 mg/mL ADI-PEG20 every three days. Imipramine was dissolved in 138 mmol/L NaCl at 30 mg/mL and administered intraperitoneally at 30 mg/kg/day. Chloroquine was dissolved in DPBS at 20 mg/mL and administered intraperitoneally at 60 mg/kg/day. Docetaxel was dissolved in 10% ethanol (Thermo Fisher Scientific), 90% corn oil (Millipore Sigma) at 5 mg/mL and administered intraperitoneally at 10 mg/kg weekly. Gemcitabine was dissolved in 0.9% (w/v) NaCl at 20 mg/mL and administered intraperitoneally at 125 mg/kg twice weekly. Mice were euthanized after 30 days of treatment or when tumors reached 1,600 mm³ for the imipramine experiment or 2,000 mm³ for the chloroquine experiment. Mice were euthanized after 21 days or when tumors reached 1,600 mm³ for the 4-drug experiment.

**Gene knockout**

**ASS1**+/− MEFs and ASS1FF/FF controls were generated as previously described (12). Atg7FF/FF and Fip200FF/FF MEFs were infected with either Ad5CMVCre or Ad5CMVempty adenoviral particles (University of Iowa, Iowa City, IA) to recombine and knock out the floxed genes or serve as negative controls, respectively. Cells were lysed for genotyping with DirectPCR Lysis Reagent (Cell; Viagen Biotech) following the manufacturer’s instructions. **ASS1** genotyping used Ass1 del Fwd, Ass1 FF Fwd, and Ass1 geno rev primers. **Atg7** genotyping used Atg7 geno Fwd, Atg7 FF Rev, and Atg7 del rev primers. Fip200 genotyping used Fip200 del Fwd, Fip200 FF Fwd, and Fip200 geno rev primers. All gene knockouts were also validated by immunoblot.

**Metabolomics**

Blood and tumors were harvested from NU/J mice harboring SKLMS1 tumors at the indicated timepoints. Blood was allowed to clot at room temperature for 30 minutes, then centrifuged at 1,500 × g for 10 minutes at 4°C. The supernatant serum was collected and immediately stored at –80°C. Tumors were flash frozen upon harvesting and stored at –80°C. Samples were shipped on dry ice for metabolomic analysis at Human Metabolome Technologies America.

**RNA sequencing**

RNA was isolated from frozen tumor samples with the Direct-zol RNA Miniprep Plus kit (Zymo Research). RNA samples were submitted to the Genome Technology Access Center at the McDonnell Genome Institute (GTAC@MGI) of Washington University in St. Louis. GTAC@MGI prepared the RNA using the KAPA RiboEase method, sequenced with an Illumina NovaSeq sequencer (Illumina), and analyzed with their standard RNA-sequencing (RNA-seq) analytic pipeline.

**Preclinical PET imaging**

Female NU/J mice harboring SKLMS1 tumors (n = 4) underwent small-animal PET imaging one day prior to ADI-PEG20 administration (NT) and 13 days after starting treatment. The mice were anesthetized with 1% isoflurane/oxygen followed by dynamic PET 0–60–minute acquisition on the Inveon PET Scanner (Siemens) after intravenous tail injection of 8–12 MBq of [18F]4-FGln. The animals were deprived of food for 4 hours prior to the [18F]4-FGln studies. The animals were maintained at 37°C during the study using a warming lamp. CT images were also acquired with the Inveon system.

The PET data were analyzed by manually drawing three-dimensional regions of interest (ROI) over the tumor identified on the PET studies with correlation to CT to confirm the tumor location. The uptake data were expressed as mean standardized uptake values (SUV) for each ROI at 50–60 minutes after injection of [18F]4-FGln.

**Quantification and statistical analysis**

Statistical tests were performed in GraphPad Prism 9 software. Differences between time series were analyzed by two-way ANOVA, using a mixed model when necessary. Grouped data were analyzed by Mann–Whitney test, Kolmogorov–Smirnov test, paired t test, or unpaired t test as appropriate. Time-to-event data were analyzed by log-rank (Mantel–Cox) test. All error bars show SD, except tumor growth error bars show SEM. All statistical details of experiments can be found in the ssure legends. P values are denoted in the following way: ns: P > 0.05; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

**Data availability**

All unique/stable reagents generated in this study are available from the corresponding author with a completed Materials Transfer Agreement. RNA-seq data have been deposited at NCBI GEO and are publicly available as of the date of publication with accession number GSE206382. All other data are available in the main text or the Supplementary Materials. This article does not report original code. Any additional information required to reanalyze the data reported in this article is available from the corresponding author upon request.
Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author.

**Results**

Arginine starvation fails to inhibit protein translation in vivo

As has been demonstrated previously in ASS1-deficient sarcoma cell lines (7, 10), our work similarly demonstrates that the proliferation of human sarcoma cell line SKLMS1 is significantly perturbed when treated with ADI-PEG20 (Fig. 1A) because of very low to no ASS1 expression. Although it slows growth, ADI-PEG20 does not cause a meaningful level of cell death in these cells (Fig. 1B). SKLMS1 can become resistant to ADI-PEG20 with long-term exposure by upregulating the expression of ASS1, and resistant cells are called LTAT (Fig. 1C).

To test the kinetics of resistance to acute ADI-PEG20 treatment of ASS1-deficient cancer cells, we monitored intracellular arginine-dependent translational capacity of SKLMS1 under arginine starvation (12). The ArgSen sensor is a constitutively expressed gene that codes for a rapidly degraded reporter protein downstream of an arginine-rich region, causing translation of the reporter to significantly slow and overall levels to drop when arginine availability limits translation (12). mApple, chosen for its ability to serve as a reporter protein for both in vitro and in vivo experiments, was cloned into the previously published lentiviral construct for the ArgSen (12). Matching previously published data using other reporters (12), ADI-PEG20 caused a rapid decrease in expression of the sensor mApple reporter in vitro compared to no treatment (Fig. 1D). In contrast, despite the fact that ADI-PEG20 slowed growth similarly in vitro and in vivo (Fig. 1A–E), no decrease in sensor expression was found in vivo (Fig. 1F). Representative sensor fluorescence images are shown in Supplementary Fig. S1A.

Metabolite measurements revealed that ADI-PEG20 was indeed very effective in vivo, decreasing arginine levels in serum to near zero within two days and suppressing them for the duration of treatment (Fig. 1G). Correspondingly, citrulline levels increased and reached a steady state within 7 days (Fig. 1H). Metabolite measurements in the tumors showed a similar but less dramatic pattern, with the tumors
losing 81% of their arginine by two days and 90% by 15 days (Fig. 1). Citrulline levels likewise increased in the tumors as in the serum (Fig. 1). These decreased arginine concentrations resulting from ADI-PEG20 treatment caused metabolic changes in the tumors. To confirm that a metabolic shift to glutamine biology was still occurring in vivo as shown previously in vitro (10), PET was used to estimate utilization of glutamine by the tumors by measuring the uptake of analogue molecule $^{18}$F-2(S,4R)-fluoroglutamine ($^{18}$F-4-FGln).

ADI-PEG20 indeed caused increased uptake of glutamine in SKLMS1 tumors (Supplementary Fig. S1B and S1C). This suggests that the metabolic shifts caused by ADI-PEG20 hold in this system, but that cells in the intratumoral environment rapidly compensate for the loss of arginine and are able to maintain arginine-dependent translation.

**ASS1 deficiency is not advantageous for spontaneous murine sarcomas**

To better understand the effects of the loss of ASS1 on tumor biology, a genetic model was made with Myf5-driven Cre expression causing recombination of floxed p53 genes in both groups and floxed Ass1 genes in only one group of a spontaneous murine sarcoma experiment, resulting in nonfunctional protein products (Fig. 2A). 59% of resulting tumors were identified morphologically as pleomorphic rhabdomyosarcomas, and 37% were osteosarcomas (Supplementary Table S1B), with images shown in Fig. 2B and C. Knocking out Ass1 did not significantly change the rate of tumor initiation in this sarcoma model (Fig. 2D). Likewise, the difference in growth rates between the two groups was not significant (Fig. 2E).

When ASS1 protein was measured by immunoblot of frozen tumors, all Ass1 null tumors had low ASS1 (Fig. 2F). A small amount of ASS1 was detected in these tumors, indicating the presence of stromal cells that did not express Myf5-Cre and therefore had an intact Ass1 gene. Eighteen of 26 (69%) Ass1 WT tumors also expressed ASS1 at a level lower than the highest-expressing Ass1-null tumor (Fig. 2F). This group of tumors was then segregated by ASS1 expression for further analysis. There was a trend toward earlier initiation in tumors with high ASS1, and when compared with Ass1-null tumors, the ASS1-high tumors appeared significantly sooner (Fig. 2G). ASS1-high tumors also had significantly higher growth rates than ASS1-low tumors (Fig. 2H).

**Cells without ASS1 grow robustly through arginine deprivation in vivo but die in vitro**

Cell lines were made from many of the spontaneous sarcomas from Fig. 2 (Supplementary Table S1A). Those cell lines with low ASS1 from both Ass1-null and WT tumors were shown to be highly sensitive to ADI-PEG20 and unable to proliferate after several hours, while many cells died within the first few days of treatment (Fig. 3A and B). Not all Ass1 WT tumor cell lines died with ADI-PEG20 treatment, as BVMO3O showed only slowed proliferation (Fig. 3A), similar to SKLMS1, likely due to its higher level of ASS1 (Supplementary Fig. S2A). ASS1-low cell lines differed in their responses to long-term ADI-PEG20 treatment depending on their genotype. Ass1-null cell lines invariably died completely when treated with ADI-PEG20, while all Ass1 WT cell lines developed resistance over weeks to months of treatment (Supplementary Table S1A).

To determine the effects of ADI-PEG20 on the proliferation of Ass1-null cells in vivo, multiple Ass1-null murine sarcoma cell lines were injected subcutaneously into either syngeneic C57BL/6J mice or NU/J mice. There, very little effect of ADI-PEG20 was found in NU/J mice in two of the three cell lines tested, whereas ADI-PEG20 seemed to slow but not stop growth in immune-competent syngeneic C57BL/6J mice (Fig. 3C and D; Supplementary Fig. S2B).

Crucially, Ass1 null tumors from all tested cell lines were able to grow robustly through ADI-PEG20 treatment in vivo while the drug remained 100% lethal to their parental cell lines in vitro, illustrating a stark contrast in the effectiveness of ADI-PEG20 between the different environments and explaining the in vivo arginine sensor data (Fig. 1F).

**EVs from ASS1-competent MEFs enable Ass1 KO tumor cell growth during arginine deprivation**

To further investigate this phenomenon, BVMA01R Ass1-null tumors that had grown through ADI-PEG20 were taken ex vivo and made into cell lines. These cells remained sensitive to ADI-PEG20 in vitro (Fig. 4A) and could not survive long-term treatment. When cultured on a feeder layer of MEFs, these cells were more resistant to death, and the shown cell line from mouse 1037 (1037 tumor cells) accepted support from the MEFs well enough to proliferate through ADI-PEG20 treatment (Fig. 4A). All tested Ass1-low cell lines showed a similar ability to accept growth support from fibroblasts during ADI-PEG20 treatment, including Ass1 WT murine sarcoma lines (Supplementary Fig. S3A and S3B). The human sarcoma cell lines SKLMS1 and SKUT1, which expresses slightly more ASS1 (10), also accepted support from human fibroblasts (Supplementary Fig. S3C and S3D). While the observed growth support was far less robust than what tumors received in vivo, this model provided an opportunity to investigate the mechanisms by which arginine auxotrophic cell proliferation could be supported by other cells in the absence of free extracellular arginine.

Coculturing 1037 tumor cells with MEFs rather than growing them on a MEF feeder layer provided a similar growth support effect (Fig. 4B). This effect could not be sustained unless the MEFs expressed ASS1 (Fig. 4B). Knockout of Ass1 in MEFs was validated in Supplementary Fig. S4A–S4D. Multiple possible mechanisms of growth support were investigated, and it was found that neither MEF-conditioned media nor inhibition of gap junctions or clathrin-mediated endocytosis could recreate or prevent the growth support phenomenon, respectively (Supplementary Fig. S5A–S5C). When MEFs were stained with a green fluorescent membrane dye, large fragments of MEF membranes were seen disconnected from their cells of origin, making contact with the tumor cells, showing that cell fragments and extracellular vesicles (EV) could likely be transferred from MEFs to tumor cells (Fig. 4C).

To test whether this was a major mechanism by which MEFs supported tumor cell growth, EVs were isolated from MEFs. Nanoparticle tracking analysis revealed that the vast majority of vesicles from both untreated and ADI-PEG20-treated MEFs had diameters between 100 and 300 nm (Fig. 4D), which corresponds to microvesicles that bud directly from the cell membrane, rather than the smaller (<100 nm diameter) exosomes. These EVs were added to 1037 tumor cells in the presence of ADI-PEG20, which protected from death and supported growth similarly to co-cultured MEFs (Fig. 4E).

In tests comparing arginine-free media to ADI-PEG20 treatment, 1037 tumor cells were able to grow modestly after approximately two days in arginine-free media monoluculture, differing significantly from ADI-PEG20 treatment, while co-culture with MEFs nonsignificantly increased proliferation (Fig. 4F). Under these arginine-free conditions, addition of EVs isolated from MEFs clearly enabled increased proliferation, to an even higher degree than with ADI-PEG20 treatment (Fig. 4G).
**Ass1 KO tumor cells macropinocytose EVs to overcome arginine deprivation**

Furthermore, the EV production inhibitor EST completely abrogated the growth-supportive effect of MEFs (Fig. 5A) while not affecting the growth of the MEFs themselves in the presence of ADI-PEG20 (Supplementary Fig. S5D), indicating that the growth support was not diminished because of decreased MEF viability. Another EV production inhibitor, imipramine, had a similar effect to EST (Fig. 5B), even though the two inhibitors target different pathways of EV production (39, 40).

As imipramine can also inhibit macropinocytosis (41), to elucidate the contributions of each of its inhibitory activities, we compared its effects to those of 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), a commonly used inhibitor of macropinocytosis. Both compounds inhibited the growth of the tumor cells at the same concentrations (Fig. 5C). However, EIPA was much more toxic to the MEFs in coculture.
Tumor growth of isolated from spontaneous murine sarcomas. (Fig. 3D). This suggests either that EIPA has other unknown toxic effects besides macropinocytosis inhibition, or that imipramine inhibits macropinocytosis less strongly at equivalent concentrations, indicating that its role in preventing EV production contributes to its anticancer activity in this context. To determine whether macropinocytosis inhibition was contributing to the inhibition of growth support by imipramine, the effect of imipramine on the uptake of fluorescently labeled membranes from MEFs into 1037 cells was measured. We found that imipramine significantly decreased the amount of membrane that 1037 cells took from the media over 24 hours when treated with ADI-PEG20 (Fig. 5E). In further experiments, MEFs with heavy arginine-labeled proteins were shown to transfer this heavy label to 1037 cells when cocultured, suggesting that 1037 cells ingest proteins originating from MEFs (Fig. 5F). This process is significantly inhibited by imipramine (Fig. 5F).

Imipramine was then tested for its ability to slow growth of BVMA01R tumors in vivo in combination with ADI-PEG20 due to its inhibition of macropinocytosis in this model. The combination of ADI-PEG20 and imipramine significantly slowed tumor growth, while either single agent did not (Fig. 5G). Although mouse weights changed statistically significantly on combination treatment, they remained mostly steady and tolerable, with a maximum average weight loss of 10% before recovering (Fig. 5H). Tumor inhibition was not due to lower ASS1 expression, as no treatment group differed significantly in tumor ASS1 levels compared with untreated (Fig. 5I).

**Autophagy/lysosomal degradation is required for cells receiving but not cells supplying growth support**

As ingested proteins must be degraded by tumor cells to obtain useful arginine, the autophagy/lysosomal degradation inhibitor chloroquine was tested with the coculture of 1037 cells and MEFs to determine whether autophagy was essential to the growth support process. Chloroquine inhibited growth of the tumor cells in coculture with MEFs and ADI-PEG20 (Fig. 6A).

To determine whether autophagy was essential for the growth support process in the tumor cells, MEFs, or both, two MEF cell lines were generated with either Atg7 or Fip200 floxed. These genes were then knocked out with Cre to inhibit autophagy specifically in the MEFs (Supplementary Fig. S4E–S4I). The effects on coculture growth support were tested, and both floxed cell lines were able to support proliferation of 1037 cells in the presence of ADI-PEG20. Atg7 KO MEFs supported growth to a lesser extent than their floxed counterparts, while Fip200 KO MEFs were completely unable to support growth (Fig. 6B and C). These results mirrored the effects of the gene knockouts on the MEFs themselves, as the Atg7 KO MEFs stayed relatively healthy in the presence of ADI-PEG20, while the Fip200 KO MEFs died rapidly due in part to extremely low ASS1 (Supplementary...
Figure 4.
EVs from ASS1-competent MEFs enable ASS1 KO tumor cell growth during arginine deprivation. A, Proliferation of ASS1 KO murine sarcoma cell line (1037) taken ex vivo after growing through ADI-PEG20 treatment in vivo. 1037 cells die when treated with ADI-PEG20, but they grow through ADI-PEG20 when cultured on feeder layers of various numbers of MMC-treated MEFs. B, Proliferation of 1037 cells when treated with ADI-PEG20 and cocultured with MEFs that have ASS1 either floxed or knocked out. See also Supplementary Fig. S4A–S4D. C, Images of 1037 cells with red nuclei cocultured with MEFs with green-stained membranes with and without ADI-PEG20. Black arrows indicate examples of large fragments of MEFs contacting 1037 cells. Images are cropped for clarity. D, Size distributions of EVs isolated from MEFs grown with and without ADI-PEG20. E, Proliferation of 1037 cells when treated with ADI-PEG20 with or without the addition of EVs isolated from MEFs. F, Proliferation of 1037 cells grown in media either without arginine or with ADI-PEG20, both with and without MEFs. G, Proliferation of 1037 cells in arginine-free media with or without the addition of EVs isolated from MEFs. Data are mean ± SD (n = 2 in A; n = 3 in B, E, F, G; n = 5 in D). Two-way ANOVA tests for A, B, E, F, G.
To separate the effects of MEF viability from the effects of MEF autophagy, GFP-ASS1 was exogenously expressed in these same cell lines, giving them ample resistance to ADI-PEG20 (Supplementary Fig. S4G–S4J). The ASS1-expressing floxed MEFs provided slightly more growth support than their Ass1 WT counterparts (Fig. 6B and C), and ASS1 expression rescued the ability of Atg7 and Fip200 KO MEFs to provide growth support (Fig. 6B and C).
When BVMA01R-derived tumors were treated in vivo with both ADI-PEG20 and chloroquine, only the combination significantly slowed tumor growth (Fig. 6D). Although mouse weights changed statistically significantly on combination treatment, they remained mostly steady and tolerable, with a maximum average weight loss of 5% before recovering (Fig. 6E). Tumor inhibition again was shown to not be due to a difference in tumor ASS1 levels among the groups, as none significantly differed from untreated (Fig. 6F). RNA-seq of these tumors revealed many differentially regulated genes (Supplementary Fig. S6A) and pathways. Relevant pathways are shown in Supplementary Fig. S6B. Notably, ADI-PEG20 greatly increased expression of genes related to cell adhesion, supporting the observation that ADI-PEG20 promoted cell–cell contacts and clustering of these cells in vitro, an effect that was more prominent in the presence of MEFs (Supplementary Fig. S6C). Phagosome and lysosome pathways were also upregulated, supporting the hypothesis that the tumor cells uptake and digest bulk nutrients from outside the cell. Eighteen of the 20 most differentially regulated pathways with ADI-PEG20 treatment are upregulated, with the cell-cycle pathway being a notable exception (Supplementary Fig. S6D). In addition, 13 of the 20 pathways are related to the immune system, which may indicate increased immune cell infiltration, a possibility that is under current investigation.

Finally, given current clinical development of ADI-PEG20 in combination with gemcitabine and docetaxel, which is now in a phase III clinical trial (NCT05712694), and to further investigate the clinical potential of chloroquine or imipramine in addition to current ADI-PEG20 combination therapies, these two drugs were separately tested with the combination of ADI-PEG20, gemcitabine, and docetaxel. While the triple treatment with ADI-PEG20 and gemcitabine/docetaxel very effectively killed ASS1 KO tumor cells in coculture with MEFs, neither chloroquine nor imipramine increased toxicity in vitro (Supplementary Fig. S7A and S7B). Therefore, we tested the four-drug combination with imipramine in vivo, which showed significantly enhanced tumor shrinkage and suppression through three weeks compared with ADI-PEG20 with GEM/DTX (Supplementary Fig. S7C and S7D). This is another clinically promising combination, but gemcitabine/docetaxel does cause some weight loss in combination with ADI-PEG20, which is slightly more severe when imipramine is added (Supplementary Fig. S7E).

**Discussion**

On the basis of the published literature describing the lack of ASS1 in many cancers and the potential advantages this condition confers,
knocking out Ass1 in addition to p53 in a murine sarcoma model was expected to result in faster initiation and/or faster growth of tumors. This was not the case, due to the naturally low levels of ASS1 in most of the Ass1 WT tumors. In retrospect, the spontaneous murine sarcoma model should have been expected to roughly recapitulate the finding of naturally low ASS1 in most human sarcomas. Muscle, the main tissue of tumor origin in this model, expresses as little ASS1 as any tissue in the body (9). This fact combined with the results of this study suggest that the main reason for a lack of ASS1 in many sarcomas is simply inheritance of suppressed ASS1 expression from their cells of origin. This interpretation conflicts with studies showing advantages of ASS1 suppression in cancer cells (8, 42). In fact, ASS1 upregulation gives an advantage to tumors in this model that opposes the prevailing thought on the topic, and is therefore a focus of further investigation.

When using arginine deprivation therapy to target ASS1-deficient cancers, it was surprising that SKLMS1 tumors did not decrease their translational capacity when treated with ADI-PEG20 in vivo. This was the first bit of evidence indicating that the in vitro and in vivo environments may prove to be crucially different in the context of arginine deprivation therapy. When all tested Ass1 KO tumors were able to grow rapidly through ADI-PEG20 treatment in multiple mouse strains, it was clear that a novel mechanism of resistance to arginine deprivation therapy must be responsible. The evidence pointed strongly toward microenvironmental metabolic support of tumor growth.

This metabolic adaptation to ADI-PEG20 was confirmed to not be intrinsic to the cancer cells, but could be recapitulated to a lesser extent in vitro by co-culturing tumor cells with ASS1-competent MEFs, further supporting the hypothesis of microenvironmental growth support. While most major routes of possible nutrient transfer were tested, experiments showed that the most important path in this system in vitro is likely the excretion of EVs and/or cell fragments from MEFs and subsequent uptake of these membrane-bound particles into tumor cells by macropinocytosis, followed by recycling of the proteins within to supply the tumor cells with arginine (Fig. 7). This recycling seems to be achieved largely through autophagy/lysosomal degradation, as shown by the results of chloroquine treatment. It was further determined that the mechanism of growth support does not require autophagy in the cells providing the support but rather the cells receiving the nutrients.

These findings result in new clinical opportunities for ADI-PEG20 combination therapies and clinical trials. Autophagy proved critical enough in this system that chloroquine had pronounced synergy in slowing tumor growth in combination with ADI-PEG20 in vivo. Imipramine, an inhibitor that theoretically targets the tumors more specifically than chloroquine in this system, largely by inhibiting macropinocytosis, also showed clear synergy with ADI-PEG20 in vivo. In addition, RNA-seq strongly suggests that ADI-PEG20 recruits immune cells to tumors. This agrees with preclinical and phase I clinical trial data showing that ADI-PEG20 increases T-cell infiltration in advanced solid tumors (43). The increased cell adhesion phenotype caused by ADI-PEG20 may also be a clinically relevant avenue for future research, as it has the potential to reduce metastasis. Arginine depletion has been shown to disturb actin filaments in endothelial tip cells, causing disordered migratory direction and decreased migration ability (44). Arginine starvation also affected human glioblastoma cell morphology, significantly inhibited their motility and invasiveness, and impaired adhesion, demonstrating another effect on the actin cytoskeleton (45). Similar results were observed with arginine depletion when examining pancreatic cancer cells (46).

This study also elucidates the magnitude of the ability of the microenvironment to metabolically support tumor growth. The system featured totally arginine-auxotrophic cancer cells, in conditions that would otherwise be lethal, growing through ADI-PEG20-induced arginine starvation robustly with the help of host cells that were able to synthesize arginine and provide it to the tumor. Some experiments even showed no apparent effect of ADI-PEG20 on the growth of the Ass1 KO tumors, indicating the magnitude to which this phenomenon.

**Figure 7.**
Overview of growth support. Tumor stromal cells produce EVs that contain proteins and other nutrients. During arginine deprivation therapy, ASS1-deficient cancer cells take up these EVs and use autophagy/lysosomal degradation to recycle the ingested proteins and produce sufficient free arginine for survival and growth. (Created with BioRender.com.)
can assist tumors. To our knowledge, there have been no reports of microenvironmental tumor growth support to this extent regarding arginine.

Overall, we have identified a new noncanonical mechanism for ADI-PEG20 resistance and a first step in metabolic adaptation to ADI-PEG20. While re-expression of ASS1 via c-Myc expression is the canonical adaptation mechanism, macropinocytosis alleviates tumors acutely as the first step in overcoming arginine starvation in arginine-auxotrophic tumors. These finds explain the negative phase III clinical trials in hepatocellular carcinoma (HCC) (47), where ADI-PEG20 was found to lower circulating arginine levels but not to improve tumor progression-free survival or overall survival. In addition, this work suggests that new clinical trials using chloroquine, imipramine, or a combination of both with ADI-PEG20 in HCC may overcome the ability of the microenvironment to compensate for ADI-PEG20-induced starvation.

As has become clear from multiple clinical trials, amino acid starvation in arginine-auxotrophic tumors requires multiple agents. Currently, ADI-PEG20 is being tested in combination with pemetrexed and cisplatin in mesothelioma (NCT02709512; ref. 48) and with docetaxel and gemcitabine in sarcoma (NCT pending; ref. 49). Although imipramine and chloroquine do not have strongly established records as effective clinical cancer therapies, this study provides evidence that they are likely to be effective in combination with certain rationally selected therapies. Our observations suggest that the addition of chloroquine or imipramine to the above regimens would prevent macropinocytosis and increase the efficacy of arginine starvation as a therapeutic. The complexity of cancer metabolic redundancies is demonstrating that development of agents such as ADI-PEG20 will require not only a full understanding of metabolic adaptations, but also that we must target the urea cycle and the adaptive pathways simultaneously.

Authors’ Disclosures

A. Baker reports personal fees from Washington University in St. Louis 2018 Angen Scholars Program Award during the conduct of the study. J.S. Bomalaski reports other support from Polaris Pharmaceuticals during the conduct of the study, other support from Polaris Pharmaceuticals outside the submitted work; in addition, J.S. Bomalaski has a patent for Polaris Pharmaceuticals issued and licensed to Polaris, and is employed by Polaris Pharmaceuticals, Inc. E. White reports Research funding from Deciphera Therapeutics. B.A. Van Tine reports grants, personal fees, and other support from Polaris during the conduct of the study; personal fees from CytokineInc. Inc., Bayer, Deciphera Pharmaceuticals, Daichi Sankyo Inc., EcorR1, Advenchen, Putnam, Salarius Pharmaceuticals, Inc., Boxer Capital LLC, Acuta Capital Partners, LLC, Audi Biosciences, Iteron Therapeutics, Inc., Total Health Conference, Apexigen Inc, PTC Therapeutics, Boehringer Ingelheim, Agenus, Regeneron Pharmaceuticals, and EcorR1 Capital, LLC, outside the submitted work. No disclosures were reported by the other authors.

Authors’ Contributions


Acknowledgments

This work was supported by NIH/NCI grants R01-CA227115 (B.A. Van Tine), and U2CA2309837 (to K.I. Shoghi), Siteman Cancer Center Support Grant P30 CA181254 (to K.I. Shoghi), Siteman Investment Program (to K.I. Shoghi), NIH instrumentation grant S10-OD018515 (to K.I. Shoghi), NIH instrumentation grant S10-OD034043 (to K.I. Shoghi), Sarcoma Foundation of America (to B.A. Van Tine), CJ’s Journey (to B.A. Van Tine), Sarcoma Alliance for Research Through Collaboration (to B.A. Van Tine), and Barnes Foundation (to B.A. Van Tine). The authors thank Dr. Jason Lewis at MSKCC for providing the precursor and standard for [18F]-4-FGln as a gift.

The publication costs of this article were defrayed in part by the payment of publication fees. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Received August 25, 2022, revised April 11, 2023; accepted June 15, 2023, published first June 20, 2023.

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