Prognostic and Therapeutic Impact of Argininosuccinate Synthetase 1 Control in Bladder Cancer as Monitored Longitudinally by PET Imaging

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Abstract

Targeted therapies have yet to have significant impact on the survival of patients with bladder cancer. In this study, we focused on the urea cycle enzyme argininosuccinate synthetase 1 (ASS1) as a therapeutic target in bladder cancer, based on our discovery of the prognostic and functional import of ASS1 in this setting. ASS1 expression status in bladder tumors from 183 Caucasian and 295 Asian patients was analyzed, along with its hypothesized prognostic impact and association with clinicopathologic features, including tumor size and invasion. Furthermore, the genetics, biology, and therapeutic implications of ASS1 loss were investigated in urothelial cancer cells. We detected ASS1 negativity in 40% of bladder cancers, in which multivariate analysis indicated worse disease-specific and metastasis-free survival. ASS1 loss secondary to epigenetic silencing was accompanied by increased tumor cell proliferation and invasion, consistent with a tumor-suppressor role for ASS1. In developing a treatment approach, we identified a novel targeted antimetabolite strategy to exploit arginine deprivation with pegylated arginine deiminase (ADI-PEG20) as a therapeutic. ADI-PEG20 was synthetically lethal in ASS1-methylated bladder cells and its exposure was associated with a marked reduction in intracellular levels of thymidine, due to suppression of both uptake and de novo synthesis. We found that thymidine uptake correlated with thymidine kinase-1 protein levels and that thymidine levels were imageable with [18F]-fluoro-L-thymidine (FLT)–positron emission tomography (PET). In contrast, inhibition of de novo synthesis was linked to decreased expression of thymidylate synthase and dihydrofolate reductase. Notably, inhibition of de novo synthesis was associated with potentiation of ADI-PEG20 activity by the antifolate drug pemetrexed. Taken together, our findings argue that arginine deprivation combined with antifolates warrants clinical investigation in ASS1-negative urothelial and related cancers, using FLT-PET as an early surrogate marker of response. Cancer Res; 74(3): 896–907. ©2013 AACR.

Introduction

Urothelial carcinoma is the fourth most common malignancy diagnosed in men, and the seventh most common cause of solid cancer-related death in the United States, accounting for 15,000 deaths per annum (1). The 5-year overall survival rate of 40% to 60% for muscle invasive bladder cancer has plateaued over the past three decades, in part, due to the frequent comorbidities in this patient group (2). Moreover, despite $4.0 billion spent each year on bladder cancer treatment in the United States alone—one of the highest expenditures in...
oncology—targeted therapies have been ineffective to date in this disease (3).

Recently, arginine auxotrophy or the absolute requirement for exogenous arginine for cellular growth has been detected in several chemoresistant solid cancers, including hepatocellular carcinoma, melanoma, mesothelioma, and prostate cancer (4–6). Arginine fuels an array of biosynthetic reactions, including proteins, nitric oxide (NO), polyamines, agmatine, and the amino acids proline and glutamate, and therefore may modulate tumorigenesis at multiple levels (7). Inactivation of the pleiotropic enzyme, argininosuccinate synthetase 1 (ASS1), with key roles in the urea cycle, citrulline–NO cycle, and arginine biosynthesis has emerged as a principal driver of tumoral arginine auxotrophy, with evidence for epigenetic silencing and hypoxia-inducible factor-1α (HIF-1α)–mediated transcriptional repression of ASS1 (5, 8, 9). Significantly, ASS1 loss has been associated with decreased overall survival in ovarian cancer and myxofibrosarcoma, and reduced metastasis-free survival in osteosarcoma, implicating a tumor-suppressor function for this metabolic gene (10–12). Thus, arginine deprivation is being tested increasingly in the clinic, modeled on the successful introduction of another amino acid depletor, namely asparaginase, in the management of acute lymphoblastic leukemia five decades ago (13). Specifically, several early-phase trials of the arginine-degrading mycoplasmal enzyme, pegylated arginine deiminase (ADI-PEG20), have led to phase II/III randomized studies in melanoma, hepatocellular cancer, and mesothelioma (14–16).

Here, we characterized ASS1 in bladder cancer based on the fact that loss of chromosome 9, including q34, the locus of ASS1, is a known early event in urothelial tumorigenesis (17). Previously, Linnenbach and colleagues provided evidence for a truncation mutation at the ASS1 locus in a ureteric carcinoma sample, while searching for tumor suppressors at 9q34 (18). Here, we show that ASS1 negativity is a common event in bladder cancer detected in approximately 40% of tumors by immunohistochemistry (IHC). Furthermore, to address a putative tumor-suppressor role, we performed additional genetic and epigenetic studies of ASS1 using a panel of bladder cancer cell lines. Finally, we studied the therapeutic consequences of ASS1 loss in bladder cancer, identifying a novel targeted antimetabolite strategy that combines arginine deprivation with the antifolate, pemetrexed. These studies were reinforced by metabolomic and positron emission tomography (PET) tracer analyses, which identify intracellular thymidine levels as a novel biomarker of early treatment response.

Materials and Methods

Primary tumor characteristics

Tissue microarrays (TMA) were constructed using a training set of biopsy samples, comprising 183 urothelial carcinomas collected at the Cancer Research UK Cambridge Research Institute (19), and an independent test set of 295 urothelial carcinomas, which were resected consecutively with curative intent in Chi Mei Medical Center, Taiwan, between January 1996 and March 2004. Institutional review boards in the United Kingdom (Cambridgeshire Local Research Ethics Committee) and Taiwan (IRB971006) ratified the tissue procurement. The diagnostic criteria were based on the updated American Joint Committee on Cancer Staging.

IHC

ASS1 IHC was performed using liver control sections as described previously (5). The scoring of thymidylate synthase (TS; 1:100, Clone EPR4545; Epitomics) and dihydrofolate reductase (DHFR;1:100, Clone EPR5285; Epitomics) was performed using an H-score, determined by the percentage and intensity of positively stained tumor cells with cases stratified according to high (no less than the median score) or low expression (less than the median score; ref. 20).

Cell lines and culture

Six bladder cancer cell lines were grown in a humidified atmosphere at 37°C and 5% CO2 in endotoxin-free RPMI medium with 10% FBS (RT112, 5637, SCaBER, 253J, and T24) or Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS (UMUC3). The arginine concentration was approximately 10-fold higher in the culture medium than found in human serum (i.e., 1 mmol/L vs. 0.06–0.1 mmol/L, respectively). Experiments were performed using cells at 70% confluency, which were harvested for analysis of ASS1 promoter methylation, constitutive ASS1 mRNA, and protein. Studies of corroborative cell lines in a nonurothelial solid cancer (mesothelioma) were performed and all cell lines were authenticated by short tandem repeat profiling (LGIC Standards).

Methylation-specific PCR

One microgram of genomic DNA was modified with sodium bisulphite using the EZ DNA methylation kit (Zymo) according to the manufacturer’s instructions. Then, 50 ng of modified DNA was amplified using the following primers designed using the ASS1 promoter sequence: UF (5′ GTAGAGGGGAAGGTTTTT); UR (5′ ACAAAAACAAATAACCCAAAA); MF (5′ GTAGAGGGGAAGGTTTTT); and MR (5′ GCACAAACAAAAATCCGGA), in which U = unmethylated and M = methylated. Control CpGenome universal methylated and unmethylated DNA were obtained from Merck Millipore.

Pyrosequencing

Methylation in the -C-phosphate-G- (CpG) islands of the ASS1 gene in the urothelial cell line panel was also analyzed using pyrosequencing technology. For pyrosequencing of the paraffin primary bladder cancer samples, PCR amplification and sequencing primers were optimized using the PyroMark Assay Design Software 2.0. Details of PCR primers and conditions are available on request.

Sequencing analysis

The coding exons and flanking sequences of ASS1 were analyzed by Sanger sequencing in forward and reverse in the panel of bladder cancer cell lines. Details of PCR primers and conditions are available on request.

Quantitative real-time reverse transcriptase PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse-transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the
manufacturer’s instructions. Multiplex PCR was performed on an ABI Prism 7500 Real-Time PCR Instrument (Applied Biosystems). Primers and TaqMan probes for ASS1 (FAM) and 18S (VIC) were obtained from Applied Biosystems (TaqMan Gene Expression Assays).

**ASS1 overexpression and knockdown**

ASS1 overexpression was performed in 253J, UMUC-3, and T24 bladder cancer cells using ASS1pIREShyg3 (or control) vector under hygromycin (200 μg/mL) selection, as described previously (10). ASS1 knockdown in 5637 bladder cancer cells was performed using the pSilencer 4.1-CMVpuro vector (Ambion) containing short hairpin RNA (shRNA) sequences targeting ASS1 (SMARTpool; Dharmacon) or a nontargeting control shRNA under puromycin (1 μg/mL) selection.

**Invasion assays**

Briefly, transwells (Corning, 3422) were coated with Matrigel (BD Bioscience, 354234) diluted 1:3 with serum-free DMEM and allowed to set for 1 hour at 37°C. Transwells were placed in the wells of a 24-well plate containing 500 μL of DMEM (PAA) with 10% serum. 3 x 10^4 cells in 200 μL of serum-free DMEM were plated into the top of the transwell, and incubated at 37°C for 48 hours. Transwells were removed from the DMEM + 10% FBS and placed into 500 μL of Trypsin/EDTA (PAA) for 1 hour at 37°C. The undersides of transwells were then washed with trypsin and the cell suspension transferred to 9.5 mL of Isoton (Coulter), and the number of cells quantified on a CASY counter. All experiments were repeated at least three times using quintuplicate readings.

**Immunoblotting**

Western blot analysis for ASS1 (BD Biosciences) was performed as described previously (5). Membranes were also probed for β-actin (Sigma-Aldrich), Hsc70, and Hsc70.

**Drug treatments**

Arginine depletion experiments were performed using ADI-PEG20 (Polaris Group) and 2% dialyzed FBS (>10 KDa, Autogen Bioclear). Cell viability was measured using the MTT assay according to manufacturer’s instructions (Roche Diagnostics). Pemetrexed (Eli Lilly and Company) was purchased from Barts Chemopharmacy. Combination drug treatments of ADI-PEG20 and pemetrexed were studied using the Chou-Talalay methodology and the effect on tumor cell apoptosis was detected using PARP cleavage (Cell Signaling Technology) and Annexin V staining (21). Proteosome inhibition and demethylation experiments were performed using MG132 (1 μmol/L; Sigma-Aldrich) and 5-aza-2’-deoxycytidine (5-Aza-dC, 0.1–1 μmol/L; Sigma-Aldrich), respectively.

**Liquid chromatography–mass spectrometry**

PBS control and ADI-PEG20 treated cells (3 x 10^6) were washed twice with phosphate buffer saline and then harvested by adding 10 mL of ice-cold 80% methanol containing 0.1% formic acid (all reagents from Fisher Scientific). Cell extracts were prepared using a standard protocol and injected into the liquid chromatography–mass spectrometry (LC-MS) system. LC was performed using the nanoACQUITY UltraPerformance (UP) LC system (Waters) and MS was executed using a Waters Q-ToF Premier operated in positive and negative electrospray ionization modes. Data acquisition and analysis were performed using the Water Masslynx software (V4.1) and mzmine software (version 2.2), respectively. Peak lists, including retention, M/Z, and peak intensities, were exported from mzmine and imported into Microsoft Office Excel 2007, revealing volcano plots and the fold change and P value for individual metabolites. Subsequent targeted metabolomic analysis was performed in the positive ion mode.

**In vitro tracer uptake assays**

Cells were plated at 1 x 10^5 cells per well the evening before the experiment. The next day cells were incubated with ADI-PEG20 (750 ng/mL). On the day of the assay, the drug media were removed and incubated in media supplemented with 2% dialyzed FBS for 1 hour. [3H] thymidine was then added at 1 μci/mL to each well and incubated for a further 1 hour. Cells were then washed twice in ice-cold PBS, and harvested with trypsin. The cell lysates were collected and added to tubes containing 2 mL of scintillation fluid and the cell-associated [3H] radioactivity was measured using the β scintillation counter (LKB Instruments). The radioactivity uptake in nmoles was normalized to the number of viable cells to give pmole uptake per 10,000 live cells and expressed relative to untreated controls. All experiments were done in triplicate and repeated 3 times.

**In vivo experiments**

UMUC-3 cells (1 x 10^6 in 0.1 mL) were implanted into the flank of CD1 nu/nu mice and developed into visible tumors by 11 days. For the in vivo [18F]-fluoro-L-thymidine (FLT)–PET studies, animals (n = 5) were injected i.v. via the tail vein with 15 to 18 MBq of [18F]-FLT and imaged using an Inveon microPET/CT (Siemens) before and 24 hours after treatment with ADI-PEG20 (5 IU). Standardized uptake values (SUV) were calculated by dividing the activity concentration in each voxel by the injected dose and dividing by the animal weight. For drug combination studies, mice were separated into four intraperitoneal (i.p.) treatment groups (n = 11 per group): PBS control (days 1 and 8), ADI-PEG20 (5 IU on days 1 and 8), pemetrexed (PEM; 100 mg/kg on days 2–4 and 9–11); and ADI-PEG20 (5 IU on days 1 and 8) plus PEM (100 mg/kg on days 2–4 and 9–11). Tumor volume and animal weights were recorded and all mice were sacrificed on day 11; tumors were collected and stored for immunohistochemical analyses. Additional [18F]-FLT microPET/CT imaging was performed in female UMUC3 bladder cancer
tumor-bearing CD1 nude mice (n = 5 per group) assessing the drug combination. Animals were imaged at baseline immediately before i.p. treatment with 5 IU ADI (ADI and PEM + ADI treatment groups) or PBS (PBS and PEM treatment groups) and imaged again 24 hours later. Immediately after the 24-hour imaging timepoint, the PEM and PEM + ADI treatment groups received 100 mg/kg PEM i.p. for 5 days whereas the others received PBS, with 18F-FLT imaging carried out on day 7.

**Statistical analysis**

GraphPad Prism version 5.0 and SPSS 14.0 were used to test results for statistical significance. The associations of ASS1...
expression level with clinicopathologic factors were evaluated using the \( \chi^2 \), Fisher exact, or Wilcoxon rank-sum test as appropriate. For all analyses, two-sided tests of significance were used with \( P < 0.05 \) considered significant.

**Results**

**Loss of ASS1 confers poor survival in bladder cancer**

First, we screened for ASS1 expression in a training set bladder cancer TMA derived from a European population revealing that 45\% (83 of 183) of biopsies were ASS1-negative. The validation TMA (Fig. 1A–F) from an Asian population yielded similar results with 37\% of bladder cancer cases (108 of 295) being ASS1-negative. ASS1 negativity conferred a poor disease-specific survival (\( P = 0.001 \)) and metastasis-free survival (\( P = 0.001 \)) on multivariate analysis (Fig. 1G and H and Table 1). Furthermore, ASS1 (253J cell lines were fully methylated, whereas the T24 line was ASS1 locus intact (data not shown). There were no mutations in ASS1 (Supplementary Table S3). Following optimization of primers for paraffin-embedded tissue, only one ASS1-positive tumor had an average methylation value of 45\% that was greater than the threshold value of 40\% (Supplementary Fig. S1). The remaining tumors with methylation values >40\% were all negative for ASS1 immunohistochemistry (data not shown; ref. 22). To determine the correlation between immunohistochemical expression of ASS1 and methylation status, tissue blocks of an independent set of 30 urothelial carcinomas with a high tumor cell content (>70\%) were selected for pyrosequencing. Following optimization of primers for paraffin-embedded tissue, only one ASS1-positive tumor had an average methylation value of 45\% that was greater than the threshold value of 40\% (Supplementary Table S1). The remaining tumors with methylation values >40\% were all negative for ASS1 immunohistochemistry (\( P < 0.001 \)), validating the role of promoter methylation in ASS1 silencing in primary bladder cancer (Supplementary Table S3).

Next, we addressed the significance of epigenetic loss of ASS1 in bladder cancer by manipulating ASS1 in our panel of urothelial cancer cell lines. Although ASS1 overexpression decreased the proliferation of 253J and UMUC-3 cells, ASS1 siRNA increased the proliferation of 5637 cells, consistent with a tumor-suppressor role for ASS1 in urothelium (Fig. 2C). We also observed a reciprocal relationship between ASS1 expression and cell invasion by performing matrigel invasion assays, confirming that ASS1 loss increased the invasiveness of urothelial tumor cells in vitro (Fig. 2D).

**ASS1-methylated bladder cancer cell lines are sensitive to ADI-PEG20**

To test the effect of arginine deprivation on bladder cancer cell line viability, we treated the panel of bladder cancer cell lines with the arginine depletor, ADI-PEG20. All ASS1-negative

<p>| Table 1. Multivariate analysis for disease-specific and metastasis-free survival in bladder cancer |</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Category</th>
<th>DSS</th>
<th>MeFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor (T)</td>
<td>Ta 1</td>
<td>1</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>T1 3.546</td>
<td>1.60–7.874</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2-4 28.571</td>
<td>3.07–250.00</td>
<td></td>
</tr>
<tr>
<td>Mitotic activity (10 HPF)</td>
<td>&lt;10 1</td>
<td>—</td>
<td>0.009&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>&gt;= 10 2.451</td>
<td>1.248–4.817</td>
<td></td>
</tr>
<tr>
<td>ASS1 expression</td>
<td>Positive 1</td>
<td>—</td>
<td>0.011&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Deficient 2.233</td>
<td>1.201–4.153</td>
<td></td>
</tr>
<tr>
<td>Nodal status (N)</td>
<td>N0 1</td>
<td>—</td>
<td>0.994</td>
</tr>
<tr>
<td></td>
<td>N1-2 1.003</td>
<td>0.468–2.175</td>
<td></td>
</tr>
<tr>
<td>Perineural invasion</td>
<td>Absent 1</td>
<td>—</td>
<td>0.160</td>
</tr>
<tr>
<td></td>
<td>Present 1.974</td>
<td>0.764–5.105</td>
<td></td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>Absent 1</td>
<td>—</td>
<td>0.481</td>
</tr>
<tr>
<td></td>
<td>Present 1.299</td>
<td>0.373–1.592</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td>Low grade 1</td>
<td>—</td>
<td>0.944</td>
</tr>
<tr>
<td></td>
<td>High grade 1.058</td>
<td>0.192–4.641</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; DSS, disease-specific survival; MeFS, metastasis-free survival.

<sup>a</sup>Statistically significant.

\[ HR \text{ (95\% CI)} \]

\[ P \text{ value} \]

\[ 0.696 \quad 0.120 \]

\[ 1.815 \quad 0.856–3.850 \]

\[ 2.737 \quad 1.419–3.847 \]

\[ 1.446 \quad 0.764–2.737 \]

\[ 1.107 \quad 0.553–1.872 \]

\[ 1.238 \quad 0.425–3.607 \]
cell lines were sensitive to ADI-PEG20 with the highly methylated lines 253J and UMUC-3 displaying the greatest sensitivity to arginine depletion; in contrast, the ASS1-positive cell lines 5637, RT112, and SCaBER were resistant to ADI-PEG20 (Fig. 3A). Moreover, forced ASS1 overexpression in ASS1-negative cell lines reduced their sensitivity to ADI-PEG20, validating the role of ASS1 loss in mediating the synthetic lethal effect to arginine deprivation (Fig. 3B).

**ADI-PEG20 inhibits pyrimidine metabolism in ASS1-negative tumor cells**

We proceeded to analyze the metabolic effects of ADI-PEG20 and confirmed a significant decrease in intracellular arginine with a corresponding increase in citrulline in ASS1-negative bladder cancer cell lines. Marked reductions in thymidine and increases in thymine and glutamine were also detected by LC/MS (Fig. 4A). In contrast, ADI-PEG20 reduced arginine levels to a minor extent in the ASS1-positive RT112 control cell line, whereas intracellular levels of thymidine, thymine, and glutamine were unaffected, indicating a nonessential role for exogenous arginine. Because of these differential effects on intracellular thymidine, we interrogated gene pathways modulated by ADI-PEG20, identifying downregulation of the folate-dependent nucleotide synthesizing enzymes TS and DHFR specifically in ASS1-negative tumor cells (data not shown). Marked suppression of DHFR and TS mRNA and protein was detected in all three ADI-sensitive bladder cancer cell lines whereas no effect was seen in the RT112 control cell line exposed to ADI-PEG20 (Fig. 4B and C). We also analyzed the thymidine salvage pathway, as a compensatory source of cellular thymidine upon suppression of de novo pyrimidine synthesis. Unexpectedly, ADI-PEG20 also reduced 3H-thymidine uptake in the arginine-auxotrophic cell lines 253J and UMUC-3 (Fig. 4D)
metastasis-free survival standardized uptake value (SUVmax) on \([18\text{F}]\)FLT-PET after 24 cancers of the bladder, lung, mesothelium, and colon (25 linked to poor clinical outcome and resistance to antifolates in bladder cancer (24). High TS expression, in particular, has been associated with poor clinical outcome and resistance to antifolates, including pemetrexed, which blocks enzymes, includ-


duction

sion

Moreover, bladder TMA analysis revealed that ASS1 loss was associated with a reciprocal increase in TS and DHFR expression. We found for the first time that upon depletion of arginine in ASS1-negative tumor cells, glutamine levels increased indicative of a block on nucleotide synthesis. Thus, our metabolomic experiments with ADI-PEG20 support the hypothesis that the ASS1 epimutation reprograms extracellular glutamine to intracellular glutamine for arginine synthesis (Supplementary Fig. S3 and S4; and Supplementary Table S1). Thus, we hypothesized that ADI-PEG20 may potentiate the cytotoxicity of pemetrexed in ASS1-negative tumor cells via modulation of folate-dependent enzymes. First, we showed that pemetrexed and ADI-PEG20 enhanced apoptosis compared with either agent alone by increasing PARP cleavage and Annexin V binding \textit{in vitro} in ASS1-methylated tumor cell lines (Fig. 5A and B). Secondly, ADI-PEG20 significantly reduced tumor growth of the UMUC-3 cell line ($P < 0.001$) in xenografted nude mice. Although pemetrexed alone had no effect, the combination was more effective than ADI-PEG20 alone ($P < 0.05$; Fig. 5C and D). Analysis of tumors from the xenograft studies confirmed reduced TS, DHFR, and TK1 levels in ADI-PEG20–treated animals (Fig. 5E). Finally, to validate our results we executed an \([18\text{F}]\)FLT microPET/CT imaging study of the combination therapy in the UMUC-3 xenograft tumor model. Our SUVmax data showing the superior efficacy of the ADI-PEG20 and pemetrexed drug combination over that of ADI-PEG20 alone are consistent with our earlier tumor volumetric measurements (Fig. 5F). Notably, there was a lack of effect on SUVmax of pemetrexed in animals that had not been pretreated with ADI-PEG20.

Discussion

Progress in the targeted therapy of advanced urothelial carcinoma has lagged behind other urological malignancies, especially prostate and renal cancer. For the first time, we have identified ASS1 as a novel bladder cancer biomarker with wider implications for the therapy and monitoring of cancers dependent on exogenous arginine for survival. We show that despite exhibiting a poorer prognosis, ASS1-negative bladder cancer is amenable to dual targeting with ADI-PEG20 and antifolates with early treatment response monitoring by \([18\text{F}]\)FLT-PET.

Our bladder cancer cell line studies reveal that unmethylated ASS1 functions as a \textit{bona fide} tumor suppressor and is consistent with recent studies in osteosarcomas and myxofibrosarcomas (11, 12). Exactly how ASS1 promoter methylation leads to a requirement for exogenous arginine, which promotes increased tumor cell proliferation and invasion is unclear. Interestingly, previous studies have associated exogenous arginine with DNA synthesis in Burkitt lymphoma cells and with increased proliferation of caco-2 colon cancer cells via a glutamine-dependent effect (30, 31). Yamauchi and colleagues proposed that under conditions of limited arginine supply intracellular glutamine is diverted for arginine synthesis as supplying exogenous arginine was shown to promote nucleotide synthesis. We found for the first time that upon depletion of arginine in ASS1-negative tumor cells, glutamine levels increased indicative of a block on nucleotide synthesis. Thus, our metabolomic experiments with ADI-PEG20 support the hypothesi


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Enhanced effect of ADI-PEG20 and pemetrexed in bladder cancer

To exploit our findings in the clinic, we used the multi-targeted antifolate pemetrexed, which blocks enzymes, including TS and DHFR, and is reported to have modest activity in bladder cancer (24). High TS expression, in particular, has been linked to poor clinical outcome and resistance to antifolates in cancers of the bladder, lung, mesothelium, and colon (25–29). Moreover, bladder TMA analysis revealed that ASS1 loss was associated with a reciprocal increase in TS and DHFR expression—both linked to poor outcome for disease-specific and metastasis-free survival—emphasizing the interdependence between arginine auxotrophy and modulation of nucleotide biosynthesis (Supplementary Fig. S3 and S4; and Supplementary Table S1). Thus, we hypothesized that ADI-PEG20 may potentiate the cytotoxicity of pemetrexed in ASS1-negative tumor cells via modulation of folate-dependent enzymes. First, we showed that pemetrexed and ADI-PEG20 enhanced apoptosis compared with either agent alone by increasing PARP cleavage and Annexin V binding \textit{in vitro} in ASS1-methylated tumor cell lines (Fig. 5A and B). Secondly, ADI-PEG20 significantly reduced tumor growth of the UMUC-3 cell line ($P < 0.001$) in xenografted nude mice. Although pemetrexed alone had no effect, the combination was more effective than ADI-PEG20 alone ($P < 0.05$; Fig. 5C and D). Analysis of tumors from the xenograft studies confirmed reduced TS, DHFR, and TK1 levels in ADI-PEG20–treated animals (Fig. 5E). Finally, to validate our results we executed an \([18\text{F}]\)FLT microPET/CT imaging study of the combination therapy in the UMUC-3 xenograft tumor model. Our SUVmax data showing the superior efficacy of the ADI-PEG20 and pemetrexed drug combination over that of ADI-PEG20 alone are consistent with our earlier tumor volumetric measurements (Fig. 5F). Notably, there was a lack of effect on SUVmax of pemetrexed in animals that had not been pretreated with ADI-PEG20.

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Progress in the targeted therapy of advanced urothelial carcinoma has lagged behind other urological malignancies, especially prostate and renal cancer. For the first time, we have identified ASS1 as a novel bladder cancer biomarker with wider implications for the therapy and monitoring of cancers dependent on exogenous arginine for survival. We show that despite exhibiting a poorer prognosis, ASS1-negative bladder cancer is amenable to dual targeting with ADI-PEG20 and antifolates with early treatment response monitoring by \([18\text{F}]\)FLT-PET.

Our bladder cancer cell line studies reveal that unmethylated ASS1 functions as a \textit{bona fide} tumor suppressor and is consistent with recent studies in osteosarcomas and myxofibrosarcomas (11, 12). Exactly how ASS1 promoter methylation leads to a requirement for exogenous arginine, which promotes increased tumor cell proliferation and invasion is unclear. Interestingly, previous studies have associated exogenous arginine with DNA synthesis in Burkitt lymphoma cells and with increased proliferation of caco-2 colon cancer cells via a glutamine-dependent effect (30, 31). Yamauchi and colleagues proposed that under conditions of limited arginine supply intracellular glutamine is diverted for arginine synthesis as supplying exogenous arginine was shown to promote nucleotide synthesis. We found for the first time that upon depletion of arginine in ASS1-negative tumor cells, glutamine levels increased indicative of a block on nucleotide synthesis. Thus, our metabolomic experiments with ADI-PEG20 support the hypothesis that the ASS1 epimutation reprograms extracellular arginine so that it is sparing for glutamine in nucleotide metabolism. Additional metabolomic flux studies are under way to explore further the interdependence of arginine and glutamine in the context of tumoral ASS1-deficiency. Exogenous arginine has also been shown to stimulate intestinal cell
Figure 4. ADI-PEG20 modulates pyrimidine metabolism in ASS1-methylated cancer cells. A, cell extracts from ASS1-negative bladder cancer lines and the RT112 control treated with ADI-PEG20 (750 ng/mL) for 24 hours were subjected to metabolomic analysis by LC/MS. Arginine decreased in all cell lines; however, the reduction was at least 1-log fold greater in the ASS1-negative tumor cells. Glutamine and thymine increased whereas thymidine decreased specifically in the ADI-treated ASS1-negative bladder cell lines with no effect in RT112 control cells. Data, mean and SE (n = 6). ADI-PEG20 downregulated TS and DHFR mRNA (B) and protein (C) by 24 hours specifically in the ASS1-methylated cell lines. In contrast, the DNA synthetic enzyme ribonucleotide reductase subunit p53R2 was unaltered, confirming the specific downregulatory effect of ADI-PEG20 on TS and DHFR proteins; β-actin was used as the loading control (50). D, [3H]thymidine uptake in bladder cancer cells after treatment with ADI-PEG20 (750 ng/mL). The cells remaining after ADI treatment decreased their uptake of thymidine by 24 hours in all ASS1-negative cell lines except the T24 bladder cell line, which showed a reduction by 72 hours (*, P < 0.05; ***, P < 0.005). In contrast, [3H] thymidine uptake was unchanged in the control RT112 cell line after ADI treatment. Data, mean and SE (n = 9). E, ADI-PEG20-mediated loss of TS and TK1 can be abrogated by inhibiting the proteasome with MG132. UMUC-3 cells were plated and incubated overnight; subsequently, cells were exposed to DMSO (Con), ADI-PEG20 (750 ng/mL), ADI-PEG20, and MG132 or MG132 (1 μmol/L) for 24 hours with immunoblotting as shown. G, [18F]-FLT microPET/CT imaging in a UMUC-3 bladder tumor-bearing CD1 nude mouse (i) before treatment showing uptake in tumor (red arrow), and increased tracer concentration in intestine (white arrow) and also gall bladder. Transverse views before (ii) and 24 hours after (iii) treatment with 5 IU ADI-PEG20 show a reduction in tumor SUVmax after treatment (3.2 vs. 1.1 after treatment), indicating a decrease in tumor cell proliferation.
motility via activation of NO and focal adhesion kinase signaling (32, 33). In particular, arginine is a dominant amino acid modulator of mTOR, a nodal pathway that integrates cell growth, proliferation, invasion, and metastasis of various cancers, including urothelial malignancy (34–36); moreover, arginine deprivation has been shown to repress mTOR and its downstream substrates p70 S6 kinase and 4E-BP1 in ASS1-negative cancer cells (6, 37). Indeed, it is becoming clear that arginine deprivation has multiple effects on gene expression and that this is likely to be determined not only by cell type but also by the status of the ASS1 gene (38).

The ADI-PEG20–induced suppression of the folate-dependent enzymes TS and DHFR and sensitization of ASS1-negative tumor cells to the cytotoxic effects of pemetrexed provide a novel metabolic approach to retargeting cytotoxic chemotherapy (39). Our results also validate the work of Cheng and Allen et al. Cancer Res; 74(3) February 1, 2014
colleagues, who showed that the irreversible TS inhibitor 5-fluorouracil was more effective when combined with pegylated human arginase in a hepatocellular carcinoma xenograft (40). It is noteworthy that the ADI-PEG20 and antifolate doublet has a wide therapeutic window in the xenograft studies. Moreover, our experience from an ongoing clinical trial of arginine depletion in mesothelioma (NCT01279967) includes a patient treated concurrently with ADI-PEG20 and the antifolate methotrexate for an unrelated condition; the combination was safe and led to a prolonged period of disease control (manuscript in preparation) (51).

Furthermore, the decreased uptake of thymidine following ADI-PEG20 treatment as shown by the ³H-thymidine and FLT tracer studies may be exploited as an early therapeutic biomarker for imaging ASS1-methylated tumors. This is in contrast to recent work with ADI-PEG20 in ASS1-negative melanoma xenografts in which there was no effect on TK1 expression and FLT-PET metabolic responses were not detected (41). These and previous results using ²F[¹⁸F]FLT-PET, RFC, reduced folate carrier; THF, tetrahydrofolate.

In conclusion, our studies linking the biology of arginine auxotrophic bladder cancer to adverse clinicopathologic variables accentuate ASS1 loss as a novel biomarker for evaluation in patients with urothelial cancer. Specifically, targeted modulation of pyrimidine synthesis—via suppression of the de novo and salvage nucleotide pathways—using arginine-depleting drugs combined with antifolates has the potential to improve the management of urothelial and related cancers with methylated ASS1.

Disclosure of Potential Conflicts of Interest
J.S. Bomalaski is executive VP Medical Affairs with Polaris Pharmaceuticals, Inc. R.C. Jackson is a consultant and advisory board member of Polaris Inc, N.R. Lemoine is the editor of Gene Therapy journal (self employed with honorarium paid by publisher) with Nature Publishing Group. No potential conflicts of interest were disclosed by the other authors.

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