Arginine Deiminase Resistance in Melanoma Cells Is Associated with Metabolic Reprogramming, Glucose Dependence, and Glutamine Addiction

Yan Long1, Wen-Bin Tsai1, Medhi Wangpaichitr2, Takashi Tsukamoto3, Niramol Savaraj2, Lynn G. Feun2, and Macus Tien Kuo1

Abstract

Many malignant human tumors, including melanomas, are auxotrophic for arginine due to reduced expression of argininosuccinate synthetase-1 (ASS1), the rate-limiting enzyme for arginine biosynthesis. Pegylated arginine deiminase (ADI-PEG20), which degrades extracellular arginine, resulting in arginine deprivation, has shown favorable results in clinical trials for treating arginine-auxotrophic tumors. Drug resistance is the major obstacle for effective ADI-PEG20 usage. To elucidate mechanisms of resistance, we established several ADI-PEG20–resistant (ADI R) variants from A2058 and SK-Mel-2 melanoma cells. Compared with the parental lines, these ADI R cell lines showed the following characteristics: (i) all ADIR cell lines showed elevated ASS1 expression, resulting from the constitutive binding of the transcription factor c-Myc on the ASS1 promoter, suggesting that elevated ASS1 is the major mechanism of resistance; (ii) the ADI R cell lines exhibited enhanced AKT signaling and were preferentially sensitive to PI3K/AKT inhibitors, but reduced mTOR signaling, and were preferentially resistant to mTOR inhibitor; (iii) these variants showed enhanced expression of glucose transporter-1 and lactate dehydrogenase-A, reduced expression of pyruvate dehydrogenase, and elevated expression of pyruvate dehydrogenase, and elevated sensitivity to the glycolytic inhibitors 2-deoxy-glucose and 3-bromopyruvate, consistent with the enhanced glycolytic pathway (the Warburg effect); (iv) the resistant cells showed higher glutamine dehydrogenase and glutaminase expression and were preferentially vulnerable to glutamine inhibitors. We showed that c-Myc, not elevated ASS1 expression, is involved in upregulation of many of these enzymes because knockdown of c-Myc reduced their expression, whereas overexpressed ASS1 by transfection reduced their expression. This study identified multiple targets for overcoming ADI-PEG resistance in cancer chemotherapy using recombinant arginine-degrading enzymes. Mol Cancer Ther; 12(11); 2581–90. ©2013 AACR.

Introduction

The amino acid L-arginine has many physiologic functions because it is a precursor for the biosyntheses of proteins, nitric oxide, polyamines, proline, glutamate, creatine, and agmatine. It also plays important role in immune response to antigen by impaired cytokine production and an arrest in T-cell proliferation (1,2). Arginine is primarily synthesized by two sequential enzymatic reactions in the urea cycle: argininosuccinate synthetase-1 (ASS1), which catalyzes the reaction of L-citrulline and aspartic acid to argininosuccinate and argininosuccinase lyase (ASL), which recycles argininosuccinate back to arginine with the production of fumaric acid, an intermediate in the tricarboxylic acid (TCA) cycle (Fig. 1).

Many advanced human tumors, including melanomas, renal cell carcinomas, hepatocellular carcinomas, mesotheliomas, prostate cancers, and small-cell lung cancers do not express ASS1 (3). ASS1-negative tumor cells therefore require arginine from the circulation for survival, whereas normal cells that synthesize arginine endogenously will survive under arginine deprivation conditions. Arginine deiminase (ADI; refs. 4–7) and human arginase 1 (4, 8, 9) two arginine-depleting recombinant proteins (Fig. 1), have been under clinical development for treating arginine-auxotrophic tumors. ADIs produced by Mycoplasma spp. and humans do not produce this enzyme. Like most recombinant bacterial proteins, it is immunogenic and has a short half-life in vivo. Pegylated ADI (ADI-PEG20) has been formulated for clinical use. Clinical trials using

Authors’ Affiliations: 1Department of Translational Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas; 2Sylvester Comprehensive Cancer Center, University of Miami, Miami, Florida; and 3Department of Neurology and Brain Science Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland

Y. Long and W.-B. Tsai contributed equally to this work.

Corresponding Author: Macus Tien Kuo, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Box 951, Houston, TX 77030. Phone: 713-834-6038; Fax: 713-834-6085; E-mail: tkuo@mdanderson.org

doi: 10.1158/1535-7163.MCT-13-0302

©2013 American Association for Cancer Research.

www.aacrjournals.org
ADI-PEG20 for treating various cancers including melanoma have produced favorable results (5, 6).

Drug resistance remains an important contributor for the treatment failure, owing to reexpression of the once-silenced ASS1. Using a cultured melanoma cell system, we previously showed that transcriptional regulation of ASS1 by ADI-PEG20 involves a switch of promoter binding, c-Myc, which functions as a positive regulator replaces hypoxia-inducible factor 1α (HIF-1α), which functions as a negative regulator (10, 11).

The current investigation was initiated to elucidate resistance mechanisms of ADI-PEG20 in melanoma cells. We developed several stable ADI-resistant (ADI-R) cell lines and found that compared with their parental counterparts, these ADI-R variants exhibited elevated glycolytic metabolic activities and were preferentially sensitive to the killing by glycolytic inhibitors. Moreover, these ADI-R cells also exhibited elevated expression of the kidney-type glutaminase (GLS1) and glutamine dehydrogenase (GDH), key enzymes for glutaminolysis and were preferentially sensitive to glutamine inhibitors. These results show that ADI resistance in arginine-auxotrophic melanoma cells is associated with metabolic reprogramming that provides potential targets for overcoming the resistance.

Materials and Methods

Reagents, antibodies, and shRNA

Reagents were obtained from the following sources: ADI-PEG20 (specific activity, 5–10 IU/mg) from Polaris Pharmaceuticals, Inc.; LY294002, 2-deoxy-D-glucose (2-DG), 3-bromopyruvate (3-BP), 6-diazo-5-oxo-L-norleucine (DON), azaserine, deferoxamine mesylate (DFX), and CoCl2 from Sigma-Aldrich; perifosine from Cayman Chemical; temsirolimus (CCI-779) from Selleckchem. Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) was prepared according to a previous report (12).

Antibodies were obtained from the following sources: mouse anti-human ASS1 monoclonal antibody from Polaris Pharmaceuticals; rabbit anti-ASL and rabbit anti-c-Myc (N262), rabbit anti-MCT1 and MCT4 antibodies from Santa Cruz Biotechnology; rabbit antiglucose transporter-1 (Glut1) from FabGennix; antibodies against lactate dehydrogenase-A (LDH-A), pyruvate dehydrogenase (PDH), acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), mTOR complex 1 (mTORC1), p70S6 kinase, p-p70S6 kinase (Thr389), 4E-BP, eIF2α, p-eIF2α (Ser51), ASCT2 (SLC15), AMPKα, and p-AMPKα (Thr172) from Cell Signaling Technology; rabbit anti-kidney type glutaminase (GLS1) and rabbit anti-GDH from Sigma-Aldrich; and rabbit anti-α-tubulin antibodies from Sigma-Aldrich.

Recombinant short hairpin RNA (shRNA) for c-Myc were purchased from Sigma-Aldrich.

Cell culture and establishment of ADI-R cells

A2058, SK-Mel-2, and A375 melanoma cells were purchased from American Type Culture Collection Center without further authentication. All cell cultures were maintained in Dulbecco’s Modified Eagle Medium containing 10% FBS in 5% CO2 atmosphere.
ADI<sup>R</sup> cell lines were established by selecting the surviving population from A2058 and SK-Mel-2 cells that were continuously exposed to ADI-PEG20 with stepwise increases in concentrations, starting from 0.1 μg/mL and 0.05 μg/mL for A2058 and SK-Mel-2, respectively. Independent ADI<sup>R</sup> cell lines were established from single cells using 96-well plate. Increased resistance in these clones were further developed by increased drug concentrations with 0.025 μg/mL increments for over 6 months, at which time the resistant cells were able to survive at 0.9 μg/mL of ADI-PEG20. Cells were routinely maintained at 0.3 μg/mL of ADI-PEG20.

Glucose uptake
Glucose transport was measured according to the procedure described previously (13). In brief, the experiments were started by adding 1 μCi of 2-DG (5–10 Ci/mmol/L) into exponentially growing A2058 cells (5 × 10<sup>5</sup> in 6-well; Corning). Cultured cells were maintained at 37°C for 20 minutes. Cells were harvested, washed three times with PBS, and solubilized in 0.1 N NaOH and the radioactivity was measured in a liquid scintillation counter.

Establishment of stable ASS1-overexpressing cell lines
The plasmid pCMV6-ASS1 carrying human full-length ASS1 cDNA sequence (GenBank, NM_000050; Origene) and empty vector (pCMV6) were transfected into A2058 and A375 cells using lipofectamine and positive clones were selected with G418.

Other procedures
Western blotting, chromatin immunoprecipitation (ChIP), cytotoxicity assay using MTT were conducted as described previously (10, 11).

Statistical analyses
The IC<sub>50</sub> values were obtained from nonlinear regression analysis of concentration–effect curves by the GraphPad Prism Software and represented by mean ± SD of three independent experiments. For analysis with only two groups (parental and ADI<sup>R</sup> cells), Student t test was used. The IC<sub>50</sub> values were plotted in reference to that of A2058, which was set at 1.0. Significance was determined when P < 0.05.

Results
Elevated ASS1 expression in ADI<sup>R</sup> cell lines
We established five independent ADI<sup>R</sup> variants from A2058 (A2058-R1 to A2058-R5) and 4 SK-Mel-2 (SK-Mel-2-R1 to SK-Mel-2-R4) lines. Cytotoxicity tests showed that all these cell lines were more resistant to ADI-PEG20 than were their respective parental cell lines. More than 95% of all ADI<sup>R</sup>-A2058 cells could survive at 0.9 μg/mL ADI-PEG20 for 48 hours treatment, whereas less than 25% of the parental cells survived (Fig. 2A). Likewise, treating SK-Mel-2 cells with 0.9 μg/mL ADI-PEG20 almost completely killed the cells, whereas more than 70% of ADI<sup>R</sup> SK-Mel-2 cells were able to survive under the same treatment (Fig. 2B).

All the ADI<sup>R</sup>-A2058 cells exhibited increased ASS1 expression (about 15-fold by densitometry, not shown) as compared with that in the drug-sensitive counterpart.
expression in the ADIR cell lines, we treated these cells and whether accumulation of HIF-1α would suppress ASS1 expression in ADIR cell lines; whereas reduced HIF-1α expression levels were seen in A2058-R2, A2058-R4, A2058-R5, and all ADIR-SK-Mel-2 lines, but not in A2058-R1 and A2058-R3 cells. The observations that no elevated expression of c-Myc and no reduction of HIF-1α expression in all ADIR cell lines prompted us to investigate whether c-Myc and HIF-1α remain important for regulating ASS1 expression in these ADIR cell lines.

Roles of c-Myc and HIF-1α in the regulation of ASS1 expression in ADIR cell lines

We previously showed that induction of ASS1 expression by ADI-PEG20 in melanoma cells was associated with the upregulation of c-Myc, which functions as a positive regulator and the downregulation of HIF-1α, which functions as a negative regulator (10, 11). Figure 2C and D shows that c-Myc expression levels were similar between all ADIR variants and their respective parental cell lines; whereas reduced HIF-1α expression levels were seen in A2058-R2, A2058-R4, A2058-R5, and all ADIR-SK-Mel-2 lines, but not in A2058-R1 and A2058-R3 cells. The observations that no elevated expression of c-Myc and no reduction of HIF-1α expression in all ADIR cell lines prompted us to investigate whether c-Myc and HIF-1α remain important for regulating ASS1 expression in these ADIR cell lines.

We chose four representative ADIR cell lines (A2058-R1, which expressed higher HIF-1α levels than the control, and A2058-R2, SK-Mel-2-R1, and SK-Mel-2-R3, which did not express detectable levels of HIF-1α). To investigate whether accumulation of HIF-1α would suppress ASS1 expression in the ADIR cell line, we treated these cells and their drug-sensitive cells (as control) with hypoxic mimics, DFX, and CoCl2 (14). Treating A2058 or SK-Mel-2 cells with DFX or CoCl2 suppressed ADI-PEG20-induced ASS1 expression (Fig. 3A, left). However, no suppression of ASS1 expression was found in A2058-R1, A2058-R2, and SK-Mel-2-R3, except SK-Mel-2-R1 cells (Fig. 3A, right). The suppression of ASS1 expression in SK-Mel-2-R1 cells by elevated HIF-1α, but not in the other ADIR cells may have been due to the lower expression levels of ASS1 in SK-Mel-2-R1 cells as compared with those in the other cell lines (Fig. 2D). These results suggest that the extent of ASS1 suppression by overexpression HIF-1α may depend upon the intrinsic ASS1 expression levels.

To investigate the role of c-Myc in regulating ASS1 expression in ADIR variants, we used c-Myc shRNA strategy. Knockdown of c-Myc expression by two independent shRNA in lentiviral vectors downregulated ASS1 expression (Fig. 3B), supporting the positive role of c-Myc in the regulation of ASS1 in the ADIR cell lines.

We also conducted ChIP assay to determine the occupancy of HIF-1α and c-Myc at the ASS1 promoter. Consistent with our previous results (10), we found that HIF-1α, but not c-Myc, binds to the ASS1 promoter in the parental A2058 (Fig. 3C, lane 1) and SK-Mel-2 cells (Fig. 3D, lane 1). In contrast, in all the ADIR variants investigated, c-Myc, not HIF-1α, binds to the ASS1 promoter (Fig. 3C and D, lanes 2 and 3). These results taken together, show that HIF-1α and c-Myc remain important in transcriptional regulation of ASS1 expression in ADIR variants.

ADIR cell lines are sensitive to PI3K/AKT inhibitors

We previously observed that A2058 and SK-Mel-2 cells treated with ADI-PEG20 activated the Ras/PI3K/AKT signaling cascade, resulting in c-Myc stabilization for ASS1 upregulation (11). Moreover, several recent reports suggested that PI3K/AKT play important roles in regulating c-Myc expression (15–17). Activation of PI3K/AKT signal is elicited by phosphorylation of AKT at threonine 308. We conducted Western blotting analyses of 5 ADIR-A2058 cell lines using anti-phosphorylated AKT antibody and found that all these cells contained activated AKT signal (Fig. 4A). Activation of AKT in these ADIR cell lines was reflected by increased sensitivities to the killing by LY294002 and perifosine, which inhibit phosphoinositide 3-kinase (PI3K) and AKT1 activities, respectively (Fig. 4B).
investigated, using cell lysate prepared from A2058 cells treated with ADI-PEG20 for 24 hours as a positive control (Fig. 4C). These results showed that mTOR is the only signal that is consistently downregulated in the ADI R cells.

**ADI R cells are associated with enhanced expression of ASL and glycolytic activities (the Warburg effect)**

We then investigated whether ADI-PEG20 resistance would alter the metabolic programs. Because ASL is directly downstream from ASS1 in the urea cycle (Fig. 1), we conducted Western blotting of its expression in five ADI R-A2058 lines. We found that ASL expression levels were elevated in all 5 ADI R cell lines (Fig. 5A). Elevated Glut1 expression in these A2058 parental cells. Elevated Glut1 expression in these the ADI R cell lines was correlated with the enhanced transport of [3H]-2-DG, a nonmetabolizable form of glucose (Fig. 5B). Compared with the parental cell lines, the ADI R variants showed higher levels of LDH-A, which catalyzes the conversion of pyruvate into lactate (Fig. 1), but lower levels of PDH (Fig. 5A). PDH is the key enzyme that catalyzes the conversion of pyruvate into acetyl CoA, an important metabolic intermediate in the TCA cycle (Fig. 1).

Monocarboxylate transporter family (MCT, SLC16) catalyzes the rapid transport across the plasma membrane of many monocarboxylates including lactate (25). Because these ADI R cells show elevated expression of LDH-A, we examined the expression levels of MCT1 and MCT4 in ADI R-A2058 cells. Results showed that levels of MCT1 were increased in A2058-R1, A2058-R2, A2058-R4, and A2058-R5, but reduced in A2058-R3; whereas levels of MCT4 were increased in A2058-R2, A2058-R3, and A2058-R5.
R5, but reduced in A2058-R4 and no change in A2058-R1 (data not shown). Thus, the expression levels of MCT1 and MCT4 are heterogeneous among the five ADI<sup>R</sup>-A2058 cells. Their roles in ADI resistance are not certain.

**ADI<sup>R</sup> cell lines are preferentially sensitive to glycolytic inhibitors**

Because ADI<sup>R</sup> cells exhibit elevated expression of Glut1 and enhanced glucose uptake, known as the Warburg effect, we asked whether these cells would be preferentially killed by 2-DG, a commonly used inhibitor of glycolysis (26–28). We found that, indeed, all the 5 ADI<sup>R</sup>-A2058 variants exhibited enhanced sensitivity to 2-DG (Fig. 5C). Moreover, we found that these five ADI<sup>R</sup> cells were also vulnerable to another glucolytic inhibitor 3-BP, a lactate/pyruvate analogue (Fig. 5C; refs. 29, 30), showing that these ADIR cells are preferentially sensitive to glycolytic inhibitors as compared with their parental cell lines.

**ADI<sup>R</sup> cells exhibit elevated glutaminolytic metabolism and increased sensitivity to glutaminase inhibitors**

Two key enzymes in glutaminolytic metabolism, glutaminase (GLS) and GDH, catalyze the two-step sequential conversion of glutamine into α-ketoglutarate via glutamate intermediate (Fig. 1). Mammals express two GLS isoforms, kidney-type (or GLS1) and liver-type (GLS2). GLS1 is expressed in a broad spectrum of cell lines. We showed elevated expression of GLS1 and GDH in all 5 ADI<sup>R</sup>-A2058 cell lines as compared with those in the parental control, indicating the increased glutaminolytic activities in these ADI<sup>R</sup> variants (Fig. 5D). These results were supported by the demonstration that all the 5 ADI<sup>R</sup> cell lines were associated with preferential sensitivity to GLS inhibitors, DON, azaserine, and BPTES (Fig. 5E). Despite increased glutaminolytic enzymes in these ADI<sup>R</sup> cell lines, we found only A2058-R4 exhibited elevated ASCT2 glutamine transporter among the 5 ADI<sup>R</sup> A2058 cells (data not shown).

**c-Myc, but not elevated ASS1, contributes to the enhanced glycolytic and glutaminolytic metabolisms in ADI<sup>R</sup> cell lines**

To investigate the kinetics of induction of these glycolytic and glutaminolytic enzymes by ADI-PEG20 in the ADI<sup>R</sup> cells, we conducted an ADI-PEG20 time-course treatment of A2058 cells from 8 to 72 hours. We found that ADI-PEG20 induced not only ASS1 and c-Myc, but also Glut1, LDH-A, and GLS1 (but not GDH) within this time frame. These results indicate that induction of ASS1, Glut1, and LDH-A, occurred very early during the development of ADI resistance and elevation of GDH levels occurred subsequently (Fig. 6A).

As elevated expression of ASS1 is the principal mechanism of ADI resistance, we investigated whether enhanced expression of these enzymes was the consequence of ASS1 upregulation in the ADI<sup>R</sup> cell lines. To this end, we used transfection to establish two independently overexpressed ASS1 cell lines in A2058 and A375 cells.
While no apparent alteration of HIF-1α levels was discernible even using prolonged exposure of the Western blot analyses (not shown). We found that expression of c-Myc, Glut1, LDH-A, GLS1, and GDH was reduced (Fig. 6B). This expression profile is directly opposite from that in the ADIR cell lines. These results show that elevated expression of these enzymes in ADI R cells was not likely due to the enhanced ASS1 expression.

We next investigated whether c-Myc plays a role in regulating the glycolytic and glutaminolytic enzymes in these ADI R cell lines, in light that c-Myc has been recognized as an important transcription regulator of 10% to 20% of total cellular genes through E-box interactions in mammalian cells (31, 32). A2058-R1 and A2058-R2 cells were treated with two independent c-Myc shRNA for 48 hours. Although the shRNA we used could only partially reduce c-Myc levels, we found reduced expression of Glut1 and GLS1 in these ADI R cells as compared with those in the scramble shRNA-treated controls, whereas levels of LDH-A and GDH were not affected. The inability of reducing LDH-A and GDH suggest that either other mechanisms are involved for the regulation of LDH-A and GDH, or regulation of these enzymes by c-Myc is more stringent than Glut1 or GLS1. In any event, these results support that c-Myc plays a role in regulating metabolic rewiring during the development of ADI-PEG20 resistance.

Expression of enzymes involved in the lipid biosynthesis in the ADI R cell lines

We also determined the expression of enzymes involved in fatty acid biosynthesis in ADI R cell lines. Compared with their parental counterpart, all the ADI R A2058 cell lines, except A2058-R4, had lower expression levels of ACC, which catalyzes the conversion of acetyl-CoA into malonyl-CoA (Fig. 1; data not shown) and all the ADIR A2058 cell lines, except A2058-R2, had lower levels of FAS, which converts malonyl-CoA into palmitoyl-CoA (data not shown). These results suggest that fatty acid metabolism may also be reduced in the ADI R cell lines compared with their parental counterpart.

Discussion

The discovery that many human malignant tumors do not express ASS1 and are arginine-auxotrophic provides a mechanistic basis for using recombinant arginine-depleting enzymes in targeted therapy of these diseases (4).
this communication, we show that in a panel of nine independently established ADI R cell lines all expressed elevated levels of ASS1, further strengthening the role of ASS1 in ADI resistance. These ADI R cell lines serve as a valuable resource for investigating the metabolic reprogramming associated with ADI resistance as summarized in Fig. 1. Several important findings are discussed below.

We found that these ADI R variants exhibited elevated AKT signaling and are preferentially sensitive to PI3K/AKT inhibitors. These findings support the important role of this pathway in ADI-PEG20 resistance (11). We also observed that expression of mTOR and its downstream signal p70S6K was downregulated in all ADI R cells. While the canonical pathway of mTOR regulation is through the PI3K/AKT pathway (33), however, downregulation of mTOR independent of this pathway has also been reported (34). The molecular mechanism for downregulation of mTOR signal in ADI resistance remains to be investigated.

We further showed that ASL was elevated in these ADI R lines, suggesting that the ADI R cells may require robust ASS1/ASL activities in the urea cycle to overcome ADI-PEG20–induced autophagy and apoptosis in the otherwise arginine-auxotrophic cancer cells (11, 35). Although ASS1 has often been considered as the rate-limiting enzyme for the biosynthesis of arginine (36), our results suggest that ADI R cells may require additional enzymes to replenish arginine under prolong arginine deprivation conditions. Our results are consistent with recent findings that several enzymes in the urea cycle may be coregulated (37), including ASS1, ASL, nitric oxide synthase (NOS), and cationic amino acid transporter 1 and may form a supermolecular complex to conduct important physiologic function (38).

We found that these ADI R cells have altered metabolic program toward aerobic glycolysis by enhancing Glut1 and LDH-A expression but reducing PDH expression, exhibiting the Warburg phenomenon. Since Warburg’s work in 1929, it has been evidenced that cancer cells, even under aerobic conditions, frequently upregulate glucose metabolism, leading to a high uptake and use of glucose, but a moderate rate of mitochondrial respiration (39, 40). This characteristic has led to the development of 2[18F]fluoro-2-deoxy-D-glucose (FDG) in positron emission tomography (PET) for the imaging of patients with primary and metastatic tumors(41). Our observations that ADI R cells exhibited elevated Warburg phenomenon suggest that it may be possible to use FDG-PET for monitoring the development of ADI resistance during cancer chemotherapy. In clinical setting, melanoma is known to be strongly PET-positive and usually become less FDG avid when tumor response to treatment. Once tumors become resistant to ADI-PEG20, melanoma will again become FDG avid. This was also observed clinically when patient failed ADI-PEG20.

Metabolic reprogramming in ADI R cell lines also include elevated expression of GLS1 and GDH, another common phenomenon in cancer cells (42, 43). These enzymes are important for producing α-ketoglutarate, an important metabolite in the TCA cycle. Tumor cells fuel their metabolism with glucose and glutamine as important bioenergetic sources for cell proliferation. The dependence of these ADI R cells to glucose and glutamine for growth was shown by the findings that these ADI R cells exhibited elevated sensitivity to the killing by glucose (2-DG and 3-BP) and glutamine inhibitors (DON, azaserine, and BPTES). While DON and azaserine have long been known to be toxic in human clinical evaluations (44),
presumably due to the presence of chemically reactive diazo group. The increased sensitivity of these ADI<sup>R</sup> cells to BPTES showed in this study may have promising therapeutic implication. New generation of GLS1 inhibitors such as BPTES derivatives, which are devoid of diazo group found in DON and azaserine (12), as well as compound 968 and its derivatives, another structurally distinct series of GLS1 inhibitor (45, 46) that are currently under clinical evaluations, may pave the way for future clinical applications.

We also found that c-Myc plays important role in the metabolic rewiring of ADI resistance. Previous studies have showed that c-Myc regulates genes involved in the glycolytic pathway by directly binding to the E-box located at the promoters of these genes (47). C-Myc can also upregulates glutamine metabolism via transcriptional repression of miR-23a and miR-23b (48). Although levels of c-Myc were not elevated in these ADI<sup>R</sup> cells, however, our kinetic analysis showed that expression of c-Myc was induced by ADI-PEG20 within the same time frame when many of these enzymes were also induced. Elevated levels of glycolytic and glutaminolytic pathways, apparently, are needed for the maintenance of ADI resistance because inhibitors to these pathways overcome the resistance. The observation that no elevated c-Myc levels in all the nine ADI<sup>R</sup> cell lines, despite their early induction, suggests that persistently high levels of c-Myc may be disadvantageous for cell growth during the development of ADI resistance.

We also discovered that elevated expression of ASS1 by transfection downregulates Glut1, LDH-A, GLS1, and GDH expression, showing that modulating ASS1 levels can influence the expression of other enzymes in distance, i.e., glycolytic and glutaminolytic pathways. While the precise mechanism(s) remain to be investigated, down-regulation of c-Myc may be responsible for the reduced expression of these enzymes in these ASS1-transfected cells, although this remains to be elucidated. Moreover, the findings of reduced levels c-Myc levels in these ASS1-transfected cells may explain why ADI<sup>R</sup> cells did not show elevated c-Myc, as this may be due to feed-back inhibition mechanism of c-Myc regulation once ASS1 is overly expressed. These possible mechanisms would further support the important roles of c-Myc in the regulation of glycolytic and glutaminolytic metabolism in melanoma cells.

Finally, the identification of metabolic reprogramming associated with ADI resistance in the arginine-auxotrophic melanoma model bears important clinical implications for targeted therapy of arginine-auxotrophic tumors. Many antitumor small molecules targeting PI3K/AKT, glycolytic, and glutaminolytic signalings have been approved for clinical use. Combination of these agents with arginine-degrading enzymes, such as ADI-PEG20 or pegylated human arginase 1 may improve the treatment efficacy of many human cancers that require arginine for survival.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Long, M.T. Kuo
Development of methodology: W.-B. Tsai
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Long, W.-B. Tsai, M. Wangpaisith, N. Savaraj, L.G. Feun
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Long, W.-B. Tsai, M.T. Kuo
Writing, review, and/or revision of the manuscript: T. Tsukamoto, N. Savaraj, L.G. Feun, M.T. Kuo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Long, T. Tsukamoto, M.T. Kuo

Acknowledgments
The authors thank Dr. Bor-Wen Wu (Polaris) for providing ADI-PEG20 and anti-ASS1 antibody.

Grant Support
The work was supported in part by ROI-CA149260 from the National Cancer Institute (to M.T. Kuo and L.G. Feun) and R21NS074151 from the National Institute of Neurological Disorders and Stroke (to T. Tsukamoto).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 22, 2013; revised July 26, 2013; accepted August 15, 2013; published OnlineFirst August 26, 2013.
Arginine Deiminase Resistance in Melanoma Cells Is Associated with Metabolic Reprogramming, Glucose Dependence, and Glutamine Addiction

Yan Long, Wen-Bin Tsai, Medhi Wangpaichitr, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0302

Cited articles
This article cites 48 articles, 15 of which you can access for free at:
http://mct.aacrjournals.org/content/12/11/2581.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/12/11/2581.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.