Activation of Ras/PI3K/ERK Pathway Induces c-Myc Stabilization to Upregulate Argininosuccinate Synthetase, Leading to Arginine Deiminase Resistance in Melanoma Cells

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Abstract

Melanomas and other cancers that do not express argininosuccinate synthetase (AS), the rate-limiting enzyme for arginine biosynthesis, are sensitive to arginine depletion with pegylated arginine deiminase (ADI-PEG20). However, ADI resistance eventually develops in tumors because of AS upregulation. Although it has been shown that AS upregulation involves c-Myc, the underlying mechanisms remain unknown. Here we show that ADI-PEG20 activates Ras signaling and the effector extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/AKT/GSK-3β kinase cascades, resulting in phosphorylation and stabilization of c-Myc by attenuation of its ubiquitin-mediated protein degradation mechanism. Inhibition of the induced cell signaling pathways using PI3K/AKT inhibitors suppressed c-Myc induction and enhanced ADI-mediated cell killing. Notably, in an animal model of AS-negative melanoma, combination therapy using a PI3K inhibitor plus ADI-PEG20 yielded additive antitumor effects as compared with either agent alone. Taken together, our findings offer mechanistic insight into arginine deprivation metabolism and ADI resistance, and they illustrate how combining inhibitors of the Ras/ERK and PI3K/AKT signaling pathways may improve ADI-PEG20 anticancer responses.

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Introduction

Malignant melanoma is the most aggressive type of skin cancer that accounts for most deaths from skin cancer, and the cure rates remains less than 10% (1, 2). Effective treatment modalities for malignant melanoma are urgently needed. It has been shown that most of malignant melanomas have abnormal urea cycle metabolism that are unable to carry out de novo synthesis of arginine (Arg) from citrulline in 2-step reactions catalyzed by argininosuccinate synthetase (AS) and argininosuccinate lyase (3). AS is the rate-limiting enzyme and malignant melanomas do not express AS and therefore require Arg from extracellular source for tumor growth. This Arg-auxotrophy provides a novel approach for using Arg-degrading enzymes to deplete Arg in the circulation to treat melanoma and other human malignancies (4). Peglated recombinant bacterial arginine deiminase (ADI-PEG20), which converts Arg to citrulline and ammonia resulting in Arg deprivation, has been under various stages of clinical evaluation for the treatment of malignant melanoma (5). This strategy has also been used in the treatments of hepatocellular carcinoma (5–8).

Although ADI-PEG20 treatments have shown promising outcomes in most studies, one important mechanism associated with treatment failure is the development of drug resistance due to reexpression of AS in the tumors. Using cultured melanoma cells, we previously showed that ADI-PEG20 treatments induced AS expression in A2058 and SK-MEL-2 cells, but not in A375 cells (9). Induction of AS expression was associated with upregulation of c-Myc and downregulation of HIF-1α. HIF-1α functions as a negative regulator by binding to the E-box at the AS promoter and suppressing AS expression before the induction. Upon ADI-PEG20 treatment, binding of HIF-1α at the E-box is replaced by c-Myc, which functions as a positive regulator for the upregulation of AS. However, the mechanisms underlying this c-Myc upregulation are unknown. In this study, we report that upregulation of c-Myc by ADI-PEG20 is due to enhanced c-Myc protein stability elicited by activation of the Ras signaling mediated protein kinase cascades. Our findings provide a plausible strategy for improving the treatment efficacy of ADI-PEG20 by intervention in this signal transduction pathway.

Materials and Methods

Reagents, antibodies, and recombinant DNA

Reagents were obtained from the following sources: ADI-PEG20 (specific activity, 5–10 IU/mg) from Polaris Pharmaceuticals Inc.; sulforhodamine B (SRB), MG-132, sodium selenite, and Ly294002 from Sigma-Aldrich; perifosine from Selleck, and PtdIns-(4, 5)-P2 from Cayman Chemical; cycloheximide (CHX)
and AKT inhibitor VIII from Calbiochem; PI-103 from Echelon Biosciences; GSK-3 siRNA from Cell Signaling, Mouse anti-β-actin, mouse anti-hemagglutinin (HA), mouse anti-Flag, and anti-α-tubulin antibodies from Sigma-Aldrich; mouse anti-AS from Polaris Pharmacologies Inc.; rabbit anti-c-Myc (N262), rabbit anti-p-T58-c-Myc, and rabbit anti-AKT (H-136) antibodies from Santa Cruz Biotechnology; mouse p-S62-c-Myc (33A12E10) antibody from Abcam; rabbit anti-phospho AKT threonine 308, mouse anti-phospho-tyrosine, anti-PTEN (A2B1), anti-USP-28, antixtacellular receptor kinase (anti-ERK), anti-phospho-ERK, and anti-glycogen synthase kinase-3 (anti-GSK-3) antibodies from Cell Signaling Technology.

Recombinant plasmid DNA encoding dominant negative (DN) forms of HA-AKT expression was described previously (10). DN GSK-3b and DN RasN17 expression plasmids were obtained from Dr. Geoffrey M. Cooper (11, 12) and HA-USP-28 recombinant DNA was from Dr. Stephen J. Elledge (13).

Cell culture, siRNA transfection, and SRB cytotoxicity assay
A2058, SK-MEL-2, and A375 melanoma cells and MDA-MB-231 breast cancer cells were purchased from American Type Culture Collection Center and were not further tested or authenticated. All cell cultures were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS at 5% CO2 atmosphere. For Arg depletion, cells were either maintained in the regular medium containing 0.3 μg/ml ADI-PEG20 or cultured in Arg-free DMEM medium containing 10% dialyzed FBS. All transfections were done using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

For the cytotoxicity assay, cells were seeded in 96-well plates (4 × 103 cells per well) and cultured with different concentrations of inhibitors with or without ADI-PEG20 for 72 hours. Cells were fixed with 50% trichloroacetic acid followed by staining with 0.4% SRB in 1% acetic acid for 30 minutes at room temperature. Plates were washed 5 times with 1% acetic acid to remove unbound dye. Bound dye was dissolved by adding 10 mmol/L unbuffered Tris base. Cell proliferation was calculated by measuring OD at 564 nm using a spectrophotometer.

Immunoprecipitation, immunoblotting, and Northern blot
Procedures for cell extract preparations and immunoprecipitation were previously described (9). Briefly, protein samples were incubated with an antibody and 50 μL 50% protein A sepharose beads. Protein A beads were collected and the immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting and visualized with an enhanced chemiluminescence kit (Thermo Scientific).

For Northern blotting, cells were harvested by TRizol reagent (Invitrogen), and total RNA was extracted according to the manufacturer’s instructions. Equal amounts of total RNA samples were separated by 2% agarose-formalddehyde gel electrophoresis, transferred to a nylon membrane, and hybridized to [α-32P]dCTP-labeled (PerkinElmer) c-Myc cDNA probe according to the standard procedures.

Mouse experiments
Female athymic NCR nu/nu-nude mice (aged 7 weeks, weight ~20 grams, from National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) were housed in a pathogen-free environment. The animals were inoculated subcutaneously with 2 × 10⁶ A2058 melanoma cells in 100 μL physiologic buffered saline (PBS) into the right flank of mice. Ten days later, when the tumor volumes reached approximately 20 mm³, the animals were randomly divided into 4 groups with 6 animals per group and the treatments were initiated by intraperitoneal injections according to the following protocol. The first group received 100 μL PBS, the second group received Ly294002 (25 mg/kg), the third group received ADI-PEG20 (4 IU or 0.625 mg/100 μL), and fourth group received Ly294002 (25 mg/kg) plus ADI-PEG20 (4 IU). Each group of animals received the same doses of drugs twice per week thereafter. Tumor size was measured by caliper. Tumor volume was calculated using the formula: (length × width²)/2. Statistical analysis was done by Student t test using Microsoft Excel 2007 program. P < 0.05 was regarded as significant. Error bars represent SEM.

Other procedures
Enzymatic activity assays for phosphatidylinositol-3 phosphate (10) and PTEN (14), and DNA fragmentation assay (15) followed the procedures previously described.

Results
ADI-PEG20 induces c-Myc protein stabilization
The enhancement of c-Myc expression by ADI-PEG20 could be regulated at the transcriptional level or at the posttranscriptional level. To distinguish between these 2 possibilities, we carried out Northern blotting and Western blotting analyses to evaluate c-Myc mRNA and protein levels, respectively. Figure 1A shows that although induction of c-Myc protein was detectable within 1 hour of treatment and continued throughout the 8 hours of treatment, no corresponding increases in c-Myc mRNA levels were seen (Fig. 1B). These results suggested that the induction mechanism is either by enhanced protein synthesis or by reduced protein degradation. To differentiate between these possibilities, we treated A2058 cells with the protein synthesis inhibitor CHX with or without ADI-PEG20. In the absence of ADI-PEG20, c-Myc protein levels were reduced rapidly with a half-life (t½) of approximately 20 minutes (Fig. 1C, top), consistent with the previous report that c-Myc is a very unstable protein with a half-life (t½) between 20 and 30 minutes (16). In the presence of ADI-PEG20, c-Myc degradation was attenuated, and more than 70% of c-Myc remained even after 4 hours of treatment (Fig. 1C, bottom). In this experiment, we purposely overexposed the blot so that c-Myc expression level at the 0 time point could be visualized, as in contrast to those shown in Fig. 1A. These results showed that ADI-PEG20 treatment induces c-Myc protein stabilization.
ADI-PEG20-induced c-Myc stabilization is due to inhibition of ubiquitin-mediated protein degradation

It has been shown that rapid c-Myc protein turnover can be mediated by the ubiquitin-dependent proteasome pathway (17, 18). To investigate whether the c-Myc stabilization induced by ADI-PEG20 was due to inhibition of ubiquitin-mediated protein degradation, we transfected A2058 cells with recombinant plasmid encoding HA-tagged ubiquitin followed by Western blotting and Northern blotting.
treating the cells with or without ADI-PEG20. Cell lysates were prepared and c-Myc was immunoprecipitated with anti-c-Myc antibody using normal rabbit IgG as a negative control. The immunoprecipitates were analyzed by Western blotting using anti-HA antibody. Figure 1D shows that a significant amount of polyubiquitinated c-Myc was present in the ADI-PEG20-treated cells; whereas almost no ubiquitinated c-Myc was detected in the ADI-PEG20-treated cells. These results showed that ADI-PEG20-induced c-Myc accumulation was due to inhibition of c-Myc ubiquitination for protein degradation.

One of the important pathways for ubiquitin-dependent regulation of c-Myc turnover is controlled by the interactions with both USP-28 and Fbw7. Fbw7 is a subunit of the E3 ubiquitin ligase complex SCF^Fbw7 that recognizes c-Myc in response to specific stimuli leading to ubiquitination and subsequent proteasome degradation of c-Myc (19). In contrast, USP-28, which forms a complex with Fbw7α and counteracts the degradation of c-Myc by removing ubiquitins conjugated by Fbw7α (20). To investigate the possible roles of USP-28/Fbw7α in ADI-PEG20–mediated c-Myc stabilization, we transfected A2058 cells with recombinant plasmids encoding Frag-Fbw7α and HA-USP-28, in the presence of proteasome inhibitor MG132, followed by treatment with or without ADI-PEG20. c-Myc was subsequently immunoprecipitated and analyzed by Western blotting with anti-c-Myc, anti-USP-28, and anti-Flag antibodies. Our results showed that ADI-PEG20 treatment resulted in a significant association between overexpressed USP-28 and c-Myc/Fbw7α complex (Fig. 1E). We next investigated the effects of ADI-PEG20 on the interactions between endogenous USP-28 and c-Myc. MDA-MB-231 cells were treated with ADI-PEG20 for either 1 or 2 hours. Cell lysates were prepared and c-Myc was immunoprecipitated with a polyclonal anti-c-Myc antibody (N-262). The precipitates were probed with anti-USP-28 antibody, anti-c-Myc antibody (N-262), and anti-c-Myc monoclonal antibody (9E10). Figure 1F shows that ADI-PEG20 treatment enhances the interaction between USP-28 and c-Myc. These results suggested the involvement of USP-28/Fbw7α in the ADI-PEG20–mediated c-Myc accumulation.

ADI-PEG20–induced c-Myc stabilization is mediated by the ERK and PI3K/AKT-GSK3β signaling pathways

c-Myc protein is targeted for ubiquitin proteasomal degradation mechanism by phosphorylation at 2 specific amino acid residues at the N-terminus, serine 62 (S62) and threonine 58 (T58). S62 is a target of ERK and T-58 is targeted by GSK-3β (21, 22). ERK-mediated phosphorylation of S62 prevents c-Myc from degradation, whereas GSK-3β–phosphorylated T-58 promotes c-Myc degradation (21, 22). We investigated the possible involvement of these pathways and found that ADI-PEG20 treatment activated ERK and enhanced c-Myc phosphorylation at S62 in association with increased c-Myc levels (Fig. 2A). To test for the causal involvement of the ERK signaling, we used the chemical inhibitor U0126, which specifically inactivates MEK1/2 (ERK kinase). Western blot analysis revealed that U0126 was effective in completely inhibiting c-Myc phosphorylation and reducing c-Myc expression levels (Fig. 2A). These results suggested that ERK is involved in the posttranslational modification and stabilization of c-Myc in response to ADI-PEG20.

Phosphorylation of c-Myc at T58 by GSK-3β, a serine/threonine kinase, is recognized by Fbw7 in the proteasomal protein degradation signal (22). It has been shown that GSK-3β itself is a target of phosphoinositide 3-kinase (PI3K)/AKT-mediated phosphorylation at Ser9 position. GSK-3β phosphorylation inactivates its ability of phosphorylating c-Myc (T58), resulting in stabilization of c-Myc. PI3K/AKT also phosphorylates GSK-3α (Ser21). We asked whether this pathway regulates c-Myc stability in response to ADI-PEG20 challenge in A2058 cells. Phosphorylation of GSK-3β(Ser9) and GSK-3α (Ser21) in the ADI-PEG20–treated cells were determined using anti–p-GSK-3α and anti–p-GSK-3β antibodies, respectively. We found that ADI-PEG20 induces phosphorylation of GSK-3β, but not GSK-3α (using lysate from 293T cells as a positive control), in a time-dependent manner (Fig. 2B). Because phosphorylated c-Myc(T58) levels were not detectable even in the unstimulated A2058 cells (using lysate from the c-Myc–transfected A2058 cells as a positive control), we turned to MDA-MB-231 cells and observed that ADI-PEG20 treatment indeed reduced p-c-Myc(T58) expression in these cells (Fig. 2B, bottom). These results showed that ADI-PEG20 treatment induces GSK-3β phosphorylation in association with reduced c-Myc(T58) phosphorylation.

We also showed that knockdown of GSK-3β using siRNA led to increased c-Myc levels even without ADI-PEG20 treatment, whereas ADI-PEG20 plus GSK-3β siRNA treatments further increased c-Myc levels (Fig. 2C). Likewise, expression of GSK-3β-DN alone enhanced the expression of c-Myc compared with empty vector–transfected cells, and together with ADI-PEG20 treatment further enhanced the expression of c-Myc in the GSK-3β-DN–transfected cells (Fig. 2D). In addition, we used a potent mammalian GSK-3 inhibitor, LiCl, which competes against the cofactor Mg\(^{2+}\) for GSK-3 activity (23). We found that LiCl treatment enhanced the expression of c-Myc and its downstream target AS in ADI-PEG20–treated MDA-MB-231 cells (Fig. 2E). Taken together, these results showed that GSK-3β plays an inhibitory role in ADI-PEG20–mediated c-Myc stabilization.

ADI-PEG20–induced phosphorylation of GSK-3β could be due to activation of PI3K/AKT signaling. To investigate the role of PI3K/Akt in ADI-PEG20–induced GSK-3β phosphorylation, we measured PI3K activity in extracts from ADI-PEG20–treated cells using phosphotidinositol as a substrate. Figure 3A shows that induction of PI3K activity occurred as early as 15 minutes and persisted throughout the 4 hours of ADI-PEG20 treatment. Activation of PI3K by ADI-PEG20 was also shown by the induction of Akt(T308) phosphorylation, a downstream effector of PI3K (Fig. 3B). We further showed that inhibition of PI3K activity with LY294002 suppressed the ADI-PEG20–induced phosphorylation of Akt(T308) and GSK-3β(Ser9) and expression levels of c-Myc (Fig. 3C). Likewise, a DN mutant form of Akt suppressed the induction of p-GSK-3β(Ser9) and the expression of c-Myc and AS in a dose-dependent manner in MDA-MB-231 cells (Fig. 3D). Activation of PI3K by ADI-PEG20 is apparently not due to downregulation of PTEN, which is a
phosphatase that negatively regulates PI3K signaling (24, 25), as we failed to detect reduction of PTEN expression levels by Western blotting within the first 4 hours of the treatment (Fig. 3E). Nor did we see reduction of PTEN phosphatase activities using sodium selenite treatment, which is known to induce PTEN activity, as a positive control (ref. 26; Fig. 3E). Overall, these results indicated that upregulation of c-Myc is through the inhibition of GSK-3β activity, which is mediated by the activation of PI3K/AKT in response to ADI-PEG20.

Involvement of Ras signaling in ADI-PEG20–induced c-Myc stabilization and cell line specificity

One important upstream activator for PI3K/Akt pathway is Ras. To investigate whether Ras is involved in ADI-induced PI3K/AKT activation that leads to c-Myc accumulation, we measured Ras activity in ADI-treated A2058 cells. Figure 4A shows that induction of Ras activity by ADI-PEG20 occurred as early as 5 minutes after A2058 cells were exposed to ADI-PEG20. Moreover, a DN mutant form of Ras (RasN17) could reduce the signal of pT308-AKT and c-Myc protein levels in A2058 transfected with HA-c-Myc plasmid as a positive control. C, effects of GSK-3β siRNA on the expression of c-Myc by ADI-PEG20. A2058 cells were transfected with GSK-3β siRNA and its control for 48 hours. Cells were treated with ADI-PEG20 for another 4 hours. Cell lysates were analyzed for indicated proteins by Western blotting. D, A2058 cells were transfected with GSK3β-DN vector for 24 hours. Cells were maintained in normal medium or medium containing 0.3 μg/mL ADI-PEG20 for 4 hours. Expression levels of c-Myc, GSK-3β, p-GSK-3β, and tubulin were analyzed by Western blotting. E, inhibition of GSK-3 activity by LiCl increases the stability of c-Myc. MDA-MB-231 cells were treated with 10 mmol/L LiCl in the absence or presence of ADI-PEG20 for 48 hours. Expression levels of AS, c-Myc, GSK-3β, p-GSK-3β, c-Myc, AS, and actin were analyzed by Western blotting.
response to ADI-PEG20 in A2058 cells (Fig. 4B). These results, collectively, showed that induction of c-Myc expression by ADI-PEG20 is due to activation of Ras/PI3K/Akt/ERK pathways in A2058 cells.

Our results described thus far were mainly carried out in the A2058 cell line. To assess the generality of these findings, we examined the effects of ADI-PEG20 in 3 additional human cancer cell lines, SK-MEL-2, MDA-MB-231, and A375. Expression of AS and c-Myc in SK-MEL-2 (9) and MDA-MB-231 cells, such as A2058 cells, can be induced by ADI-PEG20 treatment; whereas expression of c-Myc/AS is not inducible in A375 cells (9). Figure 4C shows that ADI-PEG20 treatment induced

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**Figure 3.** Roles of the PI3K/AKT signal cascade in the regulation of c-Myc expression by ADI-PEG20. A, time-course induction of PI3K activity in A2058 cells treated with ADI-PEG20. A2058 cells were treated with 0.3 μg/mL ADI-PEG20 for various time intervals. Cells were harvested. PI3K was immunoprecipitated with anti–phospho-tyrosine antibody/protein G agarose. The kinase activity was determined. –Ab, no anti-phospho-tyrosine antibody. B, protein extracts were obtained from A. The expression levels of pT308-AKT, AKT, c-Myc, PTEN, and tubulin were analyzed by Western blotting. C, effects of the PI3K inhibitor Ly294002 on ADI-PEG20–induced c-Myc expression. A2058 Cells were incubated ADI-PEG20 plus 10 μmol/L Ly294002. Protein extracts were obtained at the indicated time points. AKT phosphorylation was determined with an antibody specific to AKT phosphor-Thr308. GSK-3β phosphorylation was determined with an antibody specific to GSK-3β phosphor-Ser9. AKT, GSK-3β, or tubulin proteins were probed with their respective antibodies. D, effects of DN AKT on ADI-PEG20–induced c-Myc expression. MDA-MB-231 cells were transfected with different amounts of HA-AKT-DN vector for 24 hours. Cells were maintained in normal medium or in medium containing 0.3 μg/mL ADI for 48 hours. The expression levels of AS, c-Myc, pGSK-3β(Ser9), GSK-3β, HA, and actin were analyzed by Western blotting. E, effects of ADI-PEG20 on PTEN expression and activity. A2058 cells were treated with 0.3 μg/mL ADI-PEG20 for various time intervals. Enzymatic activities of PTEN were measured using an 1-hour treatment of sodium selenite lysate as a positive control for phosphatase activity assay. Inset shows Western blot measurement of PTEN protein levels in the treated cells.
activation of Ras, phosphorylation of AKT, phosphorylation of GSK-3β, and activation of ERK signals in A2058, SK-MEL-2, and MDA-MB-231 cells, but not in A375 cells. These effects were correlated with induced expression of c-Myc and AS proteins in these cells (Fig. 4C). This correlation suggests an important role of Ras/PI3K/Akt/GSK-3β/ERK signaling in the upregulation of c-Myc under Arg deprivation conditions in multiple cell lines.

Inhibitors of PI3K/AKT signaling can enhance ADI-PEG20–mediated cell killing through apoptosis

The results above suggested that targeting the Ras/PI3K/Akt pathway may enhance ADI-PEG20’s cell killing capacity by preventing AS expression. As a proof-of-principle, we tested 2 PI3K inhibitors, Ly294002 and PI103, both are pan-PI3K inhibitors for class I (PI3Kα/β/γ/δ; 27). We also included 2 AKT inhibitors, VIII, which is an AKT1 and AKT2 isoyme-selective inhibitor (28) and perifosine, which targets the pleckstrin homology (PH) domain of Akt, preventing its translocation to the plasma membrane (28, 29). These inhibitors may have various off-targets when high concentrations (50 μmol/L) were used. A2058 cells were maintained in normal medium, ADI-containing medium, or Arg-deficient medium, each with or without these inhibitors (10 or 5 μmol/L). As anticipated, cells cultured in Arg-deficient medium, such as those grown in the ADI-PEG20–containing medium, upregulated AS and c-Myc expression (Fig. 5A). All 4 inhibitors suppressed the induction of pT308-AKT, c-Myc, and AS expression by either ADI-PEG20 or Arg-negative culture conditions (Fig. 5A). Although each of these inhibitors shows dose-dependent anticell proliferative activities, when in combination with ADI-PEG20, each of these inhibitors showed additive effects on cell growth as compared with ADI-PEG20 or individual inhibitors alone (Fig. 5B). Furthermore, although we found that A2058 cells treated with each of these 4 inhibitors or arginine deprivation for up to 48 hours only produced minimally detectable DNA fragmentation, combination treatment with ADI-PEG20 produced significant effects on genomic DNA fragmentation (Fig. 5C). These results showed that inhibition of PI3K/AKT pathway enhances the cell killing capacity of ADI-PEG20.

PI3K inhibitor can enhance antitumor activity of ADI-PEG20 in animal tumor model

To test whether PI3K inhibitor would enhance antitumor activity of ADI-PEG20 in vivo, we treated A2058 xenografts in animals with vehicle, ADI-PEG20, Ly294002, or combination of both drugs. Ten days after treatments, we found that the average tumor volumes were 672.6 ± 80.9, 174.6 ± 38.4, 114.2 ± 17.3, and 51.6 ± 19.5 mm3, in the PBS-, and ADI-PEG20-, Ly294002-, and combination of ADI-PEG20 and ADI-PEG20)-treated groups, respectively (Fig. 6A). These corresponded to 74% (P < 0.001), 83% (P < 0.001), and 92% (P < 0.0005) reduction of tumor volumes in the ADI-PEG20-, Ly294002-, and ADI-PEG20-Ly294002-treated groups, respectively, as compared with the PBS-treated group. Representative
Figure 5. Enhanced ADI-PEG20–mediated cell killing by PI3K and AKT inhibitors in A2058 cells. A, cells were maintained in normal medium, ADI-PEG20-containing, or Arg-free medium without [dimethyl sulfoxide (DMSO) as vehicle] or without PI3K inhibitors (Ly294002, PI103) or AKT inhibitors (VIII and perifosine) for 48 hours. Expression levels of pT308-AKT, c-Myc, and AS were analyzed by Western blotting. B, effects of various PI3K and AKT inhibitors on ADI-PEG20 sensitivity. A2058 cells maintained in normal medium or medium containing 0.3 mg/mL ADI were treated with or without 5 μmol/L LY294002, 1 μmol/L PI103, 5 μmol/L AKT inhibitor VIII, and 5 μmol/L perifosine for 72 hours. The antiproliferative effects of the combination of PI3K/AKT inhibitors and ADI-PEG20 were determined by the SRB assay. Error bars represent SD from 6 independent experiments. C, DNA fragmentation assay of ADI-PEG20–induced apoptosis. DNA extracted from A2058 cells treated with 5 μmol/L LY294002, 1 μmol/L PI103, 5 μmol/L AKT inhibitor VIII, or 5 μmol/L perifosine in the absence or presence ADI-PEG20 for 24 or 48 hours or ADI-PEG20 alone was subjected to the DNA fragmentation assay. DNA samples and molecular size markers (M) were analyzed by electrophoresis on 2% agarose gels. DNA was stained with ethidium bromide and photographed.
tumor volumes from each group are shown in Fig. 6B. These results showed that Ly294002 can enhance antitumor activity of ADI-PEG20 in vivo.

Discussion
In this study, we elucidated the underlying mechanism associated with c-Myc induction by ADI-PEG20. Our results, as schematically summarized in Fig. 7, show that ADI-PEG20 activates Ras signal and its downstream kinase cascades, resulting in c-Myc stabilization. C-Myc has been recognized as one of important mammalian transcription regulator. Chromatin immunoprecipitation studies have revealed that about 10% to 20% of total cellular genes are regulated by c-Myc through E-box interactions (30, 31). These genes are involved in a vast variety of cellular functions including cell proliferation, differentiation, survival, apoptosis, metabolism, invasion, and metastasis (32, 33). Multiple regulation mechanisms starting from transcriptional initiation to protein turnover are involved in c-Myc expression in response to various extracellular challenges (18). The molecular mechanism underlying how c-Myc stabilization is induced in response to Arg deprivation as elucidated in this study exemplifies the exquisite regulation mechanism of c-Myc expression in drug resistance research.

At least 8 protein factors (including Fbw7, USP-28, Fbx29, Skp2, β-TRCP, Truss, Hect-19, and Trim32) have been reported to control c-Myc stability through ubiquitination (for review, see ref. 18). Among these, the Fbw7/USP-28 ubiquitination/deubiquitination system is the best characterized partners. Whereas Fbw7 binds to phosphorylated c-Myc, USP-28 does not bind to c-Myc directly but through interaction with Fbw7 and reverses the ubiquitination built by Fbw7 (20, 34), thereby protecting c-Myc from degradation. These observations suggest that cellular levels of USP-28 have a profound influence on Fbw7-mediated c-Myc stabilization. In this study, we found that ADI-PEG20 treatment drastically increased the association between USP-28 and c-Myc. These findings underscore the important role of USP-28 in ADI-PEG20–induced c-Myc accumulation, although it remains possible that other protein factors could also be involved.

The importance of Fbw7/USP-28 in the ADI-PEG20–dependent c-Myc regulation is also supported by the elucidation of their upstream signal transduction pathways. The role of PI3K in regulation of c-Myc has been supported by several recent reports showing that inhibition of PI3K activity resulted in amplification or overexpression of c-Myc, and that c-Myc overexpression could confer resistance to PI3K inhibitors, although the underlying mechanism was not elucidated in these reports (35–37).

We could detect activation of Ras signaling in melanoma cells within 5 minutes after ADI-PEG20 treatment. Such rapid activation suggests an intricate sensing mechanism for Arg deficiency in melanoma cells. Whether the sensing mechanism involves activation of membrane-bound receptor(s) that transmit signal to Ras resulting in c-Myc accumulation remains to be investigated (Fig. 7). The Ras pathway is a key regulator of cancer cell survival and has been shown to be constitutively active in many cancer types, including the 4 cell lines used in this study, either by BRAF(V600E) mutations (A2058 and A375) or Ras mutations (SK-MEL-2), or both (MDA-MB-231; ref. 38). Notably, we found correlations between Ras/PI3K/AKT/ERK activation and c-Myc/AS inducibility by ADI-PEG20 among 4 melanoma cell lines. Although the steady-state level of c-Myc level in A375 cells is higher than those in other 3 cell lines, the inability of c-Myc/AS induction in A375 cells is due to defective Ras upstream signal, and elucidation of this defective mechanism is currently underway in our laboratories. We previously showed that lack of c-Myc/AS induction by ADI-PEG20 in A375 cells is associated with the inability of developing resistance to ADI-PEG20 (9). Although the number of cell lines used remains small and further in-depth studies are needed, our current results suggest that Ras pathway may offer not only as a potential target for therapeutic intervention but also as a useful biomarker for predicting treatment outcomes of ADI-PEG20 in clinical setting.

Because of the importance of the Ras/PI3K/AKT/ERK pathway in many aspects of human malignancies, including tumor
growth, proliferation, invasion, and sensitivity to therapy, clinical studies have been done using inhibitors of this pathway in human cancers (39–41). Examples of these drugs are listed in Fig. 7. Of particular relevance to malignant melanoma are those targeting mutant BRAF(V600E), a recurrent mutation occurs in 50% to 60% of malignant melanomas. Clinical trials of inhibitors targeting this mutation have shown remarkable results (42); however, resistance to BRAF(V600E) almost inevitably developed after prolonged treatment, through activation of alternative/compensatory survival pathways, such as PDGFRβ or N-Ras (43), mitogen-activated protein kinase (44), and IGF-1R/Pi3K (45), and combined treatment with inhibitors of IGF-1R/Pi3K induces cell death in BRAF-resistant variants (45). The present demonstration that inhibitors of the Pi3K/AKT pathway can enhance the cell killing capacity by ADI-PEG20 suggests that intervention of this pathway may have added advantages for ADI-PEG20 cancer chemotherapy. We envision that inhibitors of BRAF(V600E), such as the inhibitors of anti-Pi3K and anti-AKT that have been shown in this study, would also enhance cell killing effects of ADI-PEG20. These approaches of simultaneously targeting 2 prevalent genetic abnormalities in melanoma (BRAF mutation and Arg-auxotrophicity) may improve the treatment efficacy of malignant melanoma.

Finally, our present study on ADI-PEG20 in melanoma cells may provide important information for the use of other Arg-degrading enzymes in cancer chemotherapy, such as recombinant arginases that convert Arg into ornithine and urea. Recombinant arginases have also been in various stages of clinical development for human cancer chemotherapy (46–49).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Tsai et al.

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Activation of Ras/PI3K/ERK Pathway Induces c-Myc Stabilization to Upregulate Argininosuccinate Synthetase, Leading to Arginine Deiminase Resistance in Melanoma Cells

Wen-Bin Tsai, Isamu Aiba, Yan Long, et al.


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