

# Uveal Melanoma Cell Growth Is Inhibited by Aminoimidazole Carboxamide Ribonucleotide (AICAR) Partially Through Activation of AMP-Dependent Kinase

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**PURPOSE.** To evaluate the effects and mechanism of aminoimidazole carboxamide ribonucleotide (AICAR), an AMP-dependent kinase (AMPK) activator, on the growth of uveal melanoma cell lines.

**METHODS.** Four different cell lines were treated with AICAR (1–4 mM). Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Cell cycle analysis was conducted by flow cytometry; additionally, expression of cell-cycle control proteins, cell growth transcription factors, and downstream effectors of AMPK were determined by RT-PCR and Western blot.

**RESULTS.** Aminoimidazole carboxamide ribonucleotide inhibited cell growth, induced S-phase arrest, and led to AMPK activation. Aminoimidazole carboxamide ribonucleotide treatment was associated with inhibition of eukaryotic translation initiation factor 4E-BP1 phosphorylation, a marker of mammalian target of rapamycin (mTOR) pathway activity. Aminoimidazole carboxamide ribonucleotide treatment was also associated with downregulation of cyclins A and D, but had minimal effects on the phosphorylation of ribosomal protein S6 or levels of the macroautophagy marker LC3B. The effects of AICAR were abolished by treatment with dipyrindamole, an adenosine transporter inhibitor that blocks the entry of AICAR into cells. Treatment with adenosine kinase inhibitor 5-iodotubercidin, which inhibits the conversion of AICAR to its 5'-phosphorylated ribotide 5-aminoimidazole-4-carboxamide-1-D-ribofuranosyl-5'-monophosphate (ZMP; the direct activator of AMPK), reversed most of the growth-inhibitory effects, indicating that some of AICAR's antiproliferative effects are mediated at least partially through AMPK activation.

**CONCLUSIONS.** Aminoimidazole carboxamide ribonucleotide inhibited uveal melanoma cell proliferation partially through activation of the AMPK pathway and downregulation of cyclins A1 and D1.

Keywords: AMPK, AICAR, melanoma, mTOR

Uveal melanoma arises from neural crest-derived melanocytes of the uveal tract<sup>1</sup> and is the most common primary intraocular malignant tumor in adults<sup>2</sup> with an incidence of four to seven individuals per 1 million/y in the United States.<sup>1,3</sup>

Clinical presentation varies depending on the size and location of the tumor. Median age at presentation is 55 years of age,<sup>4</sup> and the majority of patients are Caucasians.<sup>5</sup> Metastasis develops in up to 50% of primary uveal melanoma patients, usually through hematogenous spread.<sup>3,6</sup> Regional lymphatic dissemination occurs rarely, due to the relative lack of lymphatic drainage of the choroid.<sup>6,7</sup> The most common site of metastasis is the liver (occurring in as many as 90% of patients with metastatic uveal melanoma), and the median survival of those patients is approximately 4 to 5 months.<sup>3,8</sup> Approximately 50% of patients with liver metastasis also have

extrahepatic involvement, the most common sites being lung (30%), bone (23%), and skin (17%).<sup>2</sup> Factors predicting metastatic disease are large tumor diameter, ciliary body involvement, extrascleral extension, epithelioid melanoma histology,<sup>9</sup> vascular matrix pattern (such as closed loops), high mitotic rate, microvascular density, monosomy 3, and class 2 gene expression profile.<sup>10–14</sup>

While radical treatment of uveal melanoma consists of enucleation, the most common treatments are conservative, such as brachytherapy and external irradiation (e.g., proton beam). Survival rates and risk of metastasis are similar with either enucleation or radiation.<sup>15</sup>

Despite good local control of uveal melanoma,<sup>3,16,17</sup> the treatment of metastatic disease is still limited due to its resistance to conventional systemic chemotherapy. Many drugs,

such as imatinib, bevacizumab, and trametinib (a reversible, selective allosteric inhibitor of MEK1 and MEK2)<sup>18</sup> are presently under investigation along with intrahepatic injection or surgical intervention.<sup>3</sup> However, there is insufficient evidence that any pharmacologic treatment prolongs survival in patients with metastatic uveal melanoma.<sup>19</sup>

Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase that is a major sensor and regulator of cellular and whole-body energy levels and stress.<sup>20–24</sup> Its activity is regulated by conditions that deplete cellular ATP and elevate AMP levels (such as hypoxia, exercise, ischemia, glucose deprivation, and heat shock),<sup>25</sup> and also by some hormones such as leptin,<sup>26</sup> adiponectin,<sup>27</sup> catecholamine,<sup>28</sup> and IL-6.<sup>29</sup> Adenosine monophosphate-activated protein kinase upstream protein kinase liver kinase B1 (LKB1)<sup>30,31</sup> is a tumor suppressor that is mutated in Peutz-Jegher syndrome. Its downstream effectors also involve the tumor suppressor tuberous sclerosis complex (TSC2) and the mammalian target of rapamycin (mTOR), which are known to be important factors in cell-cycle progression and tumor formation.<sup>32,33</sup> Although many pharmacologic activators of AMPK exist, 5-aminoimidazole-1-β-4-carboxamide riboside (aminoimidazole carboxamide ribonucleotide [AICAR]) was the first compound reported to activate AMPK both in intact cells and *in vivo*.<sup>34,35</sup> Aminoimidazole carboxamide ribonucleotide is taken into cells by adenosine transporters and then converted by adenosine kinase to the monophosphorylated form, 5-aminoimidazole-4-carboxamide-1-D-ribofuranosyl-5'-monophosphate (ZMP), which mimics an increase of AMP intracellular levels. In addition to its AMPK-dependent effects, AICAR can also be converted to inosine, which acts in an AMPK-independent manner to increase cellular adenosine concentration.<sup>34,36</sup> The toxicity of AICAR is low or not apparent when given in intraperitoneal doses up to 500 mg/kg/day for 4 weeks in mice.<sup>37</sup>

Adenosine monophosphate-activated protein kinase activation has been reported to have both pro-survival and pro-apoptotic effects depending on the environment and the stimulus; for example, AMPK activation has been shown to be anti-apoptotic in situations of hyperglycemia,<sup>38</sup> glucose deprivation,<sup>39</sup> and ischemia/reperfusion injury.<sup>40</sup> Aminoimidazole carboxamide ribonucleotide-mediated activation of AMPK has been shown to inhibit proliferation and induce apoptosis in retinoblastoma cells (both *in vitro* and *in vivo*),<sup>41,42</sup> neuroblastoma cells,<sup>43</sup> childhood acute lymphoblastic leukemia cells,<sup>44</sup> glioblastoma cells,<sup>45</sup> multiple myeloma cells,<sup>46</sup> prostate cancer cells, breast cancer cells,<sup>36</sup> hepatic cancer cells,<sup>47</sup> and colon cancer cells.<sup>48</sup> Various mechanisms have been demonstrated, such as upregulation of p53,<sup>44</sup> increased expression of cell-cycle inhibitory proteins p21 and p27,<sup>36,44</sup> activation of the mitogen-activated protein kinase (MAPK)-p38 pathway,<sup>32</sup> inhibition of the Akt/mTOR/P70S6K pathway,<sup>44–46</sup> decrease of cyclins A and E,<sup>41</sup> inhibition of nuclear factor kappa-B (NF-κB) activity,<sup>36,48</sup> and decreased angiogenesis.<sup>42</sup> The various effects of AMPK on survival or growth inhibition likely depend on cell type, duration of AMPK activation, cellular events following external stimuli, and/or downstream regulated pathways of AMPK.

In the present study, we investigated the effects of AICAR on cell proliferation and its mechanism of action *in vitro* in three uveal melanoma cell lines.

## MATERIALS AND METHODS

### Chemicals

Aminoimidazole carboxamide ribonucleotide was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Dipyridamole and 5-iodotubercidin (iodo) were purchased

from Sigma-Aldrich (St. Louis, MO, USA). Aminoimidazole carboxamide ribonucleotide was dissolved in RPMI 1640 medium (Invitrogen [Life Technologies], Carlsbad, CA, USA) at a concentration of 40 mM (stock solution) and stored at –20°C until used. Dipyridamole and iodo were prepared fresh from stock solutions and diluted with growth medium. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. The following primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA): phospho-ACC (Ser-79), phospho-S6 ribosomal protein (Ser-235/236), CDK2, CDK4 (DCS156), p21 Waf1/Cip1 (12D1), p27Kip1 (D37H1), p53, LC3B, phospho-4E-BP1 (Ser-65), PCNA (PC10), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E), and β-actin (13E5).

### Cell Culture

The primary cell lines OCM 3 (aka SkMel28),<sup>49</sup> 92.1,<sup>50</sup> MEL 270,<sup>51</sup> and MEL 202<sup>52</sup> were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 µg/ml; Invitrogen), and streptomycin (100 µg/ml; Invitrogen). MEL 270 and MEL 202 were additionally supplemented with 1% minimal essential medium (MEM) vitamin solution and 1% MEM nonessential amino acids (Invitrogen). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and split when they reached approximately 90% to 95% confluence.

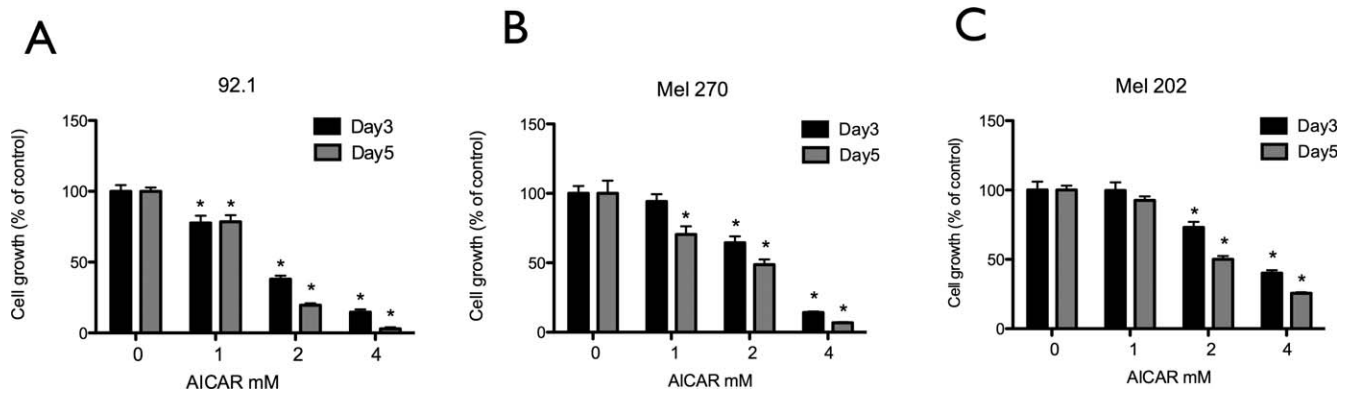
### Measurement of Cell Growth by MTT Assay

Cell viability was assessed by MTT assay. Cells were cultured in 96-well plates at a density of 4000 cells/well in 150 µL growth medium and were incubated with AICAR (1, 2, and 4 mM), dipyridamole (2 µM), or 5-iodotubercidin (0.1 µM) at 37°C for 3 and 5 days. On days 1, 3, and 5 of treatment, the culture medium was aspirated and 60 µL MTT (5 mg/mL in RPMI 1640 without phenol red; Sigma-Aldrich) was added to each well. After incubation for 2 hours at 37°C, 130 µL of dimethyl sulfoxide was added. The absorbance or optical density (OD) at 595 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). For each treatment, cell growth was evaluated as a percentage using the following equation:

$$\frac{(\text{OD}_{595} \text{ of treated sample} - \text{OD}_{595} \text{ of sample on day of culture})}{(\text{OD}_{595} \text{ of untreated sample} - \text{OD}_{595} \text{ of sample on day of culture})} \times 100 \quad (1)$$

### Cell Cycle Assessment by Flow Cytometry

Cellular DNA content was assessed by flow cytometry. Cells were cultured in 10-cm plates at a density of 2.5 million cells/plate in 10 mL growth medium, and were treated with 1 and 2 mM AICAR for 1, 3, and 5 days. After drug treatment, the cells were trypsinized, spun at 200g for 5 minutes, and washed twice with 1-mL cold PBS. While the cells were continuously vortexed, 2 mL ice-cold 75% ethanol was added slowly, and the cells were then fixed overnight. On the day of measurement, cells were spun, resuspended in 2 mL PBS with the addition of 100 µL of DNase-free RNase A (200 µL/mL; Invitrogen), and incubated at 37°C for 30 minutes. Then, 100 µL of 1 mg/mL propidium iodide (Invitrogen) was added, and the cells were incubated at room temperature for 10 minutes. The samples were read on a Becton Dickinson FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). The sub-G1 peak was quantified and represented the nonviable cell population.



**FIGURE 1.** Aminoimidazole carboxamide ribonucleotide inhibits growth of human uveal melanoma cells. Uveal melanoma cell lines 92.1 (A), MEL 270 (B), and MEL 202 (C) were treated for 3 and 5 days with various concentrations of AICAR (1–4 mM), and cell viability was measured by MTT assay. Results are expressed as percentage of growth (%) relative to control values, defined as 100%. Data represent three independent experiments, each conducted with triplicate cultures. Significance (\*) is assigned at  $P < 0.05$ .

### Western Blot Analysis

After 24 hours of incubation in the presence or absence of AICAR, medium was aspirated, and the plate was washed three times with cold PBS and kept in  $-80^{\circ}\text{C}$  overnight. On the next day, 500  $\mu\text{L}$  of 1 $\times$  lysis buffer (Cell Signaling Technology) containing protease and phosphatase inhibitor cocktail (Roche, Indianapolis, IN, USA) were added per 10-cm dish, incubated for 5 minutes on ice, and cells were scraped. Extract was centrifuged for 10 minutes at 14,000 $\times g$  in a cold microcentrifuge. The supernatant was removed, and lithium dodecyl sulfate sample buffer (Invitrogen) containing dithiothreitol (American Bioanalytical, Natick, MA, USA) was added to equal amounts of total protein from each sample and heated at  $90^{\circ}\text{C}$  for 5 minutes. Samples were loaded onto a NuPAGE 4–12% Bis-Tris Gel (Invitrogen) and then transferred to a polyvinylidene fluoride (PVDF) membrane (0.45  $\mu\text{m}$ ; Millipore, Billerica, MA, USA). The membranes were incubated overnight with primary antibody at  $4^{\circ}\text{C}$  with gentle shaking. Primary antibodies were diluted 1:1000 in 5% wt/vol BSA, Tween-20 (TBST) with exception of the antibodies for p53, CDK4 and PCNA, which were diluted in 5% nonfat dry milk, TBST. The blotted membranes were washed three times (5 minutes/wash) with TBST and incubated for 45 minutes at room temperature with horseradish peroxidase-labeled anti-rabbit or anti-mouse secondary antibody (1:100,000; Jackson ImmunoResearch, West Grove, PA, USA). The membranes were washed three times (5 minutes/wash) in TBST, and immunoreactive bands were visualized by enhanced chemoluminescence (ECL) and exposure onto Fuji RX film (Fujifilm, Tokyo, Japan) for approximately 5 minutes.

### Quantitative Real-Time RT-PCR

After 24 hours of incubation in the presence or absence of AICAR, the medium was aspirated and plates were washed with cold PBS. Cellular RNA was extracted and purified with cold PBS. Cellular RNA was extracted and purified with the RNeasy Micro kit (Qiagen, Valencia, CA, USA). Ribonucleic acid was further cleaned with an additional DNase I digestion step according to the manufacturer's instructions. Reverse transcription was performed for equal RNA amounts (4  $\mu\text{g}$ , as measured by ultraviolet spectrophotometry) with oligo dT primer (Invitrogen) and Superscript II (Invitrogen). Complementary DNA (100 ng) was used for each of the three replicates for quantitative PCR. Human cyclin A1, cyclin A2, cyclin D1, cyclin D3, cyclin E1, cyclin E2, and 18S, and  $\beta$ -actin (as endogenous controls) were amplified with commercially

designed Taqman gene expression assays (Applied Biosystems, Foster City, CA, USA) and the Taqman universal PCR master mix (Applied Biosystems). Quantitative expression data were acquired and analyzed with a Step One Plus real-time PCR system (Applied Biosystems).

### Statistical Analysis

The results are expressed as the mean  $\pm$  SEM. Data were analyzed by Student's *t*-test or ANOVA of the repeated experiments with Prism software (GraphPad Software, San Diego, CA, USA). For all analyses, significance was assigned at  $P$  less than 0.05.

## RESULTS

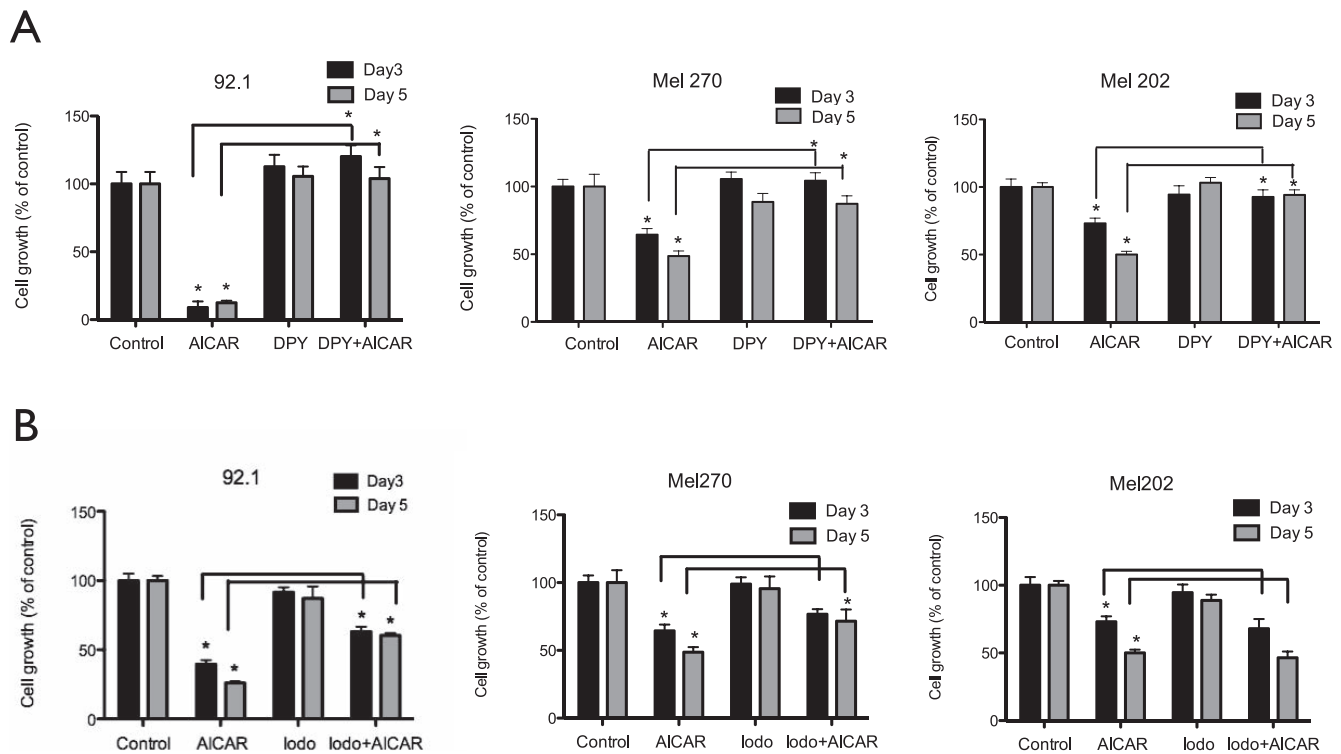
### AICAR Inhibits the Growth of Uveal Melanoma Cells

To study the effect of AICAR on the growth and metabolism of uveal melanoma cells, one skin melanoma cell line (OCM 3) and three uveal melanoma cell lines (92.1, MEL 270, and MEL 202) were treated with AICAR (1, 2, and 4 mM) for 3 and 5 days. Their metabolism and growth was evaluated using the MTT assay. Aminoimidazole carboxamide ribonucleotide inhibited their growth in a time- and dose-dependent manner ( $P < 0.05$  for all cell lines; Fig. 1, Supplementary Fig. S1).

Cellular uptake of AICAR occurs via adenosine transporters. To confirm that the inhibition of uveal melanoma cells was dependent on receptor-mediated uptake of AICAR, we pretreated cells with dipyridamole, which blocks adenosine transporters and prevents uptake of AICAR into the cells. As a negative control, dipyridamole treatment alone did not affect cell metabolism and growth. In contrast, treatment of uveal melanoma cells with dipyridamole plus AICAR abolished the inhibitory effect of AICAR in all cell lines ( $P < 0.05$ ), indicating that surface adenosine receptors are expressed on uveal melanoma cells and mediate the uptake and effects of AICAR (Fig. 2A, Supplementary Fig. S2A).

### Antiproliferative Effects of AICAR are Mediated at Least Partially via the AMPK Pathway

Since AICAR has been reported to be able to inhibit cell growth and proliferation via an AMPK-independent mechanism,<sup>53</sup> it is



**FIGURE 2.** Dipyridamole (DPY) and iodo effects on AICAR mediated uveal melanoma cell growth inhibition. Uveal melanoma cell lines 92.1, MEL 270, and MEL 202 were pretreated for 30 minutes with 2  $\mu$ M DPY (A) or 0.1  $\mu$ M iodo (B). Cells were then incubated for either 3 or 5 days without or with AICAR (2 mM). An MTT assay was performed, and results are expressed as percentage of growth (%) relative to control values, defined as 100%. Data represent three independent experiments, each conducted with triplicate cultures. Significance (\*) is assigned at  $P < 0.05$ .

important to determine whether AMPK activation coincides with the antiproliferative effects of AICAR on uveal melanoma cells. To confirm that AICAR treatment of uveal melanoma cells was associated with AMPK activation, we examined the phosphorylation of acetyl-CoA carboxylase (ACC), the downstream target of AMPK. Cells treated with AICAR (1 and 2 mM) showed an increase of phosphorylated ACC (Fig. 3A, Supplementary Fig. S3A). To confirm that ACC phosphorylation was due to intracellular AICAR, cells were pretreated with dipyridamole before AICAR. Blocking adenosine receptors and AICAR entry into the cells with dipyridamole inhibited ACC phosphorylation (Fig. 3B, Supplementary Fig. S3B). These data indicate that the AICAR-mediated inhibition of uveal melanoma cells coincides with activation of the AMPK pathway.

Other investigators have reported that once AICAR enters the cells it can be converted to either inosine or ZMP.<sup>54-56</sup> Inosine can inhibit cells via an AMPK-independent pathway, whereas ZMP activates the AMPK pathway. Aminoimidazole carboxamide ribonucleotide is converted to ZMP by adenosine kinase, but this conversion is blocked by iodo. To determine whether uveal melanoma cells inhibition by AICAR coincides with the conversion of AICAR to ZMP, we pretreated the cells with iodo prior to AICAR administration. Activation of AMPK was assessed by examination of ACC phosphorylation. Although activation of AMPK was shown to be effectively blocked by iodo treatment as judged by phosphorylated ACC immunoblots (phosphorylated ACC  $\pm$  iodo; inhibition at  $P < 0.05$ ; Fig. 3C, Supplementary Fig. S3C), a significant, but not complete reversal of AICAR-mediated uveal melanoma cell growth inhibition was observed in OCM 3, 92.1, and MEL 270 cell lines, but not MEL 202 (Fig. 2B, Supplementary Fig. S2B),

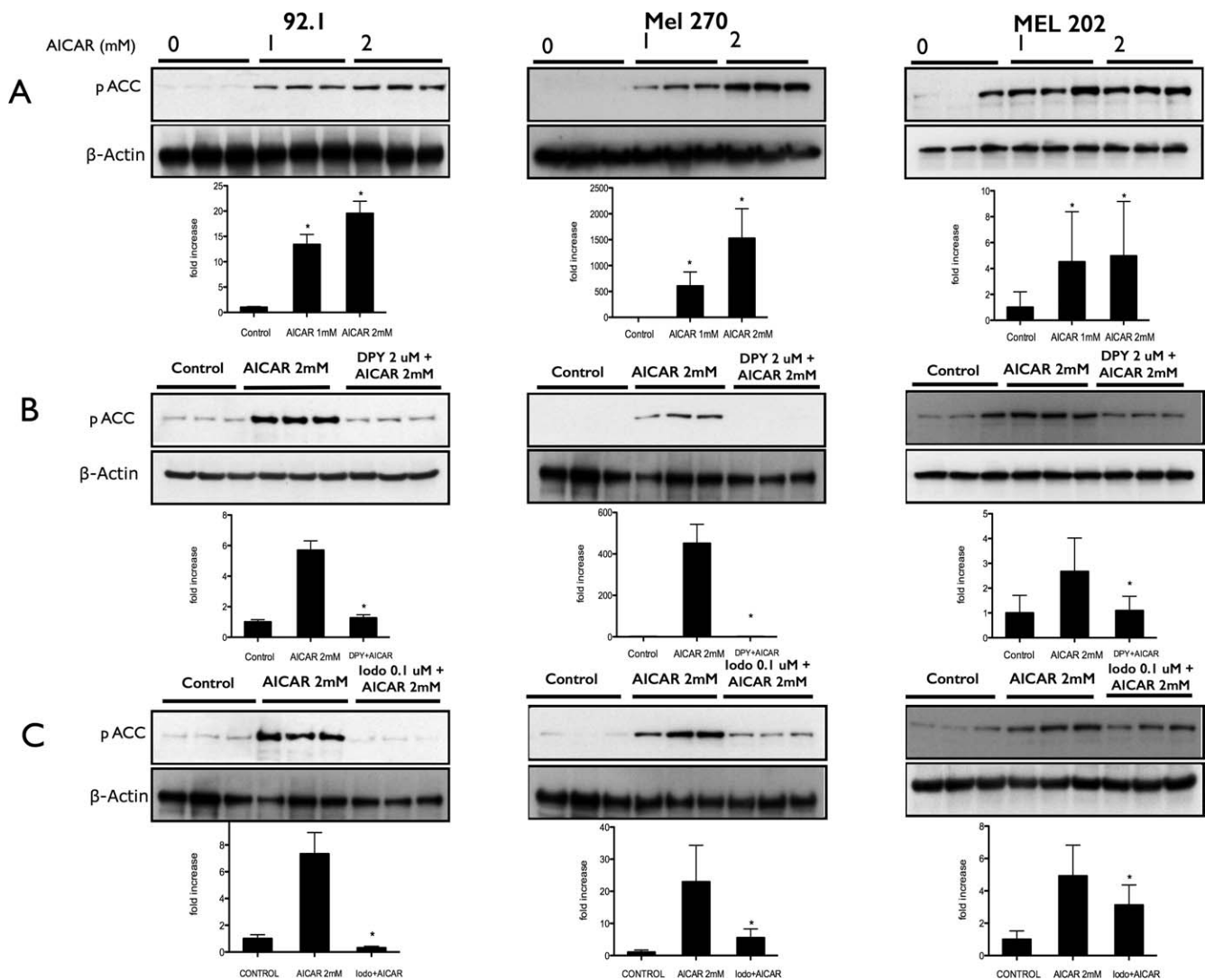
indicating that AMPK activation by ZMP is only partially responsible for the observed inhibitory effects of intracellular AICAR.

### AICAR Causes Cell Cycle Arrest in S Phase of Uveal Melanoma Cell Lines

The reported effects of AICAR on the cell cycle have been variable depending on the cell type studied.<sup>41,42,44,48,57</sup> To examine the effect of AICAR on uveal melanoma cell cycle profiles, cells were treated with AICAR (1 and 2 mM) for 1, 3, and 5 days, and the cell-cycle phase was analyzed for nuclear DNA content by propidium iodide staining and flow cytometry. Compared with control cells, AICAR treatment resulted in accumulation of cells in S phase (Fig. 4, Supplementary Fig. S4) in a dose-dependent manner.

### AICAR Decreases the Levels of Cyclins A and D in Uveal Melanoma Cells

Cell cycle progression is controlled by specific cyclins. Given the effects shown previously of AICAR on uveal melanoma cell cycle regulation, we wanted to check whether that effect was mediated by changes in the levels of the appropriate cyclins. After the cells were treated with AICAR (1 and 2 mM) for 24 hours, quantitative RT-PCR analysis showed a significant dose-dependent decrease of cyclins A1 and D1 in all cell lines; in addition to cyclin D3 in MEL 270 and cyclins A2 and E2 in MEL 202 (Fig. 5, Supplementary Fig. S5). These results suggest that in uveal melanoma cells, AICAR-induced S phase arrest might be associated with decreasing levels of cyclin proteins.



**FIGURE 3.** Aminoimidazole carboxamide ribonucleotide treatment of uveal melanoma cells is associated with activation of AMPK. (A) Western blot analysis of phosphorylated ACC (Ser-79) expression in 92.1, MEL 270, and MEL 202 cells that were treated with AICAR at a concentration of either 1 or 2 mM for 24 hours. (B) Western blot analysis of phosphorylated ACC expression in 92.1, MEL 270, and MEL 202 cells pretreated with DPY for 30 minutes before addition of AICAR at a concentration of 2 mM for 24 hours. (C) Western blot analysis of phosphorylated ACC expression in 92.1, MEL 270, and MEL 202 cells pretreated with Iodo for 30 minutes before addition of AICAR at a concentration of 2 mM for 24 hours. Density values of phosphorylated ACC bands are graphically expressed relative to control. Multiple bands represent separate biological samples. Significance (\*) is assigned at  $P < 0.05$ .

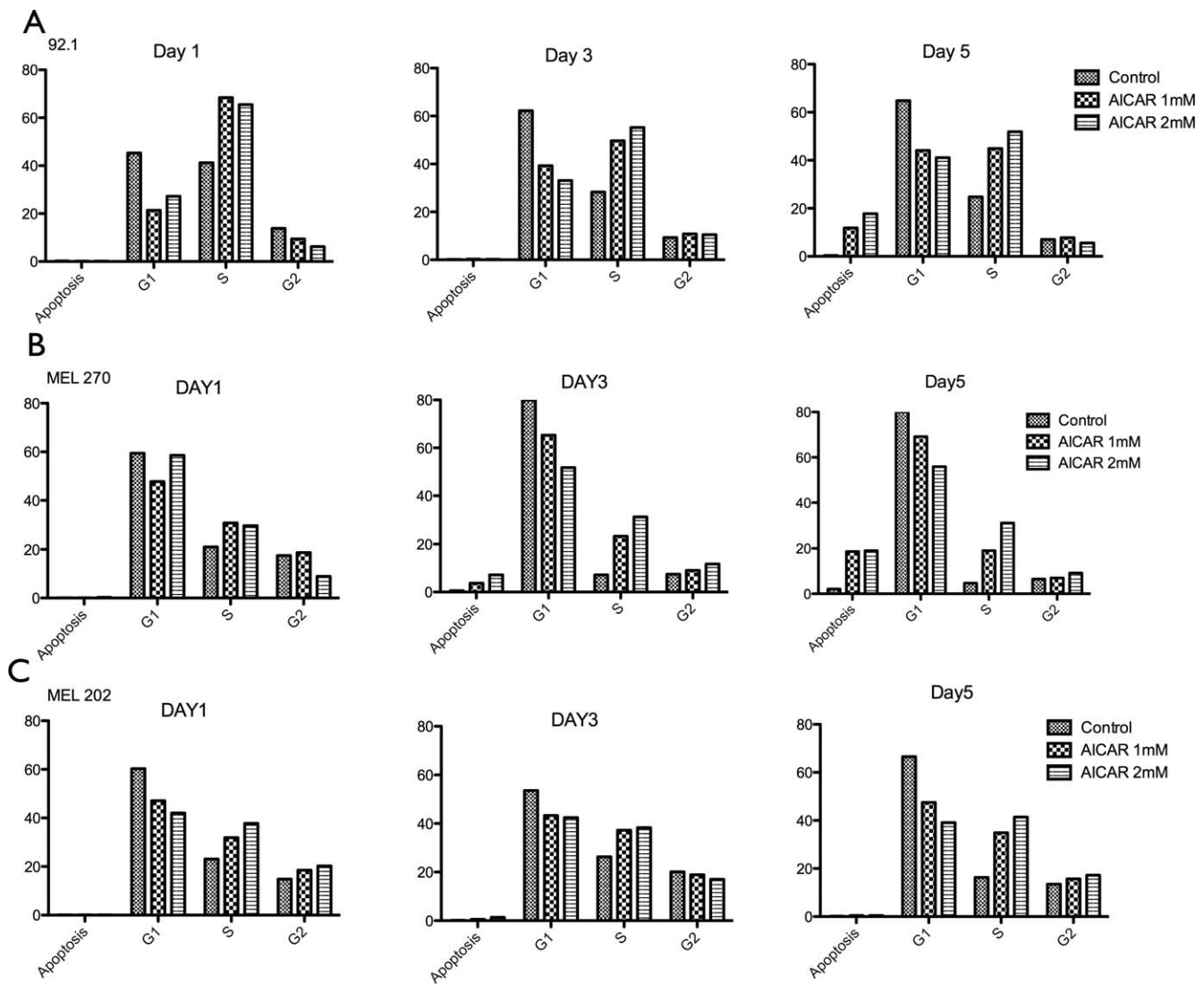
### AICAR Does Not Affect the Levels of the Cyclin-Dependent Kinases CDK2 and CDK4, CDK Inhibitor p27, p21, Tumor Suppressor Protein P53, PCNA, and MAPK Pathway

Other cell cycle progression regulators have been reported to be affected by AICAR in various cell types.<sup>36,44,46,48,57</sup> We wanted to check whether AICAR affects some of these regulators in uveal melanoma cells. We thus examined its effect on CDK2, CDK4, CDK inhibitor p27, p21, tumor suppressor protein p53, and PCNA. As shown in Figure 6 and Supplementary Figure S6, AICAR had little or no effect on the expression of the mentioned cell cycle regulators except the significant increase in p53 levels in MEL 270 cell line. In addition, we did not see change in the MAPK pathway, which has been reported to play a role in the pathogenesis of uveal melanoma.<sup>58,59</sup>

### AICAR Downregulates 4E-BP1 Phosphorylation but Not S6 Kinase or the Macroautophagy Marker LC3B in Uveal Melanoma Cells

The mTOR pathway has been demonstrated to be one of the major pathways controlling cell proliferation and autophagy. Adenosine monophosphate-dependent kinase directly and indirectly inhibits mTOR/Raptor,<sup>60</sup> directly phosphorylates Ulk1, and promotes autophagy.<sup>61–63</sup> The nonselective type of autophagy called macroautophagy is thought to be regulated and inhibited by S6 kinase, a downstream effector of mTOR.<sup>64–66</sup>

Aminoimidazole carboxamide ribonucleotide's effects on multiple cell types have been shown to be mediated through mTOR pathway and autophagy.<sup>67–70</sup> In contrast to our prior work on human retinoblastoma cells,<sup>41,42</sup> Aminoimidazole carboxamide ribonucleotide did not inhibit the phosphorylation of ribosomal protein S6, a downstream effector and a



**FIGURE 4.** Aminoimidazole carboxamide ribonucleotide blocks cell cycle progression at S phase in human uveal melanoma cells. 92.1 (A), MEL 270 (B), and MEL 202 (C) uveal melanoma cells were treated with AICAR 1 and 2 mM for 1, 3, and 5 days. After overnight fixation, cells were suspended in PBS with RNase A and propidium iodide and acquired for DNA content by flow cytometry. All the data are graphically represented as percentage of cells in apoptosis, S phase, and G<sub>2</sub>/M phase. Data represent three independent experiments.

measure of mTOR activity (Fig. 6, Supplementary Fig. S6). However, AICAR downregulated 4E-BP1 phosphorylation (another marker of mTOR activity) in OCM 3, 92.1, and MEL 270 cell lines, but not in MEL 202 ( $P < 0.05$ ; Fig. 7, Supplementary Fig. S7). In addition, the macroautophagy marker LC3B was found to be significantly increased only in OCM 3 cell line (Fig. 6, Supplementary Fig. S7). This suggests that the AICAR's effects in uveal melanoma on the mTOR pathway and autophagy are more complex than in other cell lines.

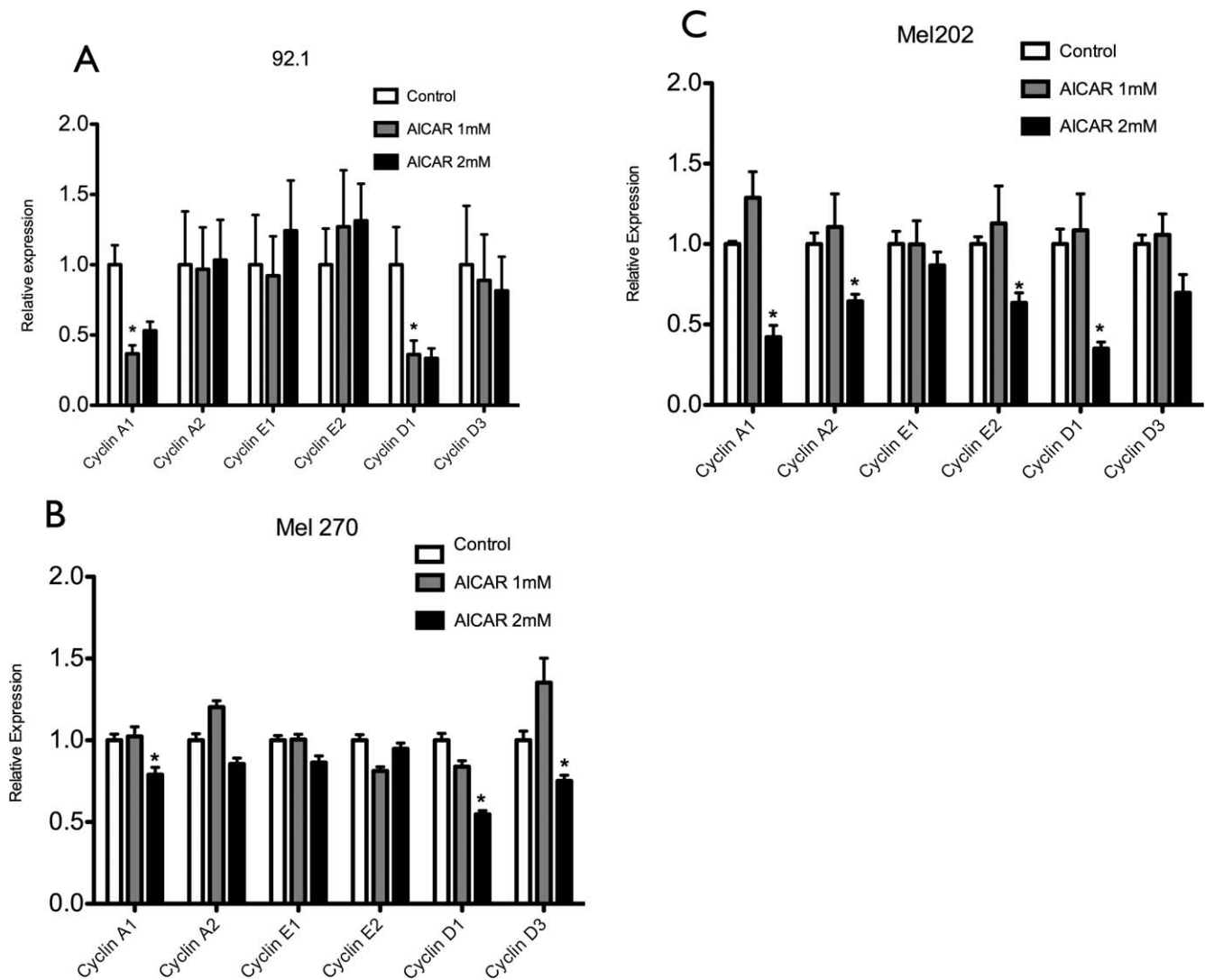
## DISCUSSION

In this study, we demonstrated that AICAR, a pharmacologic activator of AMPK, can induce S phase cell-cycle arrest and inhibit growth in three human uveal melanoma cell lines. Dipyridamole, an adenosine transporter inhibitor, abolished these AICAR-mediated effects by preventing its cellular uptake. The adenosine kinase inhibitor iodotubercidin, which inhibits the enzyme responsible for converting AICAR to ZMP, abated

AMPK activation (demonstrated by ACC phosphorylation) and blocked AICAR's growth inhibitory effects, suggesting that these effects are mediated by intrinsic mechanisms and at least partially by AMPK activation.

Previous reports from us and other laboratories indicate that the cell type determines the AICAR effects on cell cycle. Aminoimidazole carboxamide ribonucleotide's treatment of various cancer cell lines has showed arrest either in the S phase,<sup>36,46</sup> G1 phase,<sup>57</sup> and/or an increase in the sub-G0/G1 population.<sup>41,48</sup> An increase in the S-phase population was observed upon treating three uveal melanoma cell lines with AICAR, which also caused downregulation of cyclins A1 and D1. This is consistent with S phase arrest, as cyclins A1 and D1 control progression through S phase. We also observed downregulation of other cyclins in MEL 270 and MEL 202 cell lines.

The mechanisms of AICAR's inhibitory effects vary depending on the cell line being studied, and multiple mechanisms have been shown to play a role in the inhibiting effects of AICAR. Adenosine monophosphate-dependent kinase activity was upregulated and/or necessary in retinoblastoma, multiple

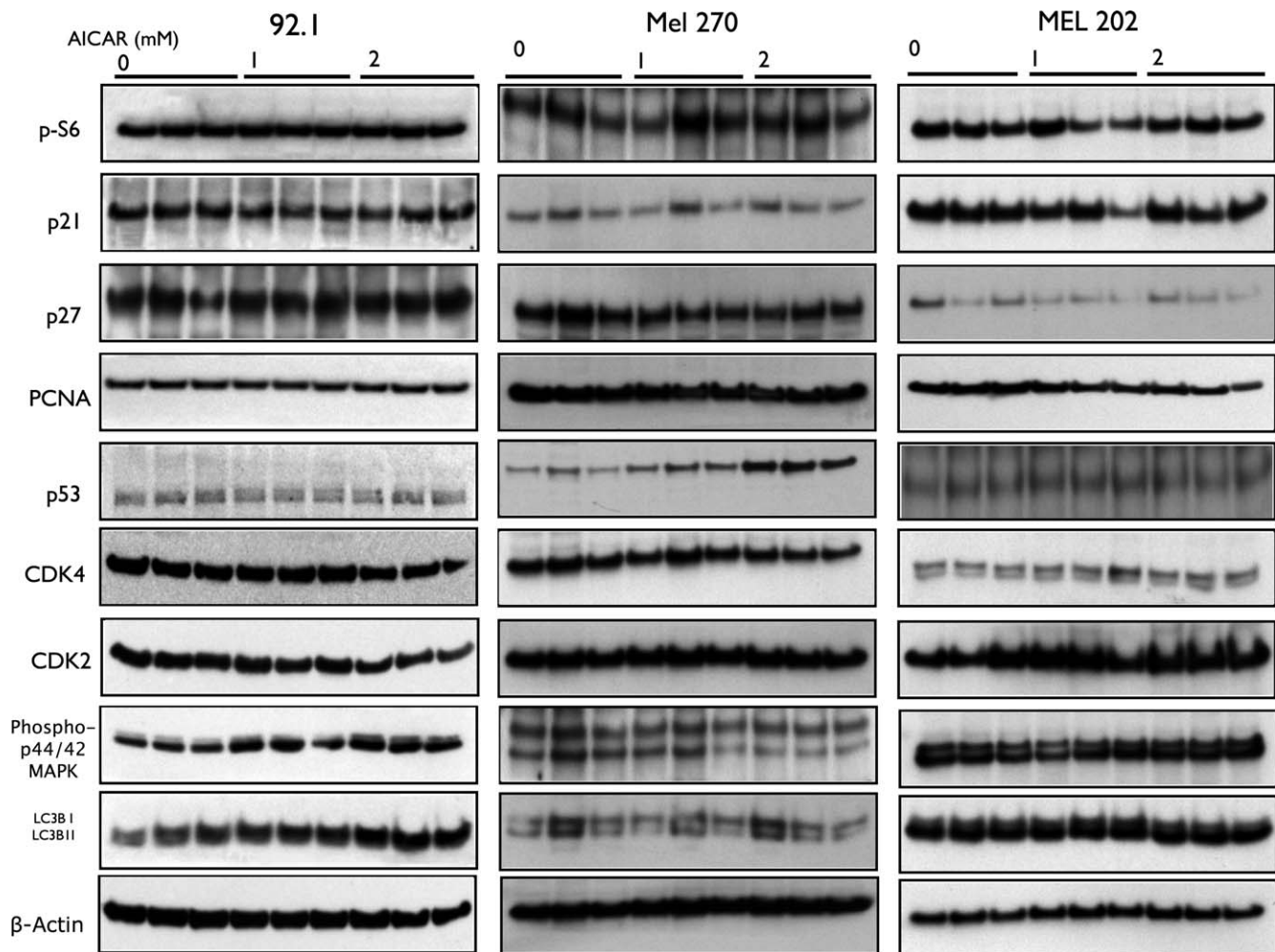


**FIGURE 5.** Aminoimidazole carboxamide ribonucleotide decreases the levels of different cyclins in uveal melanoma cells. 92.1 (**A**), MEL 270 (**B**), and MEL 202 (**C**) uveal melanoma cells were treated with AICAR at a concentration of either 1 or 2 mM for 24 hours. Quantitative RT-PCR analysis showed decrease of cyclins A1 and D1 in all cell lines, cyclin D3 in MEL 270 and cyclins A2 and E2 in MEL 202-treated cells in comparison with control cells. Significance (\*) is assigned at  $P < 0.05$ .

myeloma, colon, breast, prostate, and hepatic cancer cell lines,<sup>36,42,46–48</sup> whereas AMPK activity was nonessential in studies of Jurkat cells, myelogenous leukemia, and neuroblastoma cell lines.<sup>43,53,70</sup> Our results indicated that, in uveal melanoma cells, AMPK activity was at least partially required for the inhibitory effects of AICAR in two uveal melanoma cell lines (92.1 and MEL 270) and the skin melanoma cell line OCM 3, but not in MEL 202 cell line.

Further investigation of the growth inhibitory mechanisms of AICAR revealed more differences depending on the cell lines studied. Aminoimidazole carboxamide ribonucleotide treatment has been shown to inhibit glioblastoma cells by inhibiting lipogenesis,<sup>45</sup> triggered apoptosis by inhibiting NF- $\kappa$ B pathway in colon cancer cells,<sup>48</sup> and inhibited proliferation by upregulating the cell cycle inhibitor protein p21 in C6 glioma cells and acute lymphoblastic leukemia cells<sup>44,36</sup>; however, it inhibited p21 in retinoblastoma cells.<sup>41</sup> Aminoimidazole carboxamide ribonucleotide has been shown to increase p27 and decrease PCNA in C6 glioma cells.<sup>36</sup> In contrast to these studies, the effects we observed in AICAR-treated uveal melanoma cells did not occur through any of these mechanisms, except for the increase in p53 in MEL 270.

Aminoimidazole carboxamide ribonucleotide has been shown to be an exercise mimetic<sup>37</sup> and demonstrates antiinflammatory properties,<sup>71</sup> anticancer properties, in addition to pro-survival effects in normal cells under stress.<sup>36,43,44,46,48</sup> The mechanisms responsible for these effects are not fully understood, but they likely involve activation of AMPK. It is possible that the various effects of AICAR and AMPK depend on the specific cell type, cellular events following external stimuli, duration of AMPK activation, and/or downstream-regulated pathways of AMPK. Research on the antitumor effects of AICAR-induced AMPK activation is becoming an important area of investigation because of its link with tumor suppressors. The tumor suppressor LKB1 is an upstream activator of AMPK, and the encoding gene is mutated in Peutz-Jeghers syndrome, an autosomal dominant disease characterized by hamartomatous polyp growth and predisposition to cancers of the gastrointestinal tract. Tuberous sclerosis 2 (TSC2) is known to be downstream of activated AMPK; it forms a complex with TSC1 and inhibits mTOR, leading to negative regulation of cell growth.<sup>32</sup> Mutations of the *TSC2* gene are associated with tuberous sclerosis, which in humans is associated with



**FIGURE 6.** Aminoimidazole carboxamide ribonucleotide does not affect levels of cell cycle progression regulators in uveal melanoma cells. Western blot analysis of phospho-S6, CDK inhibitor p21, CDK inhibitor p27, PCNA, p53 (except in Mel 270), CDK4, CDK2, phospho-p44/p42 MAPK, and LC3B I, MEL 270, and MEL 202 cells treated with AICAR at a concentration of either 1 or 2 mM for 24 hours. Multiple bands represent separate biological samples.

hamartomas and an increased risk of cancers. Mammalian target of rapamycin phosphorylates 4E-BP1, leading to release of eIF4E and allowing initiation of translation. Hyperphosphorylation of 4E-BP1 has been reported to be a marker of poor prognosis and a potential target for the treatment of cutaneous melanoma, breast cancer, and astrocytoma.<sup>72-74</sup> We observed decreased phosphorylation of 4E-binding protein 1 (4E-BP1), a downstream pathway of mTOR, in three of the four cell lines tested. However, S6 kinase, another downstream effector of mTOR, was not downregulated after AICAR treatment in contrast to our previous study in retinoblastoma<sup>41,42</sup> and the study by Rattan et al.<sup>36</sup> in C6 glioma cells, suggesting that AICAR's effects in uveal melanoma on the mTOR pathway may be more complex than in other cell lines.

Adenosine monophosphate-dependent kinase activation has been reported to induce autophagy by suppressing mTOR pathway, and thus suppressing the macroautophagy inhibitor S6 kinase, and by directly phosphorylating proautophagy protein Ulk1.<sup>60,64-66</sup> The role of autophagy in cancer is still debated and can be either detrimental or protective.<sup>75</sup> Adenosine monophosphate-dependent kinase induction of autophagy has been thought to contribute to cell death in colorectal HT-29 cells,<sup>76</sup> and AICAR has been shown to induce

cell death and autophagy stimulation in chronic myelogenous leukemia cell lines.<sup>70</sup> We failed to observe any significant and consistent effects of AICAR on the autophagy marker LC3B; thus, the possibility remains that other mechanisms are responsible for the inhibition of uveal melanoma cells.

Although advances in therapy for uveal melanoma have led to significant success in local control, metastasis remains a significant problem with a lack of effective therapies. This underscores the need for the development of new targets and less toxic therapies.

In summary, our results show that AICAR, after entering the cells, inhibits uveal melanoma cell growth at least partially through activation of AMPK, inhibition of 4E-BP1 phosphorylation, and downregulation of cyclins A1 and D1. Moreover, other studies have shown that AICAR, when administered in nonchronic situations, has low toxicity, displays antiinflammatory properties, and acts as an exercise mimetic.<sup>37</sup> In addition AICAR (also known as acadesine) is already in human clinical trials for B Cell leukemia and early phase I/II study results have shown trends of efficacy; reduction of peripheral chronic lymphocytic leukemia (CLL) cells and reduction in lymphadenopathy were observed with blood levels close to 1 mM.<sup>77</sup> Together, these data indicate that AICAR has potential as a novel targeted therapy with low toxicity for uveal melanoma.



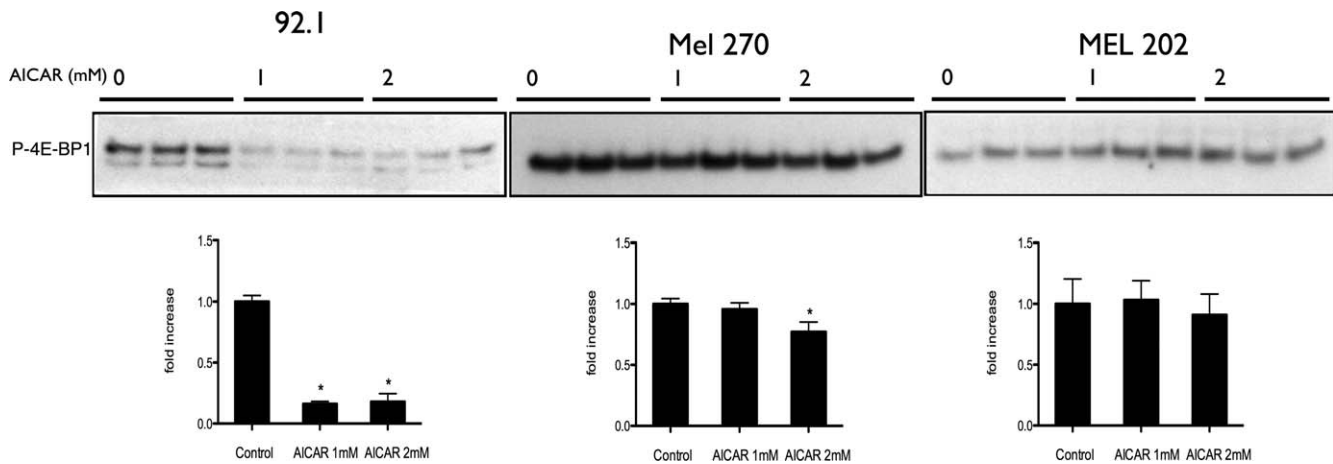


FIGURE 7. Antiproliferative effect of AICAR on uveal melanoma cells is mediated via inhibition of 4E-BP1 phosphorylation in 92.1 and Mel 270, but not in Mel 202 cells. Western blot analysis of P-4E-BP1 in 92.1, Mel 270, and Mel 202 cells treated with AICAR at a concentration of either 1 or 2 mM for 24 hours. Density values of the bands are graphically expressed relative to control. Multiple bands represent separate biological samples. Significance (\*) is assigned at  $P < 0.05$ .

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