Metabolomics Identifies Pyrimidine Starvation as the Mechanism of 5-Aminoimidazole-4-Carboxamide-1-β-Riboside-Induced Apoptosis in Multiple Myeloma Cells

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Abstract

To investigate the mechanism by which 5-aminoimidazole-4-carboxamide-1-β-ribose (AICAr) induces apoptosis in multiple myeloma cells, we conducted an unbiased metabolomics screen. AICAr had selective effects on nucleotide metabolism, resulting in an increase in purine metabolites and a decrease in pyrimidine metabolites. The most striking abnormality was a 26-fold increase in orotate associated with a decrease in uridine monophosphate (UMP) levels, indicating an inhibition of UMP synthetase (UMPS), the last enzyme in the de novo pyrimidine biosynthetic pathway, which produces UMP from orotate and 5-phosphoribosyl-α-pyrophosphate (PRPP). As all pyrimidine nucleotides can be synthesized from UMP, this suggested that the decrease in UMP would lead to pyrimidine starvation as a possible cause of AICAr-induced apoptosis. Exogenous pyrimidines uridine, cytidine, and thymidine, but not purines adenosine or guanosine, rescued multiple myeloma cells from AICAr-induced apoptosis, supporting this notion. In contrast, exogenous uridine had no protective effect on apoptosis resulting from bortezomib, melphalan, or metformin. Rescue resulting from thymidine add-back indicated apoptosis was induced by limiting DNA synthesis rather than RNA synthesis. DNA replicative stress was identified by associated H2A.X phosphorylation in AICAr-treated cells, which was also prevented by uridine add-back. Although phosphorylation of AICAr by adenosine kinase was required to induce multiple myeloma cell death, apoptosis was not associated with AMP-activated kinase activation or mTORC1 inhibition. A possible explanation for inhibition of UMP synthase activity by AICAr was a depression in cellular levels of PRPP, a substrate of UMP synthase. These data identify pyrimidine biosynthesis as a potential molecular target for future therapeutics in multiple myeloma cells.

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Introduction

AICAr (5-aminoimidazole-4-carboxamide-1-β-ribose) is a nucleoside that is taken up by cells by adenosine transporters and upon phosphorylation by adenosine kinase becomes AICA ribotide (AICAR or ZMP). It can function as an AMP mimic and activate AMP-activated kinase (AMPK; ref. 1). Because TORC1 activity can be inhibited by activated AMPK, AICAr has been studied as an antitumor agent. Several studies have shown that the tumor cytoreductive effects of AICAr can be mediated through AMPK activation and mTOR inhibition (2–4). However, cytotoxic effects do not always correlate with AMPK and mTOR activity. For example, AICAr-induced apoptosis of EGFRvIII-expressing glioblastomas does not correlate with the degree of inhibition of mTORC1 signaling. Instead, it is due to AMPK-mediated down-regulation of lipogenesis (5). In addition, AICAr can induce apoptosis in some models by AMPK-independent mechanisms (6–9).

mTOR is a potential target in multiple myeloma. Rapalogs, which primarily inhibit TORC1, induce G1 arrest in multiple myeloma cells, but not apoptosis in vitro (10). AICAr has been reported to inhibit growth in multiple myeloma cells through activation of AMPK (11). However, substantial amount of apoptosis was only shown in the 8226 cell line. AMPK activation in 8226 cells was not shown in this report and, thus, the mechanism of AICAr-induced apoptosis in multiple myeloma remains unclear. In addition, as rapalogs only induce G1 arrest in multiple myeloma cells, the ability of AICAr to induce apoptosis is unlikely to be explained simply by mTORC1 inhibition. Our preliminary experiments confirmed that AICAr induces apoptosis.
in multiple myeloma cell lines but, as previously reported (12), rapamycin only induced G1 arrest. As AICAr potentially induces several metabolic alterations that adversely affect cells, we then conducted a metabolomics screen in an attempt to pinpoint the mechanism of AICAr-induced apoptosis. We found that apoptosis was due to the inhibited activity of uridine monophosphate (UMP) synthetase (UMPS) with subsequent pyrimidine starvation.

Materials and Methods

Reagents

AICA riboside was purchased from Calbiochem. Metformin, nucleosides, dithiothreitol (DTT), S-phosphoribosyl-α-pyrophosphate (PRPP), orotate, orotidine monophosphate (OMP), and ZMP were purchased from Sigma-Aldrich. N-(phosphonacetyl)-L-aspartate (PALA) was obtained from the Developmental Therapeutics Program (NCI/NIH, Bethesda, MD). All antibodies were purchased from Cell Signaling except for anti-UMP synthetase, purchased from Abcam. Recombinant adenosine phosphoribosyltransferase (APRTase) was purchased from Origene and recombinant adenosine phosphoribosyltransferase (APRTase) was purchased from Prospec. [6-14C] orotate (50 mCi/mmol) and [8-14C] adenine (50 mCi/mmol) was purchased from MP Biomedicals. Chemical structures for bortezomib, melphalan, and metformin are shown in Supplementary Fig. S1.

Cell lines

All cell lines were obtained from American Type Culture Collection (ATCC). 8226, OPM2, U266, and MM1S cells were maintained in RPMI supplemented with 10% FBS, glutamine, nonessential amino acids, penicillin-streptomycin, and fungazone. H929 cells were maintained in RPMI media with the same supplements described above, except the media was supplemented with 0.05 mmol/L i-mercaptoethanol. HeLa cells were maintained in Dulbecco’s Modified Eagle Medium with the same supplements as 8226 cells. Cell lines were verified with short tandem repeat analysis by ATCC.

Screening for metabolites

Treated cells were harvested, washed once in PBS, frozen in liquid nitrogen, and sent to Metabolon Inc. Samples were prepared in quadruplicate. At the time of analysis, samples were extracted and prepared for analysis using Metabolon’s standard solvent extraction method. The extracted samples were split into equal parts for analysis on the gas chromatography-mass spectrometry and liquid chromatography/tandem mass spectrometry platforms. Following log transformation and imputation with minimum observed values for each compound, Welch 2-sample t tests were used to identify biochemicals that differed significantly between treated and control groups. In all, 194 biochemicals were identified. When analyzing 194 compounds, it is expected that 10 compounds meeting the cutoff for statistical significance (P ≤ 0.05) would occur by random chance.

Immunoblots

Whole-cell lysates were prepared using cell lysis buffer (Cell Signaling) supplemented with 1 mmol/L phenylmethylsulfonylfluoride immediately before use. Western blots were conducted as previously described (13).

Apoptosis assay

Apoptosis was assayed by staining for activated caspase-3 (BD Biosciences) and assessed using flow cytometry as previously described (13).

Cell-cycle analysis

Cells were incubated in 50 μg/mL propidium iodide in 0.1% sodium citrate solution for 5 minutes before running on the flow cytometer (Accrui C6). Cell-cycle profiles were analyzed using ModFitLT 3.2.

UMPS functional assay

The UMPS assay was as described (14). The reaction mixture contained 20 mmol/L Tris-HCl, pH 7.5, 2 mmol/L DTT, 5 mmol/L MgCl2, 300 μmol/L PRPP, 5 mmol/L [6-14C] orotate (50 mCi/mmol), and 0.4–0.8 μg recombinant UMPS. Reactions were incubated at 37°C for 30 minutes. Products were separated using thin layer chromatography (TLC; ref. 15). The reaction was stopped by directly spotting 5 μL of the reaction mixture onto a 20 cm × 20 cm PEI-cellulose plate and drying the sample with hot air. Plates were developed by ascending chromatography in 0.25 mol/L LiCl /0.1% formic acid. Cold standards of orotate, OMP, and UMP were used to identify spots. Plates were visualized using a Phospholmager (GE Storm 840) and spots were quantified using ImageQuant software. Orotate phosphoribosyl transferase and OMP decarboxylase activity was calculated following Traut and Jones (14).

Nucleotide extraction and high-performance liquid chromatography analysis

Cells were harvested, washed once in PBS, resuspended in 5% tricarboxylic acid, sonicated for 10 seconds, and incubated on ice for 5 minutes. Precipitated protein was removed by centrifugation (5,000 × g, 10 minutes) and the supernatant was neutralized by 5 extractions with 5 volumes of water-saturated ether. pH was adjusted to 5. Nucleotides were separated on a Phenomenex Partisil 10 SAX column (0.45 × 25 cm) equilibrated in 5 mmol/L K phosphate (pH 5) at a flow rate of 2 mL/min. Samples were eluted isocratically for 20 minutes with 5 mmol/L K phosphate buffer, followed by a linear gradient to 500 mmol/L K phosphate for 45 minutes, followed by 500 mmol/L K phosphate for an additional 15 minutes. Separation was on a Gilson high-performance liquid chromatography (HPLC) system with a Model 117 ultraviolet detector set at 260 and 280 nm. Areas of peaks were quantified using UniPoint
software and converted to molar quantities by comparison to ZMP standards.

5-Phosphoribosyl-α-pyrophosphate assay

Measurement of intracellular 5-phosphoribosyl-α-pyrophosphate (PRPP) was as described (16). Briefly, cells were treated with 250 μmol/L AICAr for 8 hours, harvested, washed once with PBS, and the pellet frozen at −80°C. Cells were thawed and resuspended in 50 100 μL 30 mmol/L Tris-HCl, pH 7.5, 0.5 mmol/L EDTA followed by heating in a boiling water bath for 45 seconds and centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was removed and used immediately. The amount of PRPP was determined on the basis of the synthesis of [8-14C]AMP from [8-14C] adenine in the presence of APRTase. Reactions were incubated for 1 hour at 37°C. TLC was conducted as described above to analyze products of the reaction.

Colorimetric orotate assay

Orotate accumulation was measured using a colorimetric assay as described (17) with modifications described by Harris and Oberholzer (18).

Statistical (ANOVA) analysis

Proc Mixed (SAS 9.2) was used to construct a repeated measures mixed effects model predicting the main outcome—percent apoptosis. Fixed effects entered in the model were cell line type, treatment, and the interaction of treatment by cell line type. Compound symmetry covariance structure was used. Marginal means of cell lines and treatments were estimated using LSmeans statement and...
differences between individual pairs of cell lines was determined using posthoc t-tests where overall main effects were significant.

Results

AICAr induces apoptosis in myeloma cells in vitro

AICAr (Fig. 1A) had cytoreductive effects on multiple myeloma lines (8226, OPM2, U266, MM1S and H929) based on cell counts (data not shown). AICAr also induced apoptosis in a concentration-dependent fashion in all 5 multiple myeloma lines identified by caspase-3 activation (Fig. 1B). On the basis of these preliminary results, further time-course experiments were carried out with either 250 µmol/L AICAr (for 8226 and U266 cells) or 500 µmol/L (for OPM2, MM1S, and H929 cells). AICAr-induced apoptosis in a time-dependent fashion (Fig. 1C). Apoptosis was first detected consistently by 48 hours. The

Figure 2. A, simplified heatmap of 8226 cells treated with 250 µmol/L AICAr (AIC) for 4 and 8 hours compared with vehicle. Red indicates an increase and green a decrease in a given metabolite over vehicle. Darker colors indicate metabolites that achieved statistically significant (P < 0.05) differences between treated and untreated group and light colors indicate metabolites that approach statistical significance (0.05 < P < 0.1). B, 8226 and MM1S cells were treated with the indicated concentration of AICAr for the indicated amount of time and Western blot probed for AMPK phosphorylation (P-AMPK), total AMPK or actin. C, primary CLL cells were incubated for 16 hours ± 500 µmol/L AICar for similar Western blot assay. 8226 and MM1S cells were incubated with 5 mmol/L metformin for 16 (8226) or 72 hours (MM1S) for similar Western blot assay. D, cells were treated either with DMSO (Con), 10 nmol/L rapamycin (R), or 500 µmol/L AICAr (A) for 8 (8226) or 24 hours (MM1S). Western blots were probed for P-p70 (Thr 389 or Ser/Thr 421/424) and actin to assess TORC1 activity.
4 of 5 cell lines studied to 25% in the cell lines. In contrast, rapamycin induced a (11). The increase in S-phase distribution ranged from 10% (21) and inhibited flux through the tricarboxylic acid cycle (22). In contrast, the largest effect of AICAr was on nucleotide metabolism (Fig. 2A). Of the 29 metabolites significantly altered by AICAr at 8 hours, 11 are within nucleotide metabolic pathways (Fig. 2A). The general trend was an increase in purine metabolites and a decrease in pyrimidine metabolites (Fig. 2A and Table 1). The number of metabolites affected by AICAr exposure at the 4-hour time point was not above the threshold of random chance, indicating this time point was too early to produce useful data (see Materials and Methods for metabolomics data analysis). In concurrently run samples of 8226 cells exposed to 10 nmol/L rapamycin for 4 or 8 hours, a larger metabolic effect was seen with significant alterations in 33 and 51 metabolites respectively (rapamycin data in Supplementary Fig. S3). As shown in the heatmap in Fig. 2A, AICAr treatment had little effect on carbohydrate, energy, amino acid, and lipid metabolism. Most of these alterations were consistent with prior literature such as decreased levels of glycolytic intermediates (21) and inhibited flux through the tricarboxylic acid cycle (22). In contrast, the largest effect of AICAr was on nucleotide metabolism (Fig. 2A). Of the 29 metabolites significantly altered by AICAr at 8 hours, 11 are within nucleotide metabolic pathways (Fig. 2A). The general trend was an increase in purine metabolites and a decrease in pyrimidine metabolites (Fig. 2A and Table 1). The most striking alteration induced by AICAr was in orotate, an intermediate in de novo pyrimidine synthesis, increasing 26-fold over vehicle control (Table 1). Although AICAr must be converted to ZMP for apoptosis (Fig. 1F) and ZMP is an AMP mimic, the finding that many of the metabolic effects of rapamycin-induced mTORC1 inhibition were not detected in AICAr-treated cells raised the question of whether AICAr-induced apoptosis was associated with AMP kinase activation and

8226 cell line was the most sensitive target with rapid induction of apoptosis. Cell-cycle analysis showed that AICAr also induced an accumulation of cells in S-phase in all 5 cell lines (Fig. 1D) consistent with a previous report (11). The increase in S-phase distribution ranged from 10% to 25% in the cell lines. In contrast, rapamycin induced a G1–S arrest without apoptosis in 4 of 5 cell lines studied (Fig. 1E). Only in OPM-2 cells was there a minimal degree of apoptosis following exposure to rapamycin. Although IGF-I is a known survival factor for multiple myeloma cells (19), it did not confer protection against AICAr-induced apoptosis while protecting against dexamethasone (Supplementary Fig. S2) suggesting a distinct mechanism of AICAr apoptosis.

AICAr becomes phosphorylated by adenosine kinase to become ZMP, which acts as a mimic of AMP. To determine whether AICAr must be converted to ZMP to induce apoptosis, cells were treated simultaneously with AICAr and iodotubercidin, an inhibitor of adenosine kinase (Fig. 1F). Treatment with iodotubercidin prevented apoptosis, indicating that AICAr must be converted to ZMP to induce apoptosis.

**Metabolomics screen**

Because AICAr can induce metabolic alterations independent of mTOR inhibition (5) and because rapamycin treatment (i.e., mTORC1 inhibition) could not reproduce the apoptosis resulting from AICAr, we conducted a high-throughput screen for alterations in metabolites selectively affected by AICAr. Preliminary time-course experiments in 8226 cells indicated that 250 μmol/L AICAr induced apoptosis between 8 and 16 hours. We, thus, profiled AICAr-treated 8226 cells at 4 and 8 hours of exposure to lessen the possibility of finding alterations that were nonspecific effects of apoptosis. After 4 or 8 hours, AICAr only altered the levels of 7 and 29 metabolites, respectively (Fig. 2A). The number of metabolites affected by AICAr exposure at the 4-hour time point was not above the threshold of random chance, indicating this time point was too early to produce useful data (see Materials and Methods for metabolomics data analysis). In concurrently run samples of 8226 cells exposed to 10 nmol/L rapamycin for 4 or 8 hours, a larger metabolic effect was seen with significant alterations in 33 and 51 metabolites respectively (rapamycin data in Supplementary Fig. S3). As shown in the heatmap in Fig. 2A, AICAr treatment had little effect on carbohydrate, energy, amino acid, and lipid metabolism. Most of these alterations were consistent with prior literature such as decreased levels of glycolytic intermediates (21) and inhibited flux through the tricarboxylic acid cycle (22). In contrast, the largest effect of AICAr was on nucleotide metabolism (Fig. 2A). Of the 29 metabolites significantly altered by AICAr at 8 hours, 11 are within nucleotide metabolic pathways (Fig. 2A). The general trend was an increase in purine metabolites and a decrease in pyrimidine metabolites (Fig. 2A and Table 1). The most striking alteration induced by AICAr was in orotate, an intermediate in de novo pyrimidine synthesis, increasing 26-fold over vehicle control (Table 1).

Although AICAr must be converted to ZMP for apoptosis (Fig. 1F) and ZMP is an AMP mimic, the finding that many of the metabolic effects of rapamycin-induced mTORC1 inhibition were not detected in AICAr-treated cells raised the question of whether AICAr-induced apoptosis was associated with AMP kinase activation and

**Table 1. AICAr-induced alteration in metabolite levels in 8226 cells**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>AICAr (fold change)</th>
</tr>
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<tbody>
<tr>
<td>Purine</td>
<td></td>
</tr>
<tr>
<td>Xanthine</td>
<td>1.31</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>1.24</td>
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<tr>
<td>Adenosine</td>
<td>1.90</td>
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<tr>
<td>AMP</td>
<td>2.20</td>
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<tr>
<td>ADP</td>
<td>1.38</td>
</tr>
<tr>
<td>GMP</td>
<td>1.72</td>
</tr>
<tr>
<td>Pyrimidine</td>
<td></td>
</tr>
<tr>
<td>Cytidine</td>
<td>0.3</td>
</tr>
<tr>
<td>Orotate</td>
<td>26.12</td>
</tr>
<tr>
<td>Uracil</td>
<td>0.41</td>
</tr>
<tr>
<td>Uridine</td>
<td>0.34</td>
</tr>
<tr>
<td>UMP</td>
<td>0.44</td>
</tr>
<tr>
<td>Nucleotide coenzymes</td>
<td></td>
</tr>
<tr>
<td>CoenzymeA</td>
<td>1.50</td>
</tr>
<tr>
<td>Flavin adenine dinucleotide (FAD)</td>
<td>1.16</td>
</tr>
<tr>
<td>Glycerolipid</td>
<td></td>
</tr>
<tr>
<td>CDP-choline</td>
<td>0.57</td>
</tr>
<tr>
<td>Glyceral 3-phosphate</td>
<td>1.48</td>
</tr>
<tr>
<td>Choline phosphate</td>
<td>1.23</td>
</tr>
<tr>
<td>Krebs cycle</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>0.71</td>
</tr>
<tr>
<td>cis-aconitate</td>
<td>0.66</td>
</tr>
<tr>
<td>Glycolysis</td>
<td></td>
</tr>
<tr>
<td>Isobarc: fructose 1,6-diphosphate</td>
<td>2.5</td>
</tr>
<tr>
<td>glucose 1,6-diphosphate</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>0.77</td>
</tr>
<tr>
<td>Cysteine, methionine, SAM, taurine metabolism</td>
<td>0.71</td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>N-formylmethionine</td>
<td>1.35</td>
</tr>
<tr>
<td>S-adenosylhomocysteine</td>
<td>1.55</td>
</tr>
<tr>
<td>Asymmetric dimethylarginine</td>
<td>1.48</td>
</tr>
</tbody>
</table>

**NOTE:** Bold numbers, \( P < 0.05. \)
mTORC1 inhibition. Unexpectedly, we could not show activation of AMPK in multiple myeloma cells. Focusing on the most AICAr-sensitive 8226 cell line, experiments using a range of AICAr concentrations (100 \(\mu\)mol/L–2 mmol/L) and incubation times (30 minutes–24 hours) failed to detect AMPK activation as monitored by phosphorylation of Thr 172 (Fig. 2B and Supplementary Fig. S4). As positive controls, our AICAr preparation successfully induced AMPK activation in primary chronic lymphocytic leukemia (CLL) cells as previously reported (2) and metformin, which indirectly activates AMPK by inhibiting mitochondrial respiration and increasing the AMP:ATP ratio (23), was capable of activating AMPK in multiple myeloma cells (Fig. 2C). Comparable attempts to show AMPK activation in AICAR-treated OPM-2 and MM1.S cells were likewise unsuccessful (Fig. 2B and Supplementary Fig. S4) while metformin was successful (Fig. 2C). These data show that although AICAr must be converted to ZMP, its mechanism of apoptosis-induction does not involve AMPK activation.

To assay effects on mTORC1, we treated cells with either rapamycin or AICAr and probed Western blots for phosphorylation of p70S6 kinase. As shown in Fig. 2D and Supplementary Fig. S4 (for OPM-2 cells), while rapamycin clearly inhibited activation of AMPK by inhibiting mitochondrial respiration and increasing the AMP:ATP ratio (23), was capable of activating AMPK in multiple myeloma cells (Fig. 2C). Comparable attempts to show AMPK activation in AICAR-treated OPM-2 and MM1.S cells were likewise unsuccessful (Fig. 2B and Supplementary Fig. S4) while metformin was successful (Fig. 2C). These data show that although AICAr must be converted to ZMP, its mechanism of apoptosis-induction does not involve AMPK activation.

Exogenous pyrimidines prevent apoptosis

The AICAR-induced accumulation of orotate identified in the screen was first confirmed by using a colorimetric assay (17). Incubation of 8226 cells with 250 \(\mu\)mol/L AICAr for 8 hours increased orotate 4.5-fold (mean of 3 experiments). Orotate is a substrate of UMPs, the last enzyme in the de novo pyrimidine biosynthetic pathway (Fig. 3). UMPs is a multifunctional enzyme that catalyzes 2 enzymatic reactions. The first reaction catalyzes the conversion of orotate to OMP using orotate phosphoribosyl transferase (ORPTase) that requires PRPP and releases PPI (reaction “1” in Fig. 3). The second reaction is catalyzed by OMP decarboxylase and converts OMP to UMP (reaction “2” in Fig. 3). The marked increase in orotate levels suggests that UMPs is inhibited by AICAr exposure. Consistent with this data is the observed associated decrease in UMP levels (Table 1), the product of the reaction (Fig. 3). All pyrimidine nucleotides can be synthesized from UMP (24), suggesting that the decrease in UMP could result in pyrimidine starvation.

Tumor cells can salvage uridine from their environment to circumvent the blockage of de novo pyrimidine biosynthesis (25, 26). Thus, to determine whether pyrimidine starvation through the inhibition of UMPs induces apoptosis, multiple myeloma cells were treated with increasing concentrations of uridine in conjunction with AICAr. The addition of uridine prevented apoptosis and prevention was related to the concentration of uridine (Fig. 4A), consistent with the observed associated decrease in UMP levels (Table 1) and the product of the reaction (Fig. 3). All pyrimidine nucleotides can be synthesized from UMP (24), suggesting that the decrease in UMP could result in pyrimidine starvation. Tumor cells can salvage uridine from their environment to circumvent the blockage of de novo pyrimidine biosynthesis (25, 26). Thus, to determine whether pyrimidine starvation through the inhibition of UMPs induces apoptosis, multiple myeloma cells were treated with increasing concentrations of uridine in conjunction with AICAr. The addition of uridine prevented apoptosis and prevention was related to the concentration of uridine (Fig. 4A). Uridine conferred complete protection as there is no statistical difference between control cells and AICAr treated cells + 100 \(\mu\)mol/L uridine. As expected, neither of the purine nucleosides, guanosine or adenosine, could prevent apoptosis (data not shown). The rescue by uridine is not due to a nonspecific increase in resistance to apoptosis as the nucleoside is incapable of preventing apoptosis induced by bortezomib, melphalan, or metformin (Fig. 4B). The finding that uridine prevents apoptosis in multiple myeloma cells treated with AICAr but not with metformin exposure is especially noteworthy, as metformin activates AMPK (Fig. 2C) and AMPK is a known mTOR inhibitor (27). These data provide...
Figure 4. A, cells were incubated with AICAr (250 µmol/L for 8226 and U266, 500 µmol/L for OPM2, MM1S, and H929) or vehicle in the presence of increasing concentrations of uridine (U) for 48 hours (8226, OPM2) or 96 hours (U266, MM1S, H929) and apoptosis assessed. Data are mean ± SD, n = 2, except for 8226.
additional evidence that AICAr-induced apoptosis is independent of effects on AMPK and mTOR.

Exogenous cytidine also prevents apoptosis although the concentration-dependence of protection is more complicated (Fig. 4C). Protection is present at low concentrations of cytidine (5–10 μmol/L) but concentrations of 50 μmol/L and above actually enhance AICAr-induced apoptosis in most of the cell lines (Fig. 4C). Enhanced apoptosis with high concentrations of cytidine did not occur in the absence of AICAr. The ability of high concentrations of cytidine to enhance apoptosis in the presence of inhibiting de novo pyrimidine biosynthesis has previously been reported and occurs when there is low cytidine deaminase activity (28–30). Cytidine deaminase converts cytidine to uridine and the presence of excessive cytidine coupled with inhibition of de novo pyrimidine synthesis enhances cell death because of unbalanced CTP/UTP ratios and aborted attempts at proliferation (30). It is possible that some multiple myeloma cell lines have a deficiency in cytidine deaminase. Nevertheless, the significant prevention of apoptosis with lower concentrations of cytidine suggests that at least some cytidine deaminase is present, allowing conversion of cytidine to uridine and some protection against pyrimidine starvation and apoptosis.

Exogenous thymidine also reduced apoptosis in all cell lines (white bars, Fig. 4D), suggesting apoptosis was induced by limiting DNA synthesis rather than RNA synthesis. Consistent with the hypothesis that AICAr induces DNA replicative stress due to limiting deoxyribonucleotide triphosphates (dNTP) is the increase in phosphorylation of H2A.X, a marker for replicative stress (Fig. 4E; ref. 31). AICAr-induced phosphorylation of H2A.X was prevented by uridine or thymidine add-back rescue as well as by a low (5 μmol/L) concentration of cytidine. The lowest panel in Fig. 4E shows the difference between adding back the apoptotic high concentration of cytidine [100 μmol/L (H)] versus low concentration [5 μmol/L (L)] with the former inducing an increased amount of H2A.X phosphorylation. As activation of caspases can also result in H2A.X phosphorylation, we repeated experiments described in Fig. 4E with the addition of the caspase inhibitor PALA (250 μmol/L) for 48 hours. Data are mean ± SD, n = 2–4 replicates.

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Figure 5. A, chemical structure of PALA. B, cells were incubated with 250 μmol/L PALA or vehicle ± 100 μmol/L uridine, 100 μmol/L cytidine, 100 μmol/L thymidine, or 100 μmol/L deoxycytidine for 48 to 96 hours. Apoptosis was assessed as described in Fig. 1. Data are mean ± SD, n = 2–4 replicates. C, OPM2 and MM1S cells were incubated with 250 μmol/L PALA or vehicle ± 5 μmol/L cytidine. Data are mean ± SD, n = 2. D, MM1S cells were treated with 250 μmol/L PALA ± 100 μmol/L uridine, 5 μmol/L cytidine, 100 μmol/L thymidine, or 100 μmol/L deoxycytidine for 48 hours. Extracts were probed for P-H2A.X (Ser 139) or actin as described above. dC, deoxycytidine.
inhibitor, ZVAD. There was a reduction in H2A.X phosphorylation, indicating at least some of this effect was a result of AICAr-induced apoptosis. However, cell-cycle analysis of AICAr-treated cells (Supplementary Fig. S5) showed that uridine rescue was associated with a prevention of S-phase arrest.

Addition of deoxycytidine over a wide range of concentrations (5–100 μmol/L) was not able to prevent AICAr-induced apoptosis (Fig. 4D) or replicative stress (Fig. 4E). The observation that thymidine can rescue cells while deoxycytidine is incapable suggests a defect in one branch of the deoxyribonucleoside salvage pathway. Multiple myeloma cells may be deficient in deoxycytidine kinase, which is required to salvage deoxycytidine into pyrimidine pools (see Discussion).

Effects of PALA
To strengthen the conclusion that pyrimidine starvation induces apoptosis in myeloma cells, cells were treated with another inhibitor of de novo pyrimidine synthesis, PALA (Fig. 5A). PALA inhibits aspartate transcarbamylase, the first catalytic reaction in the de novo pyrimidine pathway, and induces apoptosis in myeloma cells (Fig. 5B and C). In similar fashion to AICAr, PALA-induced apoptosis is also associated with replicative stress, as shown by induction of H2AX phosphorylation (Fig. 5D). The pattern of pyrimidine rescue is also similar, with PALA-induced apoptosis being prevented by uridine (Fig. 5B) and low concentrations of cytidine (Fig. 5C), whereas high cytidine concentrations enhance apoptosis (Fig. 5B). As mentioned above, since some H2AX phosphorylation could be downstream of apoptosis induction, we also assayed cell transit. Supplementary Figure S6 shows the PALA-induced S-phase accumulation was prevented by uridine add back. As with AICAr, deoxycytidine does not prevent apoptosis (Fig. 5B). Thymidine does not seem to prevent apoptosis as effectively in PALA-treated cells as previously shown in AICAr-treated cells (Fig. 5B). However, this seems to be time dependent, with thymidine more effectively preventing apoptosis at earlier timepoints (data not shown). As expected, PALA-induced H2AX phosphorylation is prevented by uridine and low concentrations of cytidine (Fig. 5D).

**Mechanism of UMPS inhibition and pyrimidine starvation**
Although unlikely, based on the long half-life of UMPS (32), one possibility for the inhibition of UMPS activity is downregulated enzyme expression. However, a Western blot analysis conducted with extracts from cells treated with AICAr for varying times showed no decrease in protein level (Fig. 6A). A second possibility is that intracellularly generated ZMP competitorily inhibits the OMP decarboxylase activity (reaction 2’ of UMPS in Fig. 3). Monophosphonucleosides are effective inhibitors of decarboxylase activity with XMP, UMP, and AMP being the most effective (14). ZMP, the phosphorylated form of AICAr, can be viewed as an analog of AMP or GMP (1). Inhibition of the decarboxylase activity can appear as inhibition of the transferase activity (accumulation of orotate) because accumulation of OMP will push the equilibrium toward orotate (33). To determine whether ZMP can inhibit UMPS activity, we conducted a functional assay using a TLC assay. ZMP inhibited recombinant UMPS activity at concentrations above 0.5 mmol/L, with the decarboxylase activity inhibited to a greater extent than the transferase activity (Table 2). As ZMP

**Table 2.** Activity of the transferase and decarboxylase activities of recombinant UMPS in the presence of varying concentrations of ZMP

<table>
<thead>
<tr>
<th>ZMP concentration (mmol/L)</th>
<th>Transferase activity</th>
<th>Decarboxylase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mmol/L ZMP</td>
<td>1.03 (0.06)</td>
<td>0.79 (0.31)</td>
</tr>
<tr>
<td>0.5 mmol/L ZMP</td>
<td>0.89 (0.22)</td>
<td>0.41 (0.15)</td>
</tr>
<tr>
<td>1.0 mmol/L ZMP</td>
<td>0.79 (0.20)</td>
<td>0.27 (0.09)</td>
</tr>
</tbody>
</table>

NOTE: Activity (mean of 4 experiments ± SD) is compared with activity in the absence of ZMP.
can accumulate in cells to the millimolar range (1, 2, 4, 34), this suggested the possibility that AICAr converted to ZMP could inhibit UMPS inside multiple myeloma cells. An HPLC analysis was, thus, conducted to measure ZMP accumulation. Although our assay was able to detect significant accumulation of ZMP in HeLa cells treated with thymidine and pemetrexed (as reported in ref. 34) to the level of 4.6 mmol/L, no ZMP accumulation was detected in 8226 cells treated with AICAr for 1 to 16 hours.

Another possible reason for UMPS inhibition is PRPP substrate limitation, first proposed by Thomas and colleagues (35). PRPP is generated at the first step of de novo purine synthesis and is a substrate for UMPS (Fig. 3). The increased levels of purine metabolites, (i.e., ADP), in AICAr-treated cells could potentially inhibit PRPP synthetase and the generation of PRPP from d-ribose as hypothesized in Fig. 3. We assessed levels of PRPP in AICAr-treated cells (8-hour exposure) by the ability of cell extracts to generate (8-13C) AMP from (8-13C) adenine in the presence of APRTase using cell extracts as a source of PRPP. Figure 6b shows a representative experiment. When exogenous PRPP is supplied in the reaction mixture along with APRTase and radiolabeled adenine (lane 6), radiolabeled AMP is generated. When cell extracts are used as a source of PRPP (lanes 1–4), a signal is generated at the same site in the TLC plates. In 3 independent experiments, the amount of AMP product was on average 3.3 times (±1.3) higher in the control cells compared with AICAr-treated cells. Thus, AICAr decreases the level of PRPP in multiple myeloma cells. This lower level of substrate could conceivably limit the activity of UMPS resulting in pyrimidine starvation.

Discussion

Our data indicate that AICAr induces multiple myeloma cell apoptosis by inhibition of UMPS activity and pyrimidine starvation in an AMPK-independent manner. A metabolomics screen showed that orotate accumulated after exposure to AICAr and that UMP levels were decreased, indicating inhibition of UMPS. Apoptosis induced by AICAr was prevented by addition of uridine. Furthermore, PALA, which also inhibits de novo pyrimidine synthesis by a different mechanism, also induced apoptosis in multiple myeloma cells that was similarly prevented by uridine. Finally, activation of AMPK was not detected in AICAr-treated cells while AICAr could activate AMPK in CLL cells and metformin could activate AMPK in multiple myeloma cells.

Our results differ from an earlier report (11) on the effects of AICAr in multiple myeloma cells. In that study, the authors concluded that the effects of AICAr were mediated via AMPK activation. They showed that AMPK became phosphorylated in U266 multiple myeloma cells following AICAr exposure. However, AICAr treatment of U266 cells in that study induced S-phase arrest with very little apoptosis and 8226 cells, the most sensitive to apoptosis in that study (as well as in ours), were not assayed for AMPK activation. In contrast, our focus on apoptosis and the most sensitive target, 8226 cells, clearly showed absence of AMPK phosphorylation/activation although significant apoptosis ensued. Quite possibly, the lack of ZMP accumulation in 8226 cells as indicated by our HPLC assay, accounts for the inability of AICAr to activate AMPK. The significant rescue afforded by uridine in the other 4 multiple myeloma cell lines also supports that their apoptotic response was mediated by an identical mechanism to that of 8226 cells. In contrast, metformin could activate AMPK in 8226 multiple myeloma cells by an independent mechanism and this was associated with apoptosis, which was unaffected by attempted uridine rescue. This suggests that AMPK activation may also mediate multiple myeloma cell apoptosis although AMPK-independent effects of metformin have been reported (36, 37). Nevertheless, it is clear that AICAr-induced apoptosis, which can be rescued by exogenous pyrimidines, can proceed without AMPK activation.

We ruled out the possibility that AICAr inhibited UMPS by downregulating its expression or by an inhibitory effect of ZMP. A more likely explanation for UMPS inhibition is decreased levels of PRPP substrate. PRPP, which is used as a substrate by UMPS in de novo pyrimidine synthesis, was significantly decreased in AICAr-treated cells. Therefore, limiting amounts of PRPP may account for inhibition of UMPS. This is consistent with previous studies in AICAr-treated Chinese hamster fibroblasts (35) or human B lymphoblasts (16). Adenosine, which our metabolomics screen identified as significantly higher in AICAr-treated cells, has been shown to induce pyrimidine starvation by a similar mechanism in some cells with an increase in orotate accumulation and a decrease in PRPP (38–41). Other purine metabolites may also depress PRPP levels. Thus, in addition to adenosine described above, the enhanced generation of ADP, which is increased by AICAr in the screen, could inhibit PRPP as previously suggested (42).

The fact that addition of thymidine can prevent most, but not all of the AICAr-induced apoptosis suggests that apoptosis is induced by replicative stress due to limiting TTP. Consistent with this notion is the finding of AICAr-induced phosphorylation of H2A.X, a marker of replicative stress, which was attenuated by uridine, cytidine and thymidine rescue. However, the fact that thymidine does not fully protect multiple myeloma cells against apoptosis compared with uridine suggests that limiting DNA replication may not fully explain all apoptosis. Pyrimidine nucleotides are required in other metabolic pathways to generate intermediates such as cytidine diphosphate choline (CDP-choline). CDP-choline is critical for the de novo phosphatidylcholine synthesis pathway and this pathway is known to be important in plasma cell development (43). As CDP-choline was found to be lower in AICAr-treated cells in our screen (Table 1), we speculate that some apoptosis may occur due to inhibition of phosphatidylcholine synthesis.
When the de novo pyrimidine synthesis pathway is inhibited, tumor cells are critically dependent upon salvage pathways. One of these enzymes is deoxycytidine kinase (dCK). After internalization, deoxycytidine, deoxyadenosine, and deoxymyoxanosine are phosphorylated by dCK in the rate-limiting step in deoxyribonucleoside salvage for generation of these 3 dNTPs. Although highly expressed in lymphocytes (44), recent gene expression profiling (45, 46) suggests dCK is poorly expressed in normal and myeloma plasma cells. This may explain the absence of protection against AICAr-induced apoptosis when deoxycytidine was used in attempted rescue add-back experiments. An additional important salvage pathway enzyme is cytidine deaminase (CDA). Cytidine and deoxycytidine are both substrates for this enzyme yielding uridine and deoxyuridine. Deoxuryridine is phosphorylated by thymidine kinase, which subsequently is converted to dTMP by thymidylate synthase for subsequent incorporation of dTTP into DNA (26). Therefore, the inability of deoxycytidine to prevent AICAr-induced apoptosis may also be due to limited conversion of deoxycytidine to deoxyuridine due to limiting CDA activity. Gene expression profiling suggests that a significant number of patients harbor multiple myeloma clones with downregulated CDA expression compared with nonmalignant plasma cells (47, 48). A relative deficiency of these salvage pathway enzymes in the multiple myeloma model may render malignant plasma cells particularly sensitive to pyrimidine starvation.

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

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Bardeleben, J.R. Reeve, S. Bassilian, A. Lichtenstein
Writing, review, and/or revision of the manuscript: C. Bardeleben, A. Lichtenstein
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Bardeleben, P.J. Frost, B. Hoang, Y. Shi
Study supervision: A. Lichtenstein

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