Metabolomics identifies pyrimidine starvation as the mechanism of 5-aminoimidazole-4-carboxamide-1-β-riboside (AICAr) induced apoptosis in multiple myeloma cells

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ABSTRACT
To investigate the mechanism by which AICAr induces apoptosis in multiple myeloma (MM) cells, we performed 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 x fold increase in orotate associated with a decrease in 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 PRPP. As all pyrimidine nucleotides can be synthesized from UMP, this suggested 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 MM 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 MM 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 MM cells.
Introduction

AICAr (5-aminoimidazole-4-carboxamide-1-β-ribooside) 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) (1). Because TORC1 activity can be inhibited by activated AMPK, AICAr has been studied as an anti-tumor agent. Several studies have demonstrated that the tumor cytoreductive effects of AICAr can be mediated through AMPK activation and mTOR inhibition (2-4). However, cytotoxic affects 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 (MM). Rapalogs, which primarily inhibit TORC1, induce G1 arrest in MM cells, but not apoptosis in vitro (10). AICAr has been reported to inhibit growth in myeloma cells through activation of AMPK (11). However, a substantial amount of apoptosis was only shown in the 8226 cell line. AMPK activation in 8226 cells was not demonstrated in this report and, thus, the mechanism of AICAr induced apoptosis in MM remains unclear. In addition, since rapalogs only induce G1 arrest in MM 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 MM 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 performed 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 UMP synthetase with subsequent pyrimidine starvation.
Material and Methods

Reagents – AICA riboside was purchased from Calbiochem. Metformin, nucleosides, dithiothreitol (DTT), 5-Phosphoribosyl-alpha-pyrophosphate (PRPP), orotate, OMP and ZMP were purchased from Sigma-Aldrich. PALA was obtained from the Developmental Therapeutics Program (NCI/NIH). All antibodies were purchased from Cell Signaling except for anti-UMP synthetase, purchased from Abcam. Recombinant UMP synthetase was purchased from Origene and recombinant APRTase was purchased from Prospec. [6-14C] orotate (50 mCi/mmol) and [8-14C] adenine (50 mCi/mmol) was purchased from MPBiomedicals. Chemical structures for bortezomib, melphalan, and metformin are shown in Supplemental Fig 1.

Cell lines – All cell lines were obtained from ATCC. 8226, OPM2, U266 and MM1S cells were maintained in RPMI supplemented with 10% fetal bovine serum, glutamine, non-essential amino acids, pen-strep and fungazone. H929 cells were maintained in RPMI media with the same supplements described above, except the media was supplemented with 0.05 mM B-mercaptoethanol. HeLa cells were maintained in DMEM with the same supplements as 8226 cells. Cell lines were verified with STR analysis by ATCC.

Screening for metabolites – Treated cells were harvested, washed once in PBS, frozen in liquid nitrogen and sent to Metabolon Inc. (Durham, NC). Samples were prepared in quadruple. 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 GC/MS and LC/MS/MS platforms. Following log transformation and imputation with minimum observed values for each compound, Welch’s two-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 cut-off for statistical significance (p<\textless;= 0.05) would occur by random chance.
Immunoblots – Whole cell lysates were prepared using cell lysis buffer (Cell Signaling) supplemented with 1 mM PMSF immediately before use. Westerns were performed 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.

UMP synthetase functional assay – The UMP synthetase assay was as described (14). The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 5 mM MgCl₂, 300 μM PRPP, 5 μM [6-¹⁴C] orotate (50 mCi/μmole) and 0.4-0.8 μg recombinant UMP synthetase. Reactions were incubated at 37 °C for 30 min. Products were separated using thin layer chromatography (TLC) (15). The reaction was stopped by directly spotting 5 μl of the reaction mixture onto a 20 cm x 20 cm PEI-cellulose plate and drying the sample with hot air. Plates were developed by ascending chromatography in 0.25M LiCl₂/0.1% formic acid. Cold standards of orotate, OMP and UMP were used to identify spots. Plates were visualized using a PhosphoImager (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 HPLC analysis – Cells were harvested, washed once in PBS, resuspended in 5% TCA, sonicated for ten seconds and incubated on ice for 5 min. Precipitated protein was removed by centrifugation (5000 x g, 10 min) and the supernatant was neutralized by 5 extractions with 5 volumes of water-saturated ether. pH was adjusted to pH=5. Nucleotides were separated on a Phenomenex Partisil 10 SAX column (0.45 X 25 cm) equilibrated in 5 mM K phosphate (pH 5) at a flow rate of 2 ml/min. Samples were eluted isocratically for 20 min with 5 mM K phosphate buffer, followed by a linear gradient to 500 mM K phosphate for 45 min, followed by 500 mM K
phosphate for an additional 15 min. Separation was on a Gilson HPLC system with a Model 117 ultraviolet detector set at 260 nm and 280 nm. Areas of peaks were quantified using UniPoint software and converted to molar quantities by comparison to ZMP standards.

PRPP assay - Measurement of intracellular PRPP was as described (16). Briefly, cells were treated with 250 μM 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 mM Tris-HCl, pH 7.5, 0.5 mM EDTA followed by heating in a boiling water bath for 45 seconds and centrifugation at 10,000 rpm for 10 min 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. The standard reaction mixture contained 30 mM Tris-HCl, pH7.5, 6 mM MgCl2, 2 mM DTT, 50 μM [8-14C] adenine (50 mCi/mmmole) and 0.1 μg recombinant APRTase. Reactions were incubated for 1 hour at 37 °C. TLC was performed 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 post-hoc t-tests where overall main effects were significant.

Results

AICAr induces apoptosis in myeloma cells in vitro
AICAr (Fig. 1A) had cytoreductive effects on MM 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 five MM lines identified by caspase 3 activation (Fig 1B). Based on these preliminary results, further time-course experiments were carried out with either 250 µM AICAr (for 8226 and U266 cells) or 500 µM (for OPM2, MM1S and H929 cells). AICAr induced apoptosis in a time-dependent fashion (Fig 1C). Apoptosis was first detected consistently by 48 hrs. The 8226 cell line was the most sensitive target with rapid induction of apoptosis. Cell cycle analysis demonstrated that AICAr also induced an accumulation of cells in S phase in all five cell lines (Fig 1D) consistent with a previous report (11). The increase in S phase distribution ranged from 10-25% 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-1 is a known survival factor for MM cells (19), it did not confer protection against AICAr-induced apoptosis while protecting against dexamethasone (supplemental figure 2) 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 (ie., mTORC1 inhibition) could not reproduce the apoptosis resulting from AICAr, we performed a high throughput screen for alterations in metabolites selectively affected by AICAr. Preliminary time course experiments in 8226 cells indicated that 250 µM 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 non-specific 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 Methods for metabolomics data analysis). In concurrently run samples of 8226 cells exposed to 10 nM rapamycin for 4 or 8 hours, a larger metabolic effect was seen with significant alterations in 33 and 51 metabolites respectively (rapamycin data in suppl figure 3). As shown in the heat map in figure 2A, AICAr treatment had little effect on carbohydrate, energy, amino acid and lipid metabolism with the exception of a few metabolites shown in Table 1. Of particular interest was the increase in phosphocholine and glycerol 3-phosphate and decrease in CDP-choline (Table 1), which suggested an inhibition of the reaction catalyzed by CTP:phosphocholine cytidylyltransferase, the rate limiting step in the major pathway of phosphatidylcholine synthesis (20)(see Discussion). In contrast, rapamycin treatment (suppl fig 3) resulted in robust effects 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 TCA 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 mTORC1 inhibition. Unexpectedly, we could not demonstrate activation of AMPK in MM cells. Focusing on the most AICAr-sensitive 8226 cell line, experiments utilizing a range of AICAr concentrations (100 μM- 2mM) and incubation times (30 mins-24 hrs) failed to detect AMPK activation as monitored by phosphorylation of Thr 172 (fig 2B and Supp. Fig. 4). As positive controls, our AICAr preparation successfully induced AMPK activation in primary 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 MM cells (Fig. 2C). Comparable attempts to demonstrate AMPK activation in AICAR-treated OPM-2 and MM1.S cells were likewise unsuccessful (Fig. 2B and Supp. Fig. 4) while metformin was successful (Fig 2C). These data demonstrate 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 suppl figure 4 (for OPM-2 cells), while rapamycin clearly inhibited phosphorylation of p70, apoptotic concentrations of AICAr did not. These data collectively demonstrate that the ability of AICAr to induce MM apoptosis and cell cycle arrest is not mediated by AMPK activation or mTORC1 inhibition. As our metabolomics screen identified orotate accumulation as the most striking abnormality, we further investigated its significance as described below.

**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 μM AICAr for 8 hours increased orotate 4.5 x fold (mean of 3 experiments). Orotate is a substrate of UMP synthetase (UMPS), the last enzyme in the *de novo* pyrimidine biosynthetic pathway (Fig. 3). UMPS is a multi-functional enzyme that catalyzes two enzymatic reactions. The first reaction catalyzes the conversion of orotate to orotidine monophosphate (OMP) using orotate phosphoribosyl transferase (OPRTase) that requires 5-phosphoribosyl-α-pyrophosphate (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 UMP synthetase is inhibited by AICAr exposure. Consistent with this data is the observed associated decrease in UMP levels (Table 1), the product of the reaction (Figure 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, in order to determine whether pyrimidine starvation through the inhibition of UMPS induces apoptosis, MM cells were treated with increasing concentrations of uridine in conjunction with AICAr. The addition of uridine prevented apoptosis and prevention was related to concentration of uridine (Fig. 4A). Uridine conferred complete protection as there is no statistical difference between control cells and AICAr treated cells+100 μM 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 non-specific 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 MM cells treated with AICAr but not with metformin exposure is especially noteworthy since metformin activates AMPK (Fig. 2C) and AMPK is a known mTOR inhibitor (27). These data provide 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 μM) but concentrations of 50 μM 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 inhibited 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 MM 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 cells 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 dNTPs is the increase in phosphorylation of H2A.X, a marker for replicative stress (Fig. 4E) (31). AICAr-induced phosphorylation of H2A.X was prevented by uridine or thymidine add-back rescue as well as by a low (5 μM) concentration of cytidine. The lowest panel in figure 4E demonstrates the difference between adding back the apoptotic high concentration of cytidine (100 μM (H)) versus low concentration (5 μM (L)) with the former inducing an increased amount of H2A.X phosphorylation. Since activation of caspases can also result in H2A.X phosphorylation, we repeated experiments described in figure 4E with the addition of the caspase 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 (Supp. Fig. 5) demonstrated that uridine rescue was associated with a prevention of S-phase arrest.

Addition of deoxycytidine (dC) over a wide range of concentrations (5-100μM) 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. MM 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, *N*- (phosphonacetyl)-L-aspartate, 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,C). In similar fashion to AICAr, PALA-induced apoptosis is also associated with replicative stress, as shown by induction of H2A.X 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) while high cytidine concentrations enhance apoptosis (Fig 5B). As
mentioned above, since some H2A.X phosphorylation could be downstream of apoptosis induction, we also assayed cell transit. Suppl. Fig. 6 demonstrates the PALA-induced S phase accumulation was prevented by uridine add back. As with AICAr, deoxycytidine (dC) does not prevent apoptosis (Fig. 5B). Thymidine does not appear to prevent apoptosis as effectively in PALA treated cells as previously shown in AICAr-treated cells (Fig 5B). However this appears to be time dependent, with thymidine more effectively preventing apoptosis at earlier timepoints (data not shown). As expected, PALA-induced H2A.X 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 UMP synthetase (32), one possibility for the inhibition of UMP synthetase activity is down-regulated enzyme expression. However, a Western blot performed with extracts from cells treated with AICAr for varying times demonstrated no decrease in protein level (Fig. 6A). A second possibility is that intracellularly generated ZMP competitively inhibits the OMP decarboxylase activity (reaction ‘2’ of UMPS in figure 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 UMP synthetase activity, we performed a functional assay using a TLC assay. ZMP inhibited recombinant UMP synthetase activity at concentrations above 0.5 mM, with the decarboxylase activity inhibited to a greater extent than the transferase activity (Table 2). Since ZMP can accumulate in cells to the millimolar range (1, 2, 4, 34), this suggested the possibility that AICAr converted to ZMP could inhibit UMP synthetase inside MM cells. An HPLC analysis was, thus, performed 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 (34)) to the level of 4.6mM, no ZMP accumulation was detected in 8226 cells treated with AICAr for 1-16 hours.
Another possible reason for UMP synthetase inhibition is PRPP substrate limitation, first proposed by Thomas (35). PRPP is generated at the first step of \textit{de novo} purine synthesis and is a substrate for UMP synthetase (Fig. 3). The increased levels of purine metabolites, (ie., ADP), in AICAr-treated cells could potentially inhibit PRPP synthetase and the generation of PRPP from D-ribose as hypothesized in figure 3. We assessed levels of PRPP in AICAr-treated cells (8 hr exposure) by the ability of cell extracts to generate (8-\textsuperscript{14}C) AMP from (8-\textsuperscript{14}C) adenine in the presence of adenosine phosphoribosyltransferase (APRTase) using cell extracts as a source of PRPP. Figure 6B demonstrates a representative experiment. When exogenous PRPP is supplied in the reaction mixture along with APRTase and radiolabeled adenine (lane 6), radio-labeled 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 to AICAr treated cells. Thus, AICAr decreases the level of PRPP in MM cells. This lower level of substrate could conceivably limit the activity of UMP synthetase resulting in pyrimidine starvation.

Discussion

Our data indicate that AICAr induces MM cell apoptosis by inhibition of UMP synthetase 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 UMP synthetase. Apoptosis induced by AICAr was prevented by addition of uridine. Furthermore, PALA, which also inhibits \textit{de novo} pyrimidine synthesis by a different mechanism, also induced apoptosis in MM 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 MM cells.

Our results differ from an earlier report (11) on the effects of AICAr in MM cells. In that study, the authors concluded that the effects of AICAr were mediated via AMPK activation. They demonstrated that AMPK became phosphorylated in U266 MM 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
demonstrated 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 four MM 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 MM 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 MM 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 UMP synthetase by down-regulating
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
UMP synthetase in de novo pyrimidine synthesis, was significantly decreased in AICAr-
treated cells. Therefore, limiting amounts of PRPP may account for inhibition of UMP
synthetase. 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 MM cells against apoptosis compared to 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 (PC) synthesis pathway and this pathway is known to be important in plasma cell development (43). Since 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 PC 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 (dC), deoxyadenosine (dA) and deoxyguanosine (dG) are phosphorylated by DCK in the rate-limiting step in deoxyribonucleoside salvage for generation of these three deoxyribonucleotide triphosphates (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. Deoxyuridine is phosphorylated by thymidine kinase which subsequently is convert 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 cytidine deaminase activity. Gene expression profiling suggests that a significant number of patients harbor MM clones with downregulated CDA expression compared to non-malignant plasma cells (47, 48). A relative deficiency of these salvage pathway enzymes in the MM model may render malignant plasma cells particularly sensitive to pyrimidine starvation.
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References

### Tables

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

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<th>Pathway</th>
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</tr>
<tr>
<td>CDP-choline</td>
<td>0.57</td>
</tr>
<tr>
<td>Glycerol 3-phosphate</td>
<td>1.48</td>
</tr>
<tr>
<td>Choline phosphate</td>
<td>1.23</td>
</tr>
<tr>
<td><strong>Krebs cycle</strong></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><strong>Glycolysis</strong></td>
<td></td>
</tr>
<tr>
<td>Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate</td>
<td>2.5</td>
</tr>
<tr>
<td>lactate</td>
<td>0.77</td>
</tr>
<tr>
<td><strong>Cysteine, methionine, SAM, taurine metabolism</strong></td>
<td></td>
</tr>
<tr>
<td>cystathionine</td>
<td>0.71</td>
</tr>
<tr>
<td>N-formylmethionine</td>
<td>1.35</td>
</tr>
<tr>
<td>S-adenosylhomocysteine (SAH)</td>
<td>1.55</td>
</tr>
<tr>
<td>assymetric dimethylarginine (ADMA)</td>
<td>1.48</td>
</tr>
</tbody>
</table>

Bold numbers (p<0.05), italicized numbers (0.05<p<0.1)
Table 2- Activity of the transferase and decarboxylase activities of recombinant UMPS in the presence of varying concentrations of ZMP.

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

Activity (mean of 4 experiments +/-SD) is compared to activity in the absence of ZMP.
Figure Legends

Figure 1 - **A)** Chemical structure of 5-aminoimidazole-4-carboxamide-1-β-ribooside (AICAr). **B)** MM lines were treated with increasing concentrations of AICAr (0, 100, 250, 500 or 1000 μM) for 48h and assessed for apoptosis by flow cytometry. Black bars = control.  **C)** Cells were treated with 250 μM AICAr (8226, U266) or 500 μM (OPM2, MM1S, H929) for the indicated times and apoptosis assessed as in A. Data are mean+/−SD apoptosis above control, n=3.  **D)** Cell cycle analysis of cells +/- AICAr 24 hrs before apoptosis is induced. Apoptosis was <11% above control in all samples. The percent of cells in G1, S and G2 phase are indicated. Data are mean+/−SD, n=2 for 8226, OPM2 and H929; n=3 for U266 and MM1S).  **E)** Cells were treated with rapamycin (0.1 nM for OPM2, 10 nM all other cell lines) or vehicle and analyzed for cell cycle distribution at 24h-72h or apoptosis between 48-96 hrs. Data are mean+/−SD, n=2.  **F)** 8226 cells were pre-treated with 100 nM iodotubercidin or vehicle for 30 mins followed by addition of 250 μM AICAr or vehicle, incubated for 48h, and assessed for apoptosis. Data are mean+/−SD, n=2.

Figure 2 – **A)** Simplified heat map of 8226 cells treated with 250 μM AICAr for 4 and 8 hours compared to 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 MM1.S 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 16h +/-500 μM AICAR for similar Western blot assay. 8226 and MM1S cells were incubated with 5 mM metformin for 16h (8226) or 72h (MM1S) for similar Western blot assay.  **D)** Cells were treated either with DMSO (Con), 10 nM Rapamycin (R), or 500 μM AICAr (A) for 8 hrs (8226) or 24 hrs (MM1S). Western blots were probed for P-p70 (Thr 389 or Ser/Thr 421/424) and actin to assess TORC1 activity.
**Figure 3**- Key steps involved in *de novo* pyrimidine synthesis and purine synthesis. Biochemicals in boxes are metabolites identified in the screen, with white arrows indicating statistically significant increases or decreases in AICAr treated cells.

**Figure 4** – **A)** Cells were incubated with AICAr (250 μM for 8226 and U266, 500 μM for OPM2, MM1S and H929) or vehicle in the presence of increasing concentrations of uridine (U), for 48h (8226, OPM2) or 96h (U266, MM1S, H929) and apoptosis assessed. Data are mean+/−SD, n=2, except for 8226, n=3. **B)** 8226 was incubated with AICAr (250 μM), bortezomib (5 nM), melphalan (10 μM) or metformin (5mM) +/- 100 μM uridine for 48h and apoptosis assessed. Data are mean+/−SD, n=2. **C)** Cells were incubated with AICAr as described above or vehicle in the presence of increasing concentrations of cytidine (C). Data are mean+/−SD, n=2 (8226,U266, MM1S) or n=3 (OPM2, H929). **D)** Cells were incubated with AICAr as described above or vehicle in the presence of 100 μM uridine (U), 100 μM thymidine (T) or 100 μM deoxycytidine (dC). Mean apoptosis and error bars were determined from three to five replicates. **E)** 8226 cells were treated with 250 μM AICAr or vehicle for the indicated time and Western blot probed for P-H2A.X (Ser 139) or actin. MM1S cells were treated with 500 μM AICAr and 100 μM of the indicated nucleoside for 72h, except in the lowest panel were C(L) is 5 μM and C(H) is 100 μM. A western blot was performed as described above.

**Figure 5**- **A)** Chemical structure of N-(phosphonacetyl)-L-aspartate (PALA). **B)** Cells were incubated with 250 μM PALA or vehicle +/- 100 μM uridine, 100 μM cytidine, 100 μM thymidine or 100 μM deoxycytidine for 48-96 hours. Apoptosis was assessed as described in Figure 1. Data are mean+/−SD, n=2-4 replicates. **C)** OPM2 and MM1S cells were incubated with 250 μM PALA or vehicle +/- 5 μM cytidine. Data are mean+/−SD, n=2. **D)** MM1S cells were treated with 250 μM PALA +/- 100 μM uridine, 5 μM cytidine, 100 μM thymidine or 100 μM deoxycytidine for 48 hours. Extracts were probed for P-H2A.X (Ser 139) or actin as described above.
Figure 6  – A) Western blot for UMPS expression in 8226 cells+/-250 μM AICAr for the indicated time. B) TLC assay for measuring PRPP in cell extracts, A (AICAr treated), C (control). The position of adenine, AMP migration and the origin is indicated. APRTase = adenosine phosphoribosyltransferase.
Figure 3

PYRIMIDINE SYNTHESIS
Dihydroorotic Acid → Orotate → OMP → UMP
+ PRPP + PPi

PURINE SYNTHESIS
D-Ribose-5-phosphate → PRPP → AICAR (ZMP) → FAICAR
+ ADP
- ATP

AICAriboside
- ADP

AMP → IMP → GMP
Figure 6

A

UMPS

actin

AIC

Time (hrs)

0  +  +  +  -  -  -  4
1  +  +  +  -  -  -  4
2  +  +  +  -  -  -  4
4  +  +  +  -  -  -  4

B

Lane:

1  2  3  4  5  6  7

Adenine

AMP

Cell extract

A  A  C  C  -  -  -

PRPP (exo)

-  -  -  -  -  +  -

APRTase

+  -  +  -  +  +  -
Metabolomics identifies pyrimidine starvation as the mechanism of 5-aminoimidazole-4-carboxamide-1-β-riboside (AICAr) induced apoptosis in multiple myeloma cells

Carolyne Bardeleben, Sanjai Sharma, Joseph R Reeve, Jr, et al.

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