RNA-dependent inhibition of ribonucleotide reductase is a major pathway for 5-azacytidine in acute myeloid leukemia

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**Running title:** RRM2 is a novel target of 5-azacytidine

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Abstract

5-azacytidine (5-azaC) is an azanucleoside approved for myelodysplastic syndrome. About 80-90% of 5-azaC is believed to be incorporated into RNA, which disrupts nucleic acid and protein metabolism leading to apoptosis. A smaller fraction (10-20%) of 5-azaC inhibits DNA synthesis through conversion to decitabine (DAC) triphosphate and subsequent DNA incorporation. However, its precise mechanism of action remains unclear. Ribonucleotide reductase (RR) is a highly regulated enzyme comprising two subunits RRM1 and RRM2 that provides the deoxyribonucleotides required for DNA synthesis/repair. Herein, we discovered for the first time that 5-azacytidine is a potent inhibitor of RRM2 in leukemia cell lines, mouse model and acute myeloid leukemia (AML) patients bone marrow mononuclear cells. 5-azaC-induced RRM2 gene expression inhibition involves its direct RNA incorporation and an attenuated RRM2 mRNA stability. Consequently, 5-azaC causes a major perturbation of dNTP pools. We also demonstrated that initial RR-mediated 5-azaC conversion to DAC is terminated through its own inhibition. In conclusion, we identify RRM2 as a novel molecular target of 5-azaC in AML. Our findings provide basis for its better clinical use either alone or in combination.
Introduction

5-azacytidine (5-azaC; Vidaza) is a pyrimidine analog that was first synthesized 45 years ago and subsequently found to be a natural product from *Streptoverticillium Ladakanus*\(^1\). Currently, 5-azaC is approved by US Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome (MDS) but it has also been widely used for the treatment of acute myeloid leukemia (AML)\(^4\). Despite decades of efforts made to delineate the mechanisms of the antileukemia activity for this compound in terms of its interference with RNA and DNA metabolism\(^5\), the precise basis of its clinical efficacy remains to be fully elucidated \(^6\).

Following cell uptake, 5-azaC is first anabolized to its nucleoside monophosphate 5-aza-CMP by uridine-cytidine kinase (UCK) and eventually phosphorylated by diphosphate kinase to its triphosphate metabolite 5-aza-CTP, which is thought to be incorporated into RNA\(^7\). Indeed, approximately 80-90% of 5-azaC is incorporated into RNA\(^7\). This ultimately disrupts mRNA and protein metabolism thereby leading to apoptosis\(^4,6\). A small fraction (10-20%) of 5-azaC disphosphate intermediate product 5-aza-CDP is instead converted to 5-aza-dCDP by ribonucleotide reductase (RR), followed by further phosphorylation to 5-azaC-dCTP (DAC-TP)\(^4,7\). DAC-TP is incorporated into DNA and covalently binds DNA methyltransferases (DNMTs), thereby inhibiting the activity of these proteins. It has been recognized that DNMTs add methyl groups to the cytosine in the context of the CpG dinucleotides in promoter region thereby promoting gene hypermethylation and epigenetic silencing. Thus 5-azaC has been classified as a hypomethylating agent. The deoxy-analog, decitabine (5-aza-2’-deoxycytidine, DAC), differs structurally from 5-azaC by having a deoxyribose and therefore does not require
RR-mediated reduction prior to DNA incorporation. At higher doses (50-100 mg/m²/day), DAC incorporates into DNA, leading to inhibition of DNA synthesis and cell death⁹,¹⁰, while at lower doses (5-20 mg/m²/day), it induces DNA demethylation and reactivates hypermethylation-associated silenced tumor suppressor genes¹¹-¹³. DAC has also been approved for the treatment of MDS.

While recent attention has focused on the hypomethylating activity of 5-azaC via its DNA pathway through the converted DAC⁴, the antileukemia activity of this compound via RNA pathway is less understood. Here we report a previously unidentified activity of 5-azaC. We showed that 5-azaC is a potent inhibitor of M2 subunits of RR (RRM2) via underlying molecular mechanisms that involve 5-azaC direct RNA incorporation and an attenuated RRM2 mRNA stability. Inhibition of RR leads to a reduced dNTP pool, which is crucial for DNA synthesis and repair. Thus, our findings support a profoundly different mode of action between 5-azaC and DAC, which may ultimately explain some difference in their clinical activities.

**Material and Methods**

**Chemicals**

5-azaC, DAC, cytidine, uridine and DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) were purchased from Sigma-Aldrich (St. Louis, MO). Triapine and GTI-2040 (LOR-2040) were obtained from The National Cancer Institute (Bethesda, MD). MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] cell viability kit was purchased from Promega (Madison, WI).
Cell Lines, cell culture and transfection

MV4-11 and K562 were obtained from American Type Culture Collection and cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, L-glutamine and penicillin-streptomycin antibiotics (Invitrogen, Carlsbad, CA). Mononuclear cells (MNCs) from bone marrow samples of 6 untreated AML patients were obtained from the Ohio State University (OSU) Leukemia Tissue Bank. MNCs were prepared and cultured as previously described. All experiments with these cells were performed in accordance with the protocols approved by the OSU institutional review board. GTI-2040 or siRRM2 (LOR-1284, Invitrogen), at the indicated final concentrations, were transfected into cells by nucleoporation (Lonza, Gaithersburg, MD).

Western blotting

Tissues or cell samples were lysed and the proteins were separated, transferred and analyzed by immunoblotting. Antibodies to RRM2 (E-16), DNMT3a and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); to DNMT1 from New England Biolabs (Ipswich, MA) and to β–actin from Sigma. The protein expression was quantified by densitometry and normalized to GAPDH.

Intracellular dNTP/NTP, DAC and DAC-TP Levels

Cells were lysed and the intracellular dNTP/NTP, DAC or DAC-TP were extracted and quantified by our previously described methods. Briefly, cell pellets were washed, deprotonized with 70% methanol and sonicated on ice bath. Cell extracts were centrifuged and the supernatant was dried under a stream of nitrogen. The residues were
reconstituted with water and centrifuged again. A 50 μL aliquot of the supernatants was injected into an ion-trap LC-MS/MS system for dNTP and DAC-TP measurements. DAC level was measured by the triple quadruple LC-MS/MS system.

**Quantitative RT-PCR**

Total RNA was isolated and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen). The cDNA templates and primers were then mixed with reagents from a SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). The primers used were as follows: RRM2+: 5’-GCCTGGGCTCAGATTTTTCTAAT-3’; RRM2-: 5’-GAACATCAGGCCGCAAGGAAAAT-3’; abl+: 5’-TGGAGATTAACACTCTAAGCATAACTAAAGGT-3’; abl-: 5’-GATGTAGTTGCTTTGGGACC-3’; GAPDH+: 5’-TCCACCCATGGCAAATTCC-3’; GAPDH-: 5’-TCGCCACTTGATTGG-3’. Reactions were carried out in triplicate in ABI StepOne Plus Real-time PCR system (Applied Biosystems), and data were analyzed by comparative CT method. The amount of RRM2 mRNA was normalized by an internal control, abl. The relative changes in the treated groups were expressed as a percentage of the untreated control (arbitrarily set at 100).

**mRNA stability**

MV4-11 cells were treated with 1µM 5-azaC. At the same time, 1µM actinomycin D (Sigma) was used to block mRNA synthesis. Cells were harvested at various time points after actinomycin D treatment. RRM2, abl and GAPDH mRNA levels were quantified by qRT-PCR. Their half-lives were calculated from the plots of their mRNA levels as a
function of time. We also performed the experiment in the presence of a potent protein synthesis inhibitor cycloheximide (Sigma). MV4-11 cells were pretreated with 10 µg/mL cycloheximide for 2 hours. Actinomycin D was added after the cells were treated with 5-azaC for 2 hours. The cells continued to be incubated with drugs for additional 4 hours and harvested for measurement of RRM2 and abl mRNAs.

**RNA reduction and hydrolysis**

MV4-11 cells were treated with 5-azaC and the total RNA was isolated and incubated with NaBH₄ or NaBD₄. The solution was then neutralized to pH 7.0 by HCl. RNA hydrolysis was performed as previously described. Briefly, a 1/10 volume of 0.1 M ammonium acetate (pH 5.3) and two units of nuclease P1 were added to 1 µg of total RNA. The mixture was incubated at 45°C for 2 hours followed by the addition of 1/10 volume of 1 M ammonium bicarbonate and 0.002 unit of venom phosphodiesterase I. The resulting mixture was incubated at 37°C for 1 hour. After adding 0.5 unit of alkaline phosphatase, the mixture was incubated at 37°C for another 1 hour. A 50 µL of resulting mixture was injected into LC-MS for quantification.

**Xenograft mouse model**

Male Nu/Nu athymic mice (4-6 weeks old) were obtained from Charles River Laboratory (Wilmington, MA). All experiments were conducted in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. MV4-11 cells were suspended with Matrigel (Becton Dickinson) and subcutaneously inoculated into mice flanks. Tumor diameters were measured and body weights were monitored.
weekly after implantation. Treatments were initiated when the average tumor size reached 100 to 150 mm$^3$. 5-azaC was injected intraperitoneally (i.p.) at the dose of 20 mg/kg, twice a week for four weeks. At the end of experiment, the tumor tissues were collected for further analysis.

**Results**

5-azaC down-regulates RRM2 expression *in vitro* and *in vivo*

We initially intended to investigate the maximal RNA effects and the associated cytotoxicity of 5-azaC by using a RRM2 antisense GTI-2040 to block its conversion to DAC. However, we discovered that RRM2 protein was completely depleted in 5-azaC-only treated (control) samples during the course of the study. We therefore hypothesized that 5-azaC itself may interfere with the synthesis of RRM2, one of the two subunits of RR, and somewhat limits its own conversion to DAC and in turn DNA incorporation. To validate this hypothesis, first we characterized the activity of 5-azaC on RRM2 expression in leukemia cell lines. Cells were exposed to 5-azaC at clinically relevant concentrations (i.e., 0.7~4.8 µM) based on previous pharmacokinetics studies$^{18}$ and a robust dose- and time-dependent decrease of RRM2 protein was observed (Figure 1A-D). In MV4-11 cells, RRM2 protein was reduced by 60% at 48 hours and completely depleted at 72 hours with 5 µM 5-azaC treatment (Figure 1A and B). RRM2 mRNA was also down-regulated by ~40% following 24 hours treatment with 5 µM 5-azaC (Figure 1C). K562 cells showed similar results, although they appeared to be less sensitive than MV4-11 cells (Figure 1A and C). At 5 µM, 5-azaC only decreased RRM2 protein by 30% and 50% after 48 or 72 hours incubation, respectively (Figure 1A).
The RRM2 downregulation was specific to 5-azaC. Its azanucleoside analogs DAC did not appreciably reduce RRM2 protein levels in MV4-11 and K562 cells (Figure 1E). In addition, we compared the potency of 5-azaC with two other established RRM2 inhibitors, triapine and GTI-2040. Consistent with the previous report\textsuperscript{19-21}, triapine dose-dependently decreased RRM2 protein level in MV4-11 but not in K562 cells (Figure 1F), whereas GTI-2040 downregulated RRM2 in both MV4-11 and K562 cells (Figure 1G). 5-azaC was more potent than triapine and GTI-2040, as shown by a leftward shift of the dose-response curve (Figure 1H). We also confirmed 5-azaC-mediated RRM2 downregulation \textit{in vivo}. MV4-11 cells were subcutaneously inoculated in athymic nu/nu mouse. Mice were intraperitoneally injected with 20 mg/kg 5-azaC twice a week, when the tumor volume reached 100–150 mm\textsuperscript{3}. This 5-azaC dose for mouse treatment is equivalent to 60 mg/m\textsuperscript{2} used in human\textsuperscript{22}. After four weeks of treatment, mice were sacrificed and RRM2 levels were tested in the engrafted tissues. We showed that 5-azaC treatment decreased RRM2 by 40\% and 70-80\% of mRNA and protein levels, respectively, compared with vehicle-treated controls (Figure 2A and B). Collectively, our data demonstrated that 5-azaC is an \textit{in vitro} and \textit{in vivo} RRM2 inhibitor.

To further demonstrate the relevance of our findings to AML, we evaluated 5-azaC effects on RRM2 expression in primary bone marrow mononuclear cells from six AML patients (Supplement Table). Cells were treated \textit{in vitro} with 5 or 10 µM 5-azaC for 24 or 48 hours. As shown in Figure 2C, 5-azaC depletes both mRNA and protein of RRM2 in samples from four patients (66\%), induces a dose- and time-dependent RRM2 decrease in one patient (17\%) and represses only RRM2 protein but not mRNA in another patient (17\%).
5-azaC perturbs the intracellular dNTPs/NTPs pool in leukemia cells

Since RRM2 catalyzes the rate-limiting step for DNA synthesis and repair by reducing ribonucleotides (NTP) to their corresponding deoxyribonucleotides (dNTP)\(^{23}\) and its level is dramatically decreased by 5-azaC, we investigated whether 5-azaC decreases the intracellular dNTPs levels. After cells exposure to 1~10 µM 5-azaC for 72 hours, the dNTPs were extracted and measured by a non-radioactive LC-MS/MS method\(^{15}\). A significant dose-dependent decrease in the intracellular levels of dTTP, dATP, dCTP and dGTP/ATP was observed. All four dNTPs levels were reduced ~70% or 60-90% by 5µM 5-azaC in MV4-11 and K562 cells, respectively (Figure 3A and B).

Conversion of 5-azaC to DAC is RRM2-mediated and self-limited

RR is believed to catalyze conversion of 5-azaC to DAC through the intermediate product 5-aza-CDP to 5-aza-dCDP, which is eventually phosphorylated to 5-aza-dCTP (DAC-TP) and incorporated into DNA\(^{4,7}\) or dephosphorylated to DAC. We therefore monitored the dynamics of conversion process by measuring the intracellular DAC and DAC-TP level after 5-azaC exposure. We found that the peak levels of DAC and DAC-TP were reached at approximately 2 hours and declined over time following 5-azaC treatment (Figure 4A and B). A second exposure to 5-azaC at 24 hours could not restore DAC/DAC-TP levels in leukemia cells, suggesting that the initial 5-azaC treatment had induced a sustained RRM2 downregulation that inhibited further conversion of 5-azaC into DAC/DAC-TP (Figure 4A and B). The role of RR in the conversion of 5-azaC into DAC/DAC-TP was validated by showing that RNAi silencing of RRM2 expression
followed by 5-azaC treatment led to a near depletion of DAC/DAC-TP levels (Figure 4A and B).

It was previously thought that the conversion of 5-azaC to DAC presumably involves multiple processes, first reduction of 5-azaCDP to DAC-DP followed by reversible dephosphorylation to DAC. However, the dephosphorylation processes have not been well defined; particularly, the enzyme that dephosphorylates the intermediate product 5-aza-dCMP to DAC has not been identified. In such a case, a relatively slow or an inefficient formation of DAC from 5-azaC would be expected. In fact, the low conversion (about 15-20%) of 5-azaC to DAC as a whole would be consistent with this scenario. We further determined the DAC/DAC-TP level in 5-azaC-treated MV4-11 cells in the presence of cytidine or uridine\textsuperscript{24,25}, the competitive inhibitors of UCK. Following this inhibition, the DAC/DAC-TP levels are significantly reduced (Figure 4D and E). These results demonstrated that UCK-RRM2-mediated pathway is a major pathway for 5-azaC conversion.

As control experiments, we incubated the heat-inactivated cell lysates with 5-azaC or DAC. As expected, there was no detectable DAC in 5-azaC-treated cell samples, since the heat inactivates the enzymes required for conversion. Additionally, DAC level was found to be stable in DAC-treated cell samples during the time course of the experiment, supporting the lack of detectable DAC was not due to its own degradation (Figure 4F).

Lack of RRM2-mediated conversion of 5-azaC to DAC limits the activity of 5-azaC on DNMTs
It has been shown that DAC/DAC-TP down-regulates DNMT1 by forming DNA-drug-enzyme complex\textsuperscript{26}. Therefore, one should expect that knockout of RRM2 expression should abolish this effect by 5-azaC, since its conversion to DAC/DAC-TP is blocked. Indeed, we observed that DNMT1 and DNMT3a proteins were not downregulated in siRRM2-treated MV4-11 cells following 5-azaC incubation (Figure 4C). To note, DNMT1 and DNMT3a protein levels remained depleted even there was no detectable (free) DAC/DAC-TP 24 hours following a second exposure of 5-azaC, indicating other mechanisms may be involved (Figure 4C).

5-azaC destabilizes RNA

To investigate the underlying mechanisms for RRM2 mRNA inhibition by 5-azaC, we first examined the effects of 5-azaC on RNA stability. We observed that 5-azaC induces a dose- and time-dependent decline in total RNA levels in both MV4-11 and K562 cell line (Figure 5A and B). However, this decrease is not due to 5-azaC cytotoxicity, since at this dose 5-azaC did not cause a significant cell death following 24 or 48 hours exposure (Supplement Figure S1). Our data thus suggest that 5-azaC destabilizes RNA and promotes its degradation. We further addressed possible mechanisms by which 5-azaC specifically down-regulates RRM2 expression. We determined the effects of 5-azaC on RRM2 mRNA stability by comparing the half-life of RRM2 mRNA ($t_{1/2}$) in the presence or absence of 5-azaC. The mRNA turnover rates were estimated by inhibiting \textit{de novo} transcription with actinomycin D and monitored the decline in mRNA level with time\textsuperscript{27}. In the absence of 5-azaC, the RRM2 mRNA turnover is rather rapid with a half-life of 4.2 hours in MV4-11 cells. Following 5-azaC treatment, RRM2 mRNA was remarkably
destabilized with a much shortened half-life of 2.5 hour (Figure 5C). The half-lives of the housekeeping controls abl and GAPDH mRNA ($t_{1/2} = 2.5$ hours for abl; $t_{1/2} = 4.2$ hours for GAPDH in control samples), however, were not significantly changed by 5-azaC ($t_{1/2} = 2.2$ hours for abl; $t_{1/2} = 4.4$ hours for GAPDH in 5-azaC-treated samples, Figure 5C). Similar results were obtained when we used another transcription inhibitor, DRB, to block mRNA synthesis$^{28}$ (Supplement Figure S2). These data suggest that the inhibition of 5-azaC on RRM2 mRNA level is due, at least partially, to its effects on mRNA stability. Furthermore, we also found protein synthesis is involved in stabilizing RRM2 mRNA, as addition of protein synthesis inhibitor cyclohexmide further decreased 5-azaC-mediated destabilization of RRM2 mRNA, although cyclohexmide itself also has similar destabilizing effect (Figure 5D). This implicates that 5-azaC and cyclohexmide exert different mechanisms to decrease RRM2 mRNA, which requires further investigation.

5-azaC incorporates into RNA

The RNA destabilization effect of 5-azaC may in part due to RNA incorporation. Previous studies have suggested that 5-azaC is incorporated into RNA$^{7,29,30}$, a potential mechanism contributing to 5-azaC’s RNA destabilizing effects. However, the provided evidence was rather indirect by measuring the incorporation of radio-labeled 5-azaC$^{7,31}$. To ascertain this possibility directly, we employed a chemical method coupled to LC-MS/MS assay. Since 5-azaC is chemically labile$^{32-34}$, it is not expected to be detectable in the total RNA acidic hydrolysate. However, 5-azaC can be readily converted to stable dihydro-5-azaC$^{35}$ and dideuterio-5-azaC with NaBH$_4$ and NaBD$_4$, respectively, in aqueous solution under mild condition and these products can be monitored by LC-
MS/MS as a means to probe 5-azaC RNA incorporation. Therefore, we first examined the LC-MS characteristics of standard dihydro-5-azaC, HD-5-azaC and dideuterio-5-azaC in the mobile phase. The total RNA isolated from the control or 5-azaC-treated MV-411 cells was treated with NaBH₄ followed by acid hydrolysis. No peak with significant intensity was observed at the retention time (~4.5 minute) and the ion transitions for 5-azaC (data not shown) or dihydro-5-azaC in the control RNA samples (Figure 6A). However, a peak with significant intensity was observed at 4.56 minute corresponding to dihydro-5-azaC with the correct ion transition in the treated samples, indicating the presence of 5-azaC in the total RNA (Figure 6B). In order to further substantiate these data, we also used NaBD₄ to reduce the blank and 5-azaC-treated total RNA. Since each of the deuterium atom increases the mass by one relative to the hydrogen atom, we would expect that the observed new peaks, if they relate to 5-azaC, would have a proportional increase in the mass unit corresponding to dihydro-5-azaC, HD-5-azaC and dideuterio-5-azaC. Following NaBD₄ treatment, similar to the NaBH₄ experiment, no significant peak was observed at 4.7 minute for any of the reduced products in the control (Figure 6C). However, a significant peak was observed at 4.7 minute with ion transitions corresponding to dihydro-5-azaC, HD-5-azaC and dideuterio-5-azaC (Figure 6D). These data indicated the presence of 5-azaC in the total RNA of the 5-azaC-treated samples and confirmed the expected 5-azaC incorporation into total RNA in a human AML cell line.

Discussion

5-azaC and DAC have long been considered as similar antitumor agents promoting DNA synthesis inhibition and hypomethylation, even they differed chemically in the ribose
moiety. Clinically, they are mainly used for treatment of hematopoietic malignancies and both are recently approved by the US FDA for the treatment of MDS. It has long been recognized that DAC possesses primarily modulating effect on DNA, whereas 5-azaC has effects on both DNA and RNA pathways, and it is believed that 5-azaC’s DNA effect was due to its conversion to DAC. More importantly, only 20% of 5-azaC is converted to DAC and the major portion of 5-azaC (~80%) acts on the RNA pathway. Despite of this, little attention has been focused on the RNA mechanism recently and this forms the basis of the present investigation. We used RRM2 antisense to inhibit 5-azaC’s conversion and its following DNA pathway, and studied its RNA effects. Unexpectedly, we discovered that RRM2 as a novel target for 5-azaC itself.

RR is a highly regulated enzyme comprising two non-identical dimeric subunits RRM1 and RRM2. RRM1/RRM2 complex serves as a major provider of dNTPs for DNA replication during S phase. Overexpression of RR is commonly found in malignant cells and increases the endogenous pool of dNTP, therefore increasing DNA synthesis rate to serve the proliferative nature of malignant cells. RR overexpression is also a potential mechanism for chemoresistance to nucleoside analogs competing for DNA incorporation. These important roles have made RRM2 an attractive target for chemotherapeutic development. Studies have demonstrated that RRM2 inhibition provides antiproliferative and antineoplastic benefits. However, several established RR inhibitors are associated with considerable limitations. For example, hydroxyurea, one of the most commonly used RRM2 inhibitors, is limited by its low affinity for RR, high hydrophilicity, short half-life and early development of resistance. Newer classes of RRM2 inhibitors, such as antisense GTI-2040 (LOR-2040) and ion chelators...
(e.g. triapine) are under development and their effectiveness is still being evaluated. We demonstrated here that 5-azaC-induced a dose- and time-dependent decrease of RRM2 protein in leukemia cells and this effect was specific to 5-azaC, but not to its reduced analog, DAC. We further compared 5-azaC with other established RRM2 inhibitors, antisense GTI-2040 and triapine, in down-regulating RRM2. 5-azaC is more potent than triapine, as indicated by a leftward shift of the dose-response curve. In addition, the \textit{in vitro} findings were confirmed in mice engrafted tumor tissues and AML patients bone marrow cells. However, since these are \textit{ex vivo} studies and the number of patients’ samples (n=6) was relatively small, it was not possible for us to correlate response to 5-azaC treatment with patients’ WHO subtype, clinical, cytogenetic or molecular characteristics (Supplement Table). With regard to the mechanisms for 5-azaC down-regulation of RRM2 mRNA level, we found that 5-azaC attenuates total RNA and RRM2 mRNA stability. The decrease in RNA stability is likely due to the incorporation of 5-azaC as confirmed by our LC-MS/MS data. We also observed a discrepancy between the decrease in RRM2 mRNA levels and protein levels, suggesting that 5-azaC may have additional transcription-independent effects on RRM2 protein, the nature of which warrants further studies.

Since formation of dNTPs from the corresponding NTPs is controlled by RR\textsuperscript{43}, enzyme activity of RR directly relates to the production of dNTPs in cells\textsuperscript{44}. A substantial amount of dNTPs is required by cells in cell cycle S phase and closely linked to the cellular growth control mechanisms. Thus, altered dNTP pools by 5-azaC-mediated RRM2 inhibition is likely to cause perturbation in DNA synthesis and replication processes thereby resulting in chromosomal instability\textsuperscript{36}. Our data indeed showed that following 5-
azaC treatment, the dTTP, dATP and dCTP pools were reduced in a dose-dependent manner.

In addition to its major effects on DNA synthesis, RRM2 is also involved in the intracellular metabolism of 5-azaC. We have shown that RRM2 expression knockout dramatically decreases the intracellular DAC and DAC-TP level and abolishes 5-azaC-mediated down-regulation of DNMTs, strongly supporting that the hypomethylation effect of 5-azaC is through its conversion to DAC. However, because 5-azaC induces strong inhibition of RRM2 expression, it is likely that 5-azaC inhibits its own further conversion to DAC over time. Indeed, we showed that following the initial conversion, both DAC and DAC-TP levels diminished to non-detectable level in about 4 hours. As there was no detectable DAC or DAC-TP even following a second dose of 5-azaC at 24 hours, we concluded that RRM2 was completely blocked by the initial 5-azaC dosing. However, the downstream DNMT1 and DNMT3a protein expression remained downregulated, suggesting a rather stable covalent complex of DNA/DNMT/DAC-TP formed following the initial 5-azaC exposure. We further extended the time course of 5-azaC treatment to evaluate its effects on DNMT protein expression. Our data suggested that 5-azaC has similar apparent DNMT-downregulating effects as DAC. Although the conversion of 5-azaC to DAC is self-limiting, the 5-azaC-derived DAC could elicit a similar DNMT downregulation as DAC that persisted over time for at least 72 hours (Supplement Figure S3). It also should be noted that the kinetics of RRM2 downregulation and DNA methylation caused by 5-azaC are likely different. Our results suggested that downregulation of RRM2 by 5-azaC is dose- and time-dependent and may require higher drug concentration and prolonged exposure than that required for DNMTs.
We also found that RRM2 participates in a direct conversion of 5-azaC to DAC, although it only accounts for a small fraction of conversion (Figure 4D). Previously, 5-azaC was believed to be converted to DAC only through the diphosphate stage and presumably by subsequent dephosphorylations. The molecular mechanism of reduction of nucleotides has been proposed\textsuperscript{45-47}, in which a binding of the nucleotide to the reductase active site is required prior to the reduction of the 2-hydroxy on the ribose moiety. The 5'-phosphate group(s) helps to anchor the nucleotide to the binding pocket. Our data indicated that the nucleoside itself (5-azaC) could also be reduced directly, which implies that 5-azaC itself could anchor the binding site sufficiently prior to the reduction.

Collectively, we found that 5-azaC is a potent and specific RRM2 inhibitor, which generates a major advancement in the pharmacology of 5-azaC and will enable efficient translation of preclinical studies to clinical effectiveness. For example, our discovery expands the therapeutic use of 5-azaC to combination regiments containing cytarabine (Ara-C) or other nucleoside analogs by increasing the Ara-CTP/dNTP ratio in favor of Ara-CTP, ultimately resulting in increased DNA incorporation of Ara-CTP and enhanced antileukemia activity. Our proposed combination study is being evaluated in a clinical trial at our institution. Furthermore, direct comparisons between 5-azaC and DAC activities are also presented and our data supports the distinction between these two compounds. 5-azaC can be converted to DAC in cells; and the process is rapid but transient and self-limited. Altogether these data may have relevance for the design and dosing of 5-azaC in regimen pursuing gene hypomethylation and RRM2 inhibition in MDS or AML.
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Authorship

Contribution: J. A. and H. W. designed the study, contributed experimental data and prepared the manuscript; P.C. developed the LC-MS/MS methods, contributed to experimental data and manuscript; Z. X. and J. W. contributed experimental data; S. L. provided technical support and helpful discussion; R.K., A.M., and W.B. provided patient samples and information; G.M. designed the study and prepared the manuscript; K.C. conceived and designed the study, prepared the manuscript and provided administrative and financial support.

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References

Figure Legends

**Figure 1. 5-azaC down-regulates RRM2 expression in vitro.** (A) RRM2 protein level was reduced by 5-azaC in a dose- and time-dependent manner in MV4-11 and K562 cells. Densitometry was performed to quantify each lane and the ratio of RRM2 over the loading control GAPDH is presented under each blot. (B) Representative densitometry plots. (C) 5-azaC down-regulates RRM2 mRNA in a dose-dependent manner. Cells were treated with serial concentrations of 5-azaC for 24 hours and collected for analysis. (D) 5-azaC down-regulates RRM2 mRNA in a time-dependent manner. MV4-11 cells were treated with 5µM 5-azaC for the indicated time and the RRM2 mRNA level was quantified. The RRM2 mRNA levels were normalized by an internal control, abl. Data are presented as the percentage of the untreated control. *p < 0.05, **p < 0.01, as compared to the control. (E) Decitabine (DAC) has minimal effects on RRM2 protein expressions. (F,G) Effects of two RRM2 inhibitors, triapine (F) and GTI-2040 (G), on RRM2 protein level. (H) Comparative plot of RRM2 protein levels in MV4-11 cells following 5-azaC, triapine or GTI-2040 treatments.

**Figure 2. 5-azaC down-regulates RRM2 expression ex vivo and in vivo.** (A, B) 5-azaC inhibits RRM2 protein (A) and mRNA (B) expression in MV4-11 tumor xenografts in mice. Athymic nu/nu mice subcutaneously inoculated with MV4-11 cells were treated with 5-azaC and the engrafted tumor tissues were subjected for analysis. (C) 5-azaC reduces RRM2 level in primary bone marrow cells from AML patients. Primary cells from six patients were cultured in vitro and treated with the indicated concentrations of 5-azaC for 24 or 48 hours. Cells were then collected for western blot and qRT-PCR
analyses. For western blot analysis, densitometry measurement was performed to quantify each lane and the ratio of RRM2 over the loading control GAPDH is presented under each blot. For qRT-PCR analysis, the data are presented as the percentage of the untreated controls. The results are mean ± SD of the representative experiment performed in triplicate. *p < 0.05, **p < 0.01 versus untreated control.

Figure 3. 5-azaC decreases intracellular dNTP pools. MV4-11 or K562 cells were treated with the indicated concentrations of 5-azaC for 72 hours and subjected for LC-MS/MS analysis for dNTP levels. 5-azaC dose-dependently reduces dTTP, dATP, dCTP and dGTP/ATP levels in MV4-11 (A) and K562 cells (B). Data are presented as the percentage of untreated control. The results are mean ± SD of a representative experiment performed in triplicate. * p < 0.05, **p < 0.01 versus untreated control.

Figure 4. RRM2 is involved in the conversion of 5-azaC to DAC in MV4-11 cells. (A-C) 5-azaC rapidly converts to DAC and DAC-TP which diminishes with time. Knockdown of RRM2 expression prevents 5-azaC conversion to DAC (A) and DAC-TP (B) and associated events (C). MV4-11 cells were transfected with siRRM2 or mock for 24 hours, followed by treatment with 10 µM 5-azaC for 2~28 hours. The arrows indicate sequences of fresh addition of 5-azaC. The intracellular DAC and DAC-TP were extracted and quantified by LC-MS/MS. Cells treated with 10 µM DAC for 2 hours serve as a positive control. Data are presented as the percentage (%) of the positive control. Knockdown of RRM2 expression inhibits 5-azaC-induced down-regulation of DNMT1 and DNMT3a (C). Western blot analysis shows that 5-azaC alone time-dependently
decreases the protein levels of DNMT1 and DNMT3a, whereas RRM2 depletion by siRRM2 abolishes its effects. Experiments were repeated and the representative results are presented. (D, E) Conversion of 5-azaC to DAC is reduced by UCK inhibitors. MV4-11 cells were treated with 10 µM 5-azaC for 2 or 4 hours. UCK was competitively inhibited by its natural substrates, cytidine or uridine. The intracellular DAC (D) and DAC-TP (E) levels were measured. Cells treated with 10 µM DAC for 2 or 4 hours serve as positive controls. (F) Stability of DAC. MV4-11 cells in PBS were heat-inactivated at 100°C for 6 minutes and then exposed to 10µM 5-azaC or DAC for 2 or 4 hours at 37°C.

**Figure 5. 5-azaC reduces stability of total RNA and RRM2 mRNA.** (A, B) 5-azaC causes a dose- and time-dependent decline in total RNA level in MV4-11 (A) and K562 cells (B). (C) 5-azaC shortens the half-life (t1/2) of RRM2 but not abl or GAPDH mRNA. MV4-11 cells were treated with 1µM actinomycin D to block the de novo transcription in the presence or absence of 5-azaC. mRNA levels of RRM2 and two internal controls, abl and GAPDH, were measured by qRT-PCR at the indicated time points after actinomycin D treatment. The data are presented as the percentage of the mRNA level measured at time 0 (without actinomycin D). Their mRNA half-lives were calculated and indicated in the plots. (D) Blockade of protein synthesis facilitates 5-azaC-induced destabilization of RRM2 mRNA. MV4-11 cells were pretreated with cycloheximide to block protein synthesis followed by treatment with 5-azaC and actinomycin D. Normalized mRNA levels are presented as the percentage of actinomycin D-only and shown as mean ± SD from triplicate experiments. * p < 0.05, as compared to actinomycin D-only; # p < 0.05, as compared to the treatment with both 5-azaC and actinomycin D.
**Figure 6. 5-azaC incorporates into RNA.** The extracted ion chromatograms (XICs) for: (A) RNA hydrolysate from blank cell sample treated with NaBH$_4$ (scale is enhanced). Arrows show approximate location of the ion peaks. (B) RNA hydrolysate from 5-azaC-treated cell sample followed by treatment with NaBH$_4$. (C) RNA hydrolysate from blank cell sample treated with NaBD$_4$ (scale is enhanced). Arrows show approximate location of the ion peaks. (D) RNA hydrolysate from 5-azaC-treated cell sample followed by treatment with NaBD$_4$. To simplify the figure, 5-azaC channel is not shown.

**Figure 7. Proposed mechanism of actions for 5-azaC in AML.** 5-azaC is majorly phosphorylated to 5-aza-CTP and incorporated into RNA. A fraction of 5-azaC intermediate product 5-aza-CDP is converted to 5-aza-dCDP by RR, followed by further phosphorylation to 5-azaC-dCTP, which is believed to incorporate into DNA. Our study identifies RRM2 as a novel target of 5-azaC. The underlying molecular mechanisms for 5-azaC’s inhibitory effects on RRM2 involve its RNA incorporation and attenuated RRM2 mRNA stability.
Figure 3

A

MV4-11

5-azaC (μM)  0  1  5  0  1  5  0  1  5

dTTP  dATP  dCTP  dGTP/ATP

B

K562

5-azaC (μM)  0  5  10  0  5  10  0  5  10

dTTP  dATP  dCTP  dGTP/ATP
RNA-dependent inhibition of ribonucleotide reductase is a major pathway for 5-azacytidine in acute myeloid leukemia

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