8-Amino-Adenosine Inhibits Multiple Mechanisms of Transcription

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Abstract

Roscovitine and flavopiridol suppress cyclin-dependent kinase 7 (CDK7) and CDK9 activity resulting in transcription inhibition, thus providing an alternative mechanism to traditional genotoxic chemotherapy. These agents have been effective in slow or nonreplicative cell types. 8-Amino-adenosine is a transcription inhibitor that has proved very effective in multiple myeloma cell lines and primary indolent leukemia cells. The objective of the current work was to define mechanisms of action that lead to transcription inhibition by 8-amino-adenosine. 8-Amino-adenosine is metabolized into the active triphosphate (8-amino-ATP) in cells. This accumulation resulted in a simultaneous decrease of intracellular ATP and RNA synthesis. When the effects of established ATP synthesis inhibitors and transcription inhibitors on intracellular ATP concentrations and RNA synthesis were studied, there was a strong correlation between ATP decline and RNA synthesis. This correlation substantiated the hypothesis that the loss of ATP in 8-amino-adenosine–treated cells contributes to the decrease in transcription due to the lack of substrate needed for mRNA body and polyadenylation tail synthesis. RNA polymerase II COOH terminal domain phosphorylation declined sharply in 8-amino-adenosine–treated cells, which may have been due to the lack of an ATP phosphate donor or competitive inhibition with 8-amino-ATP at CDK7 and CDK9. Furthermore, 8-amino-ATP was incorporated into nascent RNA in a dose-dependent manner at the 3′-end resulting in transcription termination. Finally, in vitro transcription assays showed that 8-amino-ATP competes with ATP for incorporation into mRNA. Collectively, we have concluded that 8-amino-adenosine elicits effects on multiple mechanisms of transcription, providing a new class of transcription inhibitors. Mol Cancer Ther; 9(1); 236–45. ©2010 AACR.

Introduction

Cyclin-dependent kinase (CDK) inhibitors are gaining success in the clinic as anticancer agents especially in hematologic malignancies (1). Although originally developed as cell cycle inhibitors, several of these agents were recognized as transcription inhibitors because they inhibit the activity of CDK7 and CDK9. These kinases phosphorylate serine residues at the COOH terminal domain (CTD) of RNA polymerase II, which is the enzyme responsible for synthesis of mRNA (2). Roscovitine was among the first to be tested in preclinical and clinical testings (3, 4). Flavopiridol is probably the most used transcription inhibitor, and proof-of-concept studies have shown action against CDK7 and CDK9, as well as inhibition of transcript synthesis with an immediate effect on short-lived transcripts and proteins, such as Mcl-1, cyclin D1, and cMet (5–7). Clinically, flavopiridol has been extensively used for treatment of chronic lymphocytic leukemia (8). The new agent SNS-032 (formerly known as BMS-387032) also belongs to the same category of CDK inhibitors with more potent inhibition of CDK2, CDK7, and CDK9 (9). In general, CDK inhibitors that suppress transcription provide a novel strategy for drug development.

The structure of roscovitine and other analogues clearly showed that purine and adenine provide a scaffold, which were exploited to create additional drugs and analogues (3). Our previous work determined that adenine nucleoside analogues or adenosine analogues were potent inhibitors of transcription (10–12). The two members of the carbon-8–substituted adenosine analogues that have been tested for cytotoxicity are 8-chloro-adenosine and 8-amino-adenosine. Unlike CDK inhibitors, carbon-8–substituted analogues provide different mechanisms of transcription inhibition (11). However, similar to CDK inhibitors, these analogues are also effective in slow or nonreplicating cells, such as those from multiple myeloma or indolent leukemias such as chronic lymphocytic leukemia (10, 12–14). Data from replicationally quiescent primary chronic lymphocytic leukemia cells with either CDK inhibitors or carbon-8–substituted analogues established that DNA replication was not the target of these compounds (13, 14). Furthermore, the cytotoxic mechanisms were similar with these agents in that there was an inhibition of transcription, providing a new class of transcription inhibitors. Mol Cancer Ther; 9(1); 236–45. ©2010 AACR.
transcription and a decline in short-lived transcripts and proteins, such as Mcl-1, XIAP, and cMet (5–7, 13, 14). Tumors that depend on these survival proteins succumb when protein quantities decrease below critical levels.

Whereas 8-chloro-adenosine was the first to be tested in preclinical setting and is currently in a Phase I clinical study for patients with chronic lymphocytic leukemia, the congener of 8-chloro-adenosine, 8-amino-adenosine, is more potent as shown by induction of poly(ADP-ribose) polymerase cleavage by 6 hours with 8-amino-adenosine compared with 24 hours by chlorinated adenosine. The intracellular accumulation of 8-amino-ATP is 38-fold higher than 8-chloro-ATP in myeloma cells (10, 12) and leads to a greater analogue-mediated decline in ATP pool and mRNA synthesis (6, 10, 12, 13, 15, 16).

Whereas metabolic aspects, mechanisms of action, and cytotoxic effects of 8-amino-adenosine have been investigated, information regarding how 8-amino-adenosine inhibits transcription is not available. Previous observations of 8-amino-adenosine led to the present study focused on the multiple mechanisms of transcription inhibition by 8-amino-adenosine. The results indicate that there was first a rapid decline in the intracellular ATP pool. Second, phosphorylation at the CTD of RNA polymerase II was decreased. Third, 8-amino-ATP was incorporated at the 3′-terminus in the mRNA, leading to transcription termination. Collectively, these multifaceted actions indicate that 8-amino-adenosine is a member of a new class of transcription inhibitors.

Materials and Methods

Cell Line

MM.1S cells were obtained from Drs. Steven Rosen and Nancy Krett (Robert H. Lurie Comprehensive Cancer Center, Northwestern University) and grown as a suspension culture in RPMI 1640 with L-glutamine and 10% fetal bovine serum in the presence of 5% CO2 at 37°C. This multiple myeloma cell line was derived from the peripheral blood cells of an IgA myeloma patient (17, 18). The cell line is sensitive to glucocorticoids, and a detailed characterization has been published (18). The current authors have not independently tested and authenticated these cells. Routine testing for Mycoplasma infection was done using the MycoTest kit (Invitrogen).

Materials

The drug 8-amino-adenosine and its phosphorylated metabolite 8-amino-ATP were obtained from R.I. Chemical and ChemCyte, respectively. [2-3H]8-amino-adenosine (3.8 Ci/mmol) and [5,6-3H]uridine (41.2 Ci/mmol) were purchased from Moravek Biochemicals. [α-32P]UTP (3000 Ci/mmol) was purchased from Perkin-Elmer. Flavopiridol (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment) and deoxycyformycin (Dr. V.N. Narayanan) were obtained from the National Cancer Institute.

Actinomycin D, α-amanitin, antimycin A, 2-deoxy-D-glucose, and 5,6-dichloro-l-β-D-ribofuranosylbenzimidazole (DRB) were purchased from Sigma-Aldrich.

RNA Synthesis

Exponentially growing MM.1S cells were treated with various concentrations of 8-amino-adenosine or other inhibitors. Thirty minutes before the end of the incubation time, 2 μCi [5, 6-3H]uridine was added to the cells. Samples were collected and washed before applying to glass fiber filters (Whatman) on a Millipore vacuum manifold and isolating acid-insoluble material by perchloric acid extraction. Radioactivity on the filters was quantified by liquid scintillation counting (Packard Bioscience, Perkin-Elmer Life and Analytical Sciences, Inc.). Data were expressed as percentage of untreated (control) cells.

Measurement of Intracellular Nucleotides

Ten milliliters of MM.1S cells were plated in suspension at 3 × 10⁶ cells/mL and treated with 8-amino-adenosine or other inhibitors as indicated. Cells were collected, and nucleotides were extracted using perchloric acid followed by potassium hydroxide neutralization as previously described (19, 20). To determine ATP and 8-amino-ATP concentrations, nucleotide extracts were applied to an anion exchange Partisil-10 SAX column at a flow rate of 1.5 mL/min using a Waters 2697 Separations Module (Waters Corp.). The nucleotides were separated with a 60-min concave gradient and quantitated as previously described (12).

Immunoblot Analysis

MM.1S cells were grown to a concentration of 3 × 10⁶/mL and treated with 8-amino-adenosine, DRB, or flavopiridol, as indicated. Cells were harvested and lysed in fresh working radioimmunoprecipitation assay buffer with sodium orthovanadate and one Complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche Applied Science). Protein concentration was determined by DC Protein Assay (Bio-Rad Laboratories). Colorimetric detection and quantitation were done using the PowerWave XS Microplate Spectrometer and KC4 Data Analysis software (BioTek Instruments, Inc.). Protein separated on a 4% to 12% Criterion XT gel in XT MOPS buffer (Bio-Rad Laboratories) and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore) in transfer buffer containing 20% methanol, 25 mmol/L tris base, and 190 mmol/L glycine. Membranes were blocked with blocking buffer (LI-COR Biosciences) followed by incubations with a primary antibody and then secondary antibody. The membranes were washed before visualizing and quantifying proteins using the Odyssey Infrared Imaging System and associated software (LI-COR Biosciences). Antibodies were purchased from various sources: β-actin (AC-15) from Sigma Aldrich; CDK7 (c-4) and CDK9 (c-20) from Santa Cruz Biotechnology; RNA polymerase II 8WG16, H5, and H14 from Covance; Alexa-Fluor 680...
goat anti-mouse IgG, Alexa-Fluor 680 goat anti-mouse IgM, and Alexa-Fluor 800 goat anti-rabbit IgG from Molecular Probes.


Exponentially growing MM.1S cells were treated for 1 h with various concentrations of [2-$^3$H]8-amino-adenosine. Samples were collected, extracted, and quantified as described for the RNA synthesis method (11).

Site of Nucleoside Incorporation Assay

MM.1S cells were treated with [2-$^3$H]8-amino-adenosine or [5,6-$^3$H]uridine for 2 h. RNA was isolated with Trizol LS (Invitrogen) and resuspended in diethylpyrocarbonate-treated water. RNA (20,000 dpm) was digested into internal nucleotides and 3' terminal nucleosides as previously described (11, 21). Nucleosides and nucleotides were extracted by perchloric acid and neutralized with potassium hydroxide. High-performance liquid chromatography analysis was done using a XTerra MS C18 Column (3 mm x 100 mm 3.5 μm) at a flow rate of 0.3 mL/min and monitored at an absorption of 265 nm. [5,6-$^3$H]uridine–treated samples were eluted in 100% of 20 mmol/L triethylammonium acetate buffer for 15 min. The retention times were 5.5 min for UMP and 7.5 min for uridine. 8-Amino-adenosine–treated samples were eluted using 95% buffer A (98% 80 mmol/L ammonium acetate, 0.04% acetic acid) and 5% buffer B (8% 80 mmol/L ammonium acetate, 0.04% acetic acid, 35% acetonitrile) for 30 min. The retention times were as follows: AMP, 5.2 min; adenine, 6.9 min; adenosine, 19.5 min; and 8-amino-adenosine, 23.5 min. An 8-amino-AMP standard has not been obtained; however, based on the known retention time of 8-chloro-AMP, 8-amino-AMP is predicted to elute at the same retention time as AMP. Radioactive standards were detected by an online scintillation counting. Sample fractions were collected every 20 s and quantitated by liquid scintillation counting.

In vitro Transcription Assay

α-Fetoprotein 7 plasmid (α-fetoprotein promoter driving β-galactosidase gene) and HeLa nuclear extracts were provided by Drs. Michelle Barton and Meghan Minard at the University of Texas M.D. Anderson Cancer Center (22, 23). The plasmid was digested with AgeI and calf alkaline phosphatase to limit transcription to 250 nucleotides. In vitro transcription was done in a 50-μL

Figure 1. Actions of 8-amino-adenosine on RNA synthesis (A) and intracellular ATP pool (B). A, cells were incubated with 1 μmol/L (⧫), 10 μmol/L (●), and 30 μmol/L (▲) 8-amino-adenosine, and RNA synthesis was measured using uridine incorporation assay. B, cells were incubated with 10 μmol/L 8-amino-adenosine for the indicated times, and the ATP (▪) and 8-amino-ATP (□) intracellular concentrations were measured by high-performance liquid chromatography. C, relationship between ATP decline and inhibition of RNA synthesis. Data from A and B were plotted. D, relationship between 8-amino-ATP accumulation and inhibition of RNA synthesis. Data from A and B were plotted.
reaction containing 3 mg digested DNA (5 μL), 95 μg HeLa nuclear extract in nuclear dialysis buffer (15 μL), 10 μL nuclear dialysis buffer, 71 mmol/L potassium chloride, 20 mmol/L HEPES (pH 7.9), 5 mmol/L magnesium chloride, 5 mmol/L creatine phosphate, 0.6 mmol/L ATP, 0.6 mmol/L GTP, 0.6 mmol/L CTP, 2 mmol/L DTT, 0.2 mg/mL bovine serum albumin, 10 units/mL creatine kinase, 50 μmol/L UTP, and 10 μCi α-[32P]UTP for 1 h at 30°C followed by an incubation with 10 μg TrNA and 2.5 units RNase-free DNase (Promega) and another incubation with 10 μg protease K and 2.5 μL 5% SDS/0.125 mol/L EDTA. The product was purified and resuspended in denaturing loading dye. The samples were separated in a prerun 8 mol/L urea/8% polyacrylamide gel at 600 V in 1× tris-borate-EDTA buffer. The gel was exposed to Amersham Hyperfilm MP (GE Healthcare Life Sciences, Piscataway, NJ) overnight and developed. The film was scanned and quantitations were determined using the Odyssey Infrared Imaging System software.

**Statistical Analysis**

Relationships between different parameters were obtained using GraphPad Prism Software. Student’s t test was used for analyses.

**Results**

**8-Amino-Adenosine Affects RNA Synthesis and Intracellular ATP**

To determine the actions of 8-amino-adenosine on global RNA synthesis, both time- and dose-dependent experiments were done (Fig. 1A). Whereas 1 μmol/L 8-amino-adenosine treatment resulted in ~50% inhibition of RNA synthesis after 12 hours, cells treated with 10 and 30 μmol/L 8-amino-adenosine had a 50% RNA synthesis inhibition by 2 and 4 h, respectively. RNA synthesis inhibition was in parallel to the decline in the intracellular ATP pool over time, as well as the accumulation of the metabolite 8-amino-ATP in cells (Fig. 1B). Reduction in the endogenous ATP pool was time dependent and by 6 hours of 10 μmol/L 8-amino-adenosine treatment <20% of the initial intracellular ATP level remained. Accumulation of 8-amino-ATP was efficient; at 2 and 6 hours, >3 and 6 mmol/L 8-amino-ATP were formed. Additional analyses (Fig. 1C and D) showed that there were highly significant and linear correlations between RNA synthesis inhibition and the decline in ATP pool and the accumulation of 8-amino-ATP in treated cells.

**Decline in ATP Pool Leads to RNA Synthesis Inhibition**

Because a relationship was established between the loss of RNA synthesis and the decline in the ATP pool (Fig. 1C), previously known ATP synthesis inhibitors (2-deoxy-D-glucose and antimycin A) and a RNA synthesis inhibitor (actinomycin D) were used to determine the effect of each on the ATP pool and RNA synthesis (Fig. 2A and B). 2-Deoxy-D-glucose inhibits ATP production via glycolysis in cells and cells treated with 5 mmol/L 2-deoxy-D-glucose exhibited a 45% decline in the ATP pool and 55% decrease in RNA synthesis within 4 hours. ATP generated by the electron transport pathway is inhibited at complex III by antimycin A. ATP pool and RNA synthesis were decreased by 22% and 40%, respectively, in cells treated with 2 μmol/L antimycin A. Combined treatment with 2-deoxy-D-glucose and antimycin A resulted in a 69% decrease in intracellular ATP and 90% inhibition of RNA synthesis. Actinomycin D treatment contributed to a 17% decrease in the ATP pool and 76% inhibition of RNA synthesis. 8-Amino-adenosine at 10 μmol/L had the greatest effect on the ATP pool.
and RNA synthesis, which were inhibited by 92% and 94%, respectively, at 4 hours under these conditions. To determine a relationship between ATP pool decline and RNA synthesis inhibition, data obtained with bonafide ATP inhibitors (Fig. 2A and B) were plotted with the associated decrease in RNA synthesis. These analyses (Fig. 2C) showed a statistically significant \( P < 0.0001 \) strong linear relationship \( (r^2 = 0.7065) \) between the decline of the ATP pool by ATP inhibitors and a decrease in RNA synthesis indicating that the effect of 8-amino-adenosine on the decline of the ATP pool is one mechanism of 8-amino-adenosine-mediated transcription inhibition.

**8-Amino-Adenosine Inhibits RNA Polymerase II CTD Phosphorylation**

To understand the RNA transcription inhibition mechanism by 8-amino-adenosine in MM.1S cells, immunoblots were used to evaluate total and phosphorylated RNA polymerase II. The RNA polymerase II CTD is phosphorylated at Serine-5 and Serine-2 by CDK7 and CDK9, respectively, and this is necessary for transcription initiation and elongation. Phosphorylation of the RNA polymerase II CTD at Serine-5 and Serine-2 was decreased with 8-amino-adenosine treatment, indicating that transcription initiation and elongation activity could not proceed (Fig. 3A and B). DRB and flavopiridol were used as positive controls for decreased RNA polymerase II phosphorylation. DRB is a pharmacologic inhibitor of CDK9 and thus leads to a decrease Serine-2 phosphorylation (24, 25). Flavopiridol was developed as a general CDK inhibitor and has been shown to be a potent inhibitor of CDK9 (2, 5). To rule out the possibility of a decrease in kinase levels, the total protein expression of CDK7 and CDK9 was also examined and determined to be unaffected in 8-amino-adenosine-treated cells (Fig. 3A). Thus, decline in CTD phosphorylation may be attributed to either the lack of available ATP for these kinases or 8-amino-ATP may competitively inhibit the activity of CDK7 and CDK9. There was a linear relationship between the decline in RNA synthesis and the decrease in CTD phosphorylation at Serine-5 and Serine-2 (Fig. 3C and D). These data indicate that the decline in CTD phosphorylation on 8-amino-adenosine treatment is an additional mechanism of transcription inhibition.

**8-Amino-Adenosine Is Incorporated into Nascent RNA Transcripts**

High accumulation of 8-amino-ATP in cells and its similarity to ATP suggested possible incorporation into RNA synthesis.
RNA transcripts, which may then prevent further elongation of the transcript by RNA polymerase II (11). Therefore, it was important to determine if 8-amino-adenosine incorporates into RNA. As shown in Fig. 4A, titrated 8-amino-adenosine incorporates into RNA in a dose-dependent manner. Cells were incubated with 1 and 10 μmol/L 8-amino-adenosine for 2 h. RNA was isolated and digested to determine incorporation position of uridine or 8-amino-adenosine molecules as described in Materials and Methods.

RNA transcripts, which may then prevent further elongation of the transcript by RNA polymerase II (11). Therefore, it was important to determine if 8-amino-adenosine incorporates into RNA. As shown in Fig. 4A, titrated 8-amino-adenosine incorporates into RNA in a dose-dependent manner. Additionally, >90% of the nucleoside analogue was incorporated into RNA at a 3′-terminal position, suggesting that mRNA synthesis stops on addition of 8-amino-adenosine into transcript by RNA polymerase II (Fig. 4B). Taken together, these data show that the metabolized 8-amino-adenosine is incorporated into nascent RNA transcripts resulting in transcription termination.

**In vitro Transcription Competition of ATP and 8-Amino-ATP**

To further show incorporation of 8-amino-ATP into mRNA, inhibition of further transcript elongation, and competition between 8-amino-ATP and ATP for incorporation, an *in vitro* transcription assay was used. As illustrated in Fig. 5A, the α-fetoprotein 7 DNA plasmid construct was cleaved so that only 250 nucleotides from the Lac Z gene could be transcribed on activation of the α-fetoprotein promoter. The *in vitro* transcription reaction was a cocktail of the DNA template, HeLa nuclear extract, and nucleotides. To specifically label only new RNA transcripts, α-[32P]UTP was included in the reaction mixture. The mRNA products were purified, separated by gel electrophoresis, and then exposed to film to image the results (Fig. 5B). For each experiment, the transcription inhibitor α-amanitin was added to one reaction as a negative control. The full-length 250 nucleotide RNA product was detected and quantitated. When the data from three experiments were analyzed, there seemed to be a trend to decrease mRNA product when 8-amino-ATP was used at 0.6, 1.2, and 2.4 mmol/L levels in the presence of 0.6 mmol/L ATP (Fig. 5C). Increases in ATP concentration in the transcription mixture augmented product formation. However, competition with 8-amino-ATP at a 1:1 ratio affected product generation. Again, there was a decline when 8-amino-ATP was added to the transcription mixture (Fig. 5D).

**Discussion**

The focus of this study was to identify the multiple mechanism of transcription inhibition by 8-amino-adenosine.

**Decline in ATP Pool and Effect on Transcription**

Data presented in the present work have shown that lowering intracellular ATP by previously established ATP synthesis inhibitors and 8-amino-adenosine is strongly related to a decline in RNA synthesis (Figs. 1A and 2B). 2-Deoxy-glucose inhibits ATP synthesis by competing with hexokinase in the glycolysis pathway (26). Antimycin A acts by inhibiting the complex II of the pentose phosphate pathway (27). Because ATP is the primary energy source in cells and is used as a phosphate donor for numerous enzymes, the decline in ATP likely results in transcription inhibition. More important than its role in the energetics of the cell, ATP is a substrate for mRNA synthesis including the body of the transcript and the required poly(A) tail. All adenosine-derived nucleoside analogues do not inhibit RNA synthesis and intracellular ATP pool. Cordycepin (3′-deoxyadenosine) can inhibit RNA synthesis; however, there is no effect on the intracellular ATP pool as the metabolite 3′-dATP accumulates in these MM.1S cells (28). Another congener, 8-chloro-adenosine, causes an ATP pool decline, as well as an inhibition of RNA synthesis (11, 13, 29–32). The precise mechanism by which 8-amino-adenosine causes a decline in the ATP pool has not been determined, although it is feasible that, similar to the chlorinated adenosine analogue, 8-amino-ADP is a substrate for ATP synthase and/or 8-amino-ATP may act as an inhibitor of the ATP synthase (33).
8-Amino-Adenosine and RNA Polymerase II Activity

Another mechanism of transcription inhibition by 8-amino-adenosine is the decrease in serine phosphorylation on RNA polymerase II (Fig. 3A and B). RNA polymerase II phosphorylation is a two-step process that requires a sequential phosphorylation of the CTD at Serine-5 and Serine-2 (34–38). An array of agents affects this process. DRB acts by inhibiting transcription elongation mediated by positive transcription elongation factor b (39–41). Flavopiridol, a CDK inhibitor, also suppresses transcription by inhibiting positive transcription elongation factor b (5, 7, 38, 42, 43). SNS-032 is currently being developed as a chemotherapeutic agent that inhibits CDK2, CDK7, and CDK9 and consequently decreases the transcription of short-lived oncoproteins (9, 44). Roscovitine is another CDK inhibitor that has diminutive effects on transcription (45–47).

RNA polymerase II phosphorylation decline is evident in cells within 2 hours of treatment with 8-amino-adenosine (Fig. 3A). Published work has shown that the decline in ATP pool does not lead to a global decrease in protein phosphorylation (15). Therefore, a lack of intracellular ATP substrate for the CDK7 and CDK9 proteins that mediate the phosphorylation of the RNA polymerase II CTD may not provide an appropriate rationale for this observation. Whereas there was no effect of 8-amino-adenosine incubation on total kinase level, it is possible that 8-amino-ATP can inactivate CDK7 and CDK9, which warrants future investigation.

Figure 5. Effect of 8-amino-ATP on in vitro transcription. A, schema of Assay. Step 1: the α-fetoprotein 7 plasmid was linearized by restriction enzyme digest and dephosphorylated. Step 2: in vitro transcription reaction was done using [α-32P]UTP to label nascent RNA. Step 3: nascent RNA was purified and separated by gel electrophoresis.

B, effect of various concentrations of 8-amino-ATP and ATP on in vitro transcription of 250 nucleotide RNA product. Experiments were done as described in schema. C, quantitation and normalization of the in vitro transcription reactions with 0.6 μmol/L ATP and various concentrations of 8-amino-ATP or the known RNA synthesis inhibitor α-amanitin. D, quantitation and normalization of the reactions with 0.6 or 2.4 μmol/L ATP with or without an equal concentration of 8-amino-ATP.
8-Amino-ATP Incorporation and Inhibition of mRNA Synthesis

ATP is a natural substrate for RNA polymerase II activation and incorporation into nascent RNA chains. Evidence that has been provided here shows that 8-amino-ATP is a substrate for RNA incorporation and furthermore that this incorporation at the 3′ terminus causes chain termination (Fig. 4). These effects have also been observed in 8-chloro-adenosine–treated cells (11). The chain termination is likely a consequence of the inability of the cellular machinery to proceed past the site of analogue incorporation, as well as an inability to excise the analogue from the transcript. It has been generally accepted that errors in RNA transcription result in the decay of the new transcript and the RNA transcription machinery would restart the synthesis because the rate of transcription is so great in cells that repair mechanisms would not be useful (48). However, RNA polymerases do have a 3′-5′ nuclease activity that is enhanced by cleavage stimulatory factors, which facilitate the backtracking and hydrolyzing of nascent transcripts when elongation is stalled. The polymerase is then able to reinitiate elongation (49). It is possible that the amino group may interfere with the steric of the RNA polymerase and either cause the RNA polymerase to pause at the site, unsuccessfully attempt to repair the incorporation, or perhaps cause the RNA polymerase to dissociate from the DNA.

It may be inferred that, as 8-amino-ATP accumulates in the cell, it competes with ATP for incorporation into mRNA. Evidence provided illustrates that, as the ratio of 8-amino-ATP to ATP increases, there is a decline of in vitro transcription-generated mRNA products (Fig. 5). Based on these experiments, it seems that an in vivo concentration of 8-amino-ATP must greatly exceed the concentration of ATP to fully inhibit transcription. This excess may be enhanced in 8-amino-adenosine–treated cells by the rapid decline in the ATP pool.

8-Amino-Adenosine and Polyadenylation

The stability of nascent RNA products is dependent on posttranscriptional modifications. Polyadenylation at the 3′-end of the transcript is one of these modifications.
that are critical to the stability of newly transcribed mRNA. Previous work has investigated 8-amino-ATP in the context of polyadenylation. In vitro polyadenylation assays have shown that 8-amino-ATP is a substrate for yeast poly(A) polymerase. However, once the analogue is incorporated into the poly(A) tail, further polyadenylation extension is inhibited (16). In the context of the cell, 8-amino-ATP is likely to compete with ATP as a substrate for poly(A) polymerase, as shown in the dose-dependent decrease of the poly(A) tail length (16). Similar data were obtained using the mammalian poly(A) polymerase enzyme (50). The inhibition of polyadenylation by 8-amino-ATP provides another mechanism by which 8-amino-adenosine regulates and inhibits transcription.

8-Amino-adenosine has proved to be cytotoxic in several cell lines. The cytotoxicity of this nucleoside analogue is dependent on its metabolism to the nucleoside triphosphate by adenosine kinase and other enzymes. The active metabolite has consequences in the cell that lead to RNA synthesis inhibition, decline in the ATP pool, and cell death. Present data and previous work have shown that 8-amino-adenosine can inhibit transcription by four distinct mechanisms. The decline in the ATP pool, decrease in RNA polymerase II activity due to a decline in phosphorylation, incorporation of the 8-amino-adenosine metabolite in nascent mRNA with subsequent RNA chain termination, and inhibition of polyadenylation of transcripts are all mechanisms by which 8-amino-adenosine functions to inhibit transcription (Fig. 6).

Premature chain termination may have significant consequences as it affects transcripts with short half lives. Survival proteins, such as MCL1, have short-lived transcripts, which results in a loss of protein and survival protection when transcription is impeded (31). Because of the loss of essential transcripts and their protein products, the incorporation and subsequent chain termination in the cell can have far reaching effects that lead to cytotoxicity. Further examination of this process of chain termination may provide insight into the utility of RNA chain terminators as therapeutic agents.

In conclusion, the present work provides rationales and suggestions for future studies that will lead to the development of 8-amino-adenosine as a RNA-directed nucleoside analogue for cancer therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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