Biochemical Pharmacology 79 (2010) 669–677

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Chain termination and inhibition of mammalian poly(A) polymerase by modified ATP analogues

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ARTICLE INFO

Article history:
Received 14 August 2009
Accepted 30 September 2009

Keywords:
Polyadenylation
Nucleoside analogues
RNA post-transcriptional processing
Molecular modeling
Hematological malignancies

ABSTRACT

We report the inhibition of mammalian polyadenylation by the triphosphate derivatives of adenosine analogues, 8-chloroadenosine (8-Cl-Ado) and 8-aminoadenosine (8-amino-Ado), which are under preclinical and clinical investigations for the treatment of hematological malignancies. The nucleotide substrate specificity of bovine poly(A) polymerase (PAP) towards C8-modified ATP analogues was examined using primer extension assays. Radiolabeled RNA primers were incubated with bovine PAP, and in the absence of ATP, no primer extension was observed with 8-Cl-ATP, whereas 8-amino-ATP resulted in chain termination. The effects of modified ATP analogues on ATP-dependent poly(A)-tail synthesis by bovine PAP also were determined, and incubation with analogue triphosphate resulted in significant reduction of poly(A)-tail length. To model the biochemical consequences of 8-Cl-Ado incorporation into RNA, a synthetic RNA primer containing a 3′-terminal 8-Cl-AMP residue was evaluated, and polyadenylation of the primer by bovine PAP with ATP was blocked completely. To explain these experimental observations and probe the possible structural mechanisms, molecular modeling was employed to examine the interactions between PAP and various ATP analogues. Molecular docking demonstrated that C8-modifications of ATP led to increased distance between the 3′-hydroxyl group of the RNA oligonucleotide terminus and the α-phosphate of ATP that render the molecules in an unfavorable position for incorporation into RNA. Similarly, C8-substitution with a chlorine or amino group at the 3′-terminal residue of RNA also inhibits further chain elongation by PAP. In conclusion, modified ATP analogues may exert their biological effects through polyadenylation inhibition, and thus may provide an RNA-directed mechanism of action for 8-Cl-Ado and 8-amino-Ado.

1. Introduction

Nucleoside analogues have been used extensively as antiviral and anticancer agents, and for the latter have demonstrated particular efficacy in the treatment of hematological malignancies. These analogues contain modifications in either the nucleobase, carbohydrate or in both moieties of purines and pyrimidines, and they are effective via a number of different mechanisms (reviewed in Ref. [1]. While most of the clinically established nucleoside analogues are DNA-directed, we are currently investigating for the treatment of hematological malignancies [2–5] a unique class of adenosine analogues (Fig. 1, 8-chloroadenosine; 8-Cl-Ado, and 8-aminoadenosine; 8-amino-Ado) that are RNA-directed and are modified at the C8 nucleobase position. In contrast to other analogues that largely target DNA-related events, due to the presence of a ribose sugar the mechanisms of action of these analogues appear to include decline in ATP pool and inhibition of RNA synthesis.

Both 8-Cl-Ado and 8-amino-Ado are phosphorylated intracellularly by adenosine kinase to their monophosphates and then are further phosphorylated enzymatically to their corresponding triphosphate derivatives [6,7]. In addition, our laboratory has previously demonstrated that cells treated with either 8-Cl-Ado or 8-amino-Ado exhibit a rapid decline in ATP levels in multiple myeloma cells [6], mantle cell lymphoma [8], breast cancer cell lines [9], as well as in primary CLL lymphocytes [2,3]. Mechanistic

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doi:10.1016/j.bcp.2009.09.028

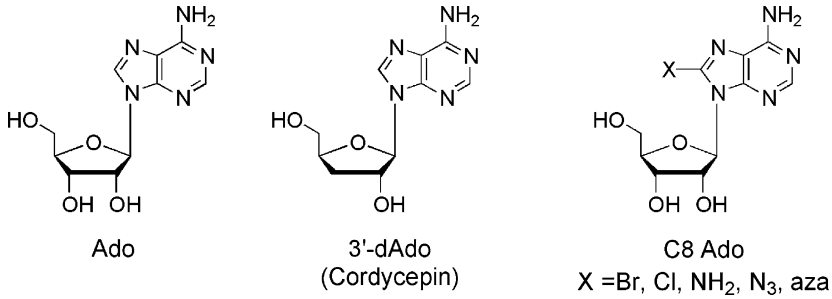

Fig. 1. Chemical structures of various adenosine analogues.

studies demonstrated that ATP generation through oxidative phosphorylation in the mitochondria may be decreased because 8-Cl-ADP acts as a substrate and 8-Cl-ATP is an allosteric inhibitor of ATP synthase [10]. Additional investigations established that 8-Cl-ATP is preferentially incorporated in mRNA species by RNA polymerase II, resulting in inhibition of transcription [5]. Additionally, the triphosphate derivatives of both analogues inhibit yeast poly(A) polymerase (PAP) in cell-free primer extension assays [4]. Collectively, these data demonstrate that these ribose analogue triphosphates have unique mechanisms of action that target mRNA transcripts.

The potential inhibition of transcription and polyadenylation by C8-modified ATP analogues, coupled with the decline in cellular ATP may result in reduced polyadenylation efficiency and a decrease in viable transcripts. Certain oncogene transcripts, such as Mcl-1 and c-Met, are rapidly turned over in the cell [11,12], and thus polyadenylation inhibition may have a significant impact on transcripts that have a short half-life. This, in turn, may exploit the observed dependency of certain cancers on specific oncogenes [13]. Our data have also shown that a known polyadenylation inhibitor, cordycepin (3′-deoxyadenosine, 3′-dAdo), induces cell death in multiple myeloma (MM) cell lines [14], demonstrating the potential utility of transcription and polyadenylation inhibitors.

Polyadenylation catalyzed by poly(A) polymerase (PAP) is one of the key steps in the post-transcriptional processing of eukaryotic mRNA precursors. 3′-End cleavage and polyadenylation *in vivo* are highly regulated processes tightly coupled to transcription (reviewed in Refs. [15,16]), and involve a complex set of multi-subunit factors [17,18]. The poly(A) tail length has been shown to correlate with mRNA stability [19] and helps to regulate mRNA degradation [20]. It is also crucial for transport of mRNA from the nucleus to the cytoplasm [21] where it functions to promote efficient translation [22,23] More recently, microRNAs also have been shown to be processed from capped and polyadenylated mRNAs [24].

PAP belongs to the superfamily of nucleotidyl transferases [25] and incorporates ATP onto the 3′ ends of RNA substrates in a template-independent manner. We previously demonstrated that a number of ATP analogues inhibit polyadenylation by yeast PAP [4]. We now extended our study to a mammalian system using bovine PAP [26]. Based on their structural similarities to ATP and our prior work with yeast PAP [4], we hypothesized that 8-Cl-ATP and 8-amino-ATP analogues may interfere with RNA polyadenylation. Our results demonstrated that the specificity of bovine PAP is nearly identical to the yeast enzyme [4]. 8-Amino-ATP was an efficient chain terminator during polyadenylation, and a 3′-terminal 8-Cl-AMP residue blocked the ability of bovine PAP to produce chain extension with ATP. In primer extension reactions including both ATP and C8-modified ATP analogues simultaneously, a concentration-dependent reduction in poly(A)-tail length was observed and thus polyadenylation inhibition may be an RNA-directed mechanism of action for these agents.

2. Materials and methods

2.1. Materials

T4 polynucleotide kinase (30 units/μL) was purchased from United States Biochemical Corp. (Cleveland, OH) and RNAsin Plus RNase Inhibitor was obtained from Promega (Madison, WI). Bovine PAP II was produced as described previously [27]. A 10-bp DNA ladder was obtained from Invitrogen (Carlsbad, CA). NAP-5 Sephadex G-25 columns were obtained from Amersham Biosciences (Piscataway, NJ), and [γ-^{32}P]ATP (7000 Ci/mmol) was purchased from MP Biomedicals (Solon, OH). Radioactive bands on polyacrylamide gels were visualized using an Amersham Biosciences Storm PhosphorImager and analyzed using Amersham Biosciences ImageQuant software.

2.1.1. ATP derivatives

ATP was obtained from Amersham Biosciences, and cordycepin triphosphate (3′-dATP) was obtained from Ambion Inc. (Austin, TX). 8-Azido-ATP, and 8-aza-ATP were purchased from TriLink Biotechnologies (San Diego, CA), and 8-Br-ATP was obtained from Sigma (St. Louis, MO). 8-Cl-ATP and 8-amino-ATP were synthesized as previously described [4].

2.1.2. RNA oligonucleotides

RNA primer 5′-UGUGCCCGA-3′ (**1**) was purchased from Dharmacon Research Inc. (Lafayette, CO) and was deprotected, and characterized using MALDI-TOF-MS (calculated $[M]^+ = 2832.8$ and found $[M]^+ = 2829.7$) as previously described [4]. Oligonucleotide 5′-UGUGCCCGA^{Cl}-3′ (**2**) was synthesized using an 8-Cl-Ado-derivatized controlled-pore glass support, purified by RP-HPLC, and characterized by MALDI-TOF-MS (calculated $[M]^- = 2865.2$ and found $[M]^- = 2865.6$) as described previously [28].

2.2. Preparation of 5′-^{32}P-radiolabeled oligonucleotides

RNA oligonucleotide **1** or **2** (20 pmol) was incubated at 37 °C for 60 min with 10.5 units of T4 polynucleotide kinase in a 20 μL reaction mixture containing 10 mM Tris acetate, 10 mM magnesium acetate, 50 mM potassium acetate, and 24 pmol of [γ-^{32}P]ATP (8.4 Ci/μL, 7000 Ci/mmol). The reaction was quenched with 2 μL of 100 mM EDTA, and the labeled DNA was purified by 7 M-20% dPAGE with 89 mM Tris, 89 mM borate, and 1 mM EDTA as the running buffer. The radiolabeled oligonucleotide was visualized by autoradiography, excised, and eluted by soaking the gel piece in 100 μL of buffer (200 mM NaCl, 10 mM Tris (pH 7.5), and 1 mM EDTA) at 25 °C for 12 h. The eluted product was purified using a NAP-5 Sephadex G-25 column and stored in double-distilled H_2O.

2.3. Bovine PAP substrate specificity assays

Radiolabeled RNA oligonucleotide **1** was incubated at 30 °C for 30 min in a 5 μL solution containing bovine PAP and a single

modified nucleoside triphosphate in the absence of ATP. The extension reactions were analyzed by 7 M-20% dPAGE. Final extension conditions were as follows: 250 μM NTP, 200 nM RNA primer **1**, 10 ng bovine PAP, 0.5 mM $MnCl_2$, 1% PVA, 10 mM Tris–HCl (pH 8.5), 0.5 U RNAsin. The triphosphates assayed were ATP, 3′-dATP, 8-Br-ATP, 8-Cl-ATP, 8-amino-ATP, 8-azido-ATP, and 8-aza-ATP.

2.4. Characterization of chain termination products

RNA primer **1** was incubated at 37 °C for 1 h in a 20 μL solution containing 200 nM RNA primer **1**, 250 μM 8-amino-ATP, 40 ng bovine PAP, 0.5 mM $MnCl_2$, 1% PVA, 10 mM Tris–HCl (pH 8.5). To remove proteins from the reaction mixture, the reactions were precipitated with ethanol and sodium acetate. Samples were desalted using C-18 ZipTips™ from Millipore (Millerica, MA) and the RNA oligonucleotides were characterized using a Voyager-DE™ STR Biospectrometry™ workstation MALDI-TOF mass spectrometer (PerSeptive Biosystems, Inc., Foster City, CA). MALDI-TOF-MS (*m*/*z*): single 8-amino-AMP termination product (5′-UGUGCCCGAA^{NH_2}-3′), calculated $[M]^+$ = 3177.0 and found $[M]^+$ = 3177.9; double 8-amino-AMP termination product (5′-UGUGCCCGAA^{NH_2}A^{NH_2}-3′), calculated $[M]^+$ = 3521.0 and found $[M]^+$ = 3506.8.

2.5. Polyadenylation of 3′-terminated 8-Cl-AMP primer 2

Radiolabeled RNA primer **2** was incubated at 30 °C for 30 min in a 10 μL solution containing 200 nM RNA primer **2**, 10 ng bovine PAP, 0.5 mM $MnCl_2$, 1% PVA, 10 mM Tris–HCl (pH 8.5), and 1 U RNAsin. Aliquots were removed at 1, 5, 15, and 30 min, and the products were analyzed by 7 M-20% dPAGE. As a control, primer **1** was assayed under the same conditions.

2.6. Polyadenylation inhibition assays with C8-modified ATP analogues

Radiolabeled RNA oligonucleotide **7** (200 nM) was incubated at 30 °C for 30 min in a 5 μL solution containing 200 nM RNA primer **1**, 5 ng bovine PAP, 0.5 mM $MnCl_2$, 1% PVA, 10 mM Tris–HCl (pH 8.5), 0.5 U RNAsin, with 0–250 μM 8-Cl-ATP or 8-amino-ATP. The extension reactions were terminated by the addition of one volume of 2× gel loading buffer, and the products were analyzed by 7 M-20% dPAGE.

2.7. Molecular modeling

Several yeast and bovine PAPs, which share high structural similarity particularly in the active sites, have previously been crystallized. The 3D structure of bovine PAP (1Q79) was retrieved from the protein database (PDB), at 2.15 Å resolution [29]. The co-crystallized ligand 3′-dATP, together with two Mn^{2+} metal ions, was used to build the 3D structures of ATP and other analogues, and they served as the starting point for energy minimization and molecular docking. The ATP analogues were subjected to correction of atom types and bonds, and then hydrogen atoms and Gasteiger–Marsili charges were added in SYBYL [30] (Tripos, St. Louis, MO). The molecules were minimized with Tripos force field using the Conj Grad method. The lowest energy conformations of these compounds were then compared.

Molecular docking was used to study the interactions between C8-modified ATP analogues and PAP protein. SYBYL was used to fix the protein side chains as well as add hydrogens and charges. All structure water molecules were maintained in the active site since they were important to coordinate the triphosphate moiety of ATP [31]. In order to evaluate the applicability of different docking programs (Gold, Glide and FlexX) [32], the co-crystallized ligand 3′-dATP was docked back into its binding pocket in PAP. Default parameters were applied unless otherwise noted. The binding site was defined as the 10 Å around the ligand 3′-dATP. We found that Gold delivered excellent docking accuracy with RMSD = 1.25 Å. The base and sugar moieties were particularly well docked but the flexible triphosphate group deviated from the crystal structures, which made the RMSD above 1 Å. These procedures were also applied to yeast PAP structure and similar results were obtained, thus the Gold program was employed in our docking studies. Unfortunately, the crystal structure of an RNA polynucleotide co-crystallized in bovine PAP is not available, however the ternary structure is available for the yeast PAP enzyme 2Q66 (a complex structure of RNA-PAP-Mg^{2+}-ATP) [31]. In order to locate the probable position of the polynucleotide in bovine enzyme, 2Q66 was aligned with 1Q79 in SYBYL [30]. The structure alignment achieved RMSD = 3.92 Å with the backbone fitting. The coordinates of the merged polynucleotide were used to define the protein active site for RNA binding and the radius was set to 10 Å [33].

The 3-D structure of the yeast PAP was carefully investigated, and we found that the aromatic ring stacking interactions between the terminal nucleoside base of the RNA and the incoming ATP base rendered the ATP in a position where its α-phosphate can be attacked by the RNA terminal 3′-hydroxyl group for the chain elongation [31]. Based on this observation, distance constraints were applied during docking in order to insure that the base-stacking interactions between the 3′-dATP and the RNA terminal nucleoside in the bovine PAP complex were retained. To this end, Gold parameters of minimum separation 1.5 Å, maximum separation 5.5 Å and spring constant 5 Å were defined for the C-5 of the terminus nucleotide and the C-5 of ATP.

The RNA oligonucleotide docking resulted in reasonable complementary interactions among the RNA, protein and 3′-dATP as well as the metal ion Mn^{2+}, and thus the docked RNA pose was used in the other docking studies of modified ATP analogues to bovine PAP.

3. Results

3.1. Bovine poly(A) polymerase nucleotide specificity

Using cell-free primer extension assays, we assessed the substrate specificity of bovine PAP [34]. Bovine PAP was incubated with various ATP analogues and a 5′-^{32}P radiolabled synthetic RNA primer (5′-UGUGCCCGA-3′, **1**) in reactions containing 200 nM RNA primer **1** and 250 μM each analogue triphosphate in the absence of ATP. The extension reactions were incubated at 30 °C for 30 min and then analyzed by 20% dPAGE (Fig. 2A). Primer extension in the presence of the natural substrate ATP produced a poly(A) tail several hundred nucleotides in length (Fig. 2A, lane 3). A known PAP chain terminator, 3′-dATP, was used as a positive control [35]. Incubation with 3′-dATP, produced a single extension product (Fig. 2A, lane 4), consistent with chain termination. Substitution at C8 resulted in a marked reduction in primer elongation by bovine PAP. Bovine PAP was unable to synthesize poly(A) tails using the halogenated derivatives, 8-Br-ATP and 8-Cl-ATP, as they do not appear to be substrates for PAP (Fig. 2A, lanes 5 and 6). In contrast, nitrogenous modification at C8 in 8-amino-ATP, 8-azido-ATP, and 8-aza-ATP, all resulted in polyadenylation termination following the incorporation of single or multiple unnatural residues (Fig. 2A, lanes 7–9).

Primer extension products and unextended primers were quantified using ImageQuant software and corrected for background noise level, then expressed as a percentage of the total signal. The extended products were classified into two categories; primers extended between one and three nucleotides and primers extended beyond three nucleotides. The remainder was considered unextended primer, and results are represented in Fig. 2B. The

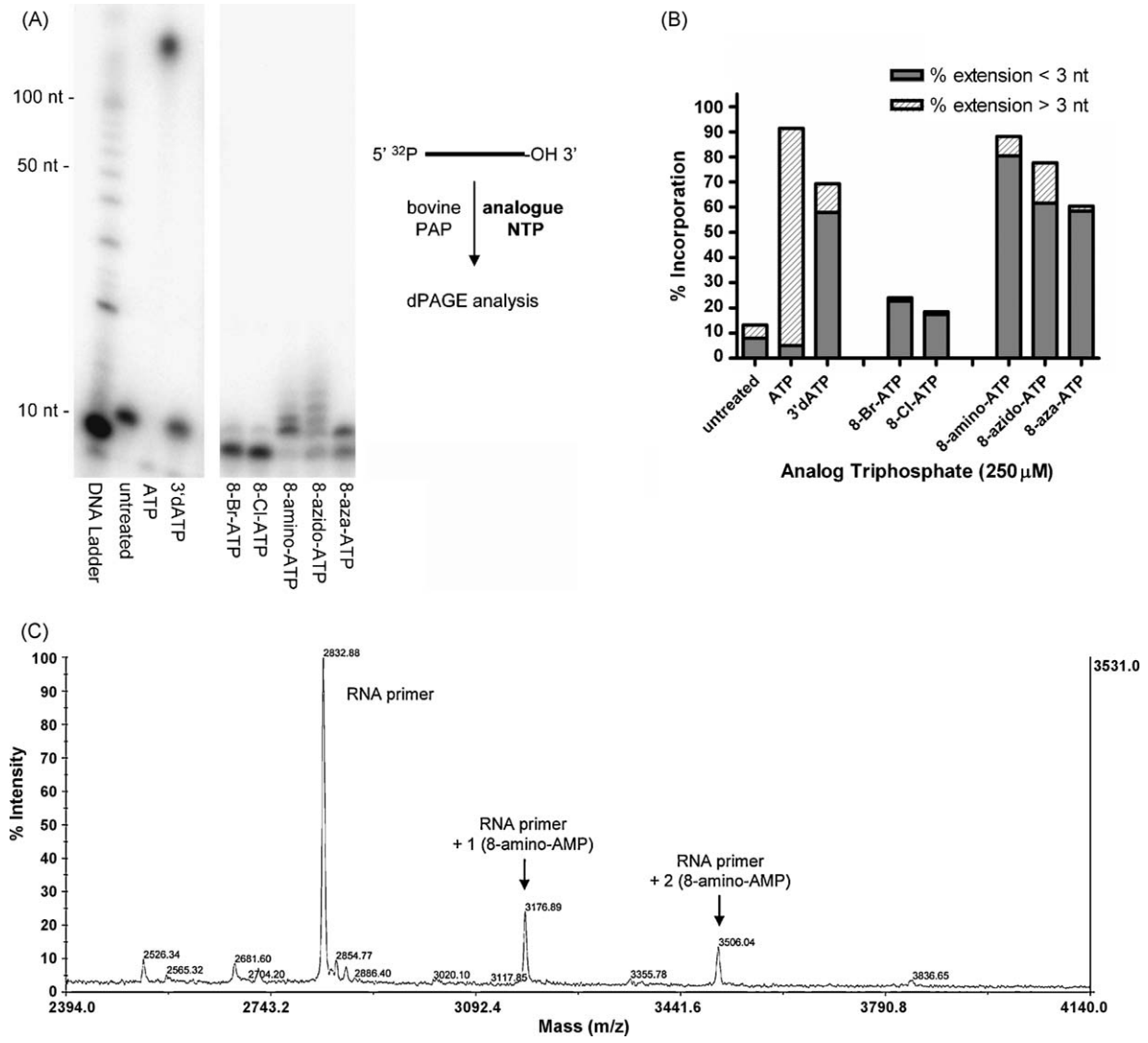

Fig. 2. Substrate specificity of bovine PAP toward various purine triphosphate analogues. (A) Elongation of RNA primer 5′-UGUGCCCGA-3′ by bovine PAP. A mixture containing 200 nM 5′-^{32}P-radiolabeled RNA primer, 1 ng bovine PAP/μL reaction, 250 μM analogue triphosphate, 10 mM Tris–HCl (pH 8.5), 0.5 mM $MnCl_2$, 1% polyvinyl alcohol was incubated at 30 °C for 30 min. The products were analyzed by 20% dPAGE. Lane 1, radiolabeled 10-bp DNA ladder; lane 2, 5′-^{32}P-radiolabeled unextended primer (no NTP); lane 3, ATP; lane 4, 3′-dATP; lane 5, 8-Br-ATP; lane 6, 8-Cl-ATP; lane 7, 8-amino-ATP; lane 8, 8-azido-ATP; lane 9, 8-aza-ATP. (B) Graphical representation of RNA primer extension by bovine PAP with various modified triphosphates. Shown is the distribution of single extension products (grey) and full extension products beyond first incorporation (hatched) as a percentage of the total counts in each lane, as determined using ImageQuant software. These experiments were conducted in triplicate with similar results. (C) MALDI-TOF mass spectrum of RNA primer **1** extension with bovine PAP and 8-amino-ATP.

analogues that resulted in a majority of primer extension of less than three nucleotides were considered chain terminators in these assays. Primer incubated with ATP resulted in >95% product that was greater than three nucleotide in length. RNA primers incubated with halogenated C8-modified analogues (8-Br-ATP and 8-Cl-ATP) remained largely as unextended primer, with a minor population (<15% over untreated) resulting in chain termination. The remaining nitrogen-modified analogues, 8-amino-ATP, 8-azido-ATP, and 8-aza-ATP, all resulted in significant incorporation (<50% over untreated) and subsequent chain termination.

3.2. Characterization of extended primers

Extended primers with 8-Cl-ATP or 8-amino-ATP were further characterized by mass spectrometry. No significant product was detected with bovine PAP and 8-Cl-ATP by mass spectrometry, which is consistent since primer extension observed by gel electrophoresis was insignificant (Fig. 2A, lane 6). Of the nitrogen-modified analogues, 8-amino-ATP resulted in the most incorporation and was further characterized as a representative using mass spectrometry. Primer **1** was incubated at 30 °C with bovine PAP and 250 μM 8-amino-ATP, then ethanol precipitated and desalted. The products then were analyzed by MALDI-TOF-MS (Fig. 2C). The masses obtained for the chain termination products were in agreement with calculated masses: 8-amino-AMP terminated (5′-UGUGCCCGAA^{NH_2}-3′), calculated mass = 3177 and found mass = 3178; double 8-amino-AMP termination product (5′-UGUGCCCGAA^{NH_2}A^{NH_2}-3′), calculated = 3521 and found $[M]^+$ = 3507.

3.3. Effect of C8-position substitution on ATP structure

In order to investigate the effect of the C8-position substitution on the ATP structure, the analogue structures were energetically minimized in SYBYL and conformational difference was observed between ATP and 8-Cl-ATP. The adenine ring of 8-Cl-ATP rotated about 90° to avoid steric hindrance between its α-phosphate and chlorine atom, as the radius of –Cl is much larger than the original –H in ATP. On the other hand, the docking results demonstrated that

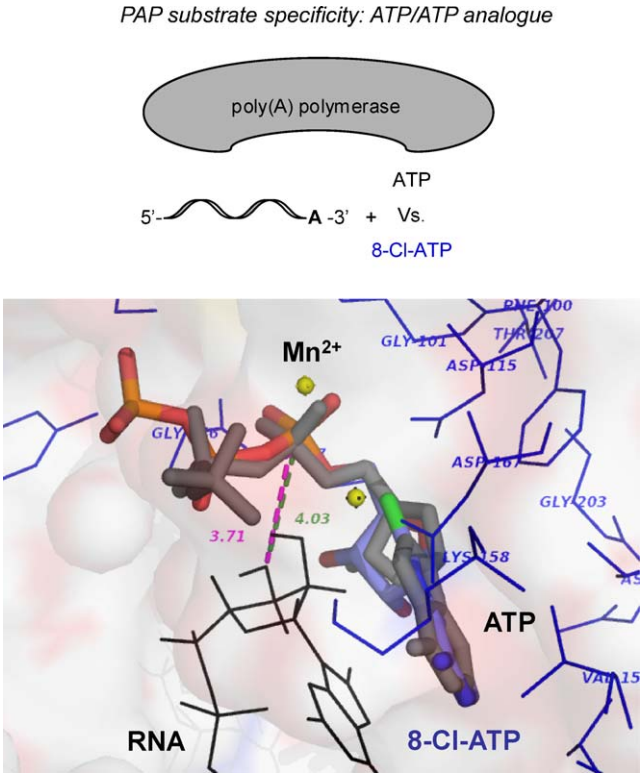

Fig. 3. The docked conformation of 8-Cl-ATP in the bovine PAP binding site, in comparison of ATP. The structure of 8-Cl-ATP is in atom type-based color code stick and that of ATP is in grey stick. The RNA is in grey line, and the transparent surface is for the PAP protein. The $D_{P_1-O_{3'}}$ is increased due to the substitution on the C8-position with chlorine atom.

the adenine base of the 8-Cl-ATP is stacked with the terminus of the poly(A) RNA substrate, and that its N6 group participates in the interactions with the residue Asp167 (Fig. 3). This base-stacking interactions contributed significantly to the binding free energy [31] of 8-Cl-ATP to the receptor. Hence, in order to diminish the steric clash caused by the addition of the chlorine atom, the α-phosphorus of 8-Cl-ATP was driven away from the RNA terminal nucleoside and the Mn^{2+} ion. The distance between the terminal 3′-hydroxyl group of the nucleotide and the α-phosphate ($D_{P_1-O_{3'}}$) of 8-Cl-ATP was 4.03 Å, which was larger than that for ATP (3.71 Å, see Fig. 3 and Table 1). In addition, the binding of 8-Cl-ATP to PAP is predicted as strong as ATP based on their Gold docking scores. The comparable binding, together with the long distance of $D_{P_1-O_{3'}}$, results in difficult nucleophilic attack of 8-Cl-ATP by the 3′-OH of the RNA terminal AMP. This could explain why 8-Cl-ATP was a poor substrate for polyadenylation by PAP.

The same procedures were applied to study the effect of other C8-substituted nucleoside analogues, including 8-Br-ATP, 8-amino-ATP, 8-azido-ATP and 8-aza-ATP. We also examined their binding modes and the distance between the 3′-hydroxyl group of the RNA terminal AMP and the α-phosphorus of the incoming ATP analogues, and the results are summarized in Table 1. The binding affinities of C8-substituted ATP analogues with PAP were comparable to ATP, and thus they may have competitive inhibitory effect on the RNA polyadenylation. For example, in the case of 8-amino-ATP, the distance of the P_1 and O_3, is larger than that of ATP, but shorter than that of 8-Cl-ATP (Fig. 3 and Table 1). This is consistent with our biochemical observations, since 8-Cl-ATP was a poor substrate for PAP, whereas 8-amino-ATP is incorporated and acts as a chain terminator. Similarly, 8-azido-ATP had a $D_{P_1-O_{3'}}$ (3.67 Å) close to that of ATP (3.71 Å), and was also observed to be an efficient substrate and chain terminator with PAP.

Table 1
The distance between the oxygen atom of the 3′-hydroxyl group of the RNA 3′-terminal nucleoside and the α-phosphate of triphosphate moiety ($D_{P_1-O_{3'}}$) resulted from molecular docking of PAP (1Q79).

Molecules	$D_{P_1-O_{3'}}$ (Å)	Gold scores
ATP	3.71	90.89
8-Cl-ATP	4.03	88.14
8-Br-ATP	3.88	88.45
8-Amino-ATP	3.80	84.43
8-Azido-ATP	3.67	76.47
8-Aza-ATP	3.84	78.46

3.4. 3′-Terminal 8-Cl-AMP residues block polyadenylation

The biochemical data presented in Fig. 2B suggest that while 8-amino-AMP terminated primers were further extended by PAP to polymerize additional 8-amino-ATP, 3′-terminal 8-Cl-AMP sites may prevent further elongation by PAP. Thus, to determine the effects of 8-Cl-AMP incorporation at 3′-terminal sites, a synthetic RNA oligonucleotide containing 3′-terminal 8-Cl-AMP was synthesized as previously described [28]. Modified primer **2** (5′-UGUGCCCGA^{Cl}-3′) was based on the same RNA sequence as primer **1** and assayed as a polyadenylation substrate with bovine PAP and ATP at 30 °C (Fig. 4A). Control primer extension with bovine PAP and unmodified RNA primer **1** was conducted under identical conditions as a control over a 30 min time course (Figure 4A, lanes 1-5). Under the same reaction conditions, RNA primer **2** containing a 3′-terminal 8-Cl-AMP residue was poorly extended by bovine PAP, and our results are in agreement with previous observations with yeast PAP [4].

The chain termination effect of RNA terminal 8-Cl-AMP and 8-amino-AMP was also modeled by modifying the RNA terminal AMP to its C8-chlorinated analogues and followed by molecular docking. The modeling indicated that the binding pose of the purine functional group of 8-Cl-AMP underwent a dramatic conformational change, thus was not able to form base-stacking interaction with the ATP (Fig. 4B). Therefore, the 3′-hydroxyl group of the terminal 8-Cl-AMP could not react with the newly bound ATP or its analogues. These results are in agreement with the primer extensions assays, where RNA primers containing 3′-terminal 8-Cl-AMP residues were not extended by PAP with ATP.

To obtain structural evidence for our observations, we used molecular modeling to examine the ligand–PAP interactions. Since a crystal structure of bovine PAP with an bound RNA oligonucleotides is not available, the yeast PAP ternary structure complex 2Q66 was employed to investigate the interactions among PAP, ATP and 5-mer poly(A) oligonucleotide and the metal ion (Mg^{2+}). Extensive contacts were observed between the substrates and the yeast PAP residues within the binding cavity. Three adenine bases of the PAP-bound RNA are completely buried in the pocket. The terminal nucleoside of the RNA shows strong interactions with ATP through hydrophobic interactions, in particular via an aromatic ring stacking effect. The 3′-hydroxyl of the RNA terminal nucleoside is located 3.2 Å away from the α-phosphorus of ATP, as if it were poised for the nucleophilic attack [31]. Thus, the distance between the 3′-hydroxyl group of the nucleotide and the α-phosphate of ATP is considered crucial for the transition state of the polyadenylation reaction [31].

3.5. Inhibition of polyadenylation by 8-Cl-ATP and 8-amino-ATP

Since cells treated with 8-Cl-Ado or 8-amino-Ado result in the accumulation of high triphosphate levels and a substantial

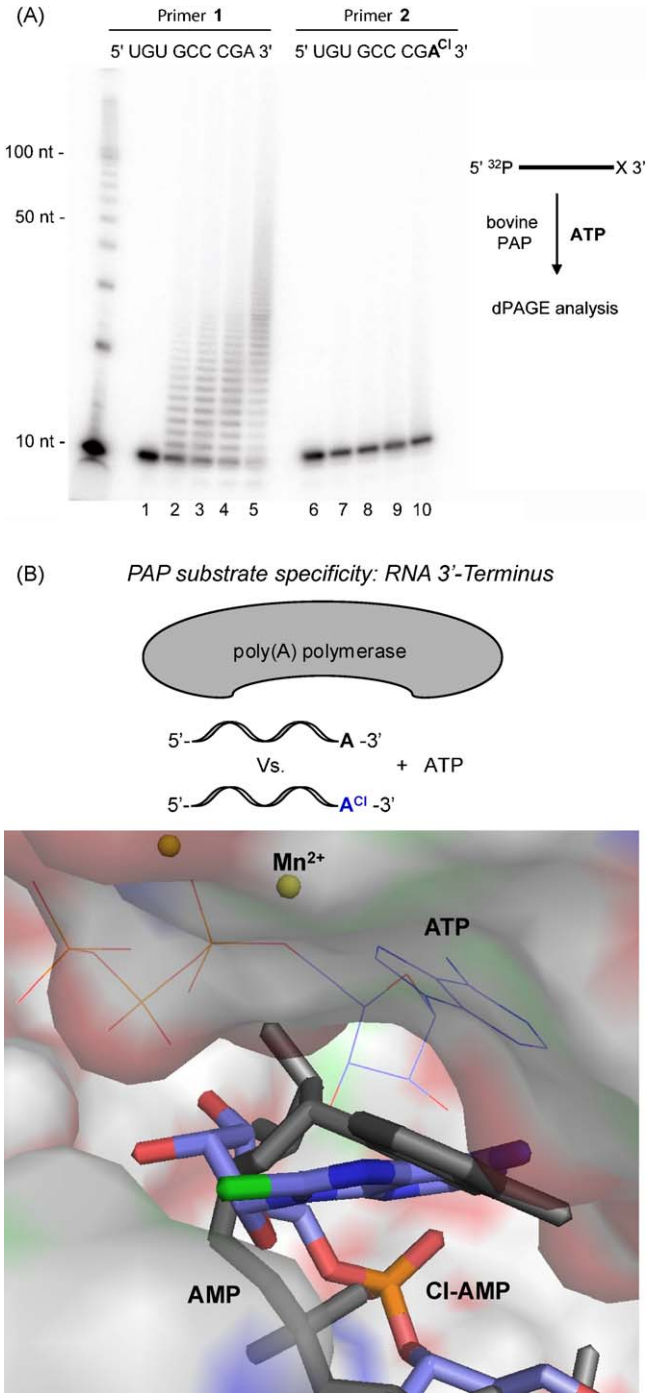

Fig. 4. 3′-Terminal 8-Cl-AMP sites block polyadenylation of RNA primers by bovine PAP and ATP. (A) Gel electrophoretic analysis of primer elongation of RNA primers with bovine PAP and ATP. A mixture containing 200 nM 5′-^{32}P-radiolabeled RNA primer, 1 ng bovine PAP/μL reaction, 250 μM analogue triphosphate, 10 mM Tris–HCl (pH 8.5), 0.5 mM $MnCl_2$, 1% polyvinyl alcohol was incubated at 30 °C for 30 min. Aliquots were removed after 0, 1, 5, 15 and 30 min (lanes 1–5 and 6–10), and the products were analyzed by 20% dPAGE, and a radiolabeled 10-bp DNA ladder was included as a marker. Lane 1–5, control primer and ATP; lanes 6–10, 8-Cl-AMP terminated primer. These experiments were conducted in triplicate and a single representative gel is shown. (B) The comparison between the chlorinated and original RNA/ATP/PAP complex. The grey stick represents the docked RNA. The atom type-based color coded stick is for docked RNA-8-Cl-AMP, and the wireframe display is ATP. The transparent surface is for the PAP protein.

reduction in ATP, we examined the effects of 8-Cl-ATP and 8-amino-ATP analogues on polyadenylation in the presence of ATP. Primer **1** was incubated at 30 °C with bovine PAP, 250 μM ATP, and increasing amounts of 8-Cl-ATP (Fig. 5A) or 8-amino-ATP (Fig. 5B), and the extension products were analyzed by 20% dPAGE. Control primer extension with bovine PAP and ATP alone resulted in the poly(A)-tail synthesis over one hundred nucleotides in length (Fig. 5A and B, lane 2). Increasing the concentration of either 8-Cl-ATP or 8-amino-ATP analogue relative to ATP resulted in a dramatic decrease in tail length (Fig. 5A and B, lanes 3–6). At equimolar concentrations of ATP and 8-amino-ATP (250 μM), the observed poly(A) tail was ~10 nucleotides or less in length (Fig. 5B, lane 7), and significantly shorter tails were also obtained with 8-Cl-ATP. These results suggest that C8-modified ATP analogues may have pronounced effects on poly(A) tail synthesis by bovine PAP. As listed in Table 1, the molecular modeling experiments indicated that the distance between the 3′-RNA terminus and 8-Cl-ATP and 8-amino-ATP was greater than with ATP (Fig. 3) and thus may inhibit polyadenylation.

4. Discussion

4.1. PAP as a therapeutic target

Dysregulation of 3′-end post-transcriptional processing of human mRNAs has been associated with a number of disease states including cancer, immunity, and inflammation (for a review, see Ref. [36]). In cancer biology, both PAP protein expression and functional activity appear to be altered in cancer cells [27,37]. PAP is overexpressed in more aggressive forms compared to the indolent forms of breast cancer, and may serve as a marker of poor prognosis in primary breast cancer [38]. The role of PAP has also been investigated in hematological malignancies, and elevated PAP function has been observed in both B-cell chronic lymphocytic leukemia (CLL) [39] and chronic myelogenous leukemia (CML) [40]. The catalytic activity of PAP has been found to be higher in acute leukemia cell extracts as compared to those from chronic leukemias, which was greater than in normal lymphocytes [27,41]. Targeting polyadenylation may be an effective RNA-directed strategy for the treatment of cancers since polyadenylation is a process that is required for cell survival. Since poly(A)-tail addition is independent of DNA synthesis, polyadenylation inhibitors may be a potential strategy for the treatment of indolent or quiescent cancers such as MM or CLL.

4.2. Yeast PAP versus bovine PAP

Yeast PAP and bovine PAP share approximately 48% homology in their protein sequence in the catalytic and central domain [42,43], and our biochemical observations indicate that they have similar substrate specificities. Our current studies demonstrated that the inhibition profile of PAP previously measured in yeast [4] is consistent in the mammalian system, and that the two enzymes share similar affinities towards modified ATPs. The active metabolites of two C8-modified adenosine analogues, 8-Cl-Ado and 8-amino-Ado, induce chain termination following incorporation into RNA oligonucleotides and also inhibit bovine PAP as triphosphate derivatives in competition with ATP as substrates. In our experiments, a number of adenosine analogues were chain terminators for bovine PAP (Fig. 2A), however, of these analogues a majority only reach cellular concentrations of below 50 μM [44–47]. In contrast, two analogues in particular, 8-Cl-Ado and 8-amino-Ado, accumulate to high triphosphate levels (>500 μM) [2,3,8,9,48]. Based on these observations, we focused our studies on examining the consequences of these two analogues and their pharmacological relevance. Both 8-Cl-ATP and 8-amino-ATP reduced poly(A)-tail length when incubated with PAP and ATP, and appear to inhibit PAP activity (Fig. 5). In addition, when incubated alone with bovine PAP, 8-amino-AMP incorporation significantly reduced further extension (Fig. 2), thus 8-amino-ATP

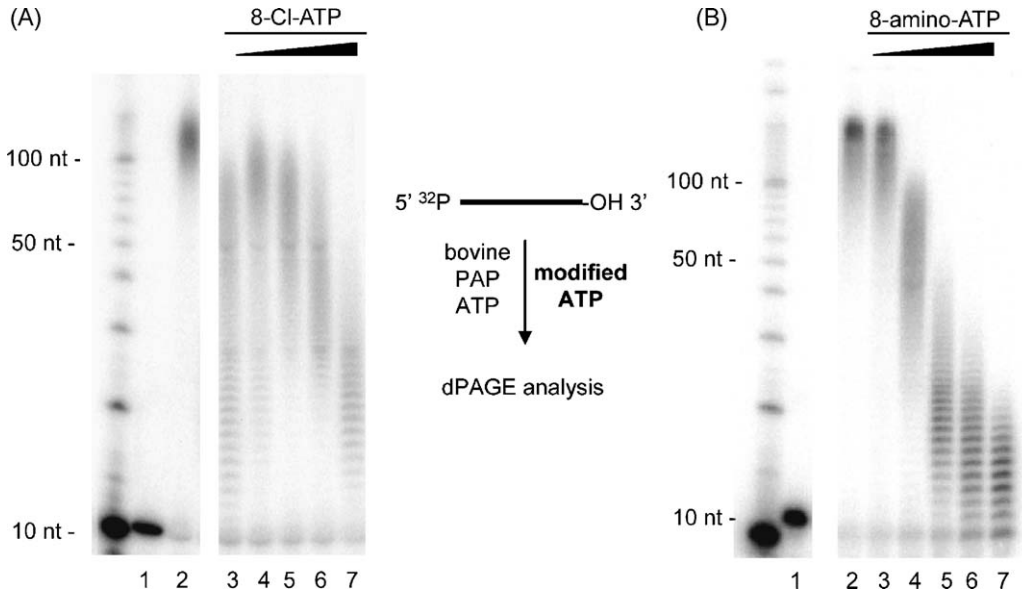

Fig. 5. Reduction of poly(A) tail length by modified ATP analogues. Gel electrophoretic analyses of an RNA primer 5′-UGUGCCCGA-3′ (**1**) with bovine PAP, 250 μM ATP, and increasing amounts of (A) 8-Cl-ATP or (B) 8-amino-ATP. A solution containing 200 nM 5′-^{32}P-radiolabeled RNA primer **1**, 1 ng bovine PAP/μL reaction, 250 μM ATP, 10 mM Tris–HCl (pH 8.5), 0.5 mM $MnCl_2$, 1% polyvinyl alcohol was incubated at 30 °C for 30 min. The products were analyzed by 20% dPAGE and a radiolabeled 10-bp DNA ladder was included as a marker. For each panel, lane 1, 5′-^{32}P-radiolabeled primer (no triphosphates); lane 2, ATP only; lanes 3–7, 250 μM ATP with 50, 100, 150, 200, or 250 μM analogue triphosphate, respectively. These experiments were conducted in triplicate with similar results.

appears to inhibit polyadenylation via enzyme inhibition as well as through chain termination.

4.3. Nucleotide specificity of PAP

Substitution at the C8-position of adenosine appears to be a critical determinant for PAP substrate selection. Although the crystal structures for PAP has been reported in vaccinia virus [49], yeast [31,50] and bovine [29,51], there still remains debate regarding the structural basis for nucleotide specificity. The first yeast PAP structure [50] did not indicate any adenine-specific contacts in the ATP region, and it was proposed that protein domain movement or glycosidic rotation of the nucleotide could align the adenine moiety of ATP into the proximity of middle or C-terminal domain residues. A more recent high-resolution crystal structure reported interactions between yeast PAP and N3, N6 and N7 of adenine, but few observations were made surrounding the C8-position [31]. Both reports suggested that steric hindrance of C2-amino group of guanine was the basis for ATP selection over GTP. However, our results in yeast [4] have shown that modification at C2 of adenine alone does not diminish PAP affinity towards modified ATP analogues. These observations are consistent with Martin et al. [29] in bovine PAP. Accordingly, in the bovine PAP crystal structure the specific amino acids interacting with the adenine group were near the N1 position of adenine [29]. Interestingly, several hydrophobic residues were observed in proximity of the ribose group of adenosine, suggesting that the substrate ribose structure may be a discriminating factor. Our previous results in yeast PAP are in agreement with this hypothesis, and substitution of hydrogen with a chlorine atom at C8 of adenine may induce a ribose conformational shift [4].

4.4. RNA primer 3′-terminus structure

Although 8-Cl-Ado appears to be a poor substrate for bovine PAP under the polyadenylation conditions used here, 8-Cl-Ado is incorporated into RNA at 3′-terminal sites, which may arise from incorporation during transcription [5]. As such, incorporation during transcription may have deleterious effects on subsequent poly(A)-tail addition, which was confirmed with our synthetically generated 8-Cl-AMP terminated RNA primer. A 3′-terminal 8-Cl-AMP residue blocks the ability for bovine PAP to add a poly(A)-tail to the primer (Fig. 3). In a biological context, there may be significant consequences since naturally occurring cleaved mammalian mRNA frequently is terminated by a CA dinucleotide at the 3′-end [52]. We have previously hypothesized that the prevention of poly(A)-tail formation following a 3′-terminal 8-Cl-AMP residue was a result of an altered sugar pucker conformation [4]. Our initial hypothesis included the possibility that the glycosidic torsion angle of the adenosine analogues under investigation were altered as a result of substitution at the C8-position. Incorporation of atoms/groups larger than a hydrogen atom at the C8-position may shift the equilibrium to favor the *syn* conformation, over the traditional *anti* conformation. However in previous work using the monophosphate derivatives of the two main analogues evaluated in this work (8-Cl-AMP and 8-amino-AMP), our 2-D NMR structural studies indicated that the substitution of a chlorine atom at C8 did not significantly shift the equilibrium between the *syn* and *anti* conformations in 8-Cl-AMP compared with AMP. Moreover, it appeared that 8-amino-AMP was shifted to predominantly favor the *anti* conformation, despite its much larger amino group at the C8 position, consistent with previous reports [57]. In our observations, it appeared that structural changes in the sugar pucker conformation were more involved with the mechanism of action of C8 substituted adenosine analogs, rather than base conformation.

Using 2-D NMR, we resolved that the sugar pucker for 8-Cl-AMP shifted from more C3′-endo towards a C2′-endo conformation, which is traditionally adopted by DNA rather than RNA and may render the sugar moiety in an unfavorable conformation for further chain extension by PAP. This observation is in agreement with the data obtained in our modeling studies, as described in the next paragraph. Chain termination observed with F-ara-ATP, Cl-F-ara-ATP and Cl-F-dATP [53] are also consistent with this hypothesis, since arabinonucleotides have been shown to adopt predominantly DNA-like conformations and favor C-2′-endo sugar pucker [54]. In the yeast model system, the last three residues of the RNA oligonucleotide was determined biochemically to be a critical

determinant for poly(A)-tail extension [55]. The high-resolution crystal structure of a catalytically inactive mutant of yeast PAP support this observation, and crystallographic analysis revealed that of the primer binding site only the region surrounding the last three residues of the 3′-terminus is highly ordered [31]. The 2′-OH groups of the last three AMP residues form direct hydrogen bonds with the enzyme, which would disfavor DNA binding. In addition, the 3′-OH of the terminal RNA residue is located only 3.2 Å from the α-phosphorus of ATP, as if in position for nucleophilic attack.

4.5. Computational modeling

Our molecular modeling supports our biochemical observations, and the C8 modified ATP analogues seem to interfere with the normal ATP binding to PAP. This effect is achieved by rendering comparable binding but making the 3′-OH of the sugar moiety less accessible to the incoming ATP, and thus inhibiting the nucleophilic attack of ATP to the oligonucleotide terminus. Several residues, including S102, D113, K228 and G246, together with Mn^{2+} and several water molecules, were shown to stabilize the triphosphate group. However upon C8-substitution of hydrogen with the bulkier chlorine atom, the 8-Cl-ATP may be pushed away from these residues, thereby putting 8-Cl-ATP in an unfavorable position for polyadenylation catalysis. This provided a possible structural mechanism as to why 8-Cl-ATP was not able to be incorporated into RNA by PAP. For both ATP and 8-Cl-ATP, the position of the purine base was largely maintained (Fig. 3), supporting the assertion that the base-stacking interactions between the ATP and the terminus of RNA were significant. Based on the analysis of the distance and the binding scores, the influence of other analogues such as 8-amino-ATP were predicted to be not as strong as 8-Cl-ATP (Table 1), which is consistent with our experimental assay: they can be incorporated but with subsequent chain termination of polyadenylation.

Polyadenylation inhibition by C8-modified adenosine analogues may have multifaceted effects which may contribute to cytotoxicity. For instance, in addition to the above-discussed mechanism, it may also have an effect on other forms of coding RNA such as microRNA primary transcripts, which have been shown to be polyadenylated [24]. Truncated generation or processing of microRNAs may also be an additional mechanism of action for these agents, and selective A-rich microRNAs may be more susceptible. Further investigations are underway in our laboratory to uncover the potential effects of adenosine analogues on microRNA expression and their biological consequences. To gain further understanding of the role of PAP in cancer biology, we are also examining PAP protein expression and post-translational modifications in hematological malignancies [56]. In summary, our current data demonstrated that C8-modified adenosine analogues indeed inhibit polyadenylation by mammalian PAP. DNA-independent therapeutic targets may be a valuable therapeutic strategy, and thus understanding the effects of adenosine analogues on PAP is the key to the further development of RNA-directed agents.

Acknowledgement

This work is supported in part by grant CA85915 from the National Cancer Institute, Department of Health and Human Services.

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