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Inhibition of mammalian ribonucleases by endogenous adenosine dinucleotides☆

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Abstract

The most potent low molecular weight inhibitors of pancreatic RNase superfamily enzymes reported to date are synthetic derivatives of adenosine 5′-pyrophosphate. Here we have investigated the effects of six natural nucleotides that also incorporate this moiety ($NADP^+$, NADPH, ATP, Ap_3A, Ap_4A, and Ap_5A) on the activities of RNase A and two of its homologues, eosinophil-derived neurotoxin and angiogenin. With eosinophil-derived neurotoxin and angiogenin, Ap_5A is comparable to the tightest binding inhibitors identified previously (K_i values at pH 5.9 are 370 nM and 100 μM, respectively); it ranks among the strongest small antagonists of RNase A as well ($K_i = 230$ nM). The K_i for NADPH with angiogenin is similar to that of Ap_5A. These findings suggest that Ap_5A and NADPH may serve as useful new leads for inhibitor design. Examination of inhibition under physiological conditions indicates that NADPH, ATP, and Ap_5A may suppress intracellular RNase activity significantly in vivo.

Keywords: RNase A; Eosinophil-derived neurotoxin; Angiogenin; Enzyme inhibitor; NADPH; ATP; Diadenosine polyphosphates

Several proteins in the mammalian pancreatic RNase superfamily have been shown to be potent biological effectors. Angiogenin (ANG) induces the formation of new blood vessels [1]. Eosinophil-derived neurotoxin (EDN, also known as RNase 2 and RNase U_s) and eosinophil cationic protein (ECP; RNase 3) are toxic to neurons and some other mammalian cells, as well as to various invading organisms such as helminths and RNA viruses (see [2,3] for reviews). The normal in vivo functions of these RNases have not yet been determined, although roles for ANG in wound healing [4], placental development [5], and the establishment of pregnancy [6] have been proposed, and EDN and ECP are thought to participate in host defense [7]. Human pancreatic RNase and RNase 4 may also have non-digestive functions [8,9].

Thus far, the unusual biological activities of three pancreatic RNase superfamily proteins have been linked with pathological processes. Evidence from mouse studies points to the critical involvement of ANG in the formation and metastatic dissemination of tumors (e.g. [10]), and an association between increased ANG expression and cancer has been observed in many clinical studies (e.g. [11]). EDN and ECP may produce some of the symptoms associated with hypereosinophilic syndromes [2]. All of the pathological effects and most of the normal activities of these RNases seem to require their enzymatic action [2,12]. Therefore, RNase inhibitors have potential applications as drugs for the treatment of human diseases and as tools to elucidate the normal roles of their targets. Potent inhibitors may also serve as reagents to neutralize adventitious RNases for in vitro applications that utilize RNA.

Efforts to develop low molecular weight RNase inhibitors have focused largely on nucleotides (see [13] for

☆ *Abbreviations:* ANG, angiogenin; EDN, eosinophil-derived neurotoxin; ECP, eosinophil cationic protein; ppA, 5′-diphosphoadenosine; ppA-3′-p, 5′-diphosphoadenosine 3′-phosphate; ppA-2′-p, 5′-diphosphoadenosine 2′-phosphate; Ap_nA, diadenosine 5′,5^n-P^1,P^n-polyphosphate.

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a review). The most effective compounds reported to date are derivatives of adenosine 5′-pyrophosphate (ppA), which inhibit bovine pancreatic RNase A, human EDN, and human RNase 4 with K_i values in the mid-to-upper nM range; although they bind less tightly to ANG, they are the most avid small nucleotide antagonists of this enzyme known. The identification of this class of inhibitor was achieved through active site mapping of RNase A [14] and ANG [15] with available nucleotides, which revealed a marked preference for a pyrophosphate vs. a phosphate at the 5′ position of adenosine and a strong contribution by a phosphate in the 3′ or 2′ position. 5′-Diphosphoadenosine 3′- and 2′-phosphate (ppA-3′-p and ppA-2′-p, respectively) were synthesized as the simplest nucleotides combining these advantageous components and were found to have the increased potencies anticipated. Subsequent work utilizing crystallographic information on RNase A complexes of these compounds led to the generation of 3′, 5′-pyrophosphate-linked nucleotides that had even higher affinity for RNase A [16].

The potent adenosine derivatives tested previously are not known to exist in nature. However, many natural nucleotides, some of which are highly abundant, incorporate the same core ppA structure (Fig. 1). These include $NADP^+$, NADPH, NAD^+, NADH, ATP, and diadenosine polyphosphates (Ap_nA; reviewed in [17]). Indeed, $NADP^+$ and NADPH even contain the ppA-2′-p structure. Here we have investigated the inhibitory potency of these nucleotides with RNase A, EDN, and ANG to obtain additional information on active site binding preferences that might be exploited for inhibitor design. We have also examined the physiological relevance of RNase inhibition by these compounds.

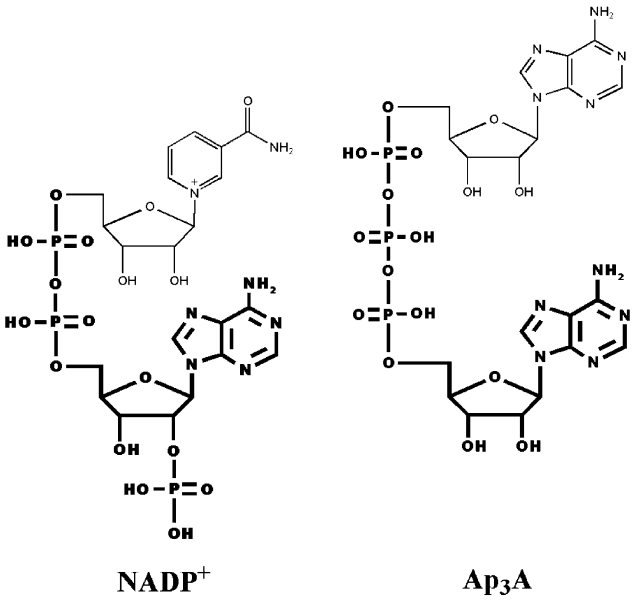

Fig. 1. Chemical structures of $NADP^+$ and Ap_3A, highlighting their structural similarities to previous adenosine nucleotide RNase inhibitors. The structures of Ap_4A and Ap_5A are identical to that of Ap_3A, except that the polyphosphate interadenosine linkers contain one and two additional phosphates, respectively.

Materials and methods

Materials. The fluorogenic RNase substrate 6-FAM–$(mA)_2$rU$(mA)_2$–Dabcyl was from Integrated DNA Technologies (Coralville, IA); 6-FAM is 6-carboxyfluorescein and mA is 2′-*O*-methyl adenosine; rU is ribouridine and Dabcyl is 4-(4-dimethylaminophenylazo)benzoyl. Other nucleotides were obtained from Sigma. Recombinant human EDN was provided by Prof. K.R. Acharya (University of Bath, UK) and the natural protein was isolated from human urine [16]. Sources of other materials and methods for nucleotide and protein quantification are described elsewhere [16,18].

Determination of K_i values. All assays were performed at 25 °C in 0.2 M Mes–NaOH (pH 5.9) containing 10 μg/ml bovine serum albumin, unless indicated otherwise. Inhibition was assessed from the dependence of k_{cat}/K_m on inhibitor concentration, [I]. Values of k_{cat}/K_m for RNase A and EDN were determined from the first-order rate constant for the full cleavage reaction at substrate concentrations well below K_m. With EDN (2.9 nM for recombinant, 1 nM for natural), the substrate 6-FAM–$(mA)_2$rU$(mA)_2$–Dabcyl was used at a concentration of 10–25 nM and the reaction was monitored fluorimetrically [19]. The same assay was used to assess inhibition of RNase A (0.5 nM) by NADPH. The effects of other inhibitors on RNase A were measured spectrophotometrically with 60 μM cytidylyl-3′,5′-guanosine as substrate [20,21]. Values of k_{cat}/K_m for ANG (10 μM) were determined with cytidylyl-3′,5′-adenosine (100 μM) in an HPLC-based assay [22]. Assays were performed in duplicate, typically with 4–6 inhibitor concentrations, and K_i values were obtained by fitting the data to the equation $(k_{cat}/K_m)_i = (k_{cat}/K_m)_0/(1 + [I]/K_i)$ with SigmaPlot, where $(k_{cat}/K_m)_i$ and $(k_{cat}/K_m)_0$ are values measured in the presence and absence of inhibitor, respectively.

Results and discussion

Inhibition of RNase A

The crystal structures of the RNase A complexes with ppA-3′-p and ppA-2′-p revealed a binding mode strikingly different from that expected on the basis of earlier structures of RNase A complexes [23]. In these structures, the 5′-β-phosphate of the inhibitor, rather than the 5′-α-phosphate, occupies the P_1 catalytic subsite and the adenosine adopts a syn, rather than the usual anti, conformation (Fig. 2). This suggested that a pyrimidine nucleoside added to the 5′-β-phosphate through its 3′ oxygen might bind in the same manner as the pyrimidine moiety of substrates, as was subsequently confirmed by X-ray crystallography [21,24]. Such adducts (e.g., dUppA-3′-p and its 5′-phospho derivative) were shown to bind 2- to 9-fold more tightly than ppA-3′-p [16].

In contrast to these 3′,5′-pyrophosphate-linked nucleotides, the NAD^+/NADP family of dinucleotides contains a 5′,5′-pyrophosphate linkage (Fig. 1). The separation between the nicotinamide group and the β-phosphate of the adenosine is therefore one carbon greater than that between the corresponding groups in

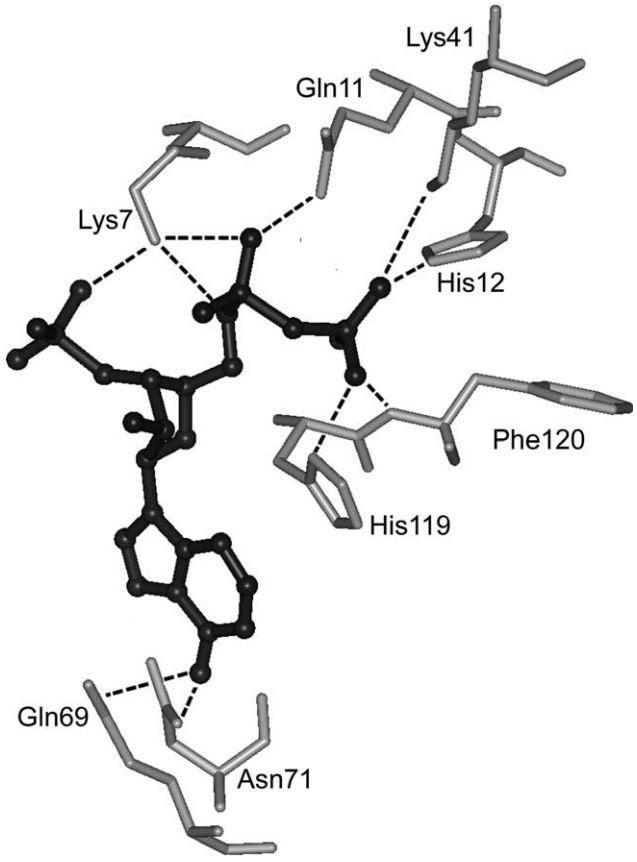

Fig. 2. Crystal structure of the complex of RNase A with ppA-3′-p showing intermolecular interactions. RNase residues are shown in gray and the inhibitor is shown in black. Hydrogen bonds are indicated by dashed lines. Drawn with Insight II (Accelrys).

the dUppA series, and the geometrical relationship also differs. Moreover, nicotinamide lacks both of the substituents of pyrimidines (O2 and N3) that hydrogen bond with RNase A. Consequently, the nicotinamide cannot substitute functionally for a pyrimidine and any interactions it might form with RNase A would be unique. Modeling of the NADPH complex from the crystal structure of the RNase A complex with ppA-2′-p (performed with the program Quanta; Accelrys, San Diego, CA) suggests several possible binding modes for the nicotinamide, all of which position it outside the pyrimidine site.

Testing of $NADP^+$ and NADPH shows that both are moderately effective against RNase A, with K_i values of 64 and 11.8 μM, respectively (Table 1). These values are 270- and 49-fold, respectively, higher than for ppA-2′-p. Some of this decrease in affinity may reflect the lower charge on the β-phosphate or less optimal interactions of this phosphate now that it is involved in an ester linkage (see [16]). It may also result from conformational adjustments of the nicotinamide nucleoside and/or the enzyme to avert steric clashes. The several-fold disadvantage of $NADP^+$ over NADPH may be due to the positive charge on the pyridine nitrogen in $NADP^+$, which is probably unfavorable in an active site designed to accommodate anions, or to less favorable interactions of the planar nicotinamide ring with RNase A.

Table 1
K_i values (μM) for inhibitors of RNase A, EDN, and ANG[a]

Inhibitor	RNase A	EDN	ANG
ppA-2′-p	0.52[b]	—	110[c]
$NADP^+$	64 ± 29	100 ± 8	387 ± 36
NADPH	11.8 ± 0.5	25 ± 3	160 ± 14
ATP	0.86 ± 0.04	20.7 ± 3.1	800[d]
Ap_3A	29 ± 6	70 ± 2	1020 ± 140
Ap_4A	2.6 ± 0.3	1.88 ± 0.46	186 ± 12
Ap_5A	0.23 ± 0.03	0.37 ± 0.06	105 ± 6

[a] All values were measured in 0.2 M Mes (pH 5.9) at 25 °C.
[b] From [14].
[c] From [13].
[d] From [15].

5′-ADP was shown previously to inhibit RNase A with a K_i of 1.2 μM [14]. We now find that 5′-ATP binds somewhat more tightly ($K_i = 0.86$ μM; Table 1), but that further extension of this molecule by attachment of a 5′-linked adenosine (to generate Ap_3A; Fig. 1) reduces affinity by 34-fold. Potency is largely restored by insertion of an additional phosphate into the internucleotide linker (to generate Ap_4A), and when the polyphosphate linker length is increased to five (generating Ap_5A), the resultant affinity ($K_i = 0.23$ μM) surpasses that of 5′-ATP, and is indistinguishable from that of ppA-3′-p. The correlation between improved K_i values and polyphosphate length may reflect multiple factors, including hydrogen bonds or Coulombic interactions of the added phosphate groups, and more favorable contacts of the adenosine as linker size grows.

Inhibition of EDN

Inhibition of EDN by nucleotides has not been studied as extensively as that of RNase A. The K_i values for ppA-3′-p and its 5′-phospho 2′-deoxyuridine derivative with natural EDN (0.25 and 0.18 μM, respectively; [13,16]) are in the same range as for RNase A, but three-dimensional structures have not been reported for these inhibitor complexes and it is not known if the binding modes are also similar. Some RNase A residues that form important interactions with the inhibitors are not conserved in EDN, indicating that the inhibitor cannot interact with the two enzymes in precisely the same manner. Moreover, the subsites near P_1 in EDN contain potential interaction partners for the inhibitors that are not present in RNase A. Crystal structures have been determined for the complexes of recombinant EDN with three nucleotides that bind much less tightly than ppA-3′-p: i.e., 3′,5′-ADP ($K_i = 32$ μM), 2′,5′-ADP ($K_i = 64$ μM), and 5′-ADP ($K_i = 92$ μM) [25]. These structures reveal considerable variability with respect to how the adenine ring is positioned and which phosphate (5′ vs. 3′ or 2′) occupies

the P_1 subsite. Thus, it is difficult to predict how any of the endogenous adenosine dinucleotides being examined here might interact with EDN.

For the present kinetic determinations, we opted to use recombinant EDN from bacteria so that the data could be directly correlated with results from ongoing crystallographic studies. The recombinant enzyme lacks the extensive N-glycosylation of natural EDN, as well as an unusual C-mannosylation at Trp7, and it contains an additional Met at its N-terminus [25]. The enzymatic activity of this protein is 3-fold lower than that of natural EDN ($k_{cat}/K_m = 6.2 \pm 0.3 \times 10^6$ and $1.8 \pm 0.2 \times 10^7\,M^{-1}\,s^{-1}$, respectively); this may reflect the close proximity of Trp7 and the N-terminus to the active site.

The K_i value for $NADP^+$ with EDN is only slightly higher than that with RNase A, and, as with RNase A, NADPH is several-fold more effective (Table 1). These similarities suggest that the modes of binding to the two RNases are analogous. In contrast with $NADP^+$ and NADPH, ATP binds much less tightly to EDN than to RNase A ($K_i = 20.7$ vs. $0.86\,\mu M$; Table 1). As with RNase A, Ap_3A is less effective than ATP, although in this case the difference is not as dramatic, and thus the K_i value for Ap_3A with EDN is only 2.4-fold higher than with RNase A. When additional phosphates are added to the polyphosphate linker, affinity improves in much the same way as with RNase A and the K_i values become similar. The value for Ap_5A, $0.37\,\mu M$, approaches those for the best previous nucleotide inhibitors. Re-testing of Ap_5A with natural EDN from urine yielded an identical K_i value, suggesting that the greater enzymatic efficiency of natural vs. recombinant EDN reflects differences in catalysis rather than binding. The similarity of the effects on EDN and RNase A binding for the progression from $n = 3$–5 in Ap_nA may signify that the interaction modes are comparable.

Inhibition of angiogenin

The affinity of human ANG for all nucleotides tested previously is ~100-fold to >10,000-fold lower than that of RNase A [13–16,26]. Structural and mutational data indicate that several factors contribute to weaker binding, including the blockage of the pyrimidine site and a poorly developed adenine-binding site (see [27]). Although crystal structures of ANG complexes with phosphate and pyrophosphate have been reported [28], ANG–nucleotide complexes have not proved amenable to crystallographic study because of unfortunate contacts with neighboring molecules in all crystal forms of ANG grown to date. The most effective nucleotide inhibitors reported thus far are ppA-2′-p and its 2′-deoxyuridine adduct dUppA-2′-p [13,15], with K_i values of 110 and $150\,\mu M$, respectively.

We find that $NADP^+$ and NADPH bind to ANG only 5- to 14-fold less tightly than to RNase A and EDN. The K_i for NADPH (Table 1) is in fact similar to those of ppA-2′-p and dUppA-2′-p, in marked contrast with results obtained for RNase A, where the nicotinamide nucleosides had a strongly negative impact on binding. Several available compounds related to NADPH were tested to explore the basis for the relatively high efficacy of this dinucleotide with ANG. NAD^+ and NADH were considerably less potent (K_i values were 3.8 and 1.2 mM, respectively), consistent with earlier findings for dUppA and dUppA-2′-p. However, the magnitude of this difference is much smaller (~8-fold vs. 55-fold), indicating that the binding modes for these dinucleotides may not be the same. Replacement of the amide group in $NADP^+$ by an acetyl group or a carboxylate improves the K_i value from 387 to $140\,\mu M$ and $250\,\mu M$, respectively. Addition of a $1,N^6$-etheno group to the adenine moiety in $NADP^+$, thereby generating a third ring, does not change affinity significantly ($K_i = 362\,\mu M$).

5′-ATP was shown previously to inhibit ANG with a K_i value of $800\,\mu M$ [15]. As with both RNase A and EDN, Ap_3A is less effective and binding strength improves as the length of the polyphosphate linker increases (Table 1). However, these improvements are much less extensive than with the other enzymes. Ap_5A ($K_i = 105\,\mu M$) is equivalent to the best previous inhibitor of ANG, ppA-2′-p.

Potency of NADPH, ATP, and Ap_5A under physiological conditions

Pancreatic RNase superfamily proteins are normally secreted into the extracellular space or sequestered into organelles, but small amounts may gain entry to the cytosol and would be highly toxic unless they are efficiently neutralized. The major cytosolic agent that affords protection against these enzymes is thought to be the RNase inhibitor protein (see [29] for a review). The effectiveness of some of the endogenous nucleotides reported here raises the possibility that these molecules might also play a role. The K_i values listed in Table 1 for inhibition of RNase A and EDN by NADPH, ATP, and Ap_5A under our standard RNase assay conditions (pH 5.9, ionic strength ~78 mM) are all well below typical bulk cytosolic concentrations of these nucleotides (e.g., ~200 μM [30], ~1 mM [31], and 7–200 μM [32,33], respectively). To investigate the in vivo relevance of these interactions, we also measured inhibition in a physiological buffer (20 mM Hepes–NaOH, 140 mM KCl, pH 7.2). The enzymatic activity of RNase A and binding of previous nucleotide inhibitors are known to become considerably weaker at pH values and ionic strengths higher than those in the standard buffer (see [13,34] and references therein).

We find that 200 μM NADPH inhibits RNase A activity only by 30% in the pH 7.2 buffer (Table 2); a full

Table 2
Inhibition of RNase A by NADPH, ATP, and Ap_5A at pH 7.2[a]

Inhibitor	[Inhibitor] (μM)	[Mg^{2+}] (mM)[b]	Inhibition (%)
NADPH	200	—	30
ATP	1000	—	84
	1000	2	40
Ap_5A	200	—	49
	200	1	21

[a] Buffer: 20 mM Hepes–NaOH, 140 mM KCl.
[b] Total Mg^{2+}, added as $MgCl_2$.

inhibition plot yields a K_i value of 476 μM, i.e., ~40-fold higher than under standard conditions. With ATP and Ap_5A, the assay buffer was supplemented with Mg^{2+}, which binds tightly to these nucleotides ($K_D \sim 10\,\mu M$ in both cases [35]) and is normally present in the cytosol at a concentration of 0.5–1 mM for the free cation [36]. One millimolar ATP inhibited RNase A by 40% in the presence of 2 mM total Mg^{2+} (~1 mM free Mg^{2+}); when no Mg^{2+} was added, 1 mM ATP decreased RNase A activity by 84%. With 200 μM Ap_5A (i.e., a concentration at the high end of the range observed) and 1 mM total Mg^{2+}, only 21% inhibition was measured, vs. 49% when Mg^{2+} was not added.

Conclusions

The endogenous dinucleotides NADPH, ATP, Ap_5A, and Ap_4A have been shown here to inhibit the enzymatic activities of RNase A and EDN with K_i values ranging from 230 nM to 25 μM under standard RNase assay conditions. Ap_5A is almost as effective as the best small nucleotide antagonist of EDN identified previously and ranks among the strongest low molecular weight inhibitors of RNase A as well. Although NADPH and Ap_5A bind less tightly to ANG, they are nonetheless equivalent to the most potent compounds reported earlier, ppA-2′-p and dUppA-2′-p. Moreover, NADPH (and $NADP^+$) show much smaller preferences for RNase A vs. ANG than all other nucleotide inhibitors tested to date. These findings suggest that Ap_5A and, in the case of ANG, NADPH may serve as useful new leads for the design of tighter-binding inhibitors.

Examination of inhibition by NADPH, ATP, and Ap_5A under physiological conditions suggests that these compounds together may be capable of suppressing the activity of pancreatic RNase superfamily enzymes in the bulk cytosol by up to several-fold. In certain locales in vivo, inhibition may be even stronger. Total or local concentrations of these nucleotides in the cytosol of some cells and in secretory granules or other organelles can be much higher (e.g., see [33]). Moreover, inhibition may be much more marked at the lower pHs often present in these organelles.

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