Evaluation of 3-deaza-adenosine analogues as ligands for adenosine kinase and inhibitors of Mycobacterium tuberculosis growth

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Objectives: Analyse a series of halogenated 3-deaza-adenosine analogues for efficacy against Mycobacterium tuberculosis H37Ra and determine if adenosine (Ado) kinase plays a role in the mechanism of action of these compounds.

Methods: The MIC as determined by microdilution broth assay provided a measure of antitubercular efficacy. MIC values were measured in M. tuberculosis strains H37Ra, SRICK1 (an Ado kinase-deficient strain of M. tuberculosis derived from H37Ra) and SRICK1 complemented with adoK, the gene which codes for Ado kinase in M. tuberculosis, in order to determine if Ado kinase played a role in the mechanism of action of these compounds. Furthermore, each compound was analysed as both a substrate and inhibitor for purified Ado kinases from M. tuberculosis and human sources.

Results: 2-Fluoro-3-deaza-adenosine, 3-fluoro-3-deaza-adenosine and 2,3-difluoro-3-deaza-adenosine exhibited antitubercular activity that was Ado kinase-dependent. Furthermore, these compounds were at least 10-fold better substrates for M. tuberculosis Ado kinase than the human homologue.

Conclusions: The Ado kinase-dependent antitubercular activity exhibited by several of the halogenated 3-deaza-adenosine analogues investigated in this study warrants further investigation of these compounds as antitubercular agents. Furthermore, substrate and inhibition studies provided insight into the Ado-binding domain of Ado kinase, indicating that steric hindrance may limit the size of exocyclic modifications at the 3-position of Ado.

Keywords: nucleoside analogues, structure-activity relationship, purines, antitubercular activity, mechanism of action

Introduction

Worldwide, tuberculosis continues to be a threat to world health, claiming more than two million lives annually.¹–³ Recently, the development of novel antitubercular drugs has been necessitated by the emergence of drug-resistant strains of Mycobacterium tuberculosis.¹–³ The purine salvage pathway is an attractive target for the development of nucleoside analogues as antitubercular drugs. Naturally occurring nucleosides are readily taken up by M. tuberculosis where enzymes in the purine salvage pathway metabolize them to nucleotides, which are utilized in RNA and DNA synthesis as well as acting as cofactors for many biochemical reactions.³ Nucleoside analogues may be useful against drug-resistant strains of M. tuberculosis because they can be similarly metabolized to nucleotides which can be cytotoxic, a mechanism of action that differs from those of currently available antitubercular drugs.

The adenosine (Ado) analogue 2-methyl-Ado (methyl-Ado) has provided proof-of-concept that nucleoside analogues can make effective antitubercular compounds. Methyl-Ado has demonstrated selective antitubercular activity with an MIC of 3 mg/L and an IC₅₀ of 80 mg/L in CEM cells. Methyl-Ado has a mechanism of action that involves phosphorylation to 2-methyl-AMP by Ado kinase.³ In order to further advance the development of nucleoside analogues as antitubercular compounds, a structure-activity relationship (SAR) was previously performed to study the

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Figure 1. Structure and numbering convention for 3-deaza-adenosine.

active site of *M. tuberculosis* Ado kinase. These studies indicated that 2-fluoro-3-deaza-Ado was a much better substrate for *M. tuberculosis* Ado kinase than 3-deaza-Ado (deaza-Ado), and that neither compound was a substrate for the human Ado kinase. 

The selective phosphorylation of this compound provided the rationale for the current *in vitro* study.

The parent compound in this work, deaza-Ado (Figure 1), has been extensively studied, demonstrating antiviral, antimarial, antibacterial, immunosuppressive and anti-inflammatory activities. The mechanism of action of deaza-Ado involves potent inhibition of S-adenosylhomocysteine hydrolase (AdoHcy hydrolase) and methylation of membrane phospholipids. Although deaza-Ado is an Ado analogue, it is not a substrate for either mammalian Ado kinase or Ado deaminase. Deaza-Ado is relatively non-cytotoxic, with antiviral activity occurring at concentrations where no cytotoxicity can be detected. Like deaza-Ado, the halogen-substituted analogues investigated in this study exhibited only moderate cytotoxicity. In the present study, six halogen-substituted analogues of deaza-Ado have been analysed for antitubercular activity and as both substrates and inhibitors of *M. tuberculosis* and human Ado kinases.

Materials and methods

Nucleoside analogues

The nucleoside analogues utilized in this work were synthesized as previously described.

Ado kinase

*Mycobacterium smegmatis* strain SRI101-pVV16/adoK, which contains the *M. tuberculosis* Ado kinase gene cloned into an Ado kinase-deficient strain of *M. smegmatis*, was used as a source of *M. tuberculosis* Ado kinase. The human Ado kinase clone 20-1 was generously provided by Dr Jozef Spychala (UNC Chapel Hill, Chapel Hill, NC, USA). Protein extracts were prepared and Ado kinases were purified as previously described.

Substrate assays

Nucleosides were assayed as substrates for *M. tuberculosis* Ado kinase, and reaction products were detected by HPLC. Assay conditions consisted of 50 mM Tris–HCl (pH 8.0), 10 mM KCl, 10 mM MgCl$_2$, 5 mM ATP, 0.01% BSA, 10 μM deoxycoformycin and 100 μM of the appropriate test compound. Human Ado kinase was assayed similarly with the following changes: assay conditions consisted of 50 mM HEPES (pH 6.0), 40 mM KCl, 1 mM MgCl$_2$, 1 mM ATP, 0.1% BSA, 10 μM deoxycoformycin and 100 μM of the appropriate test compound. Reactions proceeded at 37°C and were terminated by the addition of an equal volume of 1 M perchloric acid. Following neutralization, reactants and products were separated by HPLC using a Bio Basic anion exchange column (Thermo Electron Corp., Bellefonte, PA, USA) with a 30 min linear salt and pH gradient from 6 mM ammonium phosphate (pH 2.8) to 900 mM ammonium phosphate (pH 6). Peaks were detected as they eluted from the column by absorbance at 260 nm. All enzyme reactions were linear during the incubation period and substrate conversions were maintained at <10%.

Ado kinase inhibition assays

Assay mixtures were identical to those performed for HPLC analysis with the addition of 0.1 μM [H]$^3$Ado (4 μCi/mL) and 100 μM of the test compound. Reactions were started by the addition of enzyme, incubated for 1 h at 37°C, and stopped by the addition of 10 μL of 0.1 M EDTA. Aliquots of the reaction were applied to a DE-81 cellulose disc, dried, washed three times with 1 mM ammonium acetate (pH 5.0), rinsed with 95% ethanol and dried. Filter discs were transferred to scintillation vials with 10 mL of Complete Counting Cocktail (Research Products International, Mount Prospect, IL, USA), and radioactivity was detected with a Packard Tri-Carb model 1900 TR liquid scintillation analyser. Phosphorylation of [H]$^3$Ado was quantified by the amount of [H]AMP bound to a DE-81 cellulose disc following the reaction.

Determination of MIC

MIC values were determined in *M. tuberculosis* strains H37Ra, an Ado kinase-deficient strain derived from *M. tuberculosis* H37Ra (SRICK1) and SRICK1 complemented with the adoK gene (SRICK1::adoK) using a colorimetric microdilution broth assay as previously described. *M. tuberculosis* H37Ra is a strain that is attenuated for growth *in vivo* that was derived from the virulent H37Rv strain and is frequently utilized as a model for H37Rv. The genetic differences between these two strains are limited to mutations in several virulence genes arising from insertion sequences and random mutation. None of these genes is presumed to be involved in the mechanism of action of the nucleoside analogues represented in this study.

Results and discussion

In MIC assays, three compounds, 2-fluoro-3-deaza-Ado (3, Table 1), 3-fluoro-3-deaza-Ado (4, Table 1) and 2,3-difluoro-3-deaza-Ado (5, Table 1), were active against H37Ra. All three compounds were not active against SRICK1; however, activity was restored in SRICK1::adoK, indicating that the mechanism of action of these agents involved activation by Ado kinase. Furthermore, 2,3-difluoro-3-deaza-7-iso-Ado (6, Table 1) and 2-fluoro-3-deaza-N6-methyl-Ado (7, Table 1) exhibited activity against SRICK1::adoK, but not against H37Ra, indicating that overexpression of Ado kinase in SRICK1::adoK compensated for the poor substrate activity of these compounds, permitting them to be phosphorylated to toxic metabolites.
None of the compounds evaluated in this study had measurable antimycobacterial activity at 100 μg/mL in SRICK1, the Ado kinase-deficient strain of *M. tuberculosis*. This concentration was higher than the levels where antiviral activity and cytotoxicity were measured in previous studies and exceeded the level achieved with 2-fluoro-3-deaza-Ado in *in vivo* studies. Lack of antitubercular activity in SRICK1 at this high concentration of compound indicated that the mechanism of action of these compounds was Ado kinase-dependent and was not due to inhibition of AdoHcy hydrolase in *M. tuberculosis*. The mechanism of antiviral action of 3-deaza-Ado and some of its analogues involves inhibition of AdoHcy hydrolase and may also involve Ado-kinase-independent phosphorylation to active nucleotides. Since the compounds that we examined demonstrated Ado kinase-dependent phosphorylation and were not active in bacteria lacking Ado kinase, but were active in an Ado kinase-complemented strain, we can conclude that the mechanism of action of these compounds involves Ado kinase and is very different in *M. tuberculosis* than in human cells.

In this series, the best substrates for *M. tuberculosis* Ado kinase were 2,3-difluoro-3-deaza-Ado (5), 2-fluoro-3-deaza-Ado (3) and 3-fluoro-3-deaza-Ado (4), with specific activities of 81, 63 and 16 nmol/mg/min, respectively. For human Ado kinase, the best substrates were 2,3-difluoro-3-deaza-Ado (5), 2,3-difluoro-3-deaza-7-iso-Ado (6) and 3-fluoro-3-deaza-Ado (4), with specific activities of 6, 5 and 1 nmol/mg/min, respectively. With the exception of 2,3-difluoro-3-deaza-7-iso-Ado, compounds that were substrates for *M. tuberculosis* Ado kinase were less active with human Ado kinase. This finding is consistent with the lack of activity of deaza-Ado with mammalian Ado kinase.

2-Fluoro-3-deaza-Ado (3) was the most selective substrate for *M. tuberculosis* Ado kinase and the most promising compound in this series in terms of antimycobacterial activity, with an MIC similar to that of ethambutol (between 1 and 10 μg/mL). Previous work demonstrated that 2-fluoro-3-deaza-Ado was at best moderately cytotoxic to L1210, P388, CCRF-CEM and B16F10 cell lines with EC_{50} values of 35, 50, 100 and 40 μM, respectively. The *in vitro* activity of 2-fluoro-3-deaza-Ado (3) against *M. tuberculosis* H37Rv was also confirmed by the Tuberculosis Antimicrobial Acquisition & Coordinating Facility (TAACF), who reported an MIC of 1.6 μg/mL and a 50% inhibitory concentration (IC_{50}) against VERO cells of 24 mg/mL (A. C. Sartorelli, unpublished data). The TAACF also reported that an *in vivo* dose in mice by oral gavage of 300 mg/kg led to a serum concentration of 44 mg/L, a concentration above the measured MIC. That study concluded that in mice, oral administration of 300 mg/kg of 2-fluoro-3-deaza-Ado (3) yielded clear activity in the lung and moderate activity in the spleen (A. C. Sartorelli, unpublished data). Although 2,3-difluoro-3-deaza-Ado (5) was a slightly better substrate for *M. tuberculosis* Ado kinase and exhibited antimycobacterial activity, it was not as potent as 2-fluoro-3-deaza-Ado (3) in MIC assays. These findings suggest that the nucleotide metabolites of 2,3-difluoro-3-deaza-Ado (5) were less potent inhibitors of an unidentified downstream target(s).

The discontinuity between the ability of a compound to serve as a substrate for Ado kinase and its antimycobacterial activity is due to the prodrug nature of nucleoside analogues. For a compound to exert antimycobacterial activity, its phosphorylated metabolite(s) must be able to disrupt an essential downstream process. Therefore, the metabolites of the compound must be good inhibitors of the downstream target, even though the parent nucleoside may not be a particularly good substrate for Ado kinase. For this reason, even poor substrates for Ado kinase can still exhibit potent antimycobacterial activity, while requiring anabolism by Ado kinase.

None of the compounds in this series was a potent inhibitor of *M. tuberculosis* or human Ado kinases. The agent that demonstrated the most inhibition of *M. tuberculosis* Ado kinase was 3-chloro-3-deaza-Ado (8, Table 1). This activity provided an interesting insight into the active site of the enzyme, as little was previously known about the effects of exocyclic substitutions at the 3-position of Ado on Ado kinase activity. The difference in

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**Table 1.** Activities of 3-deaza-adenosine analogues as substrates and inhibitors of *M. tuberculosis* adenosine kinase and their antitubercular activity

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Ado kinase substrate specific activitya (nmol/mg/min)</th>
<th>Inhibition of Ado kinaseb (% of control)</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. tuberculosis</em> human</td>
<td><em>M. tuberculosis</em> human</td>
<td>H37RA</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>ND</td>
<td>ND</td>
<td>++</td>
</tr>
<tr>
<td>1. Adenosine (Ado)</td>
<td>3700 ± 500</td>
<td>2700 ± 100</td>
<td>–</td>
</tr>
<tr>
<td>2. 3-Deaza-Ado</td>
<td>1 ± 0.05</td>
<td>&lt;0.5</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>3. 2-Fluoro-3-deaza-Ado</td>
<td>63 ± 6</td>
<td>&lt;0.5</td>
<td>84 ± 4</td>
</tr>
<tr>
<td>4. 3-Fluoro-3-deaza-Ado</td>
<td>16 ± 2</td>
<td>1 ± 0.8</td>
<td>110 ± 5</td>
</tr>
<tr>
<td>5. 2,3-Difluoro-3-deaza-Ado</td>
<td>81 ± 11</td>
<td>6 ± 1.0</td>
<td>96 ± 5</td>
</tr>
<tr>
<td>6. 2,3-Difluoro-3-deaza-7-iso-Ado</td>
<td>1 ± 0.7</td>
<td>5 ± 0.4</td>
<td>103 ± 10</td>
</tr>
<tr>
<td>7. 2-Fluoro-3-deaza-N³-methyl-Ado</td>
<td>&lt;0.1</td>
<td>&lt;0.5</td>
<td>63 ± 8</td>
</tr>
<tr>
<td>8. 3-Chloro-3-deaza-Ado</td>
<td>&lt;0.2</td>
<td>&lt;0.5</td>
<td>29 ± 2</td>
</tr>
</tbody>
</table>

ND, not determined.

a Specific activity was measured with 100 μM of each compound.

b Inhibition values are the percentage of control when test compounds were used to inhibit the phosphorylation of 0.1 μM Ado. Values shown were determined with 100 μM of the indicated compound.

MIC values are denoted as follows: –, MIC > 100 mg/mL; +, MIC between 10 and 100 mg/mL; ++, MIC between 1 and 10 mg/mL.
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substrate activity between 3-fluoro-3-deaza-Ado (4) and 3-chloro-3-deaza-Ado (8) (16 and <0.2 mmol/mg/min, respectively) implied that steric hindrance may exclude exocyclic modifications as large as a chloro group at the 3-position of Ado. However, measurement of the inhibitory effects of these agents seemingly contradict this hypothesis, since it is likely that 3-chloro-3-deaza-Ado (8) binds to the active site of the enzyme in order to inhibit activity. Since the active site binds both Ado and ATP, it is possible that 3-chloro-3-deaza-Ado (8) interacts with the ATP-binding domain, thereby acting as a competitive inhibitor of ATP, or that 3-chloro-3-deaza-Ado (8) binds more tightly to the Ado-binding domain than 3-fluoro-3-deaza-Ado (4) and that this interaction makes it a better inhibitor than a substrate for this enzyme. These results indicate that a nucleophilic substitution is preferred at the 3-position of deaza-Ado, possibly to act as a hydrogen bond acceptor for substrate recognition, and that increasing the size of the nucleophile beyond the size of a fluorine atom increases the chance that the compound will be an inhibitor rather than a substrate.

Differences in Ado kinase substrate specificity in *M. tuberculosis* and human tissues provide a unique opportunity to develop antitubercular drugs with a novel mechanism of action. Thus, the present report highlights two important findings for the development of nucleoside analogues that are activated by Ado kinase. First, a 2-fluoro group can improve the activity of an Ado analogue as a substrate for Ado kinase, as evidenced by 3-deaza-Ado (2) versus 2-fluoro-3-deaza-Ado (3) and 3-fluoro-3-deaza-Ado (4) versus 2,3-difluoro-3-deaza-Ado (5). Second, exocyclic modifications at the 3-position should preserve the ability to act as hydrogen bond acceptors for substrate recognition and may be limited by the size of the substituent. The activity of 2-fluoro-3-deaza-Ado (3) provides promise that halogenated 3-deaza-Ado analogues may be developed as antimycobacterial drugs.

Acknowledgements

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Transparency declarations

None to declare.

References