Antimycobacterial activity of 2-methyl-adenosine

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Objectives: The aims of this study were to assess the in vitro activity of 2-methyl-adenosine against Mycobacterium tuberculosis and evaluate, and to intracellular efficacy, and to evaluate its effectiveness against M. tuberculosis in a persistent state model and examine its potential mechanism of action.

Methods: In vitro activity was determined by means of a colorimetric microdilution broth assay. Intracellular activity was assessed with a Mono Mac 6 human monocytic cell line. A hypoxic shift-down model was used to evaluate the effect of 2-methyl-adenosine on M. tuberculosis in a persistent state. Mechanism-of-action studies were conducted by examining the effect of 2-methyl-adenosine on the uptake of appropriate radio-labelled precursors into respective mycobacterial macromolecular components.

Results: Studies confirmed the in vitro activity of 2-methyl-adenosine against M. tuberculosis and demonstrated intracellular efficacy against M. tuberculosis within macrophages. 2-Methyl-adenosine was able to significantly affect the viability of M. tuberculosis in a hypoxic shift-down model previously described to simulate the persistent state that results during tuberculosis. Mechanism-of-action studies revealed that the immediate inhibitory effects of 2-methyl-adenosine were associated with protein and DNA synthesis and not RNA synthesis.

Conclusions: Results indicate that 2-methyl-adenosine, or similar derivatives, might be effective against M. tuberculosis infections during latency. This information should be helpful in understanding purine metabolism of M. tuberculosis and also the metabolic activity of this important human pathogen in the persistent state.

Keywords: mycobacterium, nucleoside analogues, MICs

Introduction

Although several drugs are effective against Mycobacterium tuberculosis, no new class of antimycobacterials has been developed for more than 30 years.1 With the increasing numbers of drug-resistant strains of M. tuberculosis that are appearing in the clinics and general population, it becomes apparent that new classes of antimycobacterial agents are needed in order to control, and potentially eradicate, a serious disease that has afflicted the human race for many centuries.

As the result of the NIH-sponsored Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) Program, many potentially useful compounds are being screened against M. tuberculosis (http://www.taacf.org). Although some of these compounds appear to be highly effective against M. tuberculosis, their exact mechanism of action is unknown. One of the better agents in terms of potency and selectivity that was identified by the TAACF from the compounds submitted by the Southern Research Institute was 2-methyl-adenosine (Figure 1). Many years ago other purine analogues had been shown to have activity against M. tuberculosis.2 These previous results along with our new results indicate that the evaluation of purine analogues is a promising area for the development of useful new drugs to treat this disease. Therefore, additional quantities of 2-methyl-adenosine were synthesized in order to confirm the results and to attempt to determine its general mode of action. This information will be useful because it will increase our knowledge of purine biosynthesis in mycobacteria and because it may lead to the development of new and improved antituberculous compounds. It should be noted that although purine analogues have been evaluated in the past,

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an extensive search of the literature revealed that 2-methyl-adenosine has never been tested against *M. tuberculosis*.

Recently, we demonstrated that 2-methyl-adenosine shows activity against *Mycobacterium smegmatis* and that activity probably results from the compound’s phosphorylation to methyl adenosine nucleotides and incorporation into RNA. In order to further examine its activity against *M. tuberculosis*, we have conducted the following investigation to ascertain the compound’s effect on *M. tuberculosis* by using the avirulent *M. tuberculosis*-H37Ra strain. This was done primarily to facilitate studies by precluding the use of BSL-3 facilities and thus expediting preliminary studies that will lead to the discovery of the drug target. In this study, we tested the *in vitro* activity of 2-methyl-adenosine and evaluated its intracellular activity against *M. tuberculosis*-H37Ra, using a human monocytic cell line previously described for utilization in mycobacterial drug testing. The compound was further tested for activity against *M. tuberculosis*-H37Ra in a hypoxic shift-down model, previously described by Wayne and co-workers for use in drug testing against *M. tuberculosis* incubated under conditions to simulate the persistent state. Additional studies were conducted to determine the general mechanism of action by examining the inhibition of radiolabel incorporation into macromolecular constituents.

**Materials and methods**

**Minimal inhibitory concentration (MIC)**

MICs for *M. tuberculosis*-H37Ra (ATCC 25177) were determined in our laboratories using a colorimetric microdilution broth assay reported previously. This method has been shown to be comparable to the Bactec-460 and is currently used by the NIH-sponsored TAACF Program to determine the MIC of compounds for *M. tuberculosis* (http://www.taacf.org). A frozen culture in 7H9 broth (Difco Laboratories, Detroit, MI, USA), supplemented with albumin-dextrose-catalase (ADC) enrichment (Difco) and 0.2% glycerol was thawed and diluted in broth to about 2 × 10⁶ cfu/mL and used as the inoculum. The assay used a 96-well (U-shaped) microtitre plate and a format designed to accommodate four compounds in seven two-fold dilutions. The assay plates also contained uninoculated drug and medium controls, and viability controls. Each test compound was dissolved in dimethyl sulphoxide (DMSO), and then diluted in broth at twice the desired drug concentration and 0.05 mL added to duplicate assay wells. The highest concentration of DMSO in the assay medium, 1.3%, did not affect growth. Each plate was inoculated with 0.05 mL of standardized culture and incubated at 37°C for 6 days. The REDOX indicator Alamar Blue (Acumin International, Inc., OH, USA) was added to each well as a mixture with Tween 80 and the plates incubated for an additional 18–22 h. The plates were read in an optical microtitre plate reader programmed to subtract the absorbance at 600 nm from that at 570 nm to blank out turbidity and absorbance as a result of oxidized dye. The MIC was reported as the lowest concentration of drug yielding a differential absorbance of zero or less. This approximated the colour change from blue to red that was observed visually after metabolic reduction of dyes, and represented the concentration at which no visible growth occurred. Ethambutol was used as a positive control and had an MIC range of 2–4 mg/L.

**Intracellular activity in Mono Mac 6**

The Mono Mac 6 cell line (MM6) was obtained from the German Collection of Microorganisms, Braunschweig, Germany, originally established by H. W. L. Ziegler-Heitbrock (University of Munich), and is a human acute monocytic leukaemia cell line. MM6 has been used to examine the effectiveness of antimycobacterial compounds against intracellularly replicating *M. tuberculosis*. MM6 cells were maintained in RPMI-1640 containing 10% (v/v) fetal calf serum, 2 mM L-glutamine, non-essential amino acids, 1 mM sodium pyruvic acid, and 9 µg of bovine insulin (Sigma, MO, USA) per mL (MM6 medium). Absence of mycoplasma was established with the Gen-Probe Mycoplasma Rapid Detection System (Gen-Probe, CA, USA). Viability was determined by an MTT cytotoxicity assay (Sigma), modified previously, and reported as a percentage of the non-treated cells. The LD₅₀ was determined by exposing MM6 cells to varying concentrations of 2-methyl-adenosine and plotting the % inhibition versus the log of the drug concentration.

Before infection of MM6 cells, *M. tuberculosis*-H37Ra was grown in Middlebrook 7H9 (Difco) containing 0.05% Tween 80 (Sigma) and 10% ADC (Difco). After reaching exponential phase, mycobacteria were dispersed by vortexing with glass beads and clumps were allowed to settle for 30 min. Superantigen was removed, aliquotted, frozen at −70°C, then thawed and used for infection by resuspension in appropriate culture medium. Actual cfu/mL were determined by preparation of serial dilutions in Dulbecco’s PBS (DPBS, Mediatech, Inc., Herndon, VA, USA) and plating on 7H10 agar.

Before infection, MM6 cells were adjusted to 8 × 10⁵ cells/mL in regular growth medium but with only 1% calf serum; 0.5 mL per well was dispensed in 12-well tissue culture dishes (Corning Costar, MA, USA). Mycobacteria were suspended in RPMI-1640 with 1% fetal calf serum, and the suspension was added to the MM6 cells to achieve a final ratio of 10 mycobacteria/macrophage, with a density of 4 × 10⁵ MM6 cells/1.0 mL well. After 4 h of infection, MM6 cells were collected by centrifugation (200g) and washed twice with DPBS to remove any unphagocytosed mycobacteria. MM6 cells were then re-plated at a density of 4 × 10⁶ cells/1.0 mL/well and incubated at 37°C with 5% carbon dioxide. One milliliter of fresh medium was added at day 4, and infection continued until cfu assay.

**Determination of cfu**

CFU assessment at 0 h was achieved by lysing MM6 cells with 0.25% (w/v) sodium dodecyl sulphate (SDS) in DPBS, then plating serial dilutions onto 7H10 agar plates. To decrease viscosity, 5 µL (5 U of activity) of RQ DNase (Promega, WI, USA), with MgSO₄ (5 mM), was added to each well following addition of SDS, and plates were incubated at 37°C for 20 min. The plating procedure was repeated at 7 days and cfu enumerated after 10–14 days of incubation.

**Macromolecular assays of inhibitory activity**

Procedures were designed to determine inhibitory activity by observing incorporation of radiolabelled precursors into respective macromolecular components. All procedures were developed as the result of extensive
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preliminary studies designed to evaluate immediate effects of 2-methyl-adenosine on H37Ra metabolism; time frames were based on former reports. For RNA synthesis, studies by Harshey & Ramakrishnan were used, for DNA synthesis, studies by Hiroyama & Ramakrishnan were used, and for protein synthesis, studies by Shailla et al. were used. Procedures were also based upon the time necessary for \( M. tuberculosis \) to replicate genomic DNA, i.e. 10–11 h. For protein synthesis, \( ^{14} \)Cphenylalanine was used; for RNA and DNA synthesis, incorporation of [\-\( ^{14} \)C]uracil was used. Similar procedures, using incorporation of radio-labelled precursors into TCA-pelletable material to evaluate mechanism of action for a test compound, have been published with other bacteria.

To distinguish between incorporation into RNA and DNA, RNA was hydrolysed before DNA precipitation using KOH. Radio-labelled thymidine was avoided for DNA synthesis owing to the presence of thymidine phosphorylase that hydrolyses the substrate. \(^{11,24}\) and the low thymidine kinase activity in this bacterium. \(^{25}\) To verify DNA was being used, \(^{18}\) and for protein synthesis, studies by Shaila et al. were used, \(^{17}\) for DNA synthesis, studies by Hiriyanna & Ramakrishnan were used, \(^{1,3,8,3}\) and for protein synthesis, \(^{18}\) Polymyxin B was used as a negative control. For positive controls, streptomycin sulphate, rifampicin, and ofloxacin were used for protein, RNA and DNA biosynthesis, respectively. Inhibitors were added to actively growing cultures, following addition of appropriate radio-labelled component. Samples were taken at various time intervals and processed for radiolabel incorporation by precipitation with trichloroacetic acid, followed by measurement of radioactivity in a scintillation counter. Samples were processed in duplicate and results analysed by non-linear regression (curve fit), using a one-phase exponential decay equation (Prism 3.0, GraphPad, CA, USA). Because some compounds required solvents other than water, each experiment included solvent controls to demonstrate lack of inhibitory activity (data not shown). Thus, ethanol controls were used in the case of rifampicin, NaOH was used in the case of ofloxacin, and DMSO was used in the case of 2-methyl-adenosine.

Non-replicating persistence model

To be consistent with our in vitro assay for MIC, we modified the Wayne non-replicating persistence (hypoxic shift-down) model \(^{1,1,2}\) by substituting Dubos with 7H9 (containing 0.05% Tween 80 and 10% ADC) and using \( M. tuberculosis\)-H37Ra. This procedure has also been recently used to study dormancy with \( M. tuberculosis \) in the Wayne assay. \(^{1,1,2}\) Additionally, \( M. tuberculosis \)-H37Ra cultures were set up at \( 10^3–10^6 \) cfu/mL in 12 × 75 mm tubes in a total volume of 3.0 mL (head space ratio, HSR = 0.5). Tubes were sealed with white rubber septa (Sigma) and contained 13 mm × 4 mm diameter stir bars (Fisher, PA, USA). Cultures were incubated in a 37°C incubator on a stir plate. Some cultures also contained Methylene Blue at a concentration of 1.5 mL/L to be used as an indicator of oxygen depletion. \(^{11}\) An additional tube of Methylene Blue without \( M. tuberculosis\)-H37Ra was used as a colour reference. Positive controls consisted of cultures containing metronidazole (Sigma) at 12 mg/L. When Methylene Blue-containing tubes turned colourless, cultures were incubated for an additional week, and then dosed with varying concentrations of 2-methyl-adenosine. At this time, aliquots were removed from each tube using a syringe with a 25-gauge 1.5" needle through the septa. This was done in order to obtain cfu at the time of dosing and also to adjust for addition of drug while retaining an appropriate head space ratio. Drug was also added using a syringe with a 25-gauge needle. Samples were plated on 7H10 agar (Hardy Diagnostics, CA, USA) at \( 10^5, 10^4 \) and \( 10^2 \). This procedure was repeated at 7 days following dosing. All conditions were set up in triplicate and plated in duplicate for cfu. Plates were observed 10 days following inoculation and colonies enumerated on day 18.

Results

In vitro activity of 2-methyl-adenosine against \( M. tuberculosis \) strains and cell lines

The MIC and minimal bactericidal concentration (MBC) of 2-methyl-adenosine were determined to be \( 3.1 \) and \( >100 \) mg/L, respectively, for \( M. tuberculosis\)-H37Rv by the TAACF, using the Bactec 460. Dilutions in that study were 12.5, 6.25, 3.1 mg/L, etc. The MIC and MBC of 2-methyl-adenosine for \( H37Ra \) were determined to be \( 8 \) and \( >64 \) mg/L, respectively, using the microdilution broth assay in our laboratory and dilutions of 64, 32, 16, 8, 4 mg/L, etc., as recommended by the NCCLS. These are comparable values with those obtained in the TAACF, considering the different dilutions used. The TAACF also reported an IC\(_{50}\) of \( >1000 \) mg/L for 2-methyl-adenosine using a monkey kidney cell line (http://www.taacf.org). We tested 2-methyl-adenosine in a CEM cell assay previously described by us, and found the IC\(_{50}\) to be \( 80 \) mg/L, the concentration that inhibits CEM cell growth by \( 50\% \). In addition, 2-methyl-adenosine was tested in mice at 25 mg/kg per day for up to 45 days with no toxicity and with no gross appearance of abnormal tissues (TAACF).

Through the NIH-sponsored TAACF Program, activity of 2-methyl-adenosine was also assessed against the following strains of \( M. tuberculosis \): Erdman (ATCC 35801) \(< 1.56 \) mg/L, isoniazid-resistant (ATCC 35822) \( = 3.13 \) mg/L, rifampicin-resistant (ATCC 35838) \( < 1.56 \) mg/L, ethambutol-resistant (ATCC 35837) \( < 1.56 \) mg/L, kanamycin-resistant (ATCC 35827) \( < 1.56 \) mg/L, and ciprofloxacin-resistant \( = 6.25 \) mg/L. All of these MIC values were determined with the Microplate Alamar Blue Assay (http://www.taacf.org).

Intracellular effect of 2-methyl-adenosine

Before intracellular efficacy evaluation, the LD\(_{50}\) of 2-methyl-adenosine was calculated by exposing the MM6 cells to 1.0, 2.5, 5.0, 10.0 and 50.0 mg/L, then plotting the % inhibition versus the log of the drug concentration. The LD\(_{50}\) for 2-methyl-adenosine was determined to be \( < 50 \) mg/L, with a viability loss of 38.4% at \( 50 \) mg/L. Because our macrophage model utilizes a range up to \( 8 \times \) MIC to evaluate a test compound for intracellular activity, the MIC determined by the TAACF was used as a base value for testing. This allowed the drug to be tested at \( 8 \times \) MIC without concerns regarding cell viability. Using a method similar to that previously reported by us for drug screening in this macrophage model, \(^{4,6}\) concentrations of eight-fold above the MIC (\( 8 \times \) MIC) and eight-fold below the MIC (\( < 8 \times \) MIC) concentration were also evaluated. Thus, H37Ra-infected MM6 cells were treated with 0.4, 3.1 and 25 mg/L of 2-methyl-adenosine. Treatment of H37Ra-infected MM6 cells with 2-methyl-adenosine at MIC and \( +8 \times \) MIC resulted in a decrease in cfu by 7 days, but only the reduction with \( +8 \times \) MIC was significant \(( P = 0.001, n = 3) \) (Figure 2a).

Examination of macrophage viability using the MTA cytotoxicity assay (Sigma) previously described by us, \(^{4,6}\) indicated that viability of 2-methyl-adenosine-treated cells remained at 100% of the non-treated control cells at 7 days. [Note: viabilities for both 7-day non-treated control and 2-methyl-adenosine-treated cells were 81% of...
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Figure 2. Efficacy of 2-methyl-adenosine (MeAdo) versus equivalent doses of rifampicin (RIF) using MM6 cells infected with M. tuberculosis (MTB)-H37Ra. Each point represents the mean and standard error of the mean of triplicate samples. In (a), MTB-H37Ra was treated with 2-methyl-adenosine at MIC (3.13 mg/L), -8 × MIC (0.4 mg/L) and 8 × MIC (25 mg/L). In (b), MTB-H37Ra was treated with rifampicin at MIC (0.002 mg/L), -8 × MIC (0.00025 mg/L) and 8 × MIC (0.016 mg/L). Controls were non-treated (NT).

Figure 3. Efficacy of 2-methyl-adenosine (MeAdo) tested in the ‘non-replicating persistence model’ at 3, 10, 30 and 100 mg/L. Time ‘0’ marks initiation of treatment with 2-methyl-adenosine (i.e. dosing day). At this point, Methylene Blue had turned colourless and metronidazole-treated cultures showed significant loss in viability (P = 0.05, data not shown here but discussed in text).

Efficacy of 2-methyl-adenosine on macromolecular synthesis

Three metabolic parameters were chosen to assess the effect of 2-methyl-adenosine on mycobacterial metabolism in order to obtain general information regarding mode of action. Examining incorporation of [14C]uracil into macromolecular components assessed the effect of 2-methyl-adenosine on RNA and DNA synthesis. Examining incorporation of [14C]phenylalanine into macromolecular components assessed the effect of 2-methyl-adenosine on protein synthesis. To conduct these studies, we used a range of drug concentrations representing the MIC (3 mg/L), five-fold above the MIC (15 mg/L), and 10-fold above the MIC (30 mg/L) of 2-methyl-adenosine. This was done in order to obtain a dose–response in the case of those assays that showed inhibitory activity. This concentration range was developed from extensive preliminary studies designed to establish the most informative range of inhibitory activity of 2-methyl-adenosine (data not shown). It should be noted that in those preliminary studies, we used sub-MICs of both the 2-methyl-adenosine and known inhibitors but did not observe any effects (data not shown). Equivalent dose ranges were used for known inhibitors of these parameters, such as

non-replicating persistence model

M. tuberculosis-H37Ra cells were inoculated in 7H9 as described in the Materials and methods section. cfu were determined at the initiation of the experiment and again once the cultures had reached anaerobiosis. In all sets, the cfu at initiation of the experiment were 2.3 ± 0.88 (standard error of the mean, S.E.M.) × 10^5 (n = 3) (log_{10} = 5.36) and upon anaerobiosis the cfu were 31.9 ± 4.3 (S.E.M.) × 10^5 (n = 8) (log_{10} = 6.51; Time 0, Figure 3) for all sets except those containing metronidazole. This was considered Time 0, or dosing day, for the experiment. cfu in the metronidazole cultures on dosing day were 2 ± 1.0 (S.E.M.) × 10^5 (n = 3). Thus, the metronidazole-treated cells had a significant reduction in cfu (P = 0.05) (data not shown), compared with the non-treated control cells by initiation of dosing. This significant reduction in cell viability by metronidazole was used as further confirmation that the cells had reached the persistence stage since metronidazole is active only after this stage is attained. At this time, the remaining non-treated cultures were treated with 2-methyl-adenosine at 3, 10, 30 and 100 mg/L and were incubated under anaerobic conditions for 7 days, after which cfu were again determined in all sets. Results are reported in Figure 3. A significant reduction in cfu was observed in sets treated with 2-methyl-adenosine at 30 and 100 mg/L (P ≤ 0.01, n = 6; and P ≤ 0.001, n = 5, respectively) (Figure 3). At the end of the experiment, the metronidazole-treated sets showed a significant reduction in cfu (P ≤ 0.01), compared with the non-treated control. These results demonstrate the ability of 2-methyl-adenosine to affect viability of M. tuberculosis-H37Ra in a hypoxic shift-down model previously described to simulate the persistent state of M. tuberculosis.

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rifampicin for RNA synthesis (Figure 4b), ofloxacin for DNA synthesis (Figure 5b) and streptomycin sulphate for protein synthesis (Figure 6b).

Evaluation of 2-methyl-adenosine activity on RNA synthesis revealed no reduction in [14C]uracil incorporation into RNA, even at 10 times the MIC (Figure 4a). By comparison, treatment with rifampicin at 10 times its MIC (i.e. 0.02 mg/L) had an immediate and sustained effect (Figure 4b) throughout the experimental period. This experiment was reproduced two more times with equivalent results and suggests that the mode of action of 2-methyl-adenosine is not one that directly affects RNA synthesis.

Evaluation of 2-methyl-adenosine’s effect on incorporation of [14C]uracil into DNA did not reveal a dose–response; only the 10 × MIC (30 mg/L) was effective (Figure 5a). The maximum effect was observed at 10 × MIC. This reduction was not as pronounced as with ofloxacin, a known inhibitor of DNA synthesis, at 10 × MIC (5 mg/L) (Figure 5a). The effect of 2-methyl-adenosine on DNA synthesis was confirmed by triplicate replication of the experiment. Ofloxacin demonstrated a dose–response at varying concentrations (Figure 5b). The reduction in [14C]uracil incorporation was apparent early in the experiment and persisted well below that of the non-treated control throughout the experiment (Figure 5b). This was not comparable to the results observed with 2-methyl-adenosine, in which case the reduction in [14C]uracil incorporation was not observed with the MIC or 5 × MIC (Figure 5a). In addition, the effect observed with 10 × MIC 2-methyl-adenosine was not immediately apparent, as was the case with ofloxacin (Figure 5a and b); it was not apparent until 2 h following addition of drug (Figure 5a). Known inhibitors of RNA (rifampicin) and protein synthesis (streptomycin sulphate), were evaluated and compared with ofloxacin at 10 × MIC for effect on DNA synthesis (Figure 5c).

The effect of 2-methyl-adenosine on protein synthesis was evaluated by observing incorporation of [14C]phenylalanine. A dose–response was observed for MIC (3 mg/L), 5 × MIC (15 mg/L) and 10 × MIC (30 mg/L) (Figure 6a). These experiments were repeated four times with equivalent results. A similar dose–response was observed for streptomycin sulphate, a known inhibitor of protein synthesis, when tested at MIC (1 mg/L), 5 × MIC (5 mg/L) and 10 × MIC (10 mg/L) (Figure 6b). That experiment was repeated three times to confirm results. When ofloxacin and rifampicin were tested using 10 × MIC, it was observed that ofloxacin (5 mg/L), an inhibitor of DNA synthesis, had no effect on incorporation of [14C]phenylalanine, whereas rifampicin (0.02 mg/L) had a more pronounced effect than streptomycin sulphate (Figure 6c).

Discussion

With the increasing problem of drug resistance in M. tuberculosis, it is important to seek new and improved agents to treat infections resulting from this clinically significant microbial pathogen. It is also vital to understand various biochemical pathways associated with the organism, in order to develop new strategies for drug development. Although evidence of in vitro activity for new antituberculous compounds is valuable, it is critical that the compound be effective against intracellularly replicating M. tuberculosis, and desirable if it can be used to treat latent M. tuberculosis infections.

Screening of purine analogues through the NIH-supported TAACF revealed that 2-methyl-adenosine has good in vitro activity against M. tuberculosis-H37Rv. To examine the metabolic activity of this compound in mycobacteria, we initially used a faster growing
mycobacterium, *M. smegmatis*. Results indicated that activity is the result of direct phosphorylation of 2-methyl-adenosine by adenosine kinase to metabolites that are cytotoxic to mycobacteria. Furthermore, cleavage of 2-methyl-adenosine to 2-methyl-adenine is not involved in cytotoxicity, nor is deamination to 2-methyl-inosine. Examination of lysates from *M. smegmatis* treated with 2-methyl-adenosine revealed the presence of methyl-ATP and incorporation of 2-methyl-adenosine into RNA. It was concluded that resulting methyl-ATP, or incorporation of 2-methyl-adenosine into RNA, might be responsible for activity observed against *M. smegmatis*. In this investigation, we further substantiate the in vitro activity of 2-methyl-adenosine against *M. tuberculosis*. The drug is effective intracellularly at concentrations that do not compromise macrophage viability, and shows activity against *M. tuberculosis* cultured under conditions to simulate the persistent state.

Considering the mechanism-of-action studies, it is clear that treatment with 2-methyl-adenosine has no effect on RNA synthesis. This allows us to rule out DNA-dependent RNA polymerase as a target for this compound. Its most pronounced effect was observed with protein synthesis. To a lesser degree it also affected DNA synthesis, but not equivalent to that of a known inhibitor of DNA synthesis. These observations suggest that 2-methyl-adenosine affects mycobacterial biosynthesis in a way that results in early inhibition of protein synthesis, followed by DNA synthesis. Had 2-methyl-adenosine not affected any parameter, one could assume that other pathways such as cell-wall synthesis might be affected. Such is the case when these methods are used with other antibacterial agents such as the mannopeptimycins.

Compared to positive control drugs, this scenario is more closely associated with that observed with streptomycin. Although streptomycin is not qualified as an inhibitor of DNA synthesis, it has been shown to affect DNA synthesis in bacteria at high concentrations. Fernandez & Anton demonstrated that incubation of streptomycin-resistant mutants of *Salmonella typhimurium* with streptomycin at 400 mg/L resulted in inhibition of DNA as well as protein synthesis. Studies with chloramphenicol, another inhibitor of protein synthesis, have also shown inhibition of DNA synthesis.

It is unlikely that 2-methyl-adenosine’s mode of action is identical to streptomycin, i.e. binding to a specific ribosomal subunit. This speculation is based upon the structural dissimilarity and also the fact that 2-methyl-adenosine is not bactericidal. It is more likely that 2-methyl-adenosine is affecting another parameter associated with protein synthesis and that this eventually affects DNA synthesis when high concentrations are achieved.

Although it is not possible to assign a specific target, it is plausible to suggest possible modes of action for 2-methyl-adenosine, based upon our previous and current findings. Assuming 2-methyl-adenosine is being metabolized in *M. tuberculosis* in a manner similar to *M. smegmatis*, incorporation into ATP or RNA as methyl-adenosine might affect parameters of protein synthesis that depend upon interactions with ATP or various types of RNA. These could involve such factors as aminoacyl-tRNA synthetase activity, important for accurate protein synthesis and involving ATP, as well as Shine-Dalgalorno sequences that are essential in ribosomal binding to mRNA and consisting of 5–10 nucleotide sequences rich in G and A. Substitution of adenine with 2-methyl-adenosine in either of these examples could create a steric hindrance that would disrupt or affect proper function.

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**Figure 5.** Results of [14C]uracil incorporation into DNA of *M. tuberculosis*-H37Ra. In (a), cells were treated with ofloxacin (OFX) at 10 × MIC (5 mg/L) and 2-methyl-adenosine (MeAdo) at MIC (3 mg/L), 5 × MIC (15 mg/L) and 10 × MIC (30 mg/L). In (b), cells were treated with ofloxacin at MIC (0.5 mg/L), 5 × MIC (2.5 mg/L) and 10 × MIC (5 mg/L). In (c), cells were treated with ofloxacin at 10 × MIC (5 mg/L), streptomycin sulphate (STR) at 10 × MIC (10 mg/L) and rifampicin (RIF) at 10 × MIC (0.02 mg/L). Cells were processed and cpm determined as described in the Materials and methods section. Each point represents duplicate sampling.
In conclusion, it is apparent that 2-methyl-adenosine has antimycobacterial activity that is effective in vitro and intracellularly within host macrophages. In addition, it has activity against *M. tuberculosis* in the hypoxic shift-down model that is generally associated with the persistent state that results in latent infections of *M. tuberculosis*. Although these studies do not identify the exact mode of action for 2-methyl-adenosine, they do help to define the general mode of inhibitory activity with regard to known inhibitors of RNA, DNA and protein synthesis. Whether or not 2-methyl-adenosine represents a good drug lead will have to be determined by more extensive molecular evaluations of its mechanism of action. However, it should be helpful as a tool to assist in the identification of new targets for antimycobacterial agents.

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**References**


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