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Kinetic properties of fluorinated substrate analogues on 5'-methylthioadenosine nucleosidase from *Escherichia coli*

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Summary — 5'-Deoxy-5'-[(monofluoromethyl)thio]adenosine, 5'-deoxy-5'-[(difluoro-methyl)thio]adenosine and 5'-deoxy-5'-[(trifluoro-methyl)thio]adenosine have been evaluated for their substrate and inhibitory activities toward 5'-methylthioadenosine (MTA) nucleosidase from *Escherichia coli*. Their abilities to serve as substrates of MTA nucleosidase support the concept that MTA nucleosidase plays a role in the biological activity of these compounds.

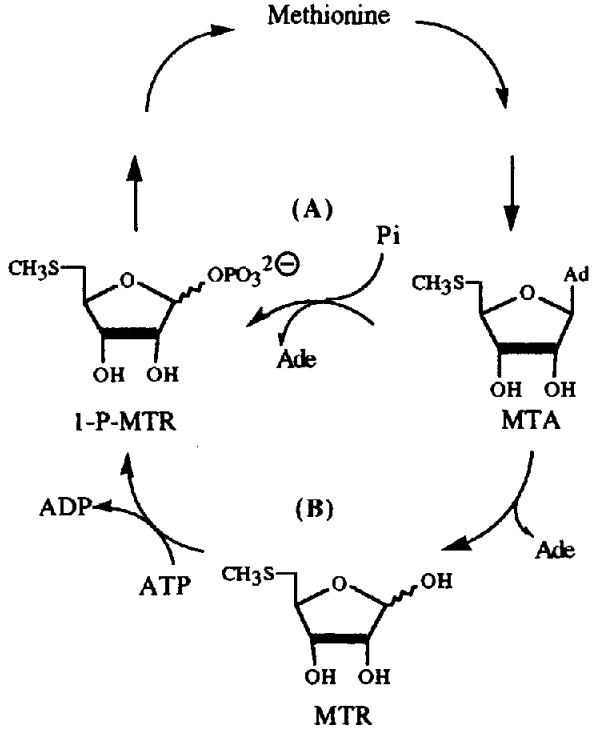
MTA nucleosidase / fluorinated substrate analogue

Introduction

5'-Methylthioadenosine (MTA) is an important metabolite of *S*-adenosylmethionine metabolism formed stoichiometrically during the biosynthesis of polyamines, spermidine and spermine [1]. Interest in MTA and its analogues has been stimulated since studies revealed that MTA is an important intermediate in methionine recycling and a fundamental component of the complex system responsible for cell growth and proliferation [2, 3]. The reaction sequence whereby MTA is converted to methionine differs in mammals and various microorganisms. In mammals direct phosphorylation is catalysed by MTA phosphorylase and converts MTA into 1-phospho-5-methylthioribose (1-P-MTR) [4]. In certain microorganisms, where MTA phosphorylase is absent, MTA is first hydrolysed by MTA nucleosidase [5–7] and the resulting 5-methylthioribose is phosphorylated by MTR kinase [8]. In both cases 1-PMTR is subsequently recycled *via* a multistep pathway to methionine [9-11] (scheme 1).

Consequently, the inhibitors of the enzymes involved in MTA catabolism, MTA phosphorylase in mammal cells [12], MTA nucleosidase [7] and MTR kinase [13-16] in various microorganisms, have been examined as potential chemotherapeutic agents.

Recently, analogues of MTA, fluorinated at the 5'-methylthio position have been synthesized, 5'-deoxy-



Scheme 1. Methionine recycling pathway, alternative route (**A** and **B**) from MTA to 1-P-MTR.

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5'-(monofluoromethylthio)adenosine MFMTA [17-19], 5'-deoxy-5'-(difluoromethylthio)adenosine DFMTA and 5'-deoxy-5'-(trifluoromethylthio)adenosine TFMTA [20]. DFMTA and TFMTA have been reported to have excellent inhibitory activity against *Plasmodium falciparum* [20], a protozoan known to use MTA nucleosidase and MTR kinase for methionine salvage [13], but the site of action of these nucleosides has not yet been elucidated.

Because the biological activity of this series of fluorinated nucleosides has not been fully explored, we aimed to examine the effect of MFMTA, DFMTA, TFMTA (fig 1) on the activity of a well-characterized and easily accessible bacterial MTA nucleosidase (EC.3.2.2.9) [5, 7] in order to check their inhibitory properties and their possible transformation into fluorinated MTR analogues.

Chemistry

MFMTA

We recently developed a short and efficient procedure for the preparation of MFMTA from an MTA derivative [18]. Accordingly, direct fluorination of N^6,N^6-dibenzoyl-2',3'-*O*-dibenzoyl-5'-deoxy-5'-(methylthio)adenosine with XeF_2 in CH_2Cl_2 at –70°C gave 5'-deoxy-5'-fluoromethylthioadenosine after removal of O- and N-benzoyl-protecting groups and purification on silica gel, in 90% yield. Although XeF_2 is expensive, the rapid and clean regiospecific fluorination observed made this preparation worthwhile.

DFMTA and TFMTA

DFMTA and TFMTA were prepared from adenosine according to the general procedure described by J Honek and coworkers *via* a 5'-deoxy-5'-thioacetate adenosine derivative [20].

The final products obtained by this method were judged after purification to be chromatographically pure and characterization by ^1H NMR and ^{19}F NMR gave the expected chemical shifts and coupling constants.

The chemical purity of MFMTA, DFMTA and TFMTA prepared in this manner and subjected to biochemical analysis was checked by HPLC.

The target compounds are all α-fluorothioether derivatives, a class of compounds which are susceptible to facile hydrolysis. Therefore it was important to determine the stability of these compounds in buffer assay prior to biochemical characterization. DFMTA and TFMTA proved to be stable in buffer assay for several days at 37°C but MFMTA undergoes gradual decomposition when stored under the same conditions for more than 6 h.

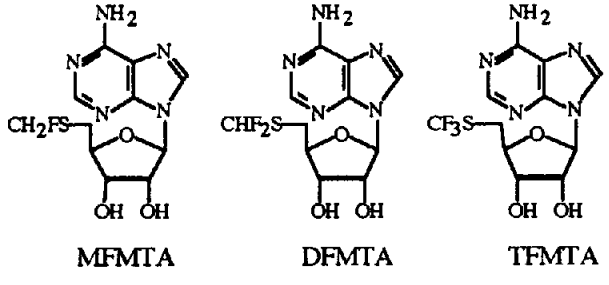

Fig 1. Structures of MTA analogues.

Substrate specificity of MTA nucleosidase

Ade formation

The ability of MFMTA, DFMTA and TFMTA to serve as substrates for MTA nucleosidase was monitored by following their hydrolysis in the presence of the purified enzyme (see *Experimental protocols*), the formation of adenine (Ade) or depletion of the fluoro compounds were easily observed by HPLC methodology.

Figure 2 is an example of an HPLC chromatogram from a reaction mixture of MTA nucleosidase and TFMTA in buffer assay. Two main peaks appeared in the chromatogram. The peak with retention time 22.9 min was the unreacted TFMTA and the peak with retention time 7.7 min was adenine, which had the same retention time as authentic Ade when examined by coinjection on HPLC. When the Ade peak was collected and subjected to chemical ionization mass spectroscopic analysis, an ion (M) at *m/z* 135 was obtained which is consistent with the structure of Ade.

Identification of sugar moiety

The respective ribosyl moieties formed during the bioconversion (preparative scale) of MFMTA, DFMTA and TFMTA with MTA nucleosidase have been identified by comparison of their migratory velocity on TLC plates (silica gel; eluent: ethylacetate/methanol 95:5; detection with 2, 3, 5-triphenyltetrazolium chloride spray) with 5-fluoromethylthioribose (MFMTR), 5-difluoromethylthioribose (DFMTR) and 5-trifluoromethylthioribose (TFMTR) used as standards and prepared by acid hydrolysis of the corresponding nucleosides. After their isolation, each sugar was subjected to chemical ionization spectroscopy analysis and ions (MH^+) were observed at *m/z* 198 (MFMTR), 216 (DFMTR) and 234 (TFMTR).

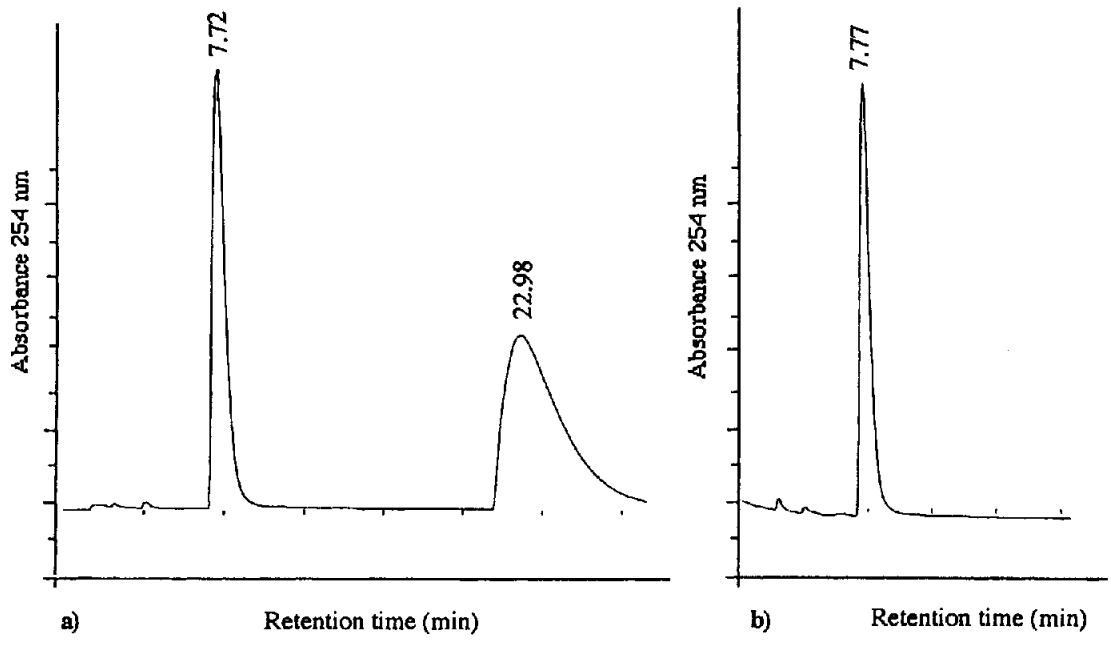

Fig 2. a) HPLC chromatogram of Ade generated upon incubation of TFMTA with MTA nucleosidase. MTA nucleosidase (1×10^{-4} U) and TFMTA (120 nmol) were incubated in 200 µl of assay buffer for 6 h. After filtration, aliquots were analysed by HPLC as described in the *Experimental protocols*. b) Coinjection of the compound collected with authentic Ade.

V_{max} determination

Using the same HPLC methodology described above, V_{max} values were determined. MTA nucleosidase was incubated with substrate saturating concentrations of each fluorinated nucleoside (6 ± 0.1 mM) in 200 µl of buffer assay. The amount of protein was adjusted so that no more than 20% of substrate was converted in 10 min. The reaction was stopped at different time intervals by placing the mixture in boiling water bath for 1 min. The protein precipitate was removed by centrifugation and the supernatant analysed by HPLC under the analytical conditions described above. Quantification of the Ade formed was carried out using a calibration curve of authentic Ade. The V_{max} (slope values from the curve: amount of Ade formed *versus* time) were respectively: 0.88, 0.72, 0.10 and 0.06 µmol/min/U for MTA, MFMTA, DFMTA and TFMTA.

Inhibition studies

The inhibition effect of MFMTA, DFMTA and TFMTA on the hydrolysis of 5'-($^3H_2$)-MTA was carried out in separate experiments. MTA nucleosidase activity was determined by measuring the formation of 5-($^3H_2$)-methylthioribose as described in the *Experimental protocols*, in the presence of each of the fluorinated methylthioadenosines. Since products of the two substrates can be distinguished from each other, the system may be treated by usual competitive inhibition relationships (fig 3). The inhibition pattern of all three fluorinated substrate analogues was competitive and the K_i values for MFMTA, DFMTA and TFMTA were found to be 0.19, 2.1 and 0.2 µM respectively; the results of the kinetic analysis are summarized in table I.

Discussion and conclusions

The inhibition constants (K_i values) determined in this study for the three fluorinated derivatives are of the same order of magnitude and when compared to the K_m value of MTA provide an indication of their good ability to compete with MTA for binding to MTA nucleosidase (table I). In the literature, studies of steric hindrance of the CF_3-group have shown it to be as bulky as an isopropyl group [21]. The K_i value 0.2 obtained for TFMTA indicates clearly that the 5'-side chain region is not relevant in the recognition mechanism of MTA nucleosidase, confirming the data reported with other analogues [6, 7]. It is noteworthy that MFMTA was more effectively metabolized by MTA

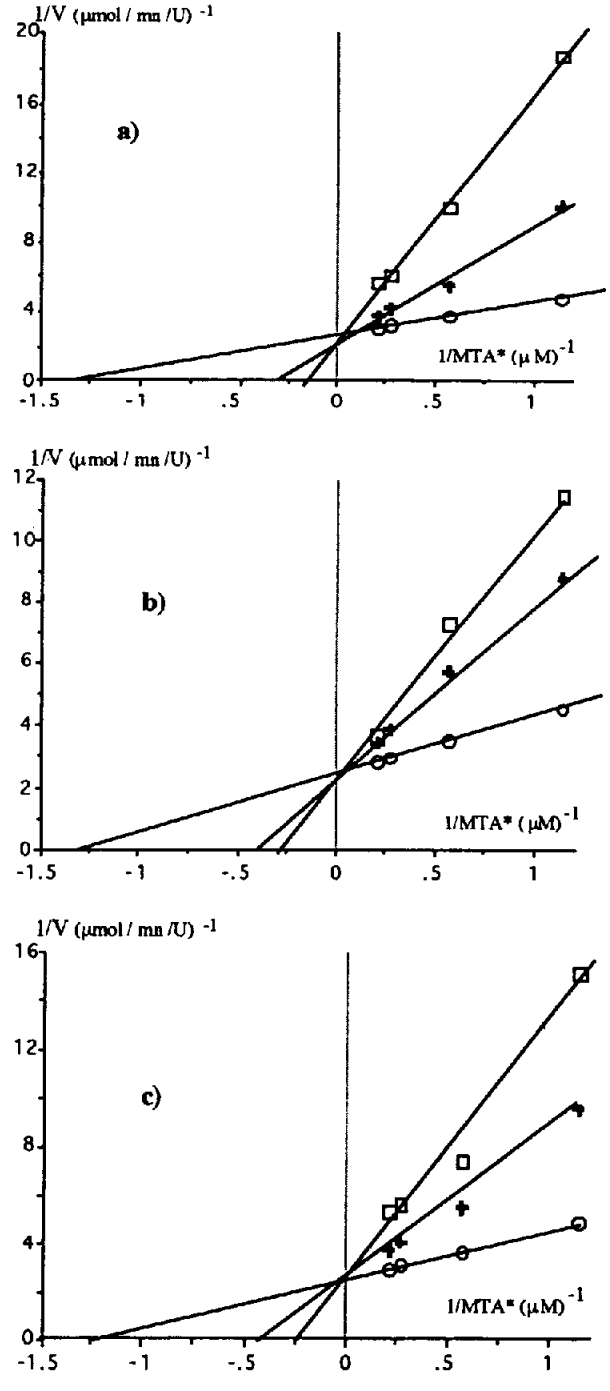

Fig 3. Double reciprocal plots of initial reaction velocity versus 5'-($^3H_2$)-methylthioadenosine concentration in the absence and the presence of two fixed concentrations of fluorinated methylthioadenosine. **a)** ○ zero, + 10 µM, □ 20.4 µM, MFMTA; **b)** ○ zero, + 0.4 µM, □ 0.5 µM, DFMTA; **c)** ○ zero, + 0.5 µM, □ 1.1 µM, TFMTA.

Table I. Kinetic parameters of *Escherichia coli* MTA nucleosidase with fluorinated MTA.

Nucleoside	K_i *(µM)*	*% rel* V_{max}[a]
MTA	0.5 (K_m)[a]	100
MFMTA	0.19	81
DFMTA	2.1	11
TFMTA	0.2	7

[a] From assays described in the *Experimental protocols.*

nucleosidase than other analogues if one considers both the K_i value and the relative substrate activity of each fluorinated analogue.

The V_{max} values observed for DFMTA and TMFTA are substantially lower than that observed for MFMTA. This might reflect the powerful electron-withdrawing properties of CHF_2S- and CF_3S-groups at the 5'-position which might decrease the depurination rate by destabilization of the carboxonium-like transition state involved in the catalytic process of MTA nucleosidase (Allart and Guillerm, unpublished results). A similar influence of electron-withdrawing groups at the 5'-position on the rate of depurination has previously been noted during acid-catalysed hydrolysis of purine nucleosides which proceeds by an A_1-type mechanism [22].

The purpose of this work was to target MTA nucleosidase with fluorinated methylthioadenosine derivatives. We have demonstrated that MFMTA, DFMTA and TFMTA are effective as inhibitors and as substrates of bacterial MTA nucleosidase and can be cleaved by the enzyme giving the corresponding fluorinated analogues of MTR.

These results must be taken into account to explain the biological activity of DFMTA and TFMTA against microorganisms where MTA nucleosidase is present [20]. Since MFMTA and DFMTA were also found to be potent inhibitors and/or substrates of MTA phosphorylase [19, 23], the significant lack of selectivity of these fluorinated nucleosides, close structural analogues of MTA, raises questions about their use as possible antibacterial and/or antiprotozoal agents.

Experimental protocols

Chemical homogeneity of the synthesized fluorinated compounds was controlled prior to use, by HPLC, using a column packed with spherisorb ODS, 2.5 µm (25 x 0.45 cm), eluent H_2O/MeOH (1v/1v).

5'-($^3H_2$)-Methylthioadenosine [24] used for the enzyme assay had a specific activity of 55 mCi/mmol; its radiochemical purity was checked by radiochromatography using a LB285 Berthold scanner equipped for CHROMA 2D. Counting was carried out with a β5 Kontron liquid scintillation counter in Beckman scintillation cocktail RV. All results were corrected for quenching by an external standard method. Mass spectra

were obtained using a Nermag (DCI/NH_3) mass spectrometer. TLC was performed on silica-gel precoated plates (Merck n° 55453).

High performance liquid chromatography

A Shimadzu SLC-6A chromatograph equipped with a spectrophotometric detector, SPD-6AV was used. The analytical column nucleosil-SA-5μ-cation exchanger resin (20 x 2.5 mm) was used. The separation between fluorinated MTA derivatives and Ade formed during enzymatic reactions was accomplished by using 0.25 M ammonium phosphate buffer, pH 4.0, with a constant pressure of 160 kgf/cm^2 at room temperature. The retention times for Ade, MFMTA, DFMTA and TFMA were respectively 7.7, 14, 15 and 22.9 min.

Enzyme purification

MTA nucleosidase was purified from *E coli* strain B according to the method of Della Regione and coworkers [7] for the first steps of purification including ammonium sulfate fractionation and DEAE-sephadex A-50 chromatography (DEAE: diethylaminoethyl). The purification was continued by FPLC (fast protein liquid chromatography) using a Mono Q HR 10/10 column equilibrated with Tris-buffer 10 mM, 1 mM dithiothreitol, pH 7.8. After loading the column with an aliquot of enzyme and washing with 20 ml of buffer the column was run with a gradient of NaCl (0–0.35 M) at a flow rate of 4 ml/min. Active fractions were pooled, concentrated by ultrafiltration and dialysed against 100 volumes of phosphate buffer (50 mM, pH 7.4, 1 mM dithiothreitol). Using this procedure the enzyme was purified 200-fold and had a specific activity of 5.5 U/mg and its apparent K_m for MTA was 0.5 μM, similar to that of highly purified enzyme [7].

Enzyme assay

MTA nucleosidase was assayed by measuring the formation of 5-($^3H_2$)-methylthioribose from 5'-($^3H_2$)-methylthioadenosine prepared by a method devised in our laboratory [24].

The standard reaction mixture used contained (in a total volume of 150 μl) 50 mM potassium phosphate buffer, pH 7.0, 10 mM dithiothreitol, 1 mg/ml bovine serum albumin, 1 mM 5'-($^3H_2$)-MTA (1.5×10^5 cpm/μmol) and the enzymatic preparation. The reaction mixture was equilibrated at 37°C and the reaction initiated by the addition of enzyme. After 10 min, the reaction was stopped by addition of 50 μl of 3 M trichloroacetic acid and the totality of the sample was loaded on a column (0.5 x 1 cm) of Dowex-50 (H^+ form) 100–200 mesh equilibrated in water. The 5-($^3H_2$)-methylthioribose produced was eluted directly into scintillation vials with 2 ml H_2O for counting. Under these conditions 5'-($^3H_2$)-MTA is quantitatively retained on the column. For kinetic and purification studies the amount of protein was adjusted so that no more than 30% of substrate was converted into methylthioribose.

Kinetic constants

The standard reaction mixture used for inhibition studies contained in a total volume of 150 μl, 5'-($^3H_2$)-MTA (0.8, 1.75, 3.5 and 4.37 μM; 6×10^8 cpm/μmol), fluorinated nucleoside (0.2–150 μM) and enzymic preparation. The assay was incubated at 37°C for 10 min. The reaction was stopped by addition of 50 μl of 3 M trichloroacetic acid. The 5-($^3H_2$)-methylthioribose produced was analysed as described above.

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