Inhibition of Growth and Purine-Metabolizing Enzymes of Trypanosomid Flagellates by \( N^6 \)-Methyladenine

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\( N^6 \)-methyladenine (6-methylaminopurine [6-MA]), a plant growth regulator and a normal constituent of nucleic acids, has been found to inhibit the growth of *Trypanosoma cruzi*, *Leishmania braziliensis*, *L. donovani*, *L. tarentolae*, *L. mexicana*, and *Crithidia fasciculata*. The extent of growth inhibition in these organisms is related to the sensitivity of guanine deaminase (guanine aminohydrolase, EC 3.5.4.3), adenine deaminase (adenine aminohydrolase, EC 3.5.4.2), and adenosine hydrolase and phosphorylase. 6-MA was not an inhibitor of the purine phosphoribosyltransferases. Of the trypanosomid flagellates tested, *Trypanosoma cruzi* was most susceptible to 6-MA. Neither adenine deaminase (as found in the leishmaniae and *C. fasciculata*) nor adenosine deaminase (as found in mammalian cells) could be demonstrated in *T. cruzi*. Guanine deaminase, which is strikingly inhibited by 6-MA in *T. cruzi*, appears to play a major role in the purine salvage pathway of this organism, as judged from growth experiments and enzyme inhibition studies. Enzyme sensitivities to 6-MA vary greatly depending upon the organism. Rabbit liver guanine deaminase was shown to be insensitive to 6-MA at the concentrations used in this study.

\( N^6 \)-Methyladenine (6-methylaminopurine [6-MA]) belongs to a group of compounds classified as cytokinins (plant growth regulators). Cytokinins have been studied for more than two decades, yet there is no clear indication as to how these agents function to influence cell growth. Their biological manifestations include induction of mitosis, cell division and enlargement, alteration of morphogenesis, promotion of seed germination, stimulation of tumor formation, and enhancement of protein and ribonucleic acid (RNA) synthesis in plants (5). The effects of cytokinins, particularly the \( N^6 \)-substituted adenine derivatives, on mammalian cells is largely unknown (8), and there appear to be no toxicological data available.

Cytokinins in fungal cells have been shown to stimulate \( Ca^{2+} \) release from a sequestering glycoprotein localized on the cytoplasmic membrane, and to enhance \( Ca^{2+} \) intake into the cells (20), and 6-MA has been shown to inhibit growth and induce sporulation in *Bacillus subtilis* (10).

The occurrence of small amounts of 6-MA in the deoxyribonucleic acid (DNA) and transfer RNA (tRNA) of bacteria, plants, and animals is well documented (2, 14, 23). Enzymes that synthesize these minor bases have been isolated and have been shown to catalyze transfer of the methyl group from \( S \)-adenosylmethionine to the appropriate nucleotide residues of the DNA and RNA polymers (6, 11–13, 22).

In a search for purine analogs that would inhibit the growth of the culture forms (epimastigotes and promastigotes) of trypanosomid flagellates, we found 6-MA to be very active especially for two important human pathogens, *Trypanosoma cruzi* and *Leishmania braziliensis*. To investigate the mechanism of action of this compound, we examined its effect on the purine salvage enzymes of a number of trypanosomids. The pathways of purine nucleotide interconversions in these parasitic organisms resemble those found in mammalian cells (see Fig. 3) except for the absence of de novo purine base biosynthesis (7, 21) and, for *Crithidia fasciculata* and the leishmaniae, the absence of adenosine-deaminating activity (16, 18, 21). For these reasons, the purine salvage pathway has become popular as a target for chemotherapeutic investigations in these organisms.

MATERIALS AND METHODS

All radioactive purines (50 mCi/mmmol; 1 Ci = 3.7 \( \times 10^{10} \) bequerels) were labeled in the 8 position and were obtained from Ameraham/Searle (Arlington Heights, Ill.). All chemicals of the defined medium used for *C. fasciculata* were obtained from Sigma Chemical Co. (St. Louis, Mo.); the ingredients of the semidefined medium of Berens et al. (1) were obtained from GIBCO Laboratories (Grand Island, N.Y.). 6-MA and rabbit liver guanase were obtained from Sigma.

*C. fasciculata*, a mosquito parasite, is the strain that has been used in this laboratory for the past 20 years (15, 17). *L. tarentolae*, a lizard parasite, was obtained through the courtesy of Larry Simpoe (University of California, Los Angeles). *L. donovani*, *L. braziliensis*, and *L. mexicana*, all human pathogens,
were kindly supplied by J. J. Marr (St. Louis University, St. Louis, Mo.). *T. cruzi* (ATCC 30013, Culbertson strain) was obtained through the courtesy of V. Iralu (Philadelphia College of Osteopathic Medicine, Philadelphia, Pa.).

*C. fasciculata* was grown at 25°C in the defined medium of Kidder and Dutta (17), in low-profile flasks when large amounts of tissue were needed for enzyme studies or in side-arm Nephelo flasks (Bellco,Vineland, N.J.) or optically clear tubes (25 by 125 mm) for the growth studies. Growth was followed by reading optical densities at 650 nm, with uninoculated medium as the standard. In all cases hemin, glucose, and inhibitor (if any) were filter sterilized and added to the bulk of the heat-sterilized medium.

*L. tarentolae* was grown at 25°C in low-profile flasks in heat-sterilized beef heart infusion (Difco Laboratories, Detroit, Mich.) to which filter-sterilized hemin (6 μg/ml) had been added, or in the filter-sterilized HO-MEM medium of Berens et al. (1), containing 8% fetal calf serum.

The other three species of *Leishmania* were grown at 25°C in disposable plastic flasks in the HO-MEM medium in an atmosphere of 5% CO₂—95% air.

*T. cruzi* was grown at 25°C in low-profile flasks in heat-sterilized trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md) to which filter-sterilized hemin (2 μg/ml) had been added. For growth studies, *T. cruzi* was grown in filter sterilized HO-MEM medium of Berens et al. (1) containing 8% calf serum.

Cell extracts were prepared from harvested, washed cells (Sorvall sealed assemblies were used on 250-ml bottles for the first sedimentation) as has been described (3). After sonication, the cell debris was removed by centrifugation at 40,000 × *g* for 1 h, and the extracts were used without further treatment (except for dilution to equalize protein concentrations) with either 50 mM phosphate buffer or 50 mM tris(hydroxymethyl)aminomethane at pH 7.5. When the human pathogens were being used, all necessary manipulations were carried out under a BIOGARD (Baker) hood.

Guanine deaminase assays contained, in a volume of 0.13 ml, 0.7 × 10⁻² to 2.3 × 10⁻² mM [₈⁻³¹P]guanine, 20 mM phosphate buffer (pH 7.5), and enzyme.

Phosphoribosyltransferases for adenine, guanine, or hypoxanthine (19) and for adenine deaminase (16) were assayed as previously described. Nucleoside phosphorylase assays were performed as were those for the hydrolyase (3), but with dialyzed enzyme preparations containing phosphate buffer. The absence of enzymatic activity in HEPES (N-2-hydroxyethylpiperazine-N'₂-2-ethanesulfonic acid) buffer indicated a nucleoside phosphorylase and not a hydrolyase.

When inhibitors were used, they were added to the enzyme-buffer solution and preincubated for 10 min at 22°C. After equilibrium was reached at 35°C, the radioactive substrate was added. The reaction was stopped by the addition of a drop of glacial acetic acid. Enzyme activities were determined by paper chromatography or by paper electrophoresis, as has been described (16).

Protein concentrations were determined by the BioRad dye method using absorbance at 595 nm.

**RESULTS**

The amount of 6-MA required for 50% inhibition of growth varies considerably with the organism tested (Table 1). *T. cruzi* exhibited the greatest susceptibility and *C. fasciculata* showed the least. This inhibition could be reversed by high levels of natural purines and derivatives. *T. cruzi*, unlike the other trypanosomids used in this study, does not possess an adenine deaminase, nor does it possess an adenosine deaminase. We investigated the possibility that 6-MA may be demethylated by the least inhibited organism, thus diluting its growth-inhibitory action. Various concentrations of 6-MA (0.5 to 2.5 mM) were incubated with sonicated extracts of *C. fasciculata* and *L. tarentolae*. No change in absorbance occurred, indicating that adenine (and from this, hypoxanthine) was not formed from 6-MA. When sonic extracts of these organisms were incubated with 6-MA at pH 6.0, 7.0, and 8.0 and chromatographed (n-butanol-acetic acid-water, 20:3:7, vol/vol) with appropriate standards, which had been added to boiled sonic extracts containing 6-MA, no trace of adenine or hypoxanthine appeared in the enzy-

<table>
<thead>
<tr>
<th>Organism</th>
<th>6-MA concn (mM) for 50% inhibition of:</th>
</tr>
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<tbody>
<tr>
<td><em>T. cruzi</em></td>
<td>Growth Absent Adenine deaminase Ribonucleoside hydrolase Adenosine</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td><em>L. braziliensis</em></td>
<td>1.6</td>
</tr>
<tr>
<td><em>L. donovani</em></td>
<td>2.2</td>
</tr>
<tr>
<td><em>L. tarentolae</em></td>
<td>2.4</td>
</tr>
<tr>
<td><em>L. mexicana</em></td>
<td>3.0</td>
</tr>
<tr>
<td><em>C. fasciculata</em></td>
<td>&gt;3</td>
</tr>
</tbody>
</table>

* Enzymes were assayed as described in the text, but with increasing levels of 6-MA.
* T. cruzi and the leishmanias were grown in the media of Berens et al. (1), and *C. fasciculata* was grown in the purine-depleted media of Kidder and Dutta (17). Growth was determined at 14 days for *T. cruzi*, at 7 days for leishmanias, and at 4 days for *C. fasciculata*. 

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matic reactions, indicating that 6-MA was not demethylated by these extracts.

6-MA was found to be a competitive inhibitor of the adenine deaminase of *C. fasciculata* and the leishmanialae and showed the greatest inhibitory action on this enzyme of *L. braziliensis*. The adenosine-cleaving enzymes of *T. cruzi* and *L. braziliensis* were more sensitive to 6-MA inhibition than were those of other leishmanialae (Table 1). The leishmanialae, unlike *C. fasciculata* (3), do not possess an adenosine hydrolase (Table 1). The ribonucleoside hydrolase of *L. braziliensis* for guanosine showed the greatest sensitivity to 6-MA (Table 1).

The most surprising finding was that 6-MA was a very effective competitive inhibitor of guanine deaminase in all the organisms tested. The guanine deaminase of *C. fasciculata* and *T. cruzi* showed the greatest affinity for guanine as substrate (Table 2; Fig. 1).

The *K*ₐ of 6-MA for *T. cruzi* was the lowest, followed by that for *L. braziliensis* (Table 1; Fig. 2). The specific activities of the guanine deaminases vary with the organism. The specific activity in *L. braziliensis* was the highest, and that in *C. fasciculata* was the lowest (Table 2). The purine phosphoribosyltransferases of these organisms were not inhibited by 6-MA.

The activity of the guanine deaminase from all of the organisms decreased 70 to 80% when heated at 55°C for 5 min. Only minimal losses occurred when cell extracts were frozen and thawed five times, except for the guanine deaminase of *L. mexicana*, which lost 75% of its activity after this treatment. All of the guanine deaminases reported here act over a broad pH range (Table 2). Therefore all enzymatic reactions were carried out at pH 7.5.

In contrast to the guanine deaminases of the trypanosomal flagellates, rabbit liver guanase, assayed at pH 8.0, was not sensitive to 6-MA (concentrations up to 4 mM). Polyacrylamide gel electrophoresis (7.5%) of *T. cruzi*, *L. tarentolae*, and rabbit liver guanase in 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 8) demonstrated that they have approximately the same migration properties.

**DISCUSSION**

6-MA is a normal constituent of DNA and transfer RNA of plant and animal cells. To date there is no evidence available suggesting any harmful effects of this compound on mammalian cells (8). It appears in human urine and is thought to be indicative of tRNA turnover (4). Methylation occurs at the polymer level (6, 11–13, 22). There is no evidence that 6-MA as the preformed base can be incorporated into DNA or tRNA (this property suggests that it is not likely to be mutagenic).

![Fig. 1. Lineweaver-Burk plot of *T. cruzi* guanine deaminase in response to varying levels of 6-MA. (A) No inhibitor; (B) 0.03 mM inhibitor; (C) 0.077 mM inhibitor.](http://aac.asm.org/)

**TABLE 2. Properties of guanine deaminase in six trypanosomal flagellates**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Sp act <em>a</em></th>
<th><em>K</em>ₐ (M)</th>
<th>pH optimum</th>
<th>6-MA <em>K</em>ₐ′ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. cruzi</em></td>
<td>2.9</td>
<td>3.8 × 10⁻⁵</td>
<td>8.5</td>
<td>9.5 × 10⁻⁶</td>
</tr>
<tr>
<td><em>L. braziliensis</em></td>
<td>9.8</td>
<td>3.33 × 10⁻⁴</td>
<td>8.0</td>
<td>7.1 × 10⁻⁵</td>
</tr>
<tr>
<td><em>C. fasciculata</em></td>
<td>2.42</td>
<td>4.2 × 10⁻⁵</td>
<td>8.5</td>
<td>1.35 × 10⁻⁴</td>
</tr>
<tr>
<td><em>L. donovani</em></td>
<td>6.25</td>
<td>1.11 × 10⁻⁴</td>
<td>7.5</td>
<td>1.4 × 10⁻⁴</td>
</tr>
<tr>
<td><em>L. tarentolae</em></td>
<td>4.88</td>
<td>1.67 × 10⁻⁴</td>
<td>8.5</td>
<td>2.1 × 10⁻⁴</td>
</tr>
<tr>
<td><em>L. mexicana</em></td>
<td>6.85</td>
<td>1.25 × 10⁻⁴</td>
<td>8.5</td>
<td>2.6 × 10⁻⁴</td>
</tr>
</tbody>
</table>

*a* Nanomoles of product per minute per milligram of protein of the supernatants of cell sonic extracts after centrifugation at 40,000 × g for 1 h. Amounts of protein in the reaction tubes were adjusted to approximate equivalence.

*b* Determined from Lineweaver-Burk plots with substrate concentrations from 0.77 × 10⁻² to 2.3 × 10⁻² mM.

*c* Determined from Dixon plots.
We have found 6-MA to be an excellent competitive inhibitor for the guanine deaminase of *T. cruzi* and *L. braziliensis* and a good inhibitor for the guanine deaminase of the other leishmaniae tested and for *C. fasciculata*.

This is the first report of the existence of a guanine deaminase in *T. cruzi*, *C. fasciculata*, *L. mexicana*, and *L. tarentolae*. Marr and co-workers have reported its presence in *L. donovani* and *L. braziliensis* (21).

Guanine deaminase competes with other enzymes for intracellular guanine and converts guanine to xanthine, but the exact importance of this enzyme is unknown at present.

The guanine deaminase of the trypanosomid pathogens appears specifically sensitive to 6-MA in contrast to mammalian (rabbit liver) guanine deaminase. This suggests a very important biochemical difference between these enzymes, which may eventually be of chemotherapeutic importance.

6-MA is a potent growth inhibitor of *T. cruzi* and is active against guanine deaminase and adenosine hydrolase. This suggests that these enzymes are crucial to the purine salvage mechanism of this organism.

We have demonstrated the absence of an adenine deaminase in *T. cruzi*. Our earlier report (18) has shown this to be an important enzyme in the purine pathway of *C. fasciculata* and leishmaniae (Fig. 3). 6-MA is a much more potent inhibitor of the adenine deaminase of *L. braziliensis* than for the other leishmaniae and *C. fasciculata*. Hartenstein and Fridovich have reported that 6-MA is a competitive inhibitor but not a substrate for the adenine deaminase of *Azotobacter vinelandii* and *Candida utilis* (9).

6-MA is a more potent growth inhibitor of *L. braziliensis* than of the other leishmaniae (Table 1). Three enzymes in the purine salvage pathway are drastically to moderately inhibited by 6-MA (guanine deaminase, adenine deaminase, and guanosine nucleoside hydrolase) (Tables 1 and 2).

In summary, 6-MA has been found to be a growth inhibitor of a number of trypanosomid flagellates, and the degree of inhibition appears to be most related to its inhibitory action on guanine deaminase and adenine deaminase. Our investigations show that although the pathogens possess a guanine deaminase, its sensitivity to 6-MA is strikingly different from that found in rabbit liver (mammalian) cells. We hope that this report will act as a stimulus for further research on the metabolism of 6-MA in animal tissues and in vivo studies of this inhibitor with the pathogenic trypanosomid flagellates.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


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