Analogues of 5-Methylthioribose, a Novel Class of Antiprotozoal Agents

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Since drug resistance and toxicity limit the use of available antiprotozoal agents, it is important that new drugs be developed as soon as possible. In this study, the method by which several protozoa degrade 5'-methylthioadenosine (MTA) was shown to differ from MTA catabolism in human cells. To exploit this metabolic difference, two analogues of methylthioribose (MTR), an MTA catabolite, were synthesized and found to be cytotoxic to *Plasmodium falciparum*, *Giardia lamblia*, and *Ochromonas malhamensis* in vitro. In contrast, these analogues had no effect on cultured mammalian cells. Analogues of MTR represent a potential new class of antiprotozoal drugs.

Parasitic protozoa cause some of the most prevalent infectious diseases of humans and produce extensive morbidity and mortality around the world (25). For example, over 600 million people are chronically infected with *Plasmodium falciparum* and at least 1.5 million malaria-related deaths occur each year (4, 15). Despite intensive research efforts, the development of new and effective antiprotozoal drugs has been difficult because of the close metabolic relationship between protozoa and human cells.

5'-Deoxy-5'-methylthioadenosine (MTA) is a naturally occurring nucleoside synthesized from S-adenosylmethionine during the production of the polyamines spermidine and spermine (23). Unlike the polyamines, MTA does not normally accumulate in cells but is rapidly degraded by one of two mechanisms (Fig. 1). In mammals (14), MTA is degraded in one step to adenine and 5-methylthioribose-1-phosphate (MTR-1-P) by MTA phosphorylase (Fig. 1, pathway A). In some other organisms (9), however, MTA is catabolized to MTR-1-P in two steps: (i) catabolism to adenine and MTR via MTA nucleosidase and (ii) conversion of MTR to MTR-1-P in an ATP-dependent reaction catalyzed by MTR kinase (Fig. 1, pathway B). In both instances, MTR-1-P is subsequently recycled to methionine through the intermediate α -ketomethylthiobutyric acid (1, 11, 19). Since MTA nucleosidase and MTR kinase are not present in mammalian cells, we hypothesized that these enzymes represent potential targets for the selective killing of organisms which contain them.

To test this hypothesis, cell extracts of *P. falciparum*, *Giardia lamblia*, and *Ochromonas malhamensis* were assayed for MTA phosphorylase (14), MTR kinase (9), and MTA nucleosidase (7) activities. *O. malhamensis*, a non-pathogenic protozoan, was studied because it can proliferate by using MTR as a sole source of methionine (21), implying that it contains MTR kinase. *Enterobacter aerogenes* (8) and *Persea americana* (avocado fruit; 10) were included as positive controls because each contains MTR kinase activity. Extracts derived from human erythrocytes and liver were also tested. Cell extracts from *P. falciparum*, *G. lamblia*, and *O. malhamensis* contained significant MTA

nucleosidase and MTR kinase activities (Table 1). MTA phosphorylase was undetectable in these samples, except in the *P. falciparum* samples, in which low activity, presumably related to residual erythrocyte enzyme, was noted. In contrast, human cells contained abundant MTA phosphorylase activity but were devoid of MTA nucleosidase and MTR kinase activities (Table 1).

To confirm the mode of MTA catabolism, the enzyme reaction mixtures were spotted onto polyethyleneimine-cellulose thin-layer chromatography plates, and the plates were developed in an aqueous solution of 1 M LiCl. Radiochromatogram scanning revealed that [¹⁴CH₃]MTR incubated with ATP and protozoal extracts (but not with extracts from mammalian cells) was converted to a new radioactive substance with a peak which comigrated with authentic MTR-1-P ($R_f = 0.50$). In similar experiments, the catabolism of MTA to MTR-1-P by mammalian extracts was found to be phosphate dependent, whereas protozoal extracts exhibited abundant MTA-cleaving activity in the absence of an exogenous phosphate source. Moreover, *P. falciparum* was tested for growth in methionine-free medium with MTR added as the sole potential source of methionine. As previously shown with *O. malhamensis* (21), and unlike mammalian cells (16), *P. falciparum* grew readily under these conditions (data not shown). The results confirm the enzyme activity data (Table 1) and demonstrate that these protozoa metabolize MTA in the two-step reaction MTA → MTR → MTR-1-P via MTA nucleosidase and MTR kinase, respectively.

Thus, since MTA nucleosidase and MTR kinase were present in protozoa but not in human cells, these enzymes appeared to be potential targets for the design of new antiprotozoal agents. Since MTA is a substrate for both MTA nucleosidase (protozoa) and MTA phosphorylase (human cells) we focused on MTR kinase and studied analogues of MTR.

MTR, 5-ethylthioribose (ETR), and 5-isobutylthioribose (iBTR) were synthesized (2, 5, 18) and tested for antiprotozoal activity (Table 2). For these experiments, cultures of *P. falciparum* (22), *G. lamblia* (12), and *O. malhamensis* (21) were grown by established techniques and were initiated at 1% parasitemia, 5×10^4 organisms per ml, and 10^5 organisms

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FIG. 1. Methionine recycling pathway. The two alternative pathways (A and B) from MTA to MTR-1-P are shown; pathway A is catalyzed by MTA phosphorylase, and pathway B is catalyzed by MTA nucleosidase and MTR kinase. Abbreviations: SAM, S-adenosylmethionine; dSAM, decarboxylated S-adenosylmethionine; MTA, 5'-methylthioadenosine; MTR, 5-methylthioribose; MTR-1-P, 5-methylthioribose-1-phosphate; KMB, α -ketomethylthiobutyric acid.

per ml, respectively. The 50% inhibitory concentration (IC_{50}) was determined by the [3H]hypoxanthine incorporation method for *P. falciparum* (6) and by enumeration on a hemacytometer for *G. lamblia* and *O. malhamensis*. The naturally occurring compound (MTR) was without effect at concentrations up to 2.5 mM, the highest tested. In contrast, both ETR and iBTR inhibited the growth of all three protozoa. ETR was uniformly more potent than iBTR and exhibited IC_{50} s of 90, 300, and 50 μM for *P. falciparum*, *G. lamblia*, and *O. malhamensis*, respectively (50 μM ETR = 9.7 $\mu g/ml$). ETR-treated malarial parasites were distorted and pycnotic with decreased numbers of all morphological forms. Moreover, regrowth of *P. falciparum* did not occur when the drug was removed after 48 h of culture, indicating that ETR was cytotoxic. In the *Giardia* experiments, decreased adherence of trophozoites was noted at drug concentrations well below the indicated IC_{50} and a lack of movement and cell lysis were apparent at concentrations above 500 μM .

TABLE 1. Enzyme activities in cell extracts

Source of extract	Enzyme activity (nmol/min per mg)		
	MTA nucleosidase ^a	MTR kinase	MTA phosphorylase
<i>E. aerogenes</i>	1.5	0.36	0
<i>P. americana</i>	1.2	0.70	0
Human erythrocytes	0	0	0.3
Human liver	0	0	1.6
<i>P. falciparum</i> ^b	0.3	0.23	0.05
<i>G. lamblia</i>	2.2	0.10	0
<i>O. malhamensis</i>	2.0	0.64	0

^a MTA nucleosidase was assayed in the absence of phosphate.

^b *P. falciparum* was purified from erythrocytes, as described by Sanderson and Walliker (17).

TABLE 2. Effect of MTR and MTR analogs on growth of protozoa in vitro

Compound	IC_{50} (μM) ^a		
	<i>P. falciparum</i>	<i>G. lamblia</i>	<i>O. malhamensis</i>
MTR	>2,500	>2,500	>2,500
ETR	90	300	50
iBTR	300	2,300	200

^a Indicated IC_{50} s are the means of three experiments for each organism (10 μM ETR = 2 $\mu g/ml$). Results between experiments did not vary by more than 15%.

Since ETR was cytotoxic to *P. falciparum* cultured in human erythrocytes, it seemed likely that the drug enters mammalian cells. To confirm that ETR is taken up by cells, formal transport studies were performed by the method of Oliver and Paterson (13). Based on the appearance of [U - ^{14}C -ribose]ETR in human erythrocytes (final mixture: 28.3% cells, 17 μM ETR), the rate of ETR uptake was determined to be 66.3 pmol/min per ml of cells.

The effect of ETR on mammalian cells in vitro was also examined. For these experiments, human and murine granulocyte-macrophage progenitor cells were grown in agar with standard sources of colony-stimulating activity (3, 20). The human promyelocytic leukemia cell line HL-60 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. ETR had no effect on the growth of human or murine bone marrow cells or the human cell line in vitro (Table 3). In preliminary experiments, oral or intraperitoneal administration of ETR to mice at doses up to 3 g/kg per day (equivalent to more than 200 g/day in an average adult human) for 7 days had no demonstrable effect on general appearance, weight, hematocrit, leukocyte count, serum chemistries, or organ histology (data not shown).

We recognize that the inhibitory concentrations of ETR against *P. falciparum* and *G. lamblia* were greater than those of many available drugs. However, the critical finding of this work was the demonstration of a metabolic difference between these protozoa and mammalian cells. ETR and iBTR are the first of a variety of potential analogs designed to exploit this difference. Additional analogs may have substantially more potency. (U.S. patent application 823,929 has been filed by the Oregon State Board of Higher Education for analogs of MTR as medicinals and biocides.)

The lack of effective therapy for many protozoal infections and the emergence of drug-resistant parasitic organisms, exemplified by multidrug-resistant strains of *P. falciparum* (24), underscore the need for mechanistically novel drugs. Since MTR analogs are unrelated in chemical structure to existing antiprotozoal compounds, they could represent a

TABLE 3. Lack of effect of ETR on mammalian cells in vitro

ETR addition	Growth of cells (mean $\pm$ SD, $n = 3$)		
	Human bone marrow ^a	Murine bone marrow ^a	HL-60 ^b
None	55 $\pm$ 6	39 $\pm$ 4	295 $\pm$ 20
5 mM	53 $\pm$ 3	38 $\pm$ 6	320 $\pm$ 25

^a Granulocyte-macrophage progenitor cells. Number of colonies per 10^5 marrow cells plated.

^b Growth (%) at 3 days.

new generation of drugs with a novel mode of action and, therefore, activity against parasites that are resistant to other drugs.

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