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Hypoxanthine Phosphoribosyltransferase from *Trypanosoma cruzi* as a Target for Structure-Based Inhibitor Design: Crystallization and Inhibition Studies with Purine Analogs

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The hypoxanthine phosphoribosyltransferase (HPRT) from *Trypanosoma cruzi* is a potential target for enzyme structure-based inhibitor design, based on previous studies which indicate that these parasites lack the metabolic enzymes required for de novo synthesis of purine nucleotides. By using a bacterial complement selection system, 59 purine analogs were assayed for their interaction with the HPRTs from *T. cruzi* and *Homo sapiens*. Eight compounds were identified from the bacterial assay to have an affinity for the trypanosomal enzyme. Inhibition constants for four of these compounds against purified recombinant trypanosomal and human HPRTs were determined and compared. The results confirm that the recombinant system can be used to identify compounds which have affinity for the trypanosomal HPRT. Furthermore, the results provide evidence for the importance of chemical modifications at positions 6 and 8 of the purine ring in the binding of these compounds to the HPRTs. An accurate three-dimensional structure of the trypanosomal enzyme will greatly enhance our understanding of the interactions between HPRTs and these compounds. Toward this end, crystallization conditions for the trypanosomal HPRT and preliminary analysis of X-ray diffraction data to a resolution of 2 Å is reported. These results represent significant progress toward a structure-based approach to the design of inhibitors of the HPRT of trypanosomes with the long-range goal of developing new drugs for the treatment of Chagas' disease.

The protozoan parasite *Trypanosoma cruzi* is the etiologic agent of Chagas' disease, which affects more than 24 million people in Latin America (13). Currently, there is no satisfactory drug for the treatment of Chagas' disease, and new drugs are urgently needed. Drugs for the treatment of diseases caused by parasites have traditionally been discovered by randomly screening either naturally occurring substances or synthetically produced chemicals for their ability to kill the parasite in culture systems. An alternative approach to discovering drugs involves the study of the biochemical and structural properties of a potential target enzyme of the parasite and the subsequent design of a compound that will inhibit the activity of that enzyme.

To identify potential target enzymes, differences in host versus parasite metabolic pathways have been investigated. Previous research has shown that protozoan parasites, including *T. cruzi*, must rely upon the salvage of exogenous purines for nucleotide synthesis (3, 16, 24). However, in humans and other mammals, purine nucleotides are both synthesized de novo (starting from amino acids, carbon dioxide, and ammonia) and salvaged from recycled preformed purine bases and nucleosides. These metabolic differences provide opportunities for potential chemotherapy targeted to the purine salvage enzymes of *T. cruzi* (22, 29).

Trypanosomes convert hypoxanthine and guanine bases to IMP and GMP, respectively, by a single enzyme, the hypoxanthine phosphoribosyltransferase (HPRT) (3, 15–17, 22). In addition, studies of human sera indicate that the most abundant purine base available to the parasite for salvage is hypoxanthine (18). Therefore, HPRT plays an important role in the salvage of metabolites and for the survival of trypanosomes in their mammalian hosts.

The HPRT from *T. cruzi* catalyzes the transfer of a monophosphorylated ribose from phosphoribosylpyrophosphate (PRPP) to the nitrogen at position 9 of the purine ring of guanine or hypoxanthine, yielding GMP or IMP. The gene encoding the HPRT from *T. cruzi* has recently been cloned and expressed in bacteria (1). Preliminary kinetic studies (1) demonstrated that the enzyme from *T. cruzi* will salvage both guanine and hypoxanthine with similar efficiency; therefore, the enzyme is also referred to as the hypoxanthine-guanine phosphoribosyltransferase (HGPRT).

In this report we describe the initiation of a target-based approach to the discovery of inhibitors targeted to the HPRT from *T. cruzi*. Initially, a recombinant screening system was used to identify compounds that bind to the trypanosomal HPRT. This screening assay is based on a technique which uses the activities of recombinant HPRTs to complement genetic deficiencies of the host bacteria (11). Results of kinetic studies of these compounds in which purified recombinant human and trypanosomal enzymes are used, further support the results from the bacterial screening assays.

Additionally, crystallization conditions were determined for the purified, recombinant trypanosomal enzyme. The crystals, which grow as hexagonal rods, diffract X rays to 2-Å resolution, and preliminary analysis reveals the space group to be $P3_121$ or $P3_221$. These results represent a significant advance toward the

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initiation of the process of inhibitor modification and design based on the three-dimensional structure of the trypanosomal enzyme.

MATERIALS AND METHODS

Source of purines and purine analogs. Hypoxanthine, guanine, GMP, IMP, allopurinol, and formycin B were from Sigma Chemical Co., St. Louis, Mo. All other purine analogs were generously provided by Burroughs Wellcome Co. (now GlaxoWellcome), Research Triangle Park, N.C.

Bacterial strains. *Escherichia coli* SØ609 (Δ*pro-gpt-lac hpt purHJ thi pup ara strA*) (21) was used for the complement selection and screening assays. Recombinant HPRTs were expressed in a *recA* mutant strain of *E. coli* SØ606 (Δ*pro-gpt-lac hpt ara thi*) (21).

Cloning and expression of recombinant HPRTs. The coding sequence for the trypanosomal HPRT was amplified from genomic DNA (strain RA) (6) by PCR with primers designed by using a previously reported sequence obtained from GenBank (accession no. L07486) (1). The sequence of the amplified gene has nine silent base substitutions and three amino acid changes with respect to the previously published sequence: lysine instead of methionine at position 23, cysteine in place of serine at position 66, and leucine instead of valine at position 86. These amino acid differences appeared in several independent PCR experiments and likely exist because of differences between the strains of the parasite rather than as a result of polymerase errors.

The coding sequence was inserted into the *Nde*I and *Pst*I sites of the pBAce expression vector (4) to create plasmid pTcPRT, which was used to transform *E. coli* SØ606. A similar construct containing cDNA encoding the human HPRT (pHumPRT) was used to transform SØ606 for production of the human enzyme in bacteria. Expression of the recombinant HPRTs was induced by phosphate starvation with semidefined medium similar to that described previously (4). Modification of the medium included the use of 150 μM phosphate, 100 μg of ampicillin per ml, and 20 μg of guanine per ml instead of 100 μM phosphate, 75 μg of ampicillin per ml, and 20 μg of adenine per ml. The bacteria were harvested by centrifugation after attaining an optical density at 600 nm of 1.3 and were resuspended in TMD buffer (100 mM Tris [pH 7.5], 8 mM $MgCl_2$, 1 mM dithiothreitol) plus Triton X-100 at 1% and lysozyme at 0.5 mg/ml to assist in cell lysis. The bacteria were lysed by repeated freeze-thaw steps in dry ice-ethanol and a 37°C water bath. The insoluble material in the bacterial lysates was separated from the soluble proteins by centrifugation at 20,000 × *g* for 30 min at 4°C. The human enzyme is resistant to high temperatures (34); therefore, lysates of bacteria expressing this enzyme were first heated to 80°C for 5 min to precipitate most of the bacterial proteins.

The recombinant HPRTs were further purified from the soluble fraction of the bacterial lysates by affinity chromatography with GMP-agarose. The procedures were modified from those published previously for the trypanosomal enzyme (1), as follows. The soluble supernatant of the lysate was loaded onto 7 to 10 ml of GMP-agarose chromatography resin (Sigma) that had been preequilibrated with TMD buffer by gently rocking in a 50-ml tube for 30 min at 4°C. The resin (with bound enzyme) was subsequently packed into an Econo-column (1.5 by 10 cm; Bio-Rad, Hercules, Calif.), and the column was washed with at least 150 ml of TMD buffer to remove proteins that do not specifically bind to the GMP-agarose. The recombinant HPRT was eluted in 2-ml fractions by using TMD buffer containing 5 mM GMP, usually in a volume of 6 to 10 ml. Finally, the GMP was removed by using a column containing 10 ml of Bio-Gel P10 polyacrylamide resin (Bio-Rad), and the purified enzyme was stored at 4°C in TMD buffer.

Bacterial screening assay. For the bacterial screening assays, the recombinant trypanosomal and human HPRTs (encoded in plasmids pTcPRT and pHum PRT, respectively) were used to complement the genetic deficiencies of the SØ609 strain of *E. coli* (21). When grown on selective medium supplemented with guanine, these bacteria may grow only if they are complemented by an active recombinant HPRT (11). By this screening assay, purine analogs were tested for their ability to bind the human and trypanosomal HPRTs on the basis of their effects on the growth of bacteria complemented by each of the recombinant enzymes.

Expression of the recombinant HPRTs is controlled by the *phoA* promoter of the pBAce expression plasmid (4). After the bacteria have depleted the phosphate in the semidefined medium, the expression levels of the recombinant HPRTs reach a significant percentage of the total soluble bacterial proteins that are expressed. The relative levels of expression are comparable for both the human and the trypanosomal enzymes (data not shown). During the early stages of bacterial growth, when phosphate levels in the medium are high, expression of the recombinant enzyme relies on the leaky *phoA* promoter, which allows sufficient quantities of the HPRTs to be produced to complement the genetic deficiency of the bacteria (11). The levels of recombinant HPRT expression during the early stages of cell growth are predicted to be in the range of 0.005 to 0.01% of the total bacterial proteins (11). These early conditions probably provide a fairly accurate simulation of the concentration of HPRTs in cells in vivo.

Bacterial cells were grown in nutrient-containing medium (NZCYM) to an optical density at 600 nm of 1.0 before harvesting, washing, and plating on semidefined medium as described previously (11). Sterile filter discs with 13 μg of test compound in dimethyl sulfoxide were distributed on plates preseeded with bacterial lawns, followed by incubation at 37°C to permit bacterial growth. Zones where the growth of bacteria was inhibited were observed, measured (starting from the edge of the disk), and tabulated. Identical plates were prepared with bacteria complemented by the human and trypanosomal HPRTs to compare the effects of the compounds on the two enzymes. Dimethyl sulfoxide alone had no effect on the bacterial growth. To assess the general bactericidal effects of these compounds, control bacteria transformed with the pBAce plasmid alone (which does not encode a recombinant HPRT) were grown on nutrient-containing medium, and the observed zones of inhibition of bacterial growth were recorded.

Enzyme assays. Enzyme activity was measured by using [^{14}C]hypoxanthine, as described previously (32). For inhibition studies, enzyme activity was measured in the presence of variable concentrations of the purine analogs (between 1 μM and 10 mM). For Dixon plot analysis (9), unlabelled hypoxanthine was used as the "inhibitor," as described above with three different concentrations of [^{14}C]hypoxanthine (13, 6.5, and 3.25 μM). Thus, the apparent K_is determined from these Dixon plots represent the apparent K_ms for hypoxanthine with the HPRTs. The 50% inhibitory concentrations (IC_{50}s) of the compounds were estimated to be the molar concentrations which reduce the activity of the enzyme to 50% of that observed in the absence of the compound. These measurements were performed under assay conditions in which the labelled substrate concentration was approximately equal to its K_m for each of the enzymes (13 μM for the trypanosomal enzyme and 6.5 μM for the human enzyme). Under these conditions, the IC_{50}s provide good approximations of the apparent K_is for the compounds, where the K_i is half of the observed IC_{50} by the following equation (27): $IC_{50} = [1 + ([S]/K_m)]K_i$, where $[S]$ is the concentration of substrate in the reaction.

Crystallization studies. Crystallization conditions were determined by hanging-drop vapor diffusion by a sparse matrix sampling method (20) and also a grid search of pH versus polyethylene glycol (PEG) concentration with commercially available reagents (Hampton Research Co., Riverside, Calif.). The best crystallization conditions for the *T. cruzi* HPRT were identified in the pH-versus-PEG screening and were refined by using finer grids of pH and PEG concentration. With the protein concentration at 10 to 12 mg/ml (as determined by the Bio-Rad Protein Assay with bovine serum albumin as the standard) in 20 mM Tris buffer (pH 8.0) and 5 mM $MgCl_2$, the protein solution was mixed with equal amounts (usually 3 μl each) of the precipitating solution comprised of 100 mM morpholineethanesulfonic acid (MES) buffer (pH 5.75 to 6.0; Sigma) and PEG 6000 (Hampton Research Co.) in the range of 9 to 12% and was equilibrated against 500 μl of this solution. The crystals grow as clusters of large hexagonal rods up to 1.0 mm in length. Crystals of similar morphology have been obtained in the presence of several different ligands, including formycin B, acyclovir, allopurinol riboside, allopurinol with PRPP, and 7-hydroxypyrazolo[4,3-*d*]pyrimidine with PRPP.

Crystals grown in the presence of formycin B and acyclovir have been characterized by using X-ray data collected at the Macromolecular Structure Group Laboratory at the University of California at San Francisco with an R-axis IIc image plate detector system equipped with a Rigaku RU200 rotating anode generator running at 50 kV and 300 mA. The two crystal forms have similar unit cell dimensions and belong to the same space group. The resolution limit of these crystals was improved by cryocrystallography and by collecting data at the Stanford Synchrotron Radiation Laboratory, beamline 7-1. All X-ray data measured have been collected from cryofrozen crystals and processed by using DENZO and SCALEPACK (25).

RESULTS

The pBAce expression system (4) enabled the generation of milligram quantities of highly purified trypanosomal HPRT. Figure 1 shows a Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel demonstrating the high-level expression of the trypanosomal HPRT in *E. coli* and its purification by affinity chromatography with GMP-agarose. Expression and purification of the human enzyme gave comparable results (data not shown). The purified recombinant HPRT of *T. cruzi* has a specific activity of 60 μmol min^{-1} mg^{-1} with hypoxanthine as the substrate. This specific activity is 10-fold greater than that determined for the purified recombinant human enzyme under the same conditions (6 μmol min^{-1} mg^{-1}).

In a previous report, we demonstrated the advantages of using bacteria to screen purine analogs for compounds which bind to the HPRTs of several different parasites (11). In the study described in this report, we extended those studies to include recombinant bacteria complemented with the HPRT from *T. cruzi* and compared these results with those for bacteria expressing the human HPRT. Fifty-nine compounds were obtained commercially or were provided by Burroughs Well-

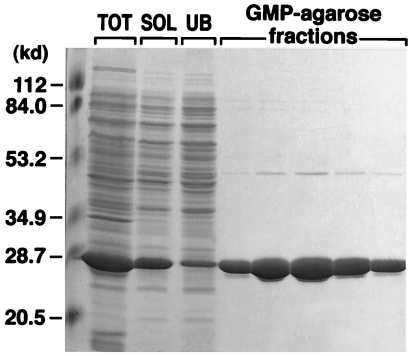
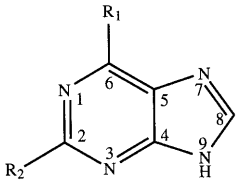


FIG. 1. High-level expression of the trypanosomal HPRT in *E. coli* and purification with GMP-agarose. The Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel shows the expression and purification of the recombinant HPRT from *T. cruzi*. Lane 1, molecular mass standards, with their masses (in kilodaltons) indicated to the left of the gel; lane TOT, total proteins from intact bacterial cells; lane SOL, soluble proteins after cell lysis; lane UB, proteins that did not bind to the GMP-agarose column. The subsequent lanes are aliquots of fractions that eluted from the column in 5 mM GMP. The recombinant HPRT migrates in the gel just below the 28.7-kDa molecular size standard.

come (now GlaxoWellcome) and were tested in the bacterial screening assay. The structures of all of these compounds have been reported previously (11). Eight compounds were identified by the assay to inhibit the growth of bacteria complemented by the recombinant trypanosomal HPRT. The structures of these compounds and their effects on bacterial growth are summarized in Table 1. Of all the compounds, 6-thiocyanopurine (compound 7) showed the most impressive zone of inhibition (Table 1). However, this compound appeared to be nonselective, because it affected the growth of cells complemented with the human HPRT about as much as it affected the growth of those expressing the trypanosomal enzyme. On the other hand, two compounds, 2-amino-6-iodopurine (compound 5) and 6-sulfinylpurine (compound 6), showed selective inhibition of growth of the bacteria complemented by the trypanosomal enzyme.

TABLE 1. Inhibition of growth of bacteria complemented by HPRTs



Compound no.	Compound substitutions			Inhibition[a]		
	R_1	R_2	Other	Control HPRT	Human HPRT	*T. cruzi* HPRT
1	-OH	-H		−	−	−
2	-OH	$-NH_2$		−	−	−
3	-OH	-OH		−	−	−
4	-I	-H		+	+	+
5	-I	$-NH_2$		−	++	+++
6	$-SOCH_3$	-H		−	+	++
7	-SCN	-H		+	+++	+++
8	-SCN	$-NH_2$		+	++	+
9	-OH	$-NH_2$	8-SH	−	−	+
10[b]	-OH	-H	9-Deaza, 8-aza	+	+	+
11	-OH	$-NHCH_2CH_3$		−	−	+

[a] +, zones of inhibition with radii that average <2 mm; ++, zones of 2 to 4 mm; +++, zones of >4 mm; −, no inhibition.
[b] Compound 10 is 7-hydroxypyrazolo[4,3-*d*]pyrimidine.

TABLE 2. Inhibition constants for compounds with purified human and trypanosomal HPRTs

Compound no.	Compound substitutions		IC_{50}	
	R_1	R_2	Human HPRT	*T. cruzi* HPRT
5	-I	$-NH_2$	2.4 mM	590 μM
6	$-SOCH_3$	-H	5.6 mM	2.4 mM
7	-SCN	-H	56 μM	12 μM
10[a]			63 ± 1 μM[b]	51 ± 4 μM[b]
Allopurinol			13 mM	2.7 mM
Formycin B			3.7 mM	2.1 mM

[a] Compound 10 is 7-hydroxypyrazolo[4,3-*d*]pyrimidine.
[b] K_is were determined for this compound by Dixon analysis (27).

Table 2 presents the IC_{50}s of several compounds identified in the bacterial assays with purified trypanosomal and human HPRTs. These assays were performed under reaction conditions in which the concentration of the competing [^{14}C]hypoxanthine was close to its K_m for each enzyme. Therefore, these IC_{50}s accurately reflect the apparent K_is for each enzyme ($K_i = IC_{50}/2$) and provide reliable comparisons of the relative affinities of the compounds for these HPRTs. Although 6-thiocyanopurine (compound 7) appeared to be nonselective in the bacterial assay, the results of kinetic studies indicate that it had a 4.6-fold selective preference for the trypanosomal enzyme versus the human enzyme (Table 2). Another compound that was slightly selective for the trypanosomal enzyme was 2-amino-6-iodopurine (compound 5). This analog of guanine had a fourfold preference in binding for the trypanosomal enzyme. However, this compound had a higher IC_{50} for the enzyme than did 6-thiocyanopurine.

Because the activity measurements were based on the conversion of radiolabelled substrate ([^{14}C]hypoxanthine) to product ([^{14}C]IMP), the addition of unlabelled hypoxanthine to the assays caused an apparent inhibition of enzyme activity. In this case, the apparent K_i represents the K_m for hypoxanthine. Thus, to test the validity of the data from the inhibition assays described above, we used hypoxanthine as an unlabelled "inhibitor." Plots of inverse activity versus inhibitor concentration (Dixon plots) give straight lines that intersect above the *x* axis (Fig. 2). The inhibitor concentration at which the lines intersect is the negative of the apparent K_i (9). On the basis of the Dixon plots, the apparent K_i for hypoxanthine was determined for the recombinant trypanosomal enzyme to be 13.5 μM (Fig. 2A). This value differs from that reported previously (6.4 μM) (1). The discrepancy may be the result of minor amino acid differences in the recombinant enzymes or may reflect slight differences in assay conditions or methods, or both. Preliminary studies in our laboratory by a spectrophotometric assay (14, 33) provide an independent determination of 12.7 ± 1.6 μM for the K_m for hypoxanthine with our purified trypanosomal enzyme (data not shown). The K_m for hypoxanthine with the human HPRT (8.8 μM) (Fig. 2B) compares well with the previously published value of 7.7 ± 0.4 μM (14).

Although 7-hydroxypyrazolo[4,3-*d*]pyrimidine (compound 10) was not initially identified in the bacterial assay as a potent inhibitor of the trypanosomal enzyme, Dixon plots confirm that this compound competitively binds the purified human and

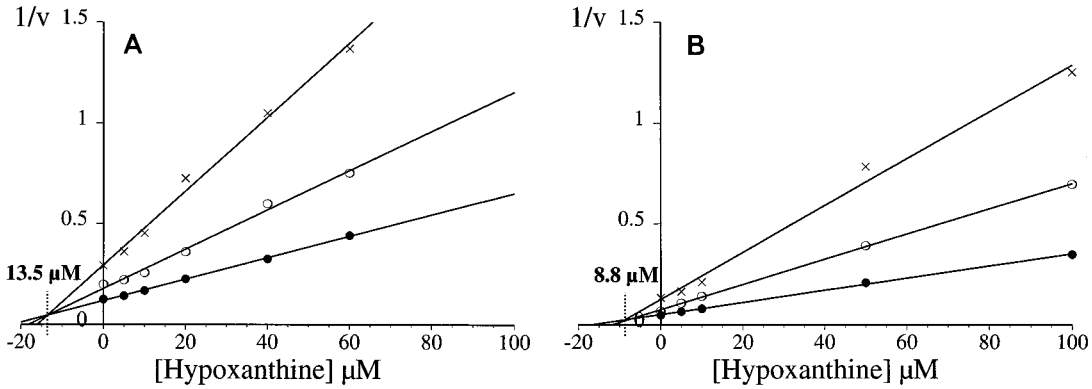


FIG. 2. Apparent K_m determination for hypoxanthine with purified trypanosomal and human HPRTs by Dixon plot analysis. The graphs are Dixon plots (9) of inhibition studies in which unlabelled hypoxanthine was used as the competitive inhibitor in activity assays for purified trypanosomal (A) and human (B) HPRTs. HPRT activity was calculated by measuring the amount of labelled IMP formed over time (plotted as inverse velocity, 1/v) at three different concentrations of ^{14}C-labelled hypoxanthine: 13 μM (●), 6.5 μM (○), and 3.25 μM (×). Because the unlabelled hypoxanthine acts as a competitive inhibitor with the [^{14}C]hypoxanthine in this assay, the K_is for hypoxanthine are equal to the K_m for this substrate with each HPRT. The K_is (in boldface type above the *x* axis) are obtained from the plots as the inverse of the concentration of inhibitor, where lines from the three substrate concentrations intersect (shown here by dashed line).

trypanosomal HPRTs with K_is of 63 and 51 μM, respectively (Table 2).

Formycin B is a nucleoside analog in which there is a carbon-carbon bond joining the purine and ribose rings. In addition, formycin B has a nitrogen at position 8 rather than a carbon. Although the apparent IC_{50} was determined to be 2.1 mM by the radioactive competition assays (Table 2), this compound was used successfully as a ligand in cocrystallization experiments with the trypanosomal HPRT. In fact, the addition of formycin B to the trypanosomal HPRT in crystallization trials permits the growth of higher-quality crystals than those obtained by using the natural product, GMP, as a ligand. This observation suggests that the conformation or electrochemistry of formycin B may help stabilize the trypanosomal enzyme.

The bacterial expression system and purification scheme provided a large quantity of the trypanosomal HPRT of sufficient quality to enable screening for crystallization conditions for this enzyme. X-ray diffraction quality crystals were obtained by using a PEG-versus-pH grid search. Typical crystals were hexagonal rods with dimensions of 0.15 by 0.15 by 0.75 mm and diffracted X-rays to better than 2.5 Å resolution on a rotating anode generator. A 100% complete data set to 2.5 Å resolution was collected at the University of California at San Francisco's Macromolecular Structure Group Laboratory by cryocrystallography. The space group has been determined to be either $P3_121$ or $P3_221$.

Crystals obtained in the presence of two different ligands, 5 mM formycin B or acyclovir, were analyzed at the Stanford Synchrotron Radiation Laboratory (beamline 7-1) where the diffraction limit was increased to 2.0 Å. The data for the crystal grown in the presence of acyclovir are summarized in Table 3 and are representative of the data in both data sets. Molecular replacement searches have been conducted by using the coordinates from both the human (10) and *Tritrichomonas foetus* (28) HPRTs as search models, for which the sequence identities to the *T. cruzi* HPRT are approximately 28 and 31% respectively. However, efforts to solve the molecular replacement by using these probes have been unsuccessful thus far. Therefore, our current approach includes attempts to identify heavy-atom derivatives of the crystals of the trypanosomal HPRT to enable the solution of the data by isomorphous replacement methods.

DISCUSSION

Although enzymes in purine salvage pathways were proposed nearly 30 years ago as possible targets for drugs in the chemotherapeutic treatment of diseases caused by parasites, as of this writing, compounds that inhibit HPRTs have yet to be used clinically in the treatment of any disease. Instead, investigators have identified a number of analogs of the natural substrates of HPRTs that are toxic to parasite and host cells after incorporation into their nucleotide pools. Unfortunately, none of these compounds provide information about the effects of inhibiting HPRT activity in parasites.

Weak competitive inhibitors of the human HPRT have been identified formerly (19). However, the concentrations of a competitive inhibitor needed to inhibit HPRT activity will probably be so high that this type of inhibitor is not likely to be useful for the treatment of a parasitic disease. What is lacking

TABLE 3. Crystallographic data for the trypanosomal HPRT

Space group	$P3_221$ or $P3_121$
Unit cell dimensions	$a = b = 95.33$ Å, $c = 76.90$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$
Resolution (Å)	2.00
Completeness	96.1%; 69.6% for 2.0- to 2.1-Å shell
Total observations	183,000
Unique reflections ($I/\sigma(I) > 0$)	25,974
R merge (on *I*)[a]	0.041
$<I/\sigma(I)>$	18.5

[a] R merge = $\Sigma\,|I_o - <I>|/\Sigma I_o$, where I_o is observed intensity and $<I>$ is the average intensity obtained from multiple observations of symmetry-related reflections. $\sigma(I)$ is the standard error of the measurement of the observed intensity.

today is a potent inhibitor or a lead compound that could be used to test the effects of inhibiting HPRT activity in a parasite. Analogs of the transition state or bisubstrate types of inhibitors might provide an approach to the design of a potent inhibitor of HPRTs (5). Even though potent inhibitors are not yet available, it should be possible to develop systems that would be useful for testing compounds targeted to the HPRTs of parasites. Furthermore, once an initial inhibitor has been identified, methods of combinatorial chemistry could be used to modify the lead compound, and a recombinant system would be useful for rapidly screening large numbers of derivatives for those which can enter bacteria and inhibit an HPRT. The requirement for crossing a bacterial membrane might result in potentially effective compounds being overlooked in such a screen, but given the large numbers of compounds to be tested, the recombinant system should be useful for identifying many compounds that are effective in inhibiting HPRT activity.

The recombinant screening system described herein is probably best suited for testing inhibitors of HPRTs that bind with apparent K_is in the submicromolar range. Unfortunately, compounds which bind to HPRTs in this concentration range are not yet available. Given the limitations of the compounds available for testing as substrates or inhibitors of HPRTs, the results described herein indicate that a recombinant screening system can be used to rapidly identify compounds which bind to the HPRT of *T. cruzi*. Also, purified human and trypanosomal recombinant enzymes were used to determine kinetic parameters for compounds identified in the recombinant bacterial assay. The inhibition data obtained for selected compounds with the purified enzymes are consistent with the results from the bacterial screening assay in that those compounds identified by screening demonstrate affinities to the HPRTs (Tables 1 and 2).

Although 6-thiocyanopurine (compound 7) showed equally impressive zones of inhibited growth (>4 mm) in the screening assays both for bacteria expressing the human HPRT and for bacteria expressing the trypanosomal HPRT (Table 1), the inhibition constants showed a 4.6-fold tighter binding affinity for the trypanosomal enzyme than for the human enzyme (Table 2). This difference in binding affinity was not predicted by the bacterial assay, possibly due to sensitivity being diminished at the more extreme levels of growth inhibition. 6-Sulfinylpurine (compound 6) demonstrates the disadvantage of testing alternate substrates, rather than inhibitors, of HPRTs in the bacterial screening assay. This compound moderately inhibited the growth of bacteria complemented by the trypanosomal enzyme (Table 1), but the IC_{50} of this compound was in the millimolar range (Table 2). The inhibition of bacterial growth may have been exaggerated by the highly toxic effects in bacteria of the nucleotide analog formed by the HPRTs. Interestingly, the twofold lower IC_{50} of this compound for the trypanosomal HPRT than for the human enzyme was accurately predicted by the bacterial assay and suggests that a more efficient turnover of the alternate substrate into the nucleotide pool in bacteria may result in an increase in the observed inhibition of growth. The problems with the interpretation of results for alternate substrates in the bacterial assay should be eliminated when true inhibitors of HPRTs are tested.

A disadvantage of the enzyme activity assays with radiolabelled substrates is that they do not discriminate between compounds acting as inhibitors or as alternate substrates; both cases will cause an observed decrease in the formation of labelled product. Therefore, an IC_{50} could reflect either (i) a true K_i if the compound is acting as a competitive inhibitor and reducing the amount of enzyme capable of catalysis by binding the active site unproductively or (ii) a K_m if the analog is acting as a substrate and forming undetectable nonlabelled product analogs. In either case, the value of the observed constant should indicate the relative potencies of the compounds and their selectivities for the trypanosomal enzyme versus the human enzyme.

Although the library of purine analogs tested in this study did not provide a useful lead compound for developing inhibitors of HPRTs, comparisons of the structures of those compounds which demonstrated affinities for the target enzymes reveal structural details that may enhance their binding to HPRTs. These structural details may be useful for incorporation into future, more potent inhibitors. All of the compounds identified by the bacterial screening assay and/or shown by kinetics to interact with the trypanosomal HPRT have electronegative atoms covalently bound to the carbon at position 6 of the purine ring (Tables 1 and 2). These results are consistent with the prediction that the major determinant in the substrate preference of HPRTs is an amino group of a lysine (K165 in the human enzyme) forming a hydrogen bond with exocyclic oxygen at position 6 of oxopurines (10). This lysine is conserved in all HPRTs sequenced to date.

All but 1 of the 59 compounds tested could theoretically be alternate substrates for HPRTs. The only compound that is predicted to be a true inhibitor of HPRTs is 7-hydroxypyrazolo[4,3-*d*]pyrimidine (compound 10). This hypoxanthine analog has a carbon rather than a nitrogen at position 9. The nitrogen at position 9 is key to the chemistry catalyzed by HPRTs, because it is the site where the ribose of PRPP is covalently linked to the purine base during the formation of IMP (10). Replacement of a nitrogen by a less reactive carbon should prevent this compound from acting as a substrate for the reaction. The K_i of compound 10 for the trypanosomal enzyme is slightly lower than that for the human HPRT. This difference in affinity may be due to the additional substitution of a nitrogen for a carbon at position 8 in the ring.

As a competitive inhibitor of HPRTs, compound 10 might be expected to inhibit the growth of bacteria in the recombinant screens. The fact that this compound went undetected in the recombinant screens is probably because it had K_i values above 50 μM for both the human and the trypanosomal enzymes. The concentration of guanine in the medium was 100 μM. Since the K_i of compound 10 is 5-fold higher than the K_m of guanine for the trypanosomal HPRT (13.3 μM; data not shown), the concentration of this inhibitor needed to compete with the available purines in the medium is predicted to be in the range of 500 μM. The use of such a high concentration is impractical and emphasizes the requirement for new strategies to create more potent inhibitors.

Allopurinol was included in our assays because it has been proposed as a potential candidate drug for the treatment of Chagas' disease (7, 12). Clinical studies demonstrated that at dosages of 600 and 900 mg/day, allopurinol was as efficacious as the currently used nitrofurans (nifurtimox and benznidazole) but showed fewer and more mild side effects in humans (12). Allopurinol is a hypoxanthine analog that is an alternate substrate of the trypanosomal HPRT and is salvaged by the enzyme and converted to the 4-hydroxypyrazolopyrimidine analog of IMP. The modified IMP (allopurinol ribotide) is subsequently converted to an AMP analog (aminopurinol ribotide) which is phosphorylated to the nucleotide triphosphate and incorporated into the parasite's RNA, causing the breakdown of mRNA synthesis (23). In our bacterial screening assays, allopurinol had no significant effect on the growth of bacteria expressing the trypanosomal HPRT. Inhibition studies with the purified recombinant enzymes demonstrate that allopurinol has an IC_{50} of greater than 2 mM for the purified

trypanosomal enzyme and an IC_{50} of greater than 10 mM for the human enzyme (Table 2). It is probable that allopurinol yields negative results in the recombinant screens because of inefficient salvage by the target HPRT. Alternatively, the bacteria could be less sensitive to the toxic effects of an allopurinol nucleotide. However, since the bacterial screening assay is intended to identify compounds that efficiently bind HPRTs, it is important that the assay yields negative results for compounds with very poor binding affinities.

Thorough comparisons of the structure of the human enzyme and the predicted structures of several parasite HPRTs do not reveal major differences in the identities of amino acids within 5 Å of the purine ring of the GMP ligand. Therefore, it is difficult to predict which amino acids are responsible for the observed differences in the selective binding of substrates or analogs among the HPRTs. These observations support the possibility that the structure of the HPRT when it is bound to a product (GMP) may be conformationally distinct from that of the enzyme when substrates are bound. Therefore, the three-dimensional structures currently available may reveal amino acids that are important for binding the product of catalysis just before its release but not those that are important for the initial binding of substrates or substrate analogs.

Ideally, additional structures of the HPRTs bound to unreacted substrates are needed to provide the missing details about the structural determinants of substrate binding. Structures of the HPRTs with both substrates bound may reveal differences between the human and trypanosomal enzymes that could be exploited in the redesign of more potent and selective inhibitors. Unfortunately, previous kinetic studies of the human and schistosomal HPRTs indicate that their mechanisms require ordered binding first of PRPP and then of the purine base (14, 33). Therefore, these enzymes may not bind the purine base alone. One possible means of obtaining a structure with unreacted purines bound to the active site is to use noncatalyzable analogs of the substrates to capture the enzyme in a conformation which more closely resembles the transition state of the reaction. Compound 10 is an excellent candidate ligand for this cocrystallization approach, because it should act as a nonsalvageable purine base. Although crystallization with PRPP alone has been difficult, cocrystallization of the trypanosomal enzyme with PRPP and compound 10 produces crystals of a quality similar to those obtained when formycin B and acyclovir are used as ligands. Therefore, these crystals may provide the solution of a structure of the HPRT with substrate analogs bound. Once the initial solution of the three-dimensional structure has been refined, additional structures of the enzyme with various ligands will provide important information regarding the binding of purine and purine nucleotide analogs to the active site of the trypanosomal HPRT.

Inhibitors that accurately mimic the transition state of the enzyme-catalyzed reaction may bind the enzyme with affinities much stronger than the affinities of the natural substrates and therefore have strong potential as lead compounds for drug design (5, 26, 31). For HPRTs, the transition state will have characteristics of both a purine ring and PRPP. Our studies of purine analogs provide new information about differences in the purine binding regions of the human and trypanosomal enzymes. This knowledge can be combined with our understanding of the kinetic mechanism and the chemistry of the reaction to design a compound that mimics details of the transition state and more completely fills the active site with a binding affinity that exceeds that of either of the substrates. Such a compound may be highly selective for the trypanosomal HPRT and therefore should reduce toxic side effects caused by unintentional interactions with other enzymes. Ultimately, iterative structural analysis (2) of the trypanosomal enzyme with lead compounds bound to the active site will permit analysis of molecular contacts between newly designed leads and the target enzyme. Such procedures have been used successfully to design inhibitors of thymidylate synthetase (30) and the human immunodeficiency virus protease (for references, see reference 8). Coupling an iterative structural approach with the bacterial screens and kinetic and inhibition studies with the recombinant enzyme will provide information that can be used to make modifications to initial lead compounds to further enhance their selective binding to the trypanosomal enzyme.

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REFERENCES

1. **Allen, T. E., and B. Ullman.** 1994. Molecular characterization and overexpression of the hypoxanthine-guanine phosphoribosyltransferase gene from *Trypanosoma cruzi*. Mol. Biochem. Parasitol. **65:**233–245.
2. **Appelt, K., R. J. Bacquet, C. A. Bartlett, et al.** 1991. Design of enzyme inhibitors using iterative protein crystallographic analysis. J. Med. Chem. **34:**1925–1934.
3. **Berens, R. L., E. C. Krug, and J. J. Marr.** 1995. Purine and pyrimidine metabolism, p. 89–117. *In* J. J. Marr and M. Müller (ed.), Biochemistry and molecular biology of parasites. Academic Press, Inc., San Diego, Calif.
4. **Craig, S. P., III, L. Yuan, D. A. Kuntz, J. H. McKerrow, and C. C. Wang.** 1991. High level expression in *Escherichia coli* of soluble, enzymatically active schistosomal hypoxanthine-guanine phosphoribosyltransferase and trypanosomal ornithine decarboxylase. Proc. Natl. Acad. Sci. USA **88:**2500–2504.
5. **Craig, S. P., III, and A. E. Eakin.** 1997. Purine salvage enzymes as targets for structure based inhibitor design. Parasitol. Today **13:**238–241.
6. **Craig, S. P., III, C. Castro, A. E. Eakin, and M. Castaneda.** 1990. *Trypanosoma* (*Schizotrypanum*) *cruzi*: repetitive DNA sequence evolution in three geographically distinct isolates. Comp. Biochem. Physiol. B Comp. Biochem. **95:**657–662.
7. **De Castro, S. L.** 1993. The challenge of Chagas' disease chemotherapy: an update of drugs assayed against *Trypanosoma cruzi*. Acta Trop. **53:**83–98.
8. **De Clercq, E.** 1995. Toward improved anti-HIV chemotherapy: therapeutic strategies for intervention with HIV infections. J. Med. Chem. **38:**2491–2517.
9. **Dixon, M.** 1953. Determination of enzyme inhibition constants. Biochem. J. **55:**170–171.
10. **Eads, J. C., G. Scapin, Y. Xu, C. Grubmeyer, and J. C. Sacchettini.** 1994. The crystal structure of human hypoxanthine-guanine phosphoribosyltransferase with bound GMP. Cell **78:**325–334.
11. **Eakin, A. E., R. Nieves-Alicea, R. Tosado-Acevedo, M. S. Chin, C. C. Wang, and S. P. Craig III.** 1995. Comparative complementation selection in bacteria enables screening for lead compounds targeted to a purine salvage enzyme of parasites. Antimicrob. Agents Chemother. **39:**620–625.
12. **Gallerano, R. H., J. J. Marr, and R. R. Sosa.** 1990. Therapeutic efficacy of allopurinol in patients with chronic Chagas' disease. Am. J. Trop. Med. Hyg. **43:**159–166.
13. **Garcia-Zapata, M. T. A., P. B. McGreevy and P. D. Marsden.** 1991. American trypanosomiasis, p. 628–637. *In* G. T. Strickland (ed.), Hunter's tropical medicine, 7th ed. The W. B. Saunders Co., Philadelphia, Pa.
14. **Giacomello, A., and C. Salerno.** 1978. Human hypoxanthine-guanine phosphoribosyltransferase; steady state kinetics of the forward and reverse reactions. J. Biol. Chem. **253:**6038–6044.
15. **Gutteridge, W. E., and M. J. Davies.** 1982. Properties of the purine phosphoribosyltransferases of *Trypanosoma cruzi*. FEMS Microbiol. Lett. **13:**207–212.
16. **Gutteridge, W. E., and M. Gaborak.** 1979. A re-examination of purine and pyrimidine synthesis in the three main forms of *Trypanosoma cruzi*. Int. J. Biochem. **10:**415–422.
17. **Hammond, D. J., and W. E. Gutteridge.** 1984. Purine and pyrimidine metabolism in the Trypanosomatidae. Mol. Biochem. Parasitol. **13:**243–261.
18. **Hartwick, R. A., A. M. Krstulovic, and P. R. Brown.** 1979. Identification and quantitation of nucleosides, bases and other UV-absorbing compounds in serum, using reversed-phase HPLC. II. Evaluation of human sera. J. Chromatogr. **186:**659–676.
19. **Jadhav, A. L., L. B. Townsend, and J. A. Nelson.** 1979. Inhibition of hypox-

anthine-guanine phosphoribosyltransferase. Biochem. Pharmacol. **28:**1057–1062.
20. **Jancarik, J., and S. H. Kim.** 1991. Sparse matrix sampling: a screening method for crystallization of proteins. J. Appl. Crystallogr. **24:**409–411.
21. **Jochimsen, B., P. Nygaard, and T. Vestergaard.** 1975. Location on the chromosome of *Escherichia coli* of genes governing purine metabolism. Mol. Gen. Genet. **143:**85–91.
22. **Marr, J. J.** 1991. Purine analogs as chemotherapeutic agents in leishmaniasis and American trypanosomiasis. J. Lab. Clin. Med. **118:**111–119.
23. **Marr, J. J., and R. L. Berens.** 1983. Pyrazolopyrimidine metabolism in the pathogenic Trypanosomatidae. Mol. Biochem. Parasitol. **7:**339–356.
24. **Marr, J. J., R. L. Berens, S. W. LaFon, and D. J. Nelson.** 1981. Purine metabolism in *T. cruzi*. Mol. Biochem. Parasitol. **3:**187–196.
25. **Otwinowski, Z.** 1993. DENZO, p. 56–62 *In* Proceedings of the CCP4 Study Weekend: Data Collection and Processing. SERC Daresbury Laboratory, Warrington, England.
26. **Schramm, V. L., B. A. Horenstein, and P. C. Kline.** 1994. Transition state analysis and inhibitor design for enzymatic reactions. J. Biol. Chem. **269:**18259–18262.
27. **Segel, I. H.** 1975. Enzyme inhibition, p. 118–120. *In* Enzyme kinetics. John Wiley & Sons, Inc., New York, N.Y.
28. **Somoza, J. R., M. S. Chin, P. J. Focia, C. C. Wang, and R. J. Fletterick.** 1996. Crystal structure of the hypoxanthine-guanine-xanthine phosphoribosyltransferase from the protozoan parasite *Tritrichomonas foetus*. Biochemistry **35:**7032–7040.
29. **Ullman, B., and D. Carter.** 1995. Hypoxanthine-guanine phosphoribosyltransferase as a therapeutic target in protozoal infections. Infect. Agents Dis. **4:**29–40.
30. **Varney, M. D., G. P. Marzoni, C. L. Palmer, et al.** 1992. Crystal-structure-based design and synthesis of benz[*cd*]indole-containing inhibitors of thymidylate synthetase. J. Med. Chem. **35:**663–676.
31. **Wolfendon, R.** 1969. Transition state analogs for enzyme catalysis. Nature **223:**704–705.
32. **Yuan, L., S. P. Craig III, J. H. McKerrow, and C. C. Wang.** 1990. The hypoxanthine-guanine phosphoribosyltransferase of *Schistosoma mansoni*. J. Biol. Chem. **265:**13528–13532.
33. **Yuan, L., S. P. Craig III, J. H. McKerrow, and C. C. Wang.** 1992. Steady-state kinetics of the schistosomal hypoxanthine-guanine phosphoribosyltransferase. Biochemistry **31:**806–810.
34. **Yuan, L., C. S. C. Wu, S. P. Craig III, A. F. Liu, J. H. McKerrow, and C. C. Wang.** 1993. Comparing human and schistosomal hypoxanthine-guanine phosphoribosyltransferases by circular dichroism. Biochim. Biophys. Acta **1162:**10–16.