Lipophilic Antifolate Trimetrexate Is a Potent Inhibitor of *Trypanosoma cruzi*: Prospect for Chemotherapy of Chagas' Disease

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***Trypanosoma cruzi*, a protozoan parasite, is the causative agent for Chagas' disease, which poses serious public health problem in Latin America. The two drugs available for the treatment of this disease are effective only against recent infections and are toxic. Dihydrofolate reductase (DHFR) has a proven track record as a drug target. The lipophilic antifolate trimetrexate (TMQ), which is an FDA-approved drug for the treatment of *Pneumocystis carinii* infection in AIDS patients, is a potent inhibitor of *T. cruzi* DHFR activity, with an inhibitory constant of 6.6 nM. The compound is also highly effective in killing *T. cruzi* parasites. The 50 and 90% lethal dose values against the trypomastigote are 19 and 36 nM, and the corresponding values for the amastigote form are 26 and 72 nM, respectively. However, as TMQ is also a good inhibitor of human DHFR, further improvement of the selectivity of this drug would be preferable. Identification of a novel antifolate selective against *T. cruzi* would open up new therapeutic avenues for treatment of Chagas' disease.**

Each year in Latin America, where 16 to 18 million people are infected with the causative parasite *Trypanosoma cruzi*, 50,000 people die of Chagas' disease. There is no efficient treatment or vaccine (5a, 8, 10). Benznidazole (*N*-benzyl-2-nitroimidazole acetamide) and Nifurtimox [3-methyl-4-(5'-nitrofurfurylideneamine) tetrahydro-4H-tiazine-1,1-dioxide], the only two drugs that are approved for clinical use, have serious limitations (8). Both drugs reduce symptoms and mortality in acute illness but are ineffective in the chronic phase of the disease. Both drugs are highly toxic. There is an urgent need for new chemotherapy to treat *T. cruzi* infection (9). We report here that trimetrexate (TMQ), an antifolate drug recently approved by FDA for the treatment of *Pneumocystis carinii* infection in AIDS patients, is a potent inhibitor of *T. cruzi*.

Antifolate drugs block the folate metabolic pathway. Folate is an essential nutrient for all organisms, as the reduced form of folate is a precursor of cofactors required for the synthesis of DNA, RNA, and protein. The enzyme dihydrofolate reductase (DHFR) catalyzes the reduction of folate to dihydrofolate (DHF) and DHF to tetrahydrofolate (THF) by use of the cofactor NADPH. The methylenated form of THF serves as a carbon donor for the synthesis of thymidylate in a reaction catalyzed by thymidylate synthase (TS). As thymidylate is essential for DNA synthesis, blocking of either DHFR or TS function causes cell death. Thus, antifolate drugs have been remarkably successful in cancer chemotherapy and in the treatment of bacterial and parasitic infections (26, 30).

Trypanosomatid parasites, including *T. cruzi* and *Leishmania* spp., are folate auxotrophs (3, 25, 31). As expected, in *Leishmania* spp. deletion of the gene encoding DHFR-TS is lethal (18). But quite surprisingly antifolates are not effective against *Leishmania* spp. and other trypanosomatids. In different species of *Leishmania*, pteridine reductase 1, or PTR1 (encoded by the gene *ptr1*), is capable of reducing folate to dihydrofolate and tetrahydrofolate. PTR1 is relatively insensitive to antifolate drugs and therefore provides a bypass mechanism for reducing folate in the presence of these drugs (4, 24). Although *ptr1*-like genes have been identified in *T. cruzi*, the biological significance of their products is unclear (29, 32). Currently available data indicate that PTR1 is not expressed in the trypomastigote and amastigote, which represent the mammalian life stages of the parasite (29). Therefore the prospect for an antifolate therapy against *T. cruzi* infection must be explored despite the disappointing failure against *Leishmania* spp.

Success of DHFR inhibitors in treating various infectious diseases is owed to the divergence in the DHFR sequence, which imparts a high degree of selectivity for certain antifolates for one organism versus others. This could be particularly important for parasitic protozoa, which, unlike humans, express DHFR as part of a bifunctional enzyme containing both DHFR and TS activity in two domains of the same polypeptide joined by a linker (13, 19). While in mammals DHFR is a monomeric protein of ~25 kDa, native bifunctional DHFR-TS in various protozoan parasites, including *T. cruzi*, is a homodimer of 110 to 140 kDa (27). There is an evidence of functional interactions between these domains, presumably via conformational changes in the domains of individual subunits of the dimer (21). Therefore, the structural and mechanistic

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distinction of protozoan DHFRs may offer a unique opportunity for drug selectivity.

Antifolates are broadly grouped into two classes. Classical antifolates, structural analogues of folic acid with a polar glutamate side chain, require a carrier-mediated active transport system for entering the cell (23). On the other hand, nonclassical antifolates lack the glutamate side chain (and are hence called lipophilic antifolates). They enter the cell via passive diffusion and therefore can be effective against these organisms (15). Consistent with this notion, the lipophilic antifolate TMQ is a potent inhibitor of *Toxoplasma gondii* and *P. carinii* (1, 2). In this communication we present evidence of the inhibitory activity of TMQ with respect to the *T. cruzi* DHFR-TS enzyme and against the trypomastigote and amastigote forms of *T. cruzi*.

MATERIALS AND METHODS

Reagents. Trimetrexate was a kind gift from MedImmune Oncology Inc. and was supplied as trimetrexate glucuronate (named Neutrexin). Restriction enzymes and the expression vector were purchased from New England Biolabs and Novagen, respectively. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Invitrogen (Gibco Cell Culture). All other chemicals were from Sigma.

Parasites. The Sylvio X-10/4 strain of *T. cruzi* was used throughout this work. Trypomastigotes were collected from the culture supernatant of infected monolayers of C2C12 cells grown in RPMI 1640 medium with 10% FBS. Trypomastigotes were converted into amastigotes by incubating in RPMI 1640 medium at acid conditions (pH 5.0) for 2 h at 37°C (34).

Cloning, expression, and purification. The coding sequence for *T. cruzi* dihydrofolate reductase thymidylate synthase (*TcDHFR-TS*; GenBank accession number L22484) was amplified by PCR using the genomic DNA as a template. The resulting PCR product (~1.6 kbp) was subcloned into the NdeI and BamHI restriction sites of the pET21a prokaryotic vector for expression of *TcDHFR-TS* protein.

Recombinant protein was expressed in the *Escherichia coli* Rosetta(DE3) pLysS strain. Overnight culture grown in LB medium containing 50 µg/ml ampicillin and 34 µg/ml chloramphenicol was diluted 1:100 into fresh LB medium containing the same concentration of antibiotics and 0.2% glucose and grown at 37°C. When the absorbance ($\lambda = 600$ nm) of the culture reached 0.7, isopropyl thio- β -D-galactoside (IPTG) was added to a final concentration of 0.4 mM and the culture was grown overnight at room temperature.

The bacterial pellet from 2 liters of culture was suspended in 50 ml of buffer A (50 mM Tris HCl, 1 mM benzamidine HCl, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol [DTT], 1 mM ethylene diaminetetraacetate [EDTA], pH 8.5), lysed by freezing and thawing, and treated with 50 µg DNase I. The suspension was clarified by centrifugation at 20,000 rpm for 30 min. DNA was precipitated by adding 0.65% streptomycin sulfate and removed by centrifugation. The cell extract was loaded on a Q-Sepharose FF column (Amersham Pharmacia), and *TcDHFR-TS* was eluted with a linear gradient of sodium chloride (0 to 0.5 M) in buffer A. Fractions containing *TcDHFR-TS* were concentrated and subjected to size exclusion chromatography on a Superdex 200 column (Amersham Pharmacia). *TcDHFR-TS* eluted as a dimeric protein was loaded onto a methotrexate (MTX)-Sepharose column (Sigma) and eluted with 2 mM DHF in buffer B (50 mM bis-Tris, 10 mM DTT, pH 7.0). To remove DHF from the preparation, *TcDHFR-TS* was dialyzed against excess of buffer C (50 mM Tris HCl, 10 mM DTT, pH 8.5) and subjected to a second anion-exchange step on a DEAE-Sepharose column (Amersham Pharmacia). Homogeneous *TcDHFR-TS* was eluted from the column using a linear gradient of sodium chloride (0 to 0.25 M) in buffer C.

Lucille White of the Southern Research Institute kindly provided us with purified recombinant human DHFR (hDHFR), which was used as a control in enzyme assays.

Enzyme activity. The DHFR activity of purified *TcDHFR-TS* and hDHFR was determined spectrophotometrically using a UV-visible spectrophotometer (DU740; Beckman Instruments). Reaction velocities were measured at 25°C for 1 min by monitoring the decrease in absorbance at 340 nm due to oxidation of NADPH to NADP⁺ and reduction of DHF to THF (coupled $\epsilon = 12,260$ M⁻¹ cm⁻¹). The DHFR activity of the *T. cruzi* enzyme was measured in 50 mM Tris HCl buffer, pH 7.0, and that of the human enzyme was measured in 50 mM

KH₂PO₄, 250 mM KCl, 5 mM β -mercaptoethanol (β -ME), pH 7.3, under optimum conditions for each enzyme. The inhibitory activity of TMQ was determined by measuring reaction velocities at several fixed concentrations (5, 10, 15, 20, 25, and 30 µM) of DHF and various concentrations (0, 6.55, 13.11, 19.66, and 26.22 nM) of TMQ. The concentration of NADPH was kept at 150 µM. The reaction was initiated by adding 1 µg of purified *TcDHFR-TS* or 3 µg of hDHFR. All measurements were repeated at least three times.

The inhibitory constant, or K_i value, for TMQ was determined from the Lineweaver-Burk plots. The concentration of inhibitor required for 50% inhibition of enzyme activity (IC₅₀) was calculated from the dependence of initial velocities on inhibitor concentration using the following equation: $V_i/V_o = (1 - C_i/IC_{50})^{-1}$, where V_i and V_o are the initial velocities in the presence and absence of inhibitor and C_i is the concentration of the inhibitor.

Antiparasitic activity assay. *T. cruzi* trypomastigotes and amastigotes were washed twice with RPMI 1640 medium, resuspended at a concentration of 10⁶/100 µl in RPMI medium with 5% FBS, incubated with varying concentrations of TMQ (0, 8, 16, 32, 64, 128, 256, 512, and 1,000 nM) at 37°C, and counted at several time points (4, 24, and 48 h) using a chemocytometer.

RESULTS

Gene cloning and expression. Automated DNA sequencing confirmed the accuracy of the *TcDHFR-TS* coding sequence inserted into the NdeI and BamHI restriction sites of the pET21a expression vector. Recombinant *TcDHFR-TS* was soluble under the conditions of induction (Fig. 1, bottom panel).

Purification of *TcDHFR-TS*. Recombinant *TcDHFR-TS* was purified to homogeneity using the protocol described in Materials and Methods. The majority of endogenous *E. coli* proteins were removed in the first anion exchange step. The enzymatically inactive oligomeric protein was separated by size exclusion chromatography in which the majority of *TcDHFR-TS* eluted as a dimer of ~110 kDa. By exploiting the high affinity of the DHFR domain for MTX, nearly homogeneous *TcDHFR-TS* was purified using affinity chromatography on an MTX-Sepharose column (Fig. 1, bottom panel). A second anion exchange step on DEAE-Sepharose column was used to remove DHF from the protein.

Enzyme inhibition studies. Fig. 1 shows the double-reciprocal plot used for calculating the K_i for TMQ inhibition of *TcDHFR-TS*. The K_i value of this inhibitor for *TcDHFR-TS* is 6.6 ± 0.6 nM, and the kinetics of inhibition suggest that TMQ is a competitive inhibitor of *TcDHFR-TS*.

The IC₅₀ values were calculated for both *TcDHFR-TS* and hDHFR and used to generate the selectivity index of the inhibitor, calculated as the ratio of the IC₅₀ value for hDHFR to the IC₅₀ for *TcDHFR-TS*. The IC₅₀ values of TMQ for hDHFR and *TcDHFR-TS* were 80.9 ± 12.5 and 20.2 ± 6.6 nM, respectively, with a resulting selectivity index of 4.0.

Antiparasitic activity assays. Given the efficacy of TMQ as an inhibitor of *TcDHFR-TS* enzyme, we tested its ability to kill *T. cruzi* parasites. After 24 h of incubation in the presence of 36 nM TMQ, there was 90% reduction in the number of live parasites (Fig. 2, white bars). These experiments were repeated three times with little variation in results. We have also observed that 64 nM drug caused a 50% reduction in the number of live trypomastigotes within 4 h after treatment. In a control experiment 100 µM TMQ had no detectable adverse effect on C2C12 mouse skeletal muscle cells in 24 h (data not shown), establishing that this level of TMQ has little cytotoxicity. Addition of 20 µM DHF partially protected the parasites from the effect of TMQ, confirming that *TcDHFR-TS* is indeed the target of TMQ.

FIG. 1. Inhibition of *TcDHFR-TS* by TMQ. Enzyme activity was measured at several fixed concentrations of DHF (5 to 30 μM) and various concentrations of TMQ (0 to 26.22 nM). Reaction rate was measured by monitoring the decrease in absorbance at 340 nM due to oxidation of NADPH (accompanying reduction of DHF). Reaction rate is decreased in the presence of increasing concentrations of the inhibitor. The line $I = 0$ represents the control, to which no inhibitor was added. Bottom panel: sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant *TcDHFR-TS* at different stages of purification. Protein samples at various stages of purification were boiled with equal volumes of $2\times$ SDS sample denaturing buffer and subjected to electrophoresis on 10% polyacrylamide gel containing 1% SDS. Ten microliters of total denatured sample was loaded in each lane. Lanes: 1, cell extract; 2, post-Q-Sepharose column; 3, dimeric pool from size exclusion chromatography on Superdex 200; 4, post-MTX-Sepharose column; 5, final protein pool from DEAE-Sepharose column step; 6, low-molecular-weight marker (molecular masses in kDa shown at the right).

To test whether TMQ could also kill the replicative mammalian stage of *T. cruzi*, trypomastigotes were transformed in amastigotes which were then incubated in the presence of various concentrations of TMQ. After 48 h of incubation, 50% of the amastigotes were killed at a concentration of TMQ of 26 nM. At a 64 nM drug concentration more than 80% of parasites were dead (Fig. 2, grey bars).

DISCUSSION

Chagas' disease poses a serious threat to public health in Latin America. Since the beginning of the 1970s, two drugs,

Nifurtimox and Benznidazole, have been used. Nifurtimox, a nitrofur, was commercialized as Lampit (Bayer), and its production has been discontinued since 1980s. The mode of action of Nifurtimox involved production of free radicals which cause oxidative damages in the parasite. Benznidazole, commercialized as Rochagan, showed high activity against *T. cruzi* parasite and may have a different mechanism of action which has not been clearly established. In general, drugs were effective in the treatment of acute infection and recent chronic infections. Unfortunately, both drugs showed serious side effects, and neither drug should be administered in patients with other complications. Moreover, these drugs were not effective

FIG. 2. Antiparasitic activity of TMQ. *T. cruzi* trypomastigotes and amastigotes were treated with various concentrations of TMQ (0 to 1,000 nM) at 37°C. As with time trypomastigotes tend to transform into amastigotes, the number of trypomastigotes was counted only up to 24 h. In the case of amastigotes counting was continued up to 48 h. The number of live parasites is presented as percentages of control (untreated) organisms, trypomastigotes (white bars), and amastigotes (grey bars). The total numbers of parasites counted in the untreated control were 3.5×10^6 and 2.1×10^6 for trypomastigotes and amastigotes, respectively. No live parasites were observed at doses higher than 128 nM.

in the treatment of chronic infection (8). A number of newer drugs have been tested more recently. Among these drugs, Allopurinol [4-hydroxypyrazolo (3,4-d) pyrimidine], an alternative substrate for the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT), has shown some efficacy and needs further evaluation (14). Development of inhibitors of cruzipain, the parasite-specific cysteine protease, has been complicated by emergence of drug-resistant parasites (11). Recent studies with drugs targeting the sterol metabolic pathway are extremely encouraging and deserve further investigation (35–37).

DHFR inhibitors have been used in chemotherapy of malaria for nearly 40 years (12). Antifolates such as trimethoprim and pyrimethamine have been used, either alone or in combination, in the treatment of other infections such as toxoplasmosis and *Pneumocystis carinii* pneumonia (PCP) (17, 40). DHFR is also a potential target for developing drugs against *Leishmania* spp. and trypanosomes (43). However, limited efforts have been devoted to investigating the effects of antifolates on *T. cruzi* infection (6, 42, 44). Results of these efforts suggested that some antifolates can inhibit the parasite but that they required relatively high doses. Classical antimicrobial antifolates such as pyrimethamine ($K_i = 0.1 \mu\text{M}$) and trimethoprim ($K_i = 1.0 \mu\text{M}$) are weak inhibitors of *T. cruzi* DHFR (16). These compounds are also poor inhibitors of leishmanial enzymes and the parasites (33). On the other hand, the classical antifolate methotrexate (MTX) is a potent inhibitor of *T. cruzi* DHFR activity ($K_i = 0.038 \text{ nM}$) but shows weak activity (50% lethal dose [LD_{50}] = $9.2 \mu\text{M}$) against the amastigotes (42). Based on the observation that the antileishmanial activity of some more potent antifolates could not be explained

in terms of their inhibitory activity for DHFR alone, it is postulated that these antifolates may target multiple folate-utilizing enzymes in the parasite (5, 18). However, the effect of antifolates against *Leishmania* spp. is severely compromised by expression of PTR1; therefore, an effective antifolate agent must be combined with inhibitors of PTR1 for successful therapeutic application. Given that there is no evidence for PTR1 expression in the infective stages of *T. cruzi* (29), antifolate drugs with a high selectivity index may offer better therapeutic potential for treatment of *T. cruzi* infection.

TMQ is a lipophilic antifolate which is currently approved for the treatment of *P. carinii* pneumonia (PCP) in AIDS patients. Although the drug of choice for the treatment of PCP is a trimethoprim-sulfamethoxazole combination, TMQ and other alternatives are often needed because of adverse effects or treatment failure (39). The data presented in this communication show that TMQ is a nanomolar inhibitor of TcDHFR-TS protein. It is important to note that TMQ has similar inhibitory activity against DHFR enzymes of *P. carinii* and *T. cruzi*; the corresponding IC_{50} values are 19.3 and 20.2 nM, respectively (22). TMQ is also a good inhibitor of *T. gondii* enzyme, with an IC_{50} value of 17 nM (22).

TMQ is a potent inhibitor of the trypomastigote and amastigote forms, the life cycle stages of *T. cruzi* in mammalian hosts. The calculated LD_{50} and LD_{90} values (doses required for killing 50% and 90% of total parasites, respectively) from our experiments are approximately 19 and 36 nM for the trypomastigotes and 26 and 72 nM for the amastigotes, respectively. The strong antiparasitic activity of TMQ against the amastigote form, a replicative mammalian stage of *T. cruzi*, is very significant. The activity of TMQ against *T. cruzi* is at least 100- to 200-fold higher than that of the currently used drugs, Benznidazole ($\text{LD}_{50} = 6 \mu\text{M}$ against trypomastigotes) and Nifurtimox ($\text{LD}_{50} = 3.4 \mu\text{M}$ against amastigotes) (7, 28). However, TMQ has not been tested in an animal model of Chagas' disease.

It should be noted that TMQ is also a good inhibitor of hDHFR and is, therefore, coadministered with Leukovorin (5-formyl THF). As mammalian cells can transport reduced folate, host cells are selectively protected by Leukovorin, which reverses the toxicity associated with inhibition of DHFR. Considering the socioeconomic condition of endemic areas and other complications associated with Leukovorin rescue, a single chemotherapeutic agent is highly desirable for treatment of Chagas' disease. Divergence in DHFRs allows preferential binding of antifolates to one DHFR over other, resulting in a high degree of selectivity for certain drugs. For example, the lipophilic antifolate trimethoprim shows 12,000-fold higher affinity for *Escherichia coli* DHFR than for hDHFR (38). Selectivity of SRI-9662, a structural analog of TMQ, for *T. gondii* enzyme (compared to human enzyme) could be enhanced from 9.1 to 97.5 by simply replacing a double bond in the linker region with a single bond (20). Considering that the primary sequence of the DHFR domain of TcDHFR-TS possesses only 24% identity to the human enzyme sequence, it is conceivable that rational design of a selective and potent inhibitor of *T. cruzi* based on TMQ would be possible. Discovery of a selective antifolate drug with potent antiparasitic activity will open up the possibility of a new therapeutic strategy for the treatment of Chagas' disease.

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