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

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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′-H11032 nitrofuranyliden) 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), 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.

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 tryptomastigote 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
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 Neotrexin). 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 L24484) 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 expression vector. Recombinant *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 (600 nm) of the culture reached 0.7, isopropyl containing the same concentration of antibiotics and 0.2% glucose and grown at 37°C. When the absorbance (600 nm) of the culture reached 0.7, isopropyl containing the same concentration of antibiotics and 0.2% glucose and grown at 37°C.
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 nitrofuran, 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...
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 (LD_{50} = 6 \mu M against trypomastigotes) and Nifurtimox (LD_{50} = 3.4 \mu 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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REFERENCES


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