Cloning, Expression, and Characterization of *Babesia gibsoni* Dihydrofolate Reductase-Thymidylate Synthase: Inhibitory Effect of Antifolates on Its Catalytic Activity and Parasite Proliferation[▽]

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Dihydrofolate reductase-thymidylate synthase (DHFR-TS) is a well-validated antifolate drug target in certain pathogenic apicomplexans, but not in the genus *Babesia*, including *Babesia gibsoni*. Therefore, we isolated, cloned, and expressed the wild-type *B. gibsoni dhfr-ts* gene in *Escherichia coli* and evaluated the inhibitory effect of antifolates on its enzyme activity, as well as on in vitro parasite growth. The full-length gene consists of a 1,548-bp open reading frame encoding a 58.8-kDa translated peptide containing DHFR and TS domains linked together in a single polypeptide chain. Each domain contained active-site amino acid residues responsible for the enzymatic activity. The expressed soluble recombinant DHFR-TS protein was approximately 57 kDa after glutathione *S*-transferase (GST) cleavage, similar to an approximately 58-kDa native enzyme identified from the parasite merozoite. The non-GST fusion recombinant DHFR enzyme revealed K_m values of 4.70 ± 0.059 (mean $\pm$ standard error of the mean) and 9.75 ± 1.64 μ M for dihydrofolic acid (DHF) and NADPH, respectively. Methotrexate was a more-potent inhibitor of the enzymatic activity (50% inhibition concentration [IC₅₀] = 68.6 ± 5.20 nM) than pyrimethamine (IC₅₀ = 55.0 ± 2.08 μ M) and trimethoprim (IC₅₀ = 50 ± 12.5 μ M). Moreover, the antifolates' inhibitory effects on DHFR enzyme activity paralleled their inhibition of the parasite growth in vitro, indicating that the *B. gibsoni* DHFR could be a model for studying antifolate compounds as potential drug candidates. Therefore, the *B. gibsoni* DHFR-TS is a molecular antifolate drug target.

Babesia gibsoni, the causative agent of canine babesiosis, is an important protozoan parasite that poses a major clinical health problem in dogs worldwide (19, 29, 43). Due to the emergence of resistance and failure of antibabesia agents to eliminate the parasite (5), novel chemotherapeutics with better potency and acceptable safety margins are required to treat the infection. To develop novel chemotherapeutics for apicomplexans related to *Babesia*, such as the *Plasmodium* parasites, many studies have focused on well-validated molecular targets, including the dihydrofolate reductase (DHFR) enzyme (10, 22, 30, 39), and to a lesser extent the thymidylate synthase (TS) enzyme (20, 25). The DHFR and TS domains are expressed as individual monofunctional enzymes in mammals and bacteria (21, 36, 41); however, in protozoans (and some plants), DHFR and TS exist as a bifunctional enzyme in which they are expressed on a single polypeptide chain (11, 23, 35). The DHFR domain is a validated target for chemotherapy of infectious diseases (42) and cancer (17) because it is important for the

proliferation of cells due to its function in DNA biosynthesis and cell replication.

The DHFR enzyme catalyzes the NADPH-dependent reduction of dihydrofolic acid (DHF) to tetrahydrofolate (4), an essential cofactor in the de novo biosynthesis of nucleotidic bases of DNA, while the TS domain catalyzes the reductive methylation of dUMP to dTMP with concomitant conversion of 5,10-methylenetetrahydrofolate to DHF (8). Therefore, the disruption of DHFR activity by antifolates depletes the reduced folate pool, thus blocking de novo dTMP biosynthesis by the TS enzyme and eventually inhibiting cell multiplication, leading to parasite death. Although antifolates inhibit the activities of bifunctional DHFR-TS enzymes of some pathogenic apicomplexan protozoans and disrupt folate metabolism (42), it has not been shown whether these drugs could also inhibit the activity of the corresponding enzyme in the genus *Babesia*, which includes the pathogen *B. gibsoni*.

Furthermore, the results of earlier studies of the inhibition of the growth of *Babesia bovis* in vitro by various antifolates (33) and the inhibition of *Babesia equi*, as well as *Babesia caballi*, by pyrimethamine (31) suggested disruption of DHFR-TS activity only. Even in a case where the *B. bovis dhfr-ts* gene had been isolated and the resistance mechanism to pyrimethamine elucidated (16), no data for the kinetic properties and inhibition profiles of the corresponding recombinant bifunctional

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enzyme by antifolates were produced. Therefore, considering the biological similarity of *B. gibsoni* to the phylogenetically closely positioned *B. bovis* and its distant cousins, the *Plasmodium* parasites (3), we would expect *B. gibsoni* to have a gene encoding the bifunctional DHFR-TS enzyme. Furthermore, we would expect this enzyme to be catalytically active and sensitive to antifolate inhibition in a manner similar to the previously studied apicomplexan parasites. If this expectation is true, then antifolates could inhibit the multiplication of *B. gibsoni* in vitro as well, thus making the compounds potential antibabesia drugs.

To test these hypotheses, we have employed an expressed sequence tag (EST) strategy to isolate and subsequently express the full-length *B. gibsoni dhfr-ts* (*Bgdhfr-ts*) gene. Furthermore, we used this recombinant protein to derive antibodies that can be used to identify and localize the native enzyme from the parasite. Additionally, we have determined the kinetic parameters of the purified recombinant DHFR-TS (rDHFR-TS) enzyme and demonstrated the inhibitory effect of three antifolates, methotrexate, pyrimethamine, and trimethoprim, on its enzymatic activity in a dose-dependent manner. Finally, we demonstrate the inhibition of parasite proliferation in vitro, in addition to the inhibition of the enzymatic activity, by these antifolates.

MATERIALS AND METHODS

Reagents and chemicals. All oligonucleotide primers were designed at the National Research Center for Protozoan Diseases Laboratory and synthesized by Sigma Co., Japan. Dimethyl sulfoxide (DMSO), acetone, acetonitrile, isopropyl- β -D-thiogalactopyranoside (IPTG), sodium pyruvate, and sodium bicarbonate were purchased from Wako Pure Chemical Industries, Japan. Other reagents and materials used in this study included Luria broth (LB) base (Invitrogen, United States), thrombin protease (Amersham Biosciences, United States), glutathione Sepharose 4B (Amersham Biosciences, United States), reduced glutathione (Roche, Germany), 0.45 μ M Millex filter unit (Corrigtwholl Co., Ireland), 48-well flat bottom tissue culture plates (Becton Dickinson and Co., United States), and 24-well multidish plates (Nunc, Denmark).

Experimental animals and parasite stock. Three adult beagle dogs (1 male and 2 female; Nihonnosan, Japan) and five 6-week-old female ddY mice (SCL, Japan) were housed, handled, and fed as described previously (1). *B. gibsoni* strain NRPCD parasites (15) were maintained in splenectomized beagles, and *B. gibsoni*-infected dog erythrocytes were collected at peak parasitemia (14%) and stored at -80°C . In vitro stock cultures of *B. gibsoni* were grown in dog erythrocytes by using a previously established method (40).

Construction of a *B. gibsoni* EST database. Total RNA was prepared from dog erythrocytes infected with the wild-type *B. gibsoni* strain NRPCD (15) by acid guanidinium thiocyanate-phenol-chloroform extraction methods (9), and the full-length cDNA library was made by using the vector-capping method (28). Briefly, the cDNA was synthesized by using the G-capping method using 5 μ g total RNA, ligated into the pGCAP1 vector, and then transformed into electrocompetent *Escherichia coli* DH12S cells (Invitrogen, United States). Ten thousand recombinant transformants were randomly selected from the library, the plasmid DNAs were purified, and the 5' ends of cDNAs sequenced by using an automated sequencer (ABI Prism 3100 genetic analyzer). Readable nucleotide sequences of 10,000 cDNA inserts were derived, and an EST database was made. From this EST database, we analyzed all partial cDNA sequences against all nonredundant databases accessed through NCBI GenBank and selected two identical cDNA clones believed to be encoding the BgDHFR-TS enzyme for further analysis.

Determination of *B. gibsoni* full-length *dhfr-ts* cDNA and bioinformatics analyses. The 5' ends of the selected cDNA clones were sequenced using the pGCAP1-2 (5'-ACTGCTCTCAGTGGATGTT-3') oligonucleotide vector primer. Then, two nucleotide primers, namely, Dfol 090-C17 (5'-TGAGGAGC CCAAGGTGAAAGTA-3') and Dfol 09-C17A (5'-ACATTGAGGGCTTCAC GATAG-3'), were designed and used to sequence the partial cDNA sequences, moving from the 5' to the 3' ends. To obtain the full-length sequence, the resulting three sets of overlapping cDNA sequence fragments were assembled

into contigs by using Genetyx software (Genetyx Corporation, Japan). The full-length cDNA and its translated polypeptide were analyzed by using Gene Runner (Hastings Software, Inc., United States) for Windows and the ProfileScan server (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>), respectively.

Cloning of *Bgdhfr-ts* cDNA. The full-length open reading frame (ORF) of the *Bgdhfr-ts* gene was PCR amplified using oligonucleotide primers 5'-*Bgdhfr-ts*F (5'-ATAGGATCCATGGCAGACTACACAGGGTG-3') and 3'-*Bgdhfr-ts*R (5'-TTACCCGGGTCAAGCGGACATTGCCATCTT-3') having BamHI and SmaI restriction sites, respectively, as underlined, and digested with BamHI and SmaI. The digested 1.5-kbp amplified cDNA was ligated into the digested pGEX-4T-1 vector and transformed to yield the pGEX-4T-1/*Bgdhfr-ts* clone (in *E. coli* DH5 α) that would express a glutathione *S*-transferase (GST)-BgDHFR-TS fusion protein in bacteria and would contain the cleavage site of thrombin protease between the GST and BgDHFR-TS domains. The pGEX-4T-1/GST-*Bgdhfr-ts* construct was transformed into *E. coli* BL21(DE3) for subsequent expression. Additionally, the above primers (5'-*Bgdhfr-ts*F and 3'-*Bgdhfr-ts*R) were used to amplify and sequence nucleotides of *B. gibsoni* genomic DNA that correspond to the ORF of the cDNA, to identify introns in this region of the genomic DNA as described earlier (2).

Expression and purification of BgDHFR-TS enzyme. A fresh 10-ml overnight culture from a single colony of the *E. coli* BL21(DE3) transformants was grown in 1.5 liters of LB base broth containing 50 μ g/ml of ampicillin at 37°C with shaking at 250 rpm until the optical density at 600 nm reached 0.4 to 0.5. Initially, the expression of the enzyme was induced by using 0.4 mM IPTG followed by expression at 37°C for 6 h. However, to obtain a sufficient amount of soluble rBgDHFR-TS enzyme, expression was optimized by induction using 1 mM IPTG and further expression at 25°C for 20 h with shaking at 250 rpm. The *E. coli* culture was centrifuged at $8,000 \times g$ for 15 min at 4°C , and the cell pellet suspended in TNE buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing 50 mg/ml lysozyme and protease inhibitors. The *E. coli* cell suspension was chilled on ice for about 2 h and sonicated for 5 min. The sonicate was immediately centrifuged at $12,000 \times g$ for 30 min at 4°C , and the clear supernatant was filtered through a 0.45- μ m-pore-size membrane with low binding affinity for proteins. The filtered supernatant was purified by using the glutathione elution method (Amersham) according to the manufacturer's instructions. The concentration of DHFR-TS protein was estimated by using a bicinchoninic acid protein assay kit (Pierce, United States), using bovine serum albumin as the standard, while the concentration of the GST fusion protein counterpart was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a bovine serum albumin standard.

Generation of anti-rBgDHFR-TS serum. Anti-GST-rBgDHFR-TS antibodies were produced in five 6-week-old female ddY mice as described previously (14), except that 250 μ g of the purified recombinant protein was administered, followed by three additional boosters of the same protein in Freund's incomplete adjuvant (Sigma Chemicals, United States). Sera were collected 14 days after the last booster and kept at -30°C until use.

Identification of native BgDHFR-TS enzyme, IFAT, and confocal laser microscopy. *B. gibsoni* lysate was prepared as described earlier (1) and separated by 12% SDS-PAGE. The native enzyme was identified by using mouse anti-GST-rBgDHFR-TS serum for Western blotting as outlined previously (49). For the negative control, both anti-GST and preimmune sera were reacted with the lysate as indicated above. The indirect fluorescent-antibody test (IFAT) and confocal laser microscopy were done as highlighted earlier (14), using anti-GST-rBgDHFR-TS as primary antibody.

Determination of rBgDHFR-TS enzyme activity and kinetic parameters. The catalytic activity of the DHFR domain was measured by using a DHFR assay kit (Sigma, United States) according to the manufacturer's protocol. The reduction in absorbance was read at 340 nm at 25°C using a DU 800 spectrophotometer interfaced with a computer using the Windows 2000 operating system running DU 800 system and application software (Beckman Coulter, Inc., United States). The readings were taken using the enzyme mechanism mode at 15-s intervals for 2.5 min. One unit was defined as the amount of enzyme required to reduce 1 μ mol of DHF per minute, based on a molar extinction coefficient of $12.3 \text{ mM}^{-1} \text{ cm}^{-1}$ at 340 nm. Thereafter, the range of recombinant enzyme concentrations (mg/ml) that gave a linear initial reaction velocity over the 2.5-min assay was determined, and one optimal value was selected for subsequent kinetic studies. To determine the steady-state kinetic parameters (K_m and the maximum rate of metabolism [V_{max}]), the concentration of DHF or NADPH was varied between 0 μ M and 120 μ M, with the other (NADPH or DHF, respectively) remaining constant at 100 μ M, and the reactions were initiated by the addition of 0.030 mg/ml of the recombinant enzyme. The kinetic parameters were calculated by using a Michaelis-Menten curve fit using a computer with Windows 2000 running

FIG. 1. Bioinformatics analysis of translated BgDHFR-TS polypeptide. (a) N-terminal DHFR and C-terminal TS-pyrimidine-hydroxymethylase (HMase) domains joined by a linker region. (b) Alignment of amino acid residues of the DHFR active site signature sequence of *B. gibsoni* with those of other apicomplexans and a plant; the tryptophan (W) residue at position 9 binds the substrate during DHFR activity. (c) Alignment of the TS active site signature sequence of the parasite with those of other apicomplexans, a plant, and humans. The cysteine residue at position 21 of the signature sequence is responsible for TS activity.

the DU 800 system and application software (Beckman Coulter, Inc., United States).

Determination of IC₅₀ for the enzyme inhibitors. The concentrations of methotrexate, pyrimethamine, and trimethoprim (Sigma, United States) required to achieve 50% inhibition of the enzyme reaction (IC₅₀) were determined at 50 μ M dihydrofolic acid and 60 μ M NADPH. Assays were started by the addition of dihydrofolic acid after preincubation of the enzyme with each inhibitor dissolved in assay buffer (DHFR assay kit; Sigma), DMSO, acetone, or acetonitrile. Non-linear curve-fitting plots of the percent inhibitions against various inhibitor concentrations was done using S-plus 6 software (Insightful Corporation, United States), and the IC₅₀s were estimated by interpolation.

In vitro growth inhibition assay of *B. gibsoni*. The parasite stock cultures (125 μ l) that had reached about 2% parasitemia were mixed with 12.5 μ l of healthy dog red blood cells suspended in 112.5 μ l of the growth medium containing methotrexate at 50 and 150 nM. For pyrimethamine and trimethoprim, 20 and 80 μ M of each drug was used for the inhibition. A 250- μ l mixture of the parasite culture per well was grown in a 48-well tissue culture plate using a previously described protocol (40). One-half volume (125 μ l) of the culture medium per well was replaced daily with an equivalent amount of fresh medium containing appropriate concentrations of the respective drugs for another two days. Thereafter, the treated parasite culture was subcultured with free healthy dog red blood cells as described above and parasite regrowth was monitored for another four days. In parallel, negative-control medium containing only assay buffer, acetone, and acetonitrile was included for methotrexate, pyrimethamine, and trimethoprim, respectively. The daily percent parasitemias of Giemsa-stained culture smears were calculated on seven to eight microscopic fields covering approximately 1,000 erythrocytes. Additionally, we examined the dynamics of blood stages (ring and merozoite forms) of the parasite in Giemsa-stained blood smears. The growth-inhibitory effect per drug concentration was monitored in triplicate and in two separate trials.

Nucleotide sequence accession number. The sequence of the *B. gibsoni dhfr-ts* gene is available in the DDBJ/EMBL/GenBank database with the accession no. AB426521.

RESULTS

Isolation of *Bgdhfr-ts* cDNA from total RNA. To be able to study the antifolate-drug target of parasitic *B. gibsoni*, we needed to isolate the wild-type *B. gibsoni dhfr-ts* gene and express the corresponding DHFR-TS enzyme in *E. coli* BL21. Thus, we employed an EST strategy to identify this gene, due to a lack of information about its isolation and sequence data. Consequently, we discovered two identical cDNA clones from the EST database that shared amino acid identity with the *B. bovis* DHFR-TS enzyme. Hence, we named the cDNA clone the *B. gibsoni* dihydrofolate reductase-thymidylate synthase gene (*Bgdhfr-ts*) and the corresponding protein the *B. gibsoni* dihydrofolate reductase-thymidylate synthase enzyme (BgDHFR-TS).

Full-length determination of *Bgdhfr-ts* cDNA and bioinformatics analyses. From the identical cDNA clones, we obtained the complete *Bgdhfr-ts* cDNA sequence. This full-length nucleotide sequence was 1,695 bp (data not shown), consisting of a 27-bp 5' untranslated region (bases 1 to 27), a 1,548-bp continuous ORF (bases 28 to 1,575), a 56-bp 3' untranslated region (bases 1,576 to 1,631), and a 64-bp poly(A) tail (bases 1,632 to 1,695). The ORF had 50.71% AT content and was sufficient to encode a protein of 58.8 kDa having 515 amino acid residues. The presence of an ATG initiation codon at the 5' end of the sequence and a TGA stop codon 27 bp 5' upstream of this initiation codon demonstrated that the 5' end of the *Bgdhfr-ts* cDNA was complete. Additionally, the presence of a TGA stop codon at position 1,575, a 3' untranslated region, and a poly(A) tail confirmed that the 3' end was complete. Hence, we were convinced that this sequence is the full-length nucleotide sequence. The nucleotide sequence within the genomic DNA that corresponds to the ORF of this cDNA contained a 42-bp intron at the 5' orientation and a 185-bp intron at the 3' orientation of the sequence.

To identify proteins that share homology with the translated BgDHFR-TS polypeptide, we performed BLASTP analysis and found that it shared significant homology with the *B. bovis* DHFR-TS enzyme, having 75% primary amino acid sequence identity (E value = 0). Furthermore, this polypeptide shared homology with the *Theileria parva* and *Theileria annulata* bifunctional enzymes, comprising 63% and 62% (E value = 0) amino acid sequence identities, respectively. In addition, because homology searches indicated that the translated polypeptide was likely an active enzyme, we were interested in identifying functional domains and active-site amino acid residues contained within these domains. Subsequently, we established that the 58.8-kDa predicted polypeptide had an N-terminal DHFR domain (9 to 185 amino acids [aa]) and a C-terminal TS domain (225 to 510 aa) joined by a 39-aa-residue linker sequence (Fig. 1a). The rBgDHFR domain contained a 9-aa active-site signature sequence that included a tryptophan residue responsible for substrate binding during enzymatic activity and a proline-tryptophan dipeptide and glycine residues conserved between the parasite and other apicomplexans and a plant (Fig. 1b). The TS domain had a 29-aa active-site signature sequence containing a cysteine residue that is likely responsible for enzymatic activity and is conserved between

FIG. 2. Characterization of BgDHFR-TS enzyme. (a) Results of 12% SDS-PAGE, electroblotting, and subsequent Coomassie blue staining of the expressed proteins and parasite merozoite lysate. Lanes: M, low-molecular-mass marker; 1, GST-rBgDHFR-TS ($3\times$ dilution); 2, rBgDHFR-TS; 3, GST; 4, merozoite lysate. (b) Results of Western blotting using mouse antiserum. Lanes: 5, approximately 58-kDa specific band from *B. gibsoni* merozoite lysate reacted with anti-rBgDHFR-TS serum; 6, negative-control serum showing no reaction. Molecular sizes of markers are indicated on the left and of bands are indicated on the right.

other apicomplexans, plants, and humans (Fig. 1c). Alignment of the DHFR active-site amino acid sequence of the cDNA with those of the corresponding genomic DNAs confirmed that mutation of the wild-type parasite sequence did not occur (data not shown).

Expression of rBgDHFR-TS enzyme in *E. coli* BL21. The next objective was to express an active rBgDHFR enzyme to be able to test the effects of antifolates on the enzymatic activity in vitro. Consequently, our strategy was to express full-length rBgDHFR-TS, rather than the DHFR domain alone, so that we could evaluate the kinetic and inhibitory profiles of a bifunctional enzyme resembling the native form. Full-length *Bgdhfr-ts* in the pGEX-4T-1 vector was subsequently transformed into the bacterial cells. Initial expression at 37°C and induction with 0.4 mM IPTG for 6 h led to protein insolubility. We attempted to solubilize the protein by using urea and then refold the enzyme, but the results of the subsequent enzyme assays demonstrated little or sometimes no DHFR activity. Therefore, we optimized the expression of the enzyme by induction with 1 mM IPTG followed by expression at 25°C for 20 h and obtained approximately 2.2 mg of purified GST-rBgDHFR-TS enzyme from 1.5 liters of culture. However, cleaving the GST tag lowered the yield of the protein because some protein remained fused with the tag. The results of SDS-PAGE analysis of the purified GST fusion enzyme revealed a single band of about 83 kDa before and 57 kDa after GST cleavage, a mass similar to the predicted molecular mass of 58.8 kDa computed from the cDNA. The expression and purification of the pGEX-4T-1 vector without the cDNA insert under the same conditions yielded the 26-kDa GST protein (Fig. 2a).

Identification of native BgDHFR-TS, IFAT, and localization of the endogenous enzyme. Antibodies directed against the BgDHFR-TS enzyme could be useful in characterizing the endogenous enzyme within the parasite; thus, we used the purified recombinant protein for inoculation in mice to generate the

FIG. 3. Results of IFAT and confocal microscopic localization of BgDHFR-TS enzyme in the parasite. Overlaid images of the protein's reaction with the anti-recombinant protein serum (a and b) and images of negative serum revealing only red staining of parasite nucleus with propidium iodide (c and d) are shown.

antibodies. Subsequently, mouse anti-rBgDHFR-TS serum reacted with the parasite merozoite lysate, resulting in a specific band corresponding to an approximately 58-kDa native enzyme, approximately the same size as that seen from the expressed protein. Mouse preimmune serum or anti-GST antibodies used as a control did not react with the merozoite lysate (Fig. 2b). Additionally, the antibodies appeared to localize the BgDHFR-TS enzyme within the cytoplasm of the *B. gibsoni* cells, as demonstrated by the detection of green fluorescent signal mainly in the parasite cytosol (Fig. 3a and b).

Enzymatic activity of rBgDHFR-TS. The presence of an active-site signature sequence, including a tryptophan residue responsible for enzymatic activity, in the predicted BgDHFR domain led us to theorize that the expressed recombinant enzyme is likely to be catalytically active. To test this putative activity, we measured the DHFR activity of the rBgDHFR domain while it was still joined to the TS domain. Indeed, we found reductase activity for both GST and non-GST fusion proteins when compared to the activity of GST alone (data not shown). Based on this enzyme activity, we proceeded to determine the steady-state kinetic parameters for the enzyme substrate and the cofactor (DHF and NADPH), respectively: cleaved (i.e., lacking GST) rBgDHFR enzyme had K_m values of 4.70 ± 0.059 (mean $\pm$ standard error of the mean) and $9.75 \pm 1.64 \mu\text{M}$ for DHF and NADPH, respectively, whereas the uncleaved (i.e., GST fusion protein) counterpart revealed K_m values of 1.60 and $17.00 \pm 0.49 \mu\text{M}$ for DHF and NADPH, respectively (Table 1). Therefore, the rBgDHFR domain retains enzymatic activity that can be used to evaluate its inhibition by antifolates.

Inhibitory effect of antifolates on rBgDHFR-TS enzyme activity. DHF, the substrate for the DHFR enzyme, shares structural similarities in its chemical structure with the antifolates (methotrexate, trimethoprim, and pyrimethamine) via their common aminopyrimidine ring (see Fig. 7). Therefore, we

TABLE 1. Kinetic parameters of the rBgDHFR-TS and GST/rBgDHFR-TS enzymes^a

Recombinant protein	DHF (μM)		NADPH (μM)	
	<i>K_m</i>	<i>V_{max}</i>	<i>K_m</i>	<i>V_{max}</i>
rBgDHFR-TS	4.70 ± 0.059	0.088 ± 0.005	9.75 ± 1.64	0.102 ± 0.03
GST-rBgDHFR-TS	1.60 ± 0.089	0.079 ± 0.045	17.00 ± 0.49	0.133 ± 0.01

^a *K_m* and *V_{max}* were determined for the substrate (DHF) and the cofactor (NADPH) in triplicate for the GST fusion enzyme and duplicate for the non-GST fusion counterpart. The mean ± standard error of the mean was calculated for each parameter.

would expect these drugs to compete for the active site in the *B. gibsoni* DHFR domain, potentially leading to an inhibitory effect. To examine this possibility, the effect of these drugs on the enzymatic activity of the purified recombinant enzymes was determined. Indeed, methotrexate, pyrimethamine, and trimethoprim all inhibited the enzymatic activity in a dose-dependent manner. Methotrexate was the most-potent inhibitor, showing an *IC*₅₀ of 68.6 ± 5.20 nM, while pyrimethamine (*IC*₅₀ = 55.0 ± 2.08 μM) and trimethoprim (*IC*₅₀ = 50 ± 12.5 μM) led to inhibition in the μM range rather than the nM range seen for methotrexate (Fig. 4a to c). Due to the slight inhibitory effect of DMSO on this enzyme, we opted to use

acetone, acetonitrile, and assay buffer as negative controls. Hence, inhibition of the enzymatic activity by these antifolates suggests that the drugs can target the endogenous enzyme in live parasites.

Inhibitory effect of antifolates on in vitro *B. gibsoni* proliferation. Based on the inhibitory effect of these antifolates on the activity of the rBgDHFR enzyme, we were interested to examine whether these drugs could also disrupt the function of the endogenous enzyme in a live parasite, thereby resulting in cell death. Consequently, we evaluated the inhibitory effect of these drugs on the parasite's growth in an in vitro culture using antifolate concentrations that included the concentration ranges of the *IC*₅₀s derived from recombinant-enzyme inhibition assays. Consistent with the findings of the recombinant-enzyme inhibition assays, methotrexate caused inhibition of the parasite's growth both at 50 and 150 nM, with only moderate inhibition observed at 50 nM, while trimethoprim and pyrimethamine resulted in moderate inhibition at 20 and 80 μM. The modest inhibition especially by pyrimethamine was demonstrated by the parasite's reemergence in cultures treated with 20 μM of the drug even after withdrawal of the treatment. These inhibitions were dose dependent, with weaker effects observed at lower drug concentrations than at higher doses. In contrast, the parasite continued to grow in the negative-control

FIG. 4. Inhibition curves of antifolates against rBgDHFR-TS enzyme activity. Triplicate inhibition curves were generated for each drug, and means ± standard errors of the means were calculated. The curves were fitted using the calculated mean values for each concentration by using S-plus 6 software (Insightful Corporation, United States).

FIG. 5. Graphs of percent parasitemia inhibition of *B. gibsoni* growth by the antifolates in vitro. Duplicate percent parasitemia inhibition was determined for each drug, and graphs were plotted using the calculated mean $\pm$ standard error of the mean values for each day using Excel software in the Windows XP operating system.

culture containing only the solvents in which each of the drugs was dissolved (Fig. 5a to c). Although the percent parasitemia was generally below 2% in control cultures, the difference in parasitemia between treated and nontreated cultures could be clearly discerned. In parallel with the inhibition of growth, we also observed during the phase that preceded the clearance of the parasite that stained blood smears of treated parasite cultures contained only ring-stage parasites, including some that were relatively large and round (Fig. 6a and b). These findings were in contrast to observations from smears of untreated cultures that revealed both the ring stage and the dividing merozoites (Fig. 6c). Taken together, it appears that antifolates at these concentrations inhibit the proliferation of the parasite.

DISCUSSION

The EST strategy is an important tool for gene discovery and drug target identification, especially for apicomplexan parasites with nuclear genomes that contain introns (45, 46, 48). This technique utilizes mRNA transcripts lacking introns to generate cDNA, thus making the expression of recombinant proteins from these parasites possible. Therefore, by using this strategy, we obtained full-length *Bgdhfr-ts* cDNA, which was similar in length to the equivalent gene in *B. bovis* (16) but was shorter than the *Plasmodium falciparum* and *Plasmodium vivax*

FIG. 6. Giemsa-stained smears of *B. gibsoni* parasites in untreated and treated cultures. Panels a and b show treated parasites (only the ring form was observed) on days 2 and 3, respectively, while panels c shows untreated parasites (both ring-form and dividing parasites were observed).

dhfr-ts genes (13, 38). We think that the difference in length of the genes and, therefore, the molecular sizes of the proteins they encode is due to the closer phylogenetic positioning of *B. gibsoni* to *B. bovis* than to their distant cousin *P. falciparum* (3).

In protozoan parasites, the DHFR and TS domains are expressed on the same polypeptide chain (23), in agreement with the findings of the current study, hence confirming our earlier expectation that the *B. gibsoni* DHFR-TS enzyme is bifunctional. Otherwise, if the DHFR domain were monofunctional, we would expect the expression of an approximately 21.3-kDa recombinant enzyme and identification of a corresponding 21-kDa native enzyme, respectively, which was not the case. The heterologous expression of the *P. falciparum* DHFR-TS enzyme in *E. coli* bacteria (18) is poor, contrary to the high expression of the corresponding *B. gibsoni* enzyme. The lower AT content (50%) of the *Bgdhfr-ts* gene than of the *P. falciparum dhfr-ts* (74.7%) (18) might explain the relatively successful expression of BgDHFR-TS. However, a high AT content alone may not satisfactorily explain the relatively efficient expression of BgDHFR-TS, because the expression of *P. falciparum* aldolase has been achieved in large amounts despite a high AT content in its corresponding gene (12).

It has been shown that the GST-fused and nonfused rat DHFR enzyme has satisfactory activity levels (47), similar to the activities of both the GST-fused and nonfused BgDHFR-TS enzyme. Therefore, it appears that fusing the enzyme with GST does not influence the enzymatic activity or even the quaternary configuration of the enzyme. Furthermore, this observation confirmed that the ORF of *Bgdhfr-ts* cDNA encoded authentic DHFR-TS. Otherwise, the negative control comprised of GST expressed from the pGEX-4T-1 vector without the cDNA insert was not enzymatically active, ruling out any possibility of the presence of *E. coli* DHFR that might have been coexpressed with the BgDHFR-TS. The K_m values of the wild-type BgDHFR-TS enzyme without GST (Table 1) were similar to those of previously characterized wild-type *P. falciparum* DHFR-TS (24, 27, 38), although a slightly higher value with respect to DHF is reported when only the DHFR domain of the wild-type *P. falciparum* enzyme was analyzed instead of the bifunctional DHFR-TS enzyme (37). However, it is difficult to tell whether the K_m of DHFR is altered when the domain is analyzed as a monofunctional enzyme. Moreover, the K_m value depends on other factors, such as pH and temperature, as well as urea and salt concentrations, that might complicate the comparison of DHFR K_m values from different studies.

Purified active rDHFR enzymes of bacteria (7) and parasites (34) have been used to screen the available antifolate libraries. In these studies, methotrexate was shown to be a stronger inhibitor of both bacterial and parasitic DHFR enzymes than pyrimethamine and trimethoprim, consistent with the inhibition trends observed in our study. However, the actual IC_{50} s for the three antifolates varied slightly from those in the previous reports. It is possible that other factors, such as urea and salt concentrations, could have contributed to the slight variations in the inhibition profiles of these drugs. Alternatively, it could just be that inhibition is species specific. The aminopteridine ring of methotrexate and the aminopyrimidine ring of pyrimethamine and trimethoprim closely resemble those of the substrate, DHF (Fig. 7). Therefore, we would expect these

FIG. 7. Chemical structures of the DHFR substrate, DHF (a), and the antifolate drugs methotrexate (b), pyrimethamine (c), and trimethoprim (d).

antifolate compounds to compete with the substrate by binding to amino acid residues in the hydrophobic pocket of the active site of the rBgDHFR enzyme and lead to the observed inhibitory effect. Methotrexate is a particularly potent inhibitor compared to pyrimethamine and trimethoprim because the molecule, by having a pteridine ring and glutamic acid, is an only slightly modified version of the normal substrate of DHFR, DHF, and so it competes effectively with the substrate for the DHFR active site.

In contrast, the relatively weaker inhibition of DHFR activity by pyrimethamine has been attributed to the steric interactions of pyrimethamine's chlorophenyl and methyl groups, which project from position 5 of 2,4-diaminopyrimidine (Fig. 7c) and thus restrict the hydrophobic binding pocket to only two amino acid residues (26). For trimethoprim, the fact that the trimethoxyphenyl ring (Fig. 7d) appears not to extend fully into the hydrophobic pocket is a possible explanation of its relatively lower potency (26). Therefore, if these antifolates bind in a similar way with the active site of rBgDHFR, then these interactions might also explain the weaker inhibition by pyrimethamine and trimethoprim than by methotrexate. Consequently, we propose that this structure-activity relationship of different classes of antifolates with the active site of DHFR could be exploited to design and identify new antifolates for possible development of antibabesias drugs, especially during the early critical screening stage before proceeding to in vitro inhibitory assays of *B. gibsoni* proliferation.

Methotrexate, pyrimethamine, and trimethoprim inhibit the growth of *P. falciparum* (44), *B. bovis* (33), *B. caballi*, and *B. equi* in vitro (31) similarly to the inhibition of the growth of *B. gibsoni* observed in our study. Thus, we provide some evidence that the *Babesia* DHFR-TS enzyme activity is essential for the parasite's survival, possibly by playing a role in the synthesis of thymidine nucleotides required for DNA biosynthesis and cell replication. Generally, the ranges of concentrations of the antifolates which inhibited *B. gibsoni*'s proliferation were within the ranges of concentrations that inhibited the recombinant enzyme. It is possible that these drugs could cross the erythrocyte membrane barrier and the parasite membrane to reach the target enzyme in the parasite cytosol. Consistent with the levels of inhibition of the recombinant enzyme's activity by the three antifolates, methotrexate was the most-potent inhibitor of *B. gibsoni* proliferation in vitro, suggesting that it could be the most-suitable antibabesia drug candidate. However, the

narrow therapeutic index and the resulting toxicity to the human host is a drawback for its use in clinical therapeutics (6). Therefore, we raise the same concern regarding the toxicity of the drug if used to treat *Babesia* infections in dogs and cattle. Nevertheless, 2,4-diamino-N10-methyl-pterotic acid, a methotrexate precursor which has antimalarial activity (32, 34) and is less toxic, might be safely used to treat *P. falciparum* infection because the parasite can metabolize this inactive precursor to active methotrexate. We hope that the same logic could apply to *Babesia* infections.

Moreover, pyrimethamine and trimethoprim were less-potent inhibitors of *B. gibsoni* proliferation than methotrexate, indicating that both antifolates might not be ideal antibabesia drugs. Nonetheless, pyrimethamine and trimethoprim have been used in combination with sulfonamides to treat malaria and bacterial infections, respectively (34). Whereas these antifolates target DHFR, sulfonamides inhibit the dihydropteroate synthase enzyme of the parasite and bacteria, providing synergy and thus enhancing their potency (34). We think that this combination might work as well for *Babesia* parasites, especially if the dihydropteroate synthase enzyme, which is the target for sulfonamides, exists in *Babesia* parasites. If this is the case, we further propose that the combination of these antifolate drugs might be a viable treatment for *B. gibsoni* infection.

After treatment with the antifolates, the presence of only the ring-form parasite, just before the parasite's clearance, made us believe that these antifolates inhibited the endogenous DHFR-TS enzyme in live parasite cells, disrupting DNA biosynthesis and cell replication and thus blocking asexual reproduction (binary fission), which serves to replenish merozoites from the ring-form parasite. In contrast, we suggest that the endogenous enzyme of the untreated parasites was not disrupted; hence, the ring-form parasite appeared to have undergone binary fission to generate new merozoites. Therefore, we hypothesize that these antifolates block the intraerythrocytic development of the parasite at the asexual reproduction stage, which involves DNA replication and cytokinesis.

In conclusion, this study reports the first isolation, cloning, and expression of the *B. gibsoni dhfr-ts* gene, providing a corresponding purified rBgDHFR-TS enzyme of approximately 57 kDa that is catalytically active. We also identify an approximately 58-kDa bifunctional native BgDHFR-TS enzyme from the parasite lysate and show that this enzyme is mainly localized in the parasite cytosol, where it is expected to be involved in folate metabolism. Additionally, the results of the study confirm the hypothesis that the inhibitory effect of these antifolate drugs on the enzymatic activity would parallel their inhibition of the parasite's growth in vitro, indicating that the *B. gibsoni* DHFR could be a model for studying antifolate compounds as potential antibabesia drugs. Consequently, we propose the screening of other available antifolate libraries, including prodrugs (precursors), using this rBgDHFR-TS enzyme and the study of the tertiary structure of the purified enzyme to facilitate the design of antifolates that can optimally target the active sites of the enzyme. These proposals will define where to proceed from our current study.

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