Precursors of methotrexate target dihydrofolate reductase-thymidylate synthase of *Babesia gibsoni* and inhibit parasite proliferation

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ABSTRACT

It is not known whether precursors of methotrexate, such as 2, 4-diamino-6-hydroxymethyl-pteridine (DAP) and 2, 4-diamino-³¹0-methyl-pteroid acid (DAMPA), could target the dihydrofolate reductase-thymidylate synthase (DHFR-TS) enzyme of *Babesia* and inhibit the parasite growth. Therefore, we have determined whether DAP and DAMPA as well as other chemically related compounds like pteroid acid (PA) and ³¹0- Trifluoropteroid acid (³¹0TFPA) could target the DHFR-TS enzyme of *B. gibsoni* and inhibit its growth. DAMPA was a more potent inhibitor of the *B. gibsoni* growth in vitro (50% inhibition concentration [IC₅₀] = 2.4 ± 0.20 µM) [mean ± standard error of the mean] than DAP (IC₅₀ = 78 ± 15 µM). Moreover, DAMPA potently inhibited enzymatic activity of recombinant DHFR-TS of *B. gibsoni* (IC₅₀ = 2.6 ± 0.15 µM) than DAP (IC₅₀ > 100 µM). In contrast, PA and ³¹0-TFPA did not inhibit the activity of the recombinant enzyme and growth of *B. gibsoni*. The inhibition of the recombinant enzyme activity by DAMPA mirrored with inhibition of the parasite growth indicating that the purified recombinant enzyme could be used for preliminary screening of some antifolate precursors. Therefore, both DAP and DAMPA inhibit growth of *B. gibsoni* by targeting the DHFR-TS enzyme of the parasite.

Key words: Methotrexate precursors; DHFR-TS enzyme; *B. gibsoni*

INTRODUCTION

*Babesia gibsoni* is one of the causative agents of canine babesiosis. Although, the infection is mainly managed by chemotherapy, available antibabesials do not eliminate the parasite from the host (Matsua et al., 2004; Wulansari et al., 2003). Furthermore, the parasite appear to develop resistance to some of these drugs (Jefferies et al., 2007). The withdrawals of these chemotherapeutic drugs from the market, because of toxicity concerns, have also limited the range of drugs available for the treatment of infection by *Babesia* including *B. gibsoni* (Vial and Gorenflot, 2006). Therefore, there is need for the discovery of effective and safe drugs for the treatment and management of the infection. One of the initial strategies for the discovery of novel drugs involves the identification and validation of their molecular target in parasites (Doering and Meijer, 2007). Although many studies have been done to identify and validate molecular drug targets in apicomplexan parasite such as *Plasmodium* (Biagini et al., 2006; Doering and Meijer, 2007; Joet et al., 2003; Joubert et al., 2009; Patel et al., 2008; Werbovetz, 2000), only a few molecular targets have been validated in the genus *Babesia* including *B. gibsoni* (Aboge et al., 2008) and *Babesia bovis* (Bork et al., 2004).
Previously, we have identified and validated, the *B. gibsoni* dihydrofolate reductase-thymidylate synthase enzyme (BgDHFR-TS) as a molecular target of 3 antifolate compounds, namely, methotrexate, pyrimethamine and trimethoprim (Aboge et al., 2008). Methotrexate, a 2, 4-diaminopteridine-based antifolate, has been shown to be particularly a more-potent inhibitor of *B. gibsoni* proliferation than pyrimethamine and trimethoprim (Aboge et al., 2008). Methotrexate has 2, 4-diaminopteridine ring, para-aminobenzoic acid and glutamic acid residues as its building blocks, and thus closely resembles the chemical structure of the dihydrofolic (DHF) acid, the substrate of the DHFR enzyme (See Fig. 3b). This makes methotrexate a more-potent inhibitor of the enzyme than pyrimethamine and trimethoprim. Therefore, we would expect methotrexate to be a prospective drug for the treatment of the parasitic infection. However, the narrow therapeutic index, and the undesirable side effects to the mammalian host is a drawback for its potential use in clinical therapeutics (Brock and Jennings, 2004). Nevertheless, 2, 4-diamino-6-hydroxymethyl-pteridine (DAP) and 2, 4-diamino,N10-methyl-pteridoic acid (DAMPA), both methotrexate precursors, inhibit the growth of *P. falciparum in vitro* (Nduati et al., 2005), and are well tolerated in non-human primates indicating that they can be safely applied in mammalian hosts (Widemann et al., 2000).

Two possible mechanisms have been suggested to be responsible for the anti-*Plasmodium* activity of DAP and DAMPA: either they can directly target DHFR-TS enzyme or they may be synthesized to the parent compound, methotrexate, and target DHFR-TS (Nduati et al., 2005). Since these compounds structurally resemble DHF, the substrate of DHFR enzyme, by having aminopteridine ring, we settled for the hypothesis that DAP and DAMPA, could directly target DHFR enzyme rather than being synthesized to the parent compound, then target the enzyme. It is known that methotrexate inhibits growth of *P. falciparum* (Walter et al., 1991) and *B. gibsoni* (Aboge et al., 2008) suggesting some resemblance in their folate metabolism. Subsequently, we would expect DAMPA and DAP to inhibit *Babesia* parasite proliferation *in vitro* in a similar manner to that of *P. falciparum*. This led us to hypothesize that DAP and DAMPA could inhibit *B. gibsoni* proliferation in a manner similar to that of *P. falciparum in vitro*. If this is true, then we further proposed that both precursors could target the DHFR-TS enzyme of *B. gibsoni* directly and then inhibit its catalytic activity. Additionally, if DAMPA, which structurally resemble pteroic acid (PA) and N10-(Trifluoroacetyl) pteroic acid (N10-TFPA) by having pteroic acid moiety actually inhibits *B. gibsoni* proliferation, then we would expect PA as well as N10-TFPA to target the enzyme and possibly inhibit the parasite proliferation, thus making these compounds prospective antibabesia drugs.

To verify these hypotheses, we have cultured *B. gibsoni in vitro* and produced a purified recombinant BgdHFR-TS (rBgdHFR-TS) enzyme, from the Bgdhfr-ts cDNA, to be able to evaluate inhibitory effects of the antifolate precursors. Thereafter, we have determined whether DAP, DAMPA, PA and N10-TFPA could inhibit the growth of *B. gibsoni in vitro*, as well as activity of the purified rBgdHFR-TS enzyme in a dose dependent manner. Finally, we have demonstrated that the inhibition of the activity of the rBgdHFR-TS enzyme paralleled the inhibition of growth of *B. gibsoni in vitro*.

**MATERIALS AND METHODS**

**Reagents and chemicals**

Aminopterin, DAP, DAMPA, PA, N10-TFPA, and methotrexate were purchased from Sigma Company, United States. Methanol, sodium pyruvate and sodium bicarbonate were obtained from Wako Pure Chemical Industries, Japan. Other reagents and materials included Luria broth (LB) Base (Invitrogen, USA), glutathione sepharose 4B (Amersham Biosciences, USA), reduced glutathione (Roche, Germany) and 0.45 μM millex® filter unit (Corrigtwohill Co., Ireland). RPMI1640 medium (Invitrogen), 48-well flat
bottom tissue culture plates (Becton Dickinson and Co. USA) and 12-well multidish plates (Nunc, Denmark) were used for the parasite culture.

**Preparing stock solutions of the inhibitor compounds**

Stock solutions, 10 mM each, of DAP, DAMPA, PA and $\text{N}^{10}$-TFPA were prepared using 28% methanol containing 50 mM sodium hydroxide, while stock solution of methotrexate (10 mM) was prepared using 1 x assay buffer supplied with DHFR assay kit (Sigma, USA). The stock solutions were used immediately for the inhibition assays.

**Experimental animals**

Six adult-beagles dogs including 1 male and 5 female dogs (Nihonnosan, Japan), were fed once per day, and given water *ad libitum*. The dogs were housed in a P2 level facility, and the experiments were conducted in accordance with the Stipulated Regulations for the Care and Use of Research Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

**Expression and purification of rBgDHFR-TS enzyme**

The *Bgdhfr-ts* cDNA was used to produce the rBgDHFR-TS enzyme in *E. coli* BL21 (DE3) as outlined in our previous publication (Aboge et al., 2008). Briefly, the *Bgdhfr-ts* cDNA was prepared from the total RNA of the parasite. The open reading frame of the gene was cloned into pGEX-4T-1 vector and expressed as glutathione S-transferase (GST) fusion protein. The rBgDHTR-TS protein was purified and expression confirmed by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% gel. At first, inhibition of enzymatic activity was verified by aminopterin since it belongs to the same chemical group as methotrexate, which is known to have antibabesia effect (Aboge et al., 2008; Nott and Bagnara, 1993).

**Cultivation of *B. gibsoni* stock**

*B. gibsoni*-Oita strain was obtained from the Department of Infectious Diseases, School of Veterinary Medicine, Azabu University, Japan. A 2 ml-parasite stock culture was grown in a 12-well flat bottom tissue culture plate, in a humidified CO$_2$ incubator (Brand Bio-Lab) at 37°C in the presence of 5% carbon dioxide. The 2 ml-culture mixture contained fresh dog erythrocytes suspended at 5% hematocrit, in RPMI 1640 medium (2.05 mM L-glutamine, 25 mM Heps buffer, 23.8 mM NaHCO$_3$, 0.91 mM sodium pyruvate, penicillin G at 100 units/ml, streptomycin at 100 µg/ml) having 20% dog serum. Then, 1 ml of old culture-medium was replaced with 1 ml of fresh medium daily, and after 3 or 4 days, the old parasite cultures were sub-cultured in fresh dog erythrocytes containing fresh complete medium at 5% hematocrit. Antibabesia effect of aminopterin on this parasite culture was also evaluated.

**Inhibition assays of *B. gibsoni* growth in vitro**

To prepare a working concentration for preliminary inhibition assays, 10 mM of each of the compounds (DAP DAMPA, PA and $\text{N}^{10}$-TPA) were diluted in fresh culture medium containing 20% dog serum to get a final concentration of 100 µM. To evaluate the growth inhibition of *B. gibsoni*, 200 µl of fresh culture medium containing 200 µM of each of the test compounds were dispensed into individual wells of a 48-well plate. Thereafter, 20 µl of fresh canine erythrocytes was resuspended in the mixture, and then 200 µl of stock cultures of *B. gibsoni* having a parasitemia of approximately 0.5% were added into each well. Thus, the final assay mixture had an initial parasitemia of 0.25% and an approximate hematocrit value of 5% as well as a final concentration of 100 µM for each of the compounds. The assay mixture was incubated in a humidified CO$_2$ incubator under the same conditions described above. The old culture media containing the respective compounds were replaced daily with 350 µl of fresh media having the inhibitors for another 2 days. Then, the treated parasite was sub-cultured in fresh dog erythrocytes resuspended in a fresh culture
The target of antifolate precursors in Babesia

medium without the compounds for another 5 days. In parallel, negative control medium, which contained only 2% methanol and 2.5 mM NaOH was included for all the tested compounds. On the 7th day, percent parasitemia of Giemsa-stained blood smears was calculated on 7 to 8 microscopic fields covering approximately 1,000 erythrocytes. The inhibition of growth of B. gibboni per drug concentration was monitored in duplicate and in 2 separate trials. The percent parasitemia inhibitions (PPI) for the test compounds were calculated relative to the negative control (solvents without inhibitors) by using the formulae: PPI = 100 − [PP_{test compound}/PP_{no inhibitor} X 100]. The PP_{test compound} and PP_{no inhibitor} represent percent parasitemia in the presence of the test compounds and percent parasitemia in the presence of solvents without inhibitors, respectively.

**Inhibition of catalytic activity of the rBgDHFR-TS enzyme**

To screen the inhibitory effect of DAP, DAMPA, PA and N10-TFPA on the enzymatic activity, 100 μM of each of the compounds were initially incubated with 0.025 mg/ml of the rBgDHFR-TS enzyme solution. Negative control contained the solvent (2% methanol mixed with 2.5 mM sodium hydroxide) in which these compounds were dissolved. Additionally, 100 μM of methotrexate was included as reference positive control. The catalytic activity was determined by using DHFR assay kit (Sigma, USA) according to the manufacturer’s protocol. The reaction rate of the enzyme was measured by using a DU 800 spectrophotometer interfaced with Windows 2000 computer running DU 800 system and application software (Beckman coulter, Inc., USA). The percent inhibition of enzyme activity (PIEA) for the test compounds were calculated relative to the negative control (solvents without inhibitors) by using the formulae: PIEA = 100 − [ERR_{test compound}/ERR_{no inhibitor} X 100], where ERR_{test compound} and ERR_{no inhibitor} represent enzyme reaction rate in presence of the test compounds and enzyme reaction rate in presence solvents without inhibitors, respectively.

**Determination of IC_{50} of DAP and DAMPA**

The concentrations of DAP and DAMPA required to achieve 50% inhibition of the enzyme reaction (IC_{50}) were determined at 50 μM dihydrofolic acid and 60 μM NADPH by using the DHFR assay kit. The final concentrations of DAP used for the determination of IC_{50} included 0, 25, 50, 75, 100 and 125 μM, while those for DAMPA were 0, 1, 2, 4, 6, 8 and 10 μM. The assays were started by the addition of DHF acid after pre-incubation of the enzyme with each inhibitor dissolved in 2% methanol having 2.5 mM sodium hydroxide. The IC_{50} values of the compounds for the PIEA were estimated by interpolation on the linear segments of inhibition curves by using the S-plus 6 software (Insightful Corporation, USA).

Additionally, the IC_{50}s of DAP and DAMPA for the PPI of B. gibboni in vitro were determined. We also determined whether or not the inhibition of rBgDHFR-TS enzyme activity by DAMPA would mirror the percent parasitemia inhibition of the parasite within same range of concentrations of the drug.

**Nucleotide sequence accession number**

Nucleotide sequence data of Bgdhfr-ts cDNA reported in this paper is available in the GenBank™, EMBL and DDBJ databases under the accession number AB426521.

**RESULTS AND DISCUSSION**

One of the strategies for discovery of antiparasitic drugs is based on identification and validation of molecular drug targets in the parasitic pathogens (Geyer et al., 2005; Sahu et al., 2008; Werbovetz, 2000). Some of these drug targets include enzymes involved in folate metabolism of the parasites (Yuthavong et al., 2006). The strategy requires in vitro cultures of parasite in addition to availability of purified active target enzyme and in vivo animal models, when possible, for screening of lead compounds (Fidock et al., 2004).
On this basis, we cultured *B. gibsoni* in vitro for subsequent in vitro assay of antifolate precursors and achieved a parasitemia of between 4 and 5%. The growth of *Babesia* in this culture was inhibited by aminopterin (IC$_{50}$ = 82.7 ± 14.7 nM) in a manner similar to methotrexate (Aboge et al., 2008), with which it belongs to the same chemical group. Similarly, the purified 83-kDa rBgDHFR-TS enzyme was enzymatically active and aminopterin inhibited its catalytic activity (Fig. 1). The IC$_{50}$ for the enzymatic inhibition was 63.17 ± 0.6003 nM falling within the nanomolar inhibition range as for the parasite culture. Thus the inhibition profiles indicated that the assay systems could be used to evaluate further, the inhibitory effects of antifolate precursors and their related compounds, on both the enzyme target as well as the parasite pathogen.

![Graph showing inhibition of DHFR-TS enzyme activity](image)

**Figure 1.** Preliminary evaluation of in vitro drug assay using the DHFR-TS enzyme of *B. gibsoni*. The rBgDHFR-TS enzyme activity was screened in the absence and presence of inhibitor-aminopterin. Inset is the 83-kDa GST fusion recombinant enzyme.

Next, we were interested in knowing whether DAP, DAMPA, $^\text{31}$ TFPA and PA could inhibit the growth of *B. gibsoni* by targeting the BgDHFR-TS enzyme. Subsequently, we treated in vitro cultures of the parasite and the rBgDHFR-TS enzyme with each of these compounds at 100 μM. This concentration was arrived at based on an initial preliminary in vitro assay, and thus it formed the maximum dose for the inhibition screens. A flow chart for the evaluation of inhibitory effects of DAP, DAMPA, $^\text{31}$ TFPA and PA on the parasite growth and the enzyme activity is outlined in Figure 2. We found that DAMPA markedly inhibited the growth of the parasite at 100 μM while DAP caused moderate inhibition only at this concentration (Table 1). Thus, DAP and DAMPA inhibit *B. gibsoni* growth in a manner similar to inhibition of the growth of *P. falciparum* in vitro (Nduati et al., 2005). These compounds are precursors of
methotrexate, which is known to inhibit the growth of both *P. falciparum* (Nduati et al., 2008) and *B. gibsoni* (Aboge et al., 2008) by disrupting DHFR-TS enzyme involved in folate metabolism.

**Figure 2.** Schematic diagram illustrating the evaluation of inhibitory effects of DAP, DAMPA, PA and N10-TFPA on *B. gibsoni* growth and the activity of rBgDHFR-TS enzyme. The IC₅₀ of the compounds, which inhibited the parasite growth and enzymatic activity by more than 50% was determined at 100µM for each of the compounds.

**Table 1.** Inhibition of the growth of *B. gibsoni* by methotrexate, DAMPA, DAP, PA and N¹⁰-TFPA. The solvents in which the compounds were dissolved were used as negative control. The inhibition assay by these compounds was done at 100 µM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% parasitemia inhibition (PPI)</th>
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<tr>
<td>Methotrexate</td>
<td>100</td>
</tr>
<tr>
<td>DAMPA</td>
<td>100</td>
</tr>
<tr>
<td>DAP</td>
<td>76.30 ± 2.80</td>
</tr>
<tr>
<td>PA</td>
<td>36.65 ± 0.25</td>
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<tr>
<td>N¹⁰-TFPA</td>
<td>7.55 ± 1.85</td>
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Therefore, the inhibition of *B. gibsoni* growth by DAMPA and DAP made us to hypothesize that these compounds could target the BgDHFR-TS enzyme. To confirm this putative hypothesis, the purified
rBgDHFR-TS enzyme was incubated with these compounds, and then the inhibition of activity of the enzyme was determined at 100 µM. Consistent with the inhibition of growth of the parasite, DAMPA potently inhibited the enzymatic activity, while DAP caused moderate inhibition only at this concentration (Table 2). Therefore, these findings confirmed our previous expectation that DAP and DAMPA could inhibit the activity of the rBgDHFR-TS enzyme in a manner similar to inhibition of the parasite growth, hence supporting our hypothesis that these compounds directly target the BgDHFR-TS enzyme. Previously, it has been suggested that DAP and DAMPA inhibit Plasmodium growth by either directly targeting DHFR-TS enzyme, or by synthesizing these precursors to methotrexate, which then targets DHFR-TS enzyme (Nduati et al., 2005). Whereas these 2 suggestions might be true for the malaria parasite because the synthesis of pteridine compounds from their precursors is known to take place in the parasite, it is still uncertain whether this pteridine pathway exists in Babesia. Consequently, the inhibition of B. gibsoni growth by the methotrexate precursors could be solely by direct targeting of DHFR-TS rather than these compounds being synthesized to methotrexate by Babesia and then target the enzyme.

**Table 2.** Inhibition of the activity of rBgDHFR-TS enzyme by methotrexate, DAMPA, DAP, PA and N10-TFPA. All the compounds were used at a concentration of 100 µM.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% inhibition of enzyme activity (PIEA)</th>
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<tr>
<td>Methotrexate</td>
<td>95.9 ± 3.80</td>
</tr>
<tr>
<td>DAMPA</td>
<td>92.4 ± 2.21</td>
</tr>
<tr>
<td>DAP</td>
<td>46.7 ± 9.40</td>
</tr>
<tr>
<td>PA</td>
<td>17.3 ± 4.20</td>
</tr>
<tr>
<td>N10-TFPA</td>
<td>26.6 ± 6.10</td>
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DAMPA was a more potent inhibitor of both the recombinant enzyme activity and the growth of B. gibsoni than DAP. One possible explanation for this finding is the closer resemblance of DAMPA to the substrate, DHF, than DAP. Although both DAP and DAMPA have 2, 4-diaminopteridine ring, DAMPA has an extra para-aminobenzoic acid group (Fig. 3c and d) and thus have a chemical structure that closely resemble the substrate, DHF, resulting in the observed potent inhibition. It appears that the inclusion of para-aminobenzoic acid group is crucial for the more potent inhibition. The parent compound, methotrexate, which potently inhibits activity of rBgDHFR-TS enzyme and the growth of B. gibsoni (Aboge et al., 2008), only differs from DAMPA by addition of the glutamic acid residue.

From the above inhibition results, we argued that PA and N10-TFPA could inhibit B. gibsoni growth in a manner similar to that of DAMPA since the compound structurally resembles PA and N10-TFPA by having pteric acid moiety (Fig. 3e to f). If PA and N10-TFPA actually inhibit the parasite growth then, it would be convincing to argue that they can target DHFR-TS of B. gibsoni, and thus inhibit the activity of the rBgDHFR-TS enzyme. Contrary to our expectations, both PA and N10-TFPA did not inhibit growth of B. gibsoni. In addition, these compounds did not inhibit activity of the DHFR-TS enzyme indicating that PA and N10-TFPA do not target this enzyme. The chemical structures of PA and N10-TFPA closely resemble
that of DAMPA by having pteroic acid group (Fig. 3d to f). Therefore, we would have expected PA and \( ^{N10}-\text{TFPA} \) to inhibit growth of *B. gibsoni* as well as the enzyme activity in a manner similar to DAMPA inhibition. However, the observation was contrary to our anticipation suggesting that the structural differences in the substitution of either the amino or the hydroxyl groups on the outer pteridine/quinazoline rings could play a role in this difference in inhibition profiles. DAMPA has 2 amino groups in the outer quinazoline/pteridine ring, while PA and \( ^{N10}-\text{TFPA} \) have 1 amino group only and a hydroxyl group, hence, the replacement of one of the amino groups with hydroxyl group in either outer quinazoline or pteridine ring could be a possible explanation for the lack of inhibition property of PA and \( ^{N10}-\text{TFPA} \). Moreover, it is known that 2, 4-diaminoquinazoline based compounds with 2 NH\(_2\) groups in the quinazoline ring inhibit, *P. falciparum* growth *in vitro* (Ommeh *et al.*, 2004) a finding that appear to support the above theory.

**Figure 3.** The chemical structures of the DHFR substrate, DHF (a), and the antifolate drug, methotrexate (b) as well as the structures of 2, 4-diamino-6-hydroxymethyl-pteridine (c) and 2, 4-diamino-N10-methyl-pteridoic acid (d). The chemical structures of \( ^{N10}-(\text{Trifluoroacetyl})\) pteroic acid (e) and pteroic acid (f), which are pteroic acid-based aminoquinazolines are shown. 2, 4-diaminopridine ring (I), para-aminobenzoic acid, (II) and glutamic acid residue (III) are the building blocks of methotrexate.

Since DAP and DAMPA were the only compounds that caused inhibition of the parasite growth and the enzyme activity by more than 50% at an initial screening concentration of 100 \( \mu \text{M} \), we determined the 50% inhibition concentrations [IC\(_{50}\)] of these compounds. DAMPA was a more-potent inhibitor of *B. gibsoni* growth, with an IC\(_{50}\) of 2.4 ± 0.2 \( \mu \text{M} \), while DAP was about 30 times less potent, revealing an IC\(_{50}\) of 78 ± 15 \( \mu \text{M} \) (Fig. 4a and b). For the enzymatic inhibition by DAMPA, the IC\(_{50}\) was about 2.6 ± 15 \( \mu \text{M} \) (Fig. 4c) compared that of DAP, which was more than 100 \( \mu \text{M} \). The IC\(_{50}\) of DAMPA (2.4 ± 0.2 \( \mu \text{M} \)) achieved by
the inhibition of the growth of *B. gibsoni* was about 20 times lower than the mean plasma concentration of DAMPA (51 µM) achieved after intravenous infusion in non human primates (Widemann *et al.*, 2000). Therefore, if DAMPA were to be administered by the same route to dogs infected by *B. gibsoni*, then, sufficient plasma concentration well above the IC₅₀ of the compound could be achieved. However, it would be of interest to know how the plasma concentration would change, if DAMPA is administered via the relatively safer oral route. Furthermore, the compound is rapidly eliminated via the kidney after conjugation to glucuronide in plasma (Widemann *et al.*, 2000) suggesting that not enough concentration of the drug could be attained in the erythrocytes infected by *Babesia* parasite. Nevertheless, if a higher concentration of DAMPA can be attained within infected erythrocytes, especially those levels higher than the IC₅₀ observed in the current study but lower than the mean plasma concentration (51 µM), then, this compound might be a good candidate for further studies as a prospective antibabesial drug. Moreover, DAMPA is a methotrexate precursor, which is well tolerated in non-human primates suggesting that they can be safely applied in mammalian hosts (Widemann *et al.*, 2000).

**Figure 4.** The inhibitory effects of DAMPA and DAP on the growth of *B. gibsoni* (a and b). Panel c shows the inhibition of the activity of rBgdHFR-TS enzyme by DAMPA. Duplicate inhibition curves were generated for each compound, 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, USA).
Given that DAMPA was a more potent inhibitor of the Babesia growth and rBgDHFR-TS enzyme activity, we were particularly interested in knowing whether inhibition of the enzyme activity by DAMPA would parallel the inhibition of the parasite growth. Consequently, we found that there inhibition of the recombinant enzyme activity paralleled inhibition of the growth of the parasite by DAMPA. The IC\textsubscript{50} of enzymatic inhibition was $2.6 \pm 0.15 \ \mu M$ very close to that of the inhibition of the parasite growth, $2.6 \pm 0.15 \ \mu M$. This close correlation is particularly important because, in the enzyme assay mixture, inhibitor can directly target the enzyme without necessarily crossing any membrane barrier. However, in the Babesia culture, the inhibitor has to cross the erythrocyte membrane and the parasite membrane to be able to target the BgDHFR-TS enzyme in the parasite cytoplasm. Therefore, this correlation indicates that DAMPA could cross the erythrocyte membrane barrier and the parasite membrane to reach the drug target in the parasite cytoplasm. Furthermore, because the inhibitory effect of DAMPA on the recombinant enzyme activity paralleled its inhibition of the B. gibsoni growth in vitro, the purified rBgDHFR-TS enzyme could be used for preliminary screening of some of the antifolate drugs. This could facilitate the initial selection of potent antifolates for subsequent evaluation for their inhibition of the parasite proliferation by ensuring that only lead compounds are selected for subsequent assays. We also confirm our previous proposal that the rBgDHFR-TS enzyme could be used to screen some of the available precursors of antifolate libraries (Aboge et al., 2008).

In summary, we have shown that the methotrexate precursors, such as DAP and DAMPA, inhibit the growth of B. gibsoni in vitro, whereas PA and \textsuperscript{10}TFPA, which are also pterico acid based compounds like DAMPA do not inhibit the parasite growth. Additionally, we have demonstrated that the inhibitory effect of DAMPA on the recombinant enzyme activity would parallel its inhibition of the parasite growth in vitro indicating that this methotrexate precursor inhibits the growth of B. gibsoni by directly targeting DHFR-TS enzyme of B. gibsoni. Therefore, this study provides an insight into the molecular and biochemical mechanism of antibabesial effect of DAMPA and DAP, both methotrexate precursors, which are well tolerated in mammalian cells.

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