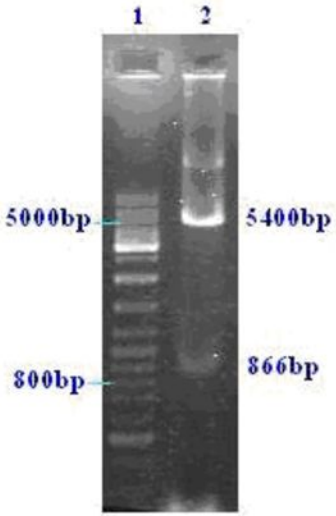

Figure 2

Inhibition of pteridine reductase 1 (PTR1) expression in *Leishmania* promastigotes using a full-length antisense construct

Farnaz Kheirandish[1 and 2], Mojgan Bandehpour[3], Nushin Davoudi[4], Nariman Mosaffa[5], Fereydoun Mahboudi[4], *Bahram Kazemi [1 and 3], Ali Haghighi[1], Mehdi Mohebali[6]

1- Parasitology Dept., Shahid Beheshti University, M.C., Tehran, I.R. Iran

2- Dept. of Parasitology, Lorestan University of Medical Sciences, Khorramabad, I.R. Iran

3- Cellular and Molecular Biology Research Center, Shahid Beheshti University, M.C., Tehran, I.R. Iran

4- Biotechnology Dept., Institute Pasteur of Iran, Tehran, Iran

5- Immunology Dept., Shahid Beheshti University, M.C., Tehran, I.R. Iran

6- Parasitology Dept., Tehran University of Medical Sciences, Tehran, Iran

Abstract: *Leishmania* exhibit many unusual features, one of which is the pteridine metabolic pathway essential for the growth for these parasites. *Leishmania* have evolved a complex and versatile pteridine salvage network capable of scavenging a wide array of conjugated and unconjugated pteridines.

The *L. major PTR1* gene was cloned into pcDNA3 digested with KpnI and BamHI. The gene was cloned antiparallel to the promoter and named pcDNA-Rptr. *L. major* promastigotes were divided into two groups. One group was transfected with 50 μg of pcDNA-Rptr, whereas the other group was electroporated with pcDNA3. Mentioned cells were cultured and plated onto semi-solid media.

Western blotting was performed on extracts from transfected promastigotes of *L. major* using an leishmania major PTR1 antibody. The PTR1 protein was not expressed in pcDNA-Rptr–tansfected promastigotes, 'Our results indicate that our system may be useful for studying the pteridine salvage pathway in *Leishmania* as a possible drug target.

Key words: Pteridine reductase 1, antisense, *Leishmania*, expression, transfection

Introduction:

Leishmania are protozoan parasites that cause a spectrum of diseases termed leishmaniasis, which vary in severity from mild cutaneous to fatal visceral disease. *Leishmania* exhibit many unusual features, including the pteridine metabolic pathway, which is essential for growth. Thus, all parts of this pathway should be excellent targets for chemotherapeutic attack. As pteridine auxotrophs, *Leishmania* absolutely require an exogenous source in order to infect their mammalian hosts [1-3].

To overcome this problem, *Leishmania* have evolved a complex and versatile pteridine salvage network capable of scavenging a wide array of conjugated and unconjugated pteridines, notably folate and biopterin, respectively [4]. Folate and biopterin serve as co-factors only in their fully reduced tetrahydro forms, H4-folate and H4-biopterin, respectively.

In *Leishmania* and mammalian cells, H4-folate is generated from folate and dihydrofolate (H2-folate) by the NADPH-dependent enzyme dihydrofolate reductase (DHFR) [5].

In *Leishmania* and other protozoa, DHFR occurs as a bifunctional enzyme, joined to thymidylate synthase (DHFR-TS) [6]. The principal role of H4-folate is to serve as an essential co-factor in the *de novo* biosynthesis of thymidylate in *Leishmania* [7]. In mammalian cells, H4-biopterin is synthesized *de novo* or salvaged through DHFR-mediated reduction of H2-biopterin [8].

In contrast, in *Leishmania* the *de novo* biopterin synthetic pathway is absent and DHFR-TS shows no activity with biopterin or H2-biopterin. Instead, reduced biopterin is generated through the action of pteridine reductase 1 (PTR1), which sequentially reduces oxidized biopterin to H2-biopterin and then to H4-biopterin [3,9-11].

PTR1 was identified as the gene within the *Leishmania* H region that, if overexpressed by gene amplification or DNA transfection, confers methotrexate (MTX) resistance [3,10]. The predicted PTR1 protein shows homology to a large family of aldo/keto reductases and short-chain dehydrogenases, including several enzymes involved in pteridine metabolism, such as sepiapterin reductase [3] and dihydropteridine reductase (DHPR) [12].

Despite the requirement of *Leishmania* for reduced folate and biopterin for growth, currently available anti-pteridines have not shown much promise clinically against leishmaniasis, even though they have proved effective against other protozoan infections' [13]. Thus, there is great need for continued efforts and research in this area. In this study, we focus on the inhibition of *PTR1* gene expression for the purposes of treating leishmaniasis.

Materials and Methods

DNA extraction and gene amplification: *L. major* was grown in NNN medium and cultured in RPMI-1640 enriched with 10% fetal bovine serum. *Leishmania* promastigotes were harvested by centrifugation and DNA was extracted. A set of primers (PTR F, 5′-GGA TCC ATG ACT GCT CCG ACC-3′; PTR R, 5′-GGT ACC TCA GGC CCG GGT AAG-3′) was designed based on the published *L. major ptr1* sequence (GenBank Accession No. L01699) with BamHI and KpnI restriction sites on the 5′-ends of the forward and reverse primers, respectively. The *PTR1* coding region was amplified from genomic DNA and the PCR product was ligated to a 3′ T-tailed, EcoRV-digested pBluescript.

Construction of the antisense *PTR1* gene: The *Leishmania major PTR1* gene (accession code EF113119) was cloned into pBluescript in the KpnI and BamHI recognition sites, hereafter referred to as pBSC-ptr [14]. This was used as the source of the *L. major PTR1* gene. Recombinant pBluescript was digested with KpnI and BamHI enzymes, after which the *PTR1* gene DNA fragment was purified using a Fermentas DNA purification kit (cat. No. k0513) and subcloned into pcDNA3 digested with KpnI and BamHI. The eukaryotic expression vector pcDNA3 harbors a gene encoding neomycin resistance. The recombinant plasmid was transformed into the *E. coli* TOP10 strain. Because the *PTR1* gene is cloned antiparallel to the promoter, this expression plasmid is referred to as either pcDNA-Rptr or antisense.

Transfection of *Leishmania* promastigotes: *L. major* was cultured in liquid medium 199 (Sigma, UK) supplemented with 10% defined heat-inactivated fetal bovine serum

(Biosera), 10 mM adenine (Sigma, UK), 40 mM HEPES (Sigma, UK), 0.25% hemin (Sigma, UK), 100 mg/ml streptomycin (Biosera) and 100 IU/ ml penicillin (Biosera).

Late logarithmic phase *L. major* promastigotes were harvested by centrifugation (1500×*g*, 10 min) and resuspended at a concentration of 5×10^7/ml in ice-cold transfection buffer (21 mM HEPES, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose). The promastigotes were divided into two groups: one group was transfected with 50 μg of pcDNA-Rptr (antisense), whereas the other group was electroporated with pcDNA3 using a BioRad Gene Pulser at 450 V and 450 μF capacitance under conditions described previously [15- 17]. Transfected and electroporated cells were cultured for 48 h in drug-free medium 199 and subsequently plated onto semi-solid medium 199 containing 40 μg/ml G418 (Sigma, UK) as a selective antibiotic [18,19]. After two weeks, single colonies were mass cultured into medium 199 supplemented with 40 μg/mL neomycin.

Inhibition of PTR1 expression: Transfected and untransfected *L. major* promastigotes were collected by centrifugation at 3000 g for 10 min and washed twice with 1x TBS (150 mM NaCl, 10 mM Tris pH 7.5). Cells were suspended and sonicated in lysis buffer (50 mM Tris, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF). Lysates were centrifuged at 1000 g for 10 min and the supernatants were taken for analysis. Western blot analysis was performed as described previously [20]. Briefly, 10^6 of each transfected and untransfected *L. major* promastigotes were harvested and lysed by sonication. Protein samples were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and incubated with a 1:500 dilution of rabbit anti-PTR1 antibody [14] for 1 hour at 37 ºC. Then, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-

rabbit IgG (1:5000) as the secondary antibody. Antibody binding was visualized using diaminobenzidine (DAB) for one hour at 37 ºC.

Results

Figure 1 shows the PCR product of the *L. major* PTR1 gene. The gene was cloned into pBluescript, digested with Bam HI and KpnI, and subcloned into pcDNA3 as antisense (pcDNA-Rptr). To confirm the identity of the recombinant plasmid, pcDNA-Rptr was digested with the restriction enzymes KpnI and BamHI. An 866-bp insert and a 5.4-kb vector fragment were observed by electrophoresis [Fig. 2]. When pcDNA3 was digested with KpnI and BamHI, only the 5.4-kb vector fragment was observed [Fig. 3], indicating that the target fragment was inserted into the multiple cloning site of pcDNA3.

Cells after transfection: Twenty-four hours after transfection, some dead cells were observed. After selection with 40 μg/mL G418, untransfected cells died, and transfected cells survived. The cell clones were isolated 10-14 days after G418 selection.

Inhibition of *PTR1* expression by antisense: Transfected and untransfected promastigotes were mass cultured. Promastigotes were harvested by centrifugation and lysed by sonication. Protein samples were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and analyzed using a rabbit anti-PTR1 primary antibody. The PTR1 protein was expressed in untransfected promastigotes [Fig. 4, lane 1], but not in pcDNA3 Rptr–transfected promastigotes [Fig. 4, lane 2], indicating that the gene was inhibited by the antisense construct.

Discussion

The properties of *PTR1* suggest several mechanisms to explain how its overproduction creates MTX resistance. Because PTR1 binds MTX tightly, it may sequester MTX from DHFR-TS [11]. In addition, PTR1 exhibits a broad specificity for pteridine substrates and reduces folate to the H2- and H4 forms [21]. PTR1 may mediate MTX resistance through its ability to reduce folate to generate H2 folate, which is known to be extremely effective in relieving the inhibition of DHFR-TS by MTX *in vitro* [22-24].

Deletion of the *PTR1* gene is lethal to the promastigote in the presence of MTX. This lethal effect can be countered by the provision of reduced pterins, but not folate [5]. Others have already done this, so we are doing it as well. In this study, pcDNA-Rptr was prepared and confirmed; the vector contains the *PTR1* gene oriented antiparallel to the promoter. *L. major* promastigotes were transfected with pcDNA-Rptr or pcDNA3, and lysates were examined by western blot analysis, which showed that the expression of the *PTR1* gene was inhibited by pcDNA-Rptr successfully. Our data are similar to those in a study by Chen et al., in which they used full-length antisense RNA to inhibit the expression of the gp63 gene in *Leishmania amazonensis* [25]. Although the targeted gene deletion in *L. major GP63* reported by Joshi et al. [26, 27] is considered in the present investigation, our data demonstrate that inhibition occurs in the cytosol, as Dumas et al. reported in their study using cytosolic antisense RNA to regulate the expression of noncoding RNA in amastigotes [28]. Liang et al. demonstrated that small nucleolar RNA (snoRNA) genes can be silenced in *L. major*, *Leptomonas collosoma* and *Trypanosoma brucei*. Silencing is achieved in *Leptomonas collosoma* and *L. major* by expressing an

antisense transcript complementary to the snoRNA gene, resulting in the accumulation of small interfering RNA (siRNA). The siRNA then eliminates the mature snoRNA [29]. Other scientists have used antisense RNA to inhibit beta-tubulin synthesis in the *Leishmania donovani* amastigote [30], as well as a mini-exon sequence to inhibit amastigote growth [31-34]. In contrast to these previous studies, the present work is the first report on the inhibition of *Leishmania PTR1* by a full-length antisense construct. Our results demonstrate that *PTR1* antisense RNA can efficiently block *PTR1* mRNA and protein expression in *L. major* promastigotes. This is a model for gene inhibition of *Leishmania*They were confirmed previously in materiale and methods, and the approach that we report here may prove useful for creating similar model systems for studying other infectious organisms and cancers involving a single gene.

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Figure legends

Figure 1. Electrophoresis of PCR products on a 1% agarose gel.

Lane 1: The 866-bp PCR product of *Leishmania major ptr1* gene

Lane 2**:** 100-bp DNA ladder marker

Figure 2: Identification of recombinant plasmid pcDNA-Rptr using agarose gel electrophoresis.

Lane 1:100-bp DNA ladder marker

Lane 2: pcDNA-Rptr digested with KpnI and BamHI

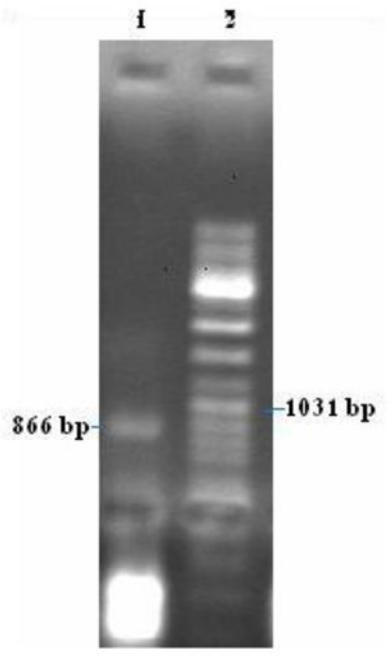
Figure 3: 1% agarose gel electrophoresis

Lane 1: pcDNA3 digested with KpnI and BamHI

Lane 2: 100-bp DNA ladder marker

Figure 4: Western blot analysis to demonstrate lack of expression of PTR1 protein in antisense-transfected promastigotes' Lane 1: Lysate of untransfected promastigotes (without antisense).

Lane 2: Lysate of pcDNA-Rptr-transfected promastigotes (with antisense)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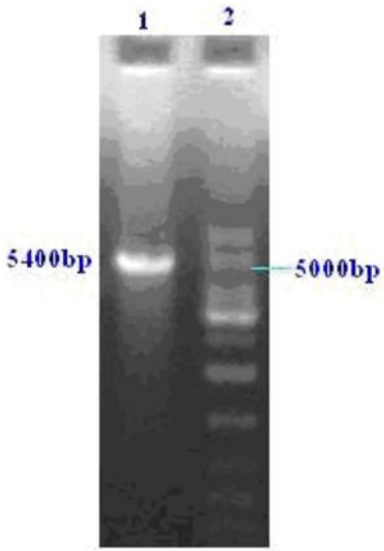
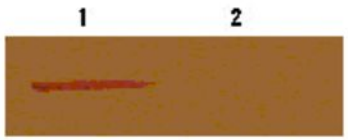


Figure 3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