RESEARCH ARTICLE

WILEY

The potential therapeutic role of PTR1 gene in non-healing anthroponotic cutaneous leishmaniasis due to *Leishmania tropica*

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Abstract

Background: Drug resistance is a common phenomenon frequently observed in countries where leishmaniasis is endemic. Due to the production of the pteridine reductase enzyme (PTR1), drugs lose their efficacy, and consequently, the patient becomes unresponsive to treatment. This study aimed to compare the in vitro effect of meglumine antimoniate (MA) on non- healing *Leishmania tropica* isolates and on MA transfected non-healing one to PTR1.

Methods: Two non-healing and one healing isolates of *L. tropica* were collected from patients who received two courses or one cycle of intralesional MA along with bi-weekly liquid nitrogen cryotherapy or systemic treatment alone, respectively. After confirmation of *L. tropica* isolates by polymerase chain reaction (PCR), the recombinant plasmid pcDNA-rPTR (antisense) was transfected via electroporation and cultured on M199. Isolates in form of promastigotes were treated with different concentrations of MA and read using an enzyme-linked immunosorbent assay (ELISA) reader and the half inhibitory concentration (IC_{50}) value was calculated. The amastigotes were grown in mouse macrophages and were similarly treated with various concentrations of MA. The culture glass slides were stained, and the mean number of intramacrophage amastigotes and infected macrophages were assessed in triplicate for both stages.

Results: All three transfected isolates displayed a reduction in optical density compared with the promastigotes in respective isolates, although there was no significant difference between non-healing and healing isolates. In contrast, in the clinical form (amastigotes), there was a significant difference between non-healing and healing isolates ($p < 0.05$).

Conclusion: The results indicated that the PTR1 gene reduced the efficacy of the drug, and its inhibition by antisense and could improve the treatment of non-healing cases. These findings have future implications in the prophylactic and therapeutic modality of non- healing *Leishmania* isolates to drug.

1 | INTRODUCTION

Leishmaniasis is a major public health challenge caused by protozoan parasites of the genus *Leishmania*. The disease exists on four continents with a prevalence of 12 million cases[1,2] in an at-risk population of over 1 billion.[3] The clinical manifestations are commonly in the form of visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), or mucocutaneous infections (MCL), which is primarily strongminded by the causative *Leishmania* parasites and the host's immune response to the infection.[4] Cutaneous leishmaniasis is among the most abundant and most neglected disfiguring and stigmatizing tropical diseases causing nearly 1-1.5 million new cases of active

sores annually.[1,2] However, ignoring to include co-morbid major depressive disorder (MDD) has been recognized to be a substantial but disregarded contributor to global disease burden.[5] Therefore, with the presence of co-morbid MDD in both acute and scarred CL, the overall global impact reported in 2016 was seven times greater than the previous figure.[5,6]

Two Old World CLs are induced by *L. major* and *L. tropica*, the causative agents of zoonotic CL (ZCL) and anthroponotic CL (ACL), respectively, in the six most greatly affected countries.[3] At present, no efficient and approved human vaccine is available. The control measures against the biological vectors and animal reservoirs are not effective due to the implication of a considerable number of incriminated

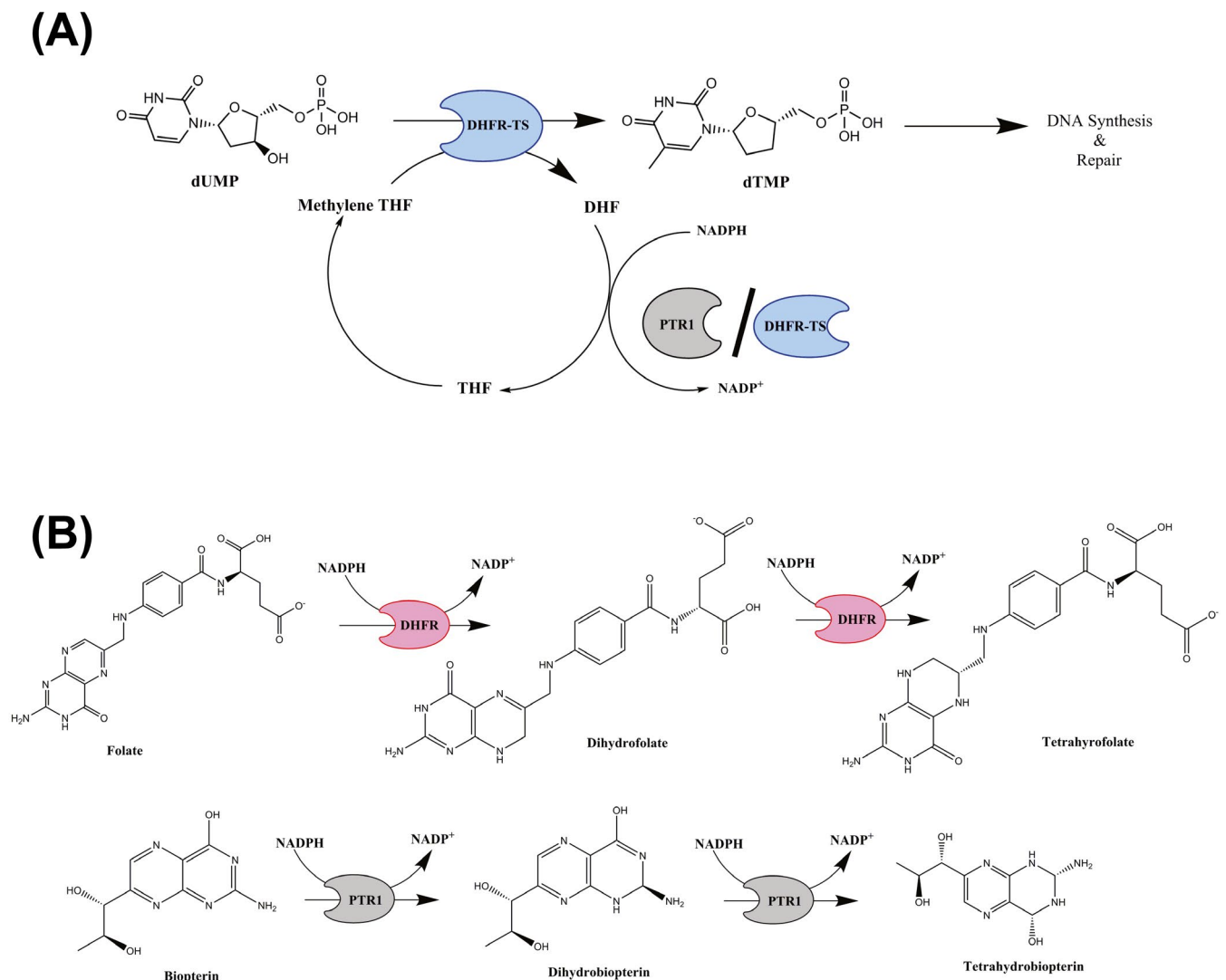**FIGURE 1** The catalytic pathways of PTR1 and DHFR. A, PTR1 and DHFR both take the fully oxidized pteridine via dihydro intermediate to the tetrahydro product. DHPR, PTR1, and DHFR prefer NADPH as the cofactor. B, DHFR-TS synthesizes dTMP while converting methylene THF to DHF which is converted back to THF by DHFR-TS. PTR1 converts H2 biopterin to H4 biopterin. PTR1 can reduce both pterins and folates
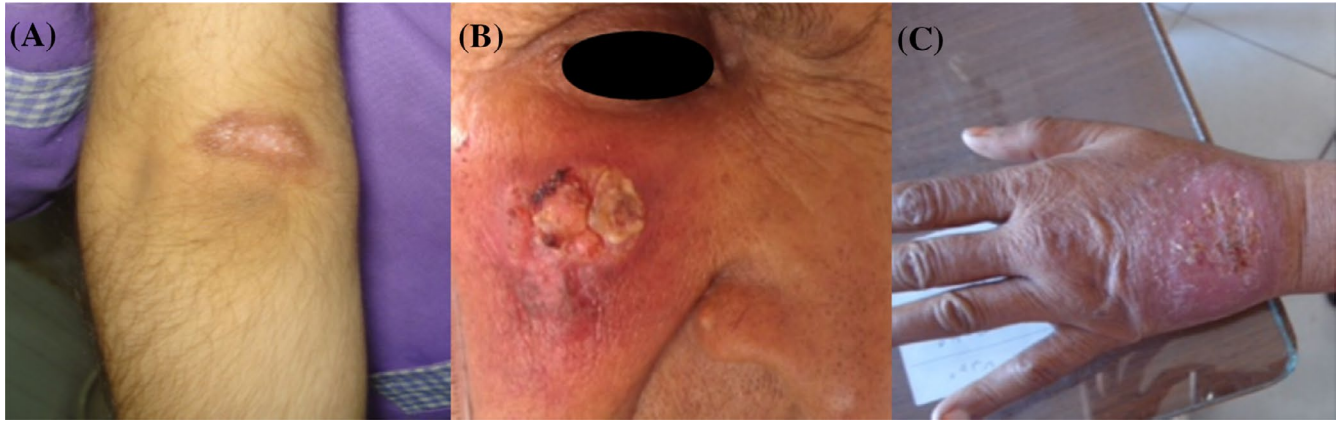
FIGURE 2 Representative images of the ACL patients. A, the skin lesion displaying a healed isolate. B, C: cutaneous lesions exhibiting non-healing isolates

TABLE 1 Baseline demographic and clinical features of unresponsive and unresponsive cases with anthroponotic cutaneous leishmaniasis

No.	Patient	Age(year)	Location	Size (cm)	Type
1	Male	30	Hands	2.4	Unresponsive (Nodular)
1	Female	33	Face	2.1 × 2	Unresponsive (Nodular)
1	Male	28	Hands	1.4	Responsive (Ulcerative)

species.[1] Chemotherapy is the major approach to control the disease, and this strategy relies on two preparations of pentavalent antimonials (SbV), meglumine antimoniate (MA), and sodium stibogluconate (Pentostam). Although developed 72 years ago, the use of these drugs continues to be the main treatment in endemic areas. However, resistance against SbV has been significantly increased.[7-9] For the past few decades, MA has been the drug of choice for the treatment of CL.[10] Although this drug has been the first-line therapy, varying reports of unresponsive patients (10% - 59%) with CL have been documented in Iran including Kerman,[10] Bam,[11-13] Mashhad,[14] Fars,[15] and Yazd.[16]

Reports have demonstrated that the growth of organisms associated with the *Trypanosome* family relies on folate-pteridine.[17] Members of the *Trypanosomatidae* family, as well as *Leishmania*, synthesize enzymes to neutralize drug cure. One such enzyme is pteridine reductase 1 (PTR1).[18] PTR1 is similar to dihydrofolate reductase (DHFR),[19] which represents a part in activating the thymidylate synthase enzyme (TS) and catalyzes the conversion of deoxyuridylate to deoxythymidylate. Furthermore, DHFR reduces dihydrofolate (H2-folate) to tetrahydrofolate (H4-folate) which serves as a cofactor of thymidylate synthase (Figure 1A).[20] Thus, DHFR-TS has a major role in deoxyribonucleic acid (DNA) synthesis, and if it is inhibited, the result would be the death of the microorganism.[17] The application of drugs to curb metabolism aiming at controlling cell proliferation is an important breakthrough. One of the mechanisms that these drugs use is blocking DHFR-TS.[17] Since PTR1 activity is similar to DHFR, both enzymes decrease the effect of these drugs (Figure 1B).[19] Because of the creation of enzymes including PTR1 by *Leishmania* parasites, the drug significantly loses its effectiveness, and eventually leading [18]

ACL is restricted to humans; thus, rigorous surveillance, early, and effective treatment are necessary as important measures to control the disease.[10] In this study, PTR1 gene expression was inhibited by mRNA antisense to measure the likelihood of using gene expression inhibition in the therapy of leishmaniasis. This modality could be unique and used as a preventive and therapeutic measure against the ACL non-healing isolates.

2 | MATERIALS AND METHODS

2.1 | Ethical consideration

The protocol was granted approval by the Ethics Committees of the Leishmaniasis Research Center and Kerman University of Medical Sciences (IR.KMU.REC.1390.166). All CL cases received treatment freely without charge; their written informed consent was obtained and their data were kept confidential.

2.2 | Sampling and parasites

Two non-healing and one healing isolates (Figure 2A) of *L. tropica* were randomly selected and used in the following experiments (Table 1). The non-healing isolates (Figure 2B,C) were collected from patients who had received two courses of intralesional or systemic treatment with no recovery after three months of follow-up examination.

Skin tissues were taken from the periphery of cutaneous lesions by a scalpel and blade, smeared on glass slides, air-dried, fixed, and stained by standard Giemsa. Slide smears were diagnosed by direct microscopic examination and identified to species-level by nested-PCR technique. Parasites were grown in NNN medium and

subcultured in RPMI1640 with 10% heated fetal bovine serum (FBS). DNA extraction was performed on *Leishmania* promastigotes and harvested in the late logarithmic phase.

2.3 | Construction of the antisense

A pair of primers PTR F and PTR R (PTR F, 5′-GGA TCC ATG ACT GCT CCG ACC-3′; PTR R, 5′-GGT ACC TCA GGC CCG GGT AAG-3′) were designed according to the PTR1 construction with *BamHI* and *KpnI* restriction locations recognized on the 5′-ends of the primers. The PTR1 coding region was amplified from genomic DNA, and the PCR product was ligated to a 3′ T-tailed, EcoRV-digested pBluescript and was then sequenced. Recombinant pBluescript containing the *L. tropica* PTR1 gene (accession no. EF113119) was digested with *KpnI* and *BamHI* enzymes and purified and then subcloned into pcDNA3 to be then digested with *KpnI* and *BamHI*. The recombinant plasmid was transformed into the *Escherichia coli* (*E. coli*) TOP10 strain. The PTR1 gene was cloned antiparallel to the sense.[21]

2.4 | Transfection

Leishmania tropica was grown in the monophasic medium 199 (Sigma-Aldrich, UK) supplemented with 10% well-defined heated FBS (Biosera, South Korea), 10 mmol/L adenine (Sigma, UK), 40 mmol/L HEPES (Sigma, UK), 0.25% hemin (Sigma, UK), 100 μg/ml streptomycin (Biosera, South Korea), and 100 IU/ml penicillin (Sigma-Aldrich, UK). Late log-form *L. tropica* promastigotes were collected by centrifugation at 1500 g for 10 min and accustomed to 5×10^7 ml in transfection buffer (ice-cold). The promastigotes were transfected with 50 μg of pcDNA-rPTR (antisense) by means of a Gene Pulser (Bio-Rad, UK) at 450 V and 450 μF as previously described and transfected promastigotes were cultured in medium 199.[22]

2.5 | Drug preparation

Meglumine antimoniate (MA, Rhône Poulenc, France) was provided through the provincial health system. MA was stored at ambient temperature and diluted in medium to make two concentrations of 0.512 mmol/L/ml and 2.04 mmol/L/ml.[23,24]

2.6 | Treatment schedule and unresponsive patients

The treatment regimen was based on the leishmaniasis guideline approved by the national expert committee.[25] MA was either administered intralesionally (IL) once a week for 12 weeks accompanied by biweekly cryotherapy (liquid nitrogen-six times), or MA alone

intramuscularly (20 mg/kg/day for 21 days). Unresponsive patients with ACL were the ones who had received two courses of MA (IL in combination with cryotherapy or had received intramuscular (IM) alone, as mentioned above) with no re-epithelialization of the skin lesion after three months of follow-up assessment.

2.7 | Promastigote assay

The susceptibility level of promastigotes was assessed based on the method described elsewhere.[23] Successive dilutions of MA in RPMI1640 (pH, 7.2) (Table 2) were prepared in a 96-well micro-titer plate. Promastigotes (10^5) were gathered at log-phase, and 100 μl of medium was added to each well, which was then incubated at 24°C ± 1°C for a normal time of 72 h. Promastigotes were cultivated in a medium with no drug and used as the untreated control group, and a medium with no organism was used as blank. All experiments were implemented in triplicate. The colorimetric cell viability analysis was completed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, UK) in sterile PBS, and 10 μl of MTT solution was added to each well, and each well was incubated at 24°C ± 1°C for 3 h. The reaction was stopped by isopropanol alcohol and read by a spectrophotometer (ELX800) at 492 nm. The IC_{50} value was calculated as follows:

$$\text{Log } IC_{50} = \log x1 + \left\{ \frac{(y1 - y02)}{y1 - y2} \right\} \times \{ \log(x2) - \log(x1) \}$$

2.8 | Amastigote assay

Murine macrophages were gathered from a BALB/c mouse by injecting 6 ml of RPMI1640 culture medium into the mouse peritoneal cavity and extracted macrophages were washed thoroughly and resuspended in RPMI1640. The viability test was done according to the method described elsewhere[24]. First, 90 μl of trypan blue staining solution (0.2%) in physiological saline containing 0.01% sodium azide to 10 μl of cell suspension (10^6 cells/ml) was added. After 3 min, the cells were calculated under a compound microscope, and viability was assessed. Then, 200 μl of the cells (10^6 cells/ml) was added to 8-chamber slides (Lab-Tek, Nalge Nunc International NY, USA) and incubated at 37°C and 5% CO2 for 2 h. Metacyclic promastigotes were added to macrophages, and they were incubated at 37°C with 5% CO_2 for 24 h. Then, MA concentrations in medium (Table 3) were added to the slides and incubated at 37°C for 72 h. Dried slides were fixed with methanol, stained by Giemsa, and studied under a light microscope (Nikon, Japan). Macrophages containing amastigotes with no drugs and macrophages without amastigotes were used as negative and blank control groups, respectively. Drug action was estimated by examining the number of intramacrophage amastigotes counting 100 macrophages. Every experiment was performed thrice.

TABLE 2 Comparison of optical density (OD) and IC$_{50}$ values among wild, transfected and non-transfected promastigotes (nonclinical stage) by colorimetric assay

Concentration of MA(mmol/L/ml)	Type	OD 490 nm	IC$_{50}$ ± SD	p value
0.512	Isolate 1(wild and transfected)	0.043	0.44 ± 0.051	*
	Isolate 2 (resistant and transfected)	0.065	0.44 ± 0.051	
	Isolate (resistant and transfected)	0.065	0.44 ± 0.051	
	Resistance isolate 1	0.094	0.71 ± 0.028	
	Resistance isolate 2	0.098	0.71 ± 0.028	
	Resistance isolate 3	0.096	0.71 ± 0.028	
2.04	Isolate (wild and transfected)	0.023	0.44 ± 0.051	*
	Isolate 2 (resistant and transfected)	0.050	0.44 ± 0.051	
	Isolate 3 (resistant and transfected)	0.020	0.44 ± 0.051	
	Resistance isolate 1	0.089	0.71 ± 0.028	
	Resistance isolate 2	0.089	0.71 ± 0.028	
	Resistance isolate 3	0.089	0.71 ± 0.028	

*Not-significant.

TABLE 3 Comparison of the mean infection rate and number of amastigotes per macrophage treated with various concentrations of meglumine antimonite (MA) among transfected and non-transfected parasites

Concentration of MA (mmol/L/ml)	Isolate	Mean infection rate (macrophage)	Mean number of amastigotes in each macrophage
0.512	Healing isolates	1.6 ± 1.1	2.6 ± 0.5
	Non-healing isolates	9 ± 2.6	15.6 ± 2.7
	Healing and non- healing isolates[a]	0.039; $p < 0.05$	0.01; $p < 0.05$
2.04	Healing isolates	1 ± 0	1 ± 0
	Non- healing isolates	5 ± 1	6.6 ± 2
	Healing and non- healing isolates[a]	0.042; $p < 0.05$	0.02; $p < 0.05$

[a]The effect of MA at 0.512 and 2.04 mmol/L concentrations in both healing and non-healing isolates was performed. There was a significant difference among different isolates ($p < 0.05$).

2.9 | Statistical analysis

The SPSS statistical software was used to analyze the data. The IC$_{50}$ values of MA for both promastigote and amastigote forms were determined and compared using t-test, and $p < 0.05$ was defined as statistically significant.

3 | RESULTS

3.1 | Construction of the antisense

The antisense of the pteridine reductase 1 gene in the plasmid pcDNA was confirmed by PCR using universal forward and reverse primers. Figure 3A shows an 860-bp amplicon representing the PTR1 gene.

3.2 | Transfection

After electroporation to verify the transfer of the antisense to the parasite, PCR with cytomegalovirus (CMV) promotor primers were carried out. Figure 3B displays the 660-bp band indicating the presence of antisense in the parasites.

3.3 | Promastigote assay

In promastigote assay, different concentrations of MA showed a reduction in optical density (OD) as measured by MTT assays. Isolates in form of promastigotes were treated with different concentrations of MA and read by an ELISA reader. The IC$_{50}$ value was calculated by the probit test in SPSS. All three transfected isolates including two non-healing and the one healing isolates showed a
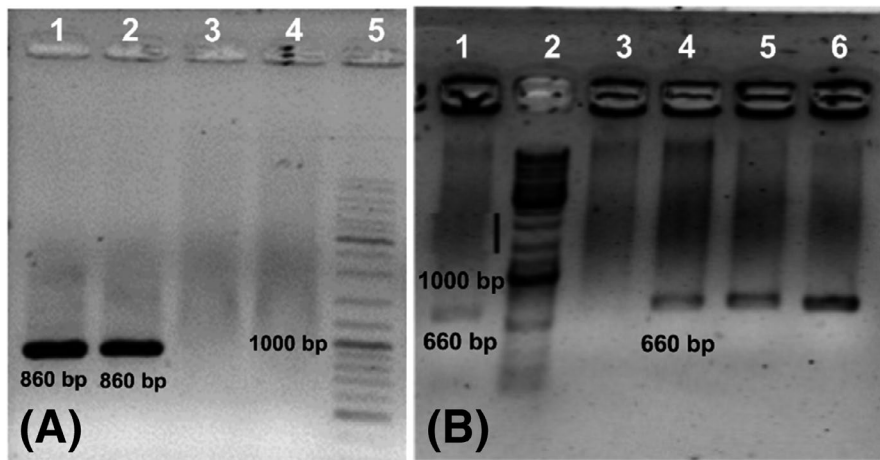
FIGURE 3 A, The antisense pteridine reductase 1 gene in the plasmid pcDNA. Lane 5; DNA size marker. Lanes 3, 4; negative controls. Lanes 1, 2; 860 bp of pteridine reductase 1 gene. B, Confirmation of transfectants after electroporation by PCR. Lane 2 DNA size marker. Lane 1; positive control. Lane 3 negative control. Lanes4, 5, 6; transfectants
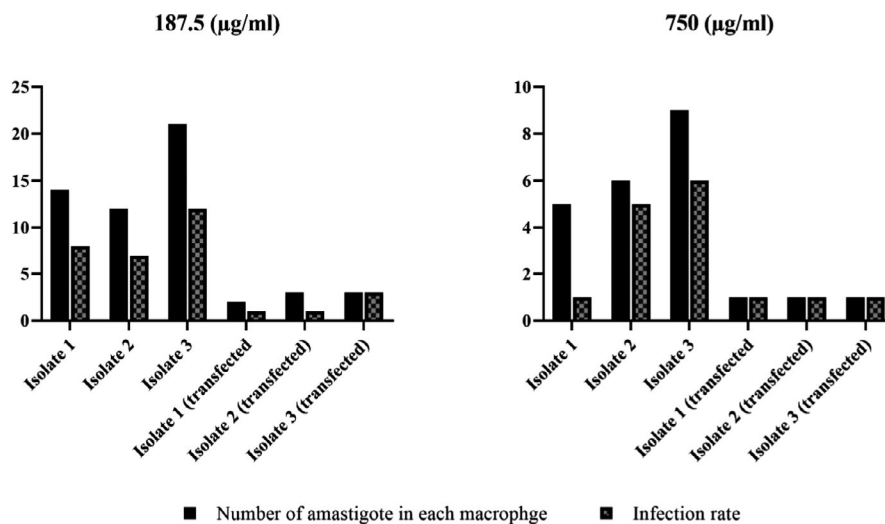

FIGURE 4 The mean number of infected macrophages and the number of amastigotes per macrophage

decrease in optical density compared to the promastigotes in corresponding non-healing isolates, albeit with no significant difference (Table 2).

3.4 | Amastigote assay

The effect of MA was assessed by the mean infection rate (MIR) of each macrophage and also by the mean number of intramacrophage amastigotes in both groups of transfected and non-transfected parasites (Figure 4A,B). In the amastigote stage, there was a significant difference in healing strains as compared to the non-healing isolates ($p < 0.05$; Table 3).

4 | DISCUSSION

Leishmaniasis caused by the *Leishmania* species is a commonly found infectious disease in over 101 countries and territories in tropical and subtropical regions. This disease remains to be a serious global health problem with a growing social and medical burden.[1-3] CL is endemic in 18 out of 31 provinces of Iran.[25] In Iran, the majority of cases infected

with ACL have been treated with MA, although a significant number of patients remain refractory.[10-16] Since ACL induced by *L. tropica* is anthroponotic, it is transmitted by the bite of the female *Phlebotomus sergenti* only from human-to-human. Therefore, control strategies are essentially focused toward a robust surveillance system, early detection, and prompt and effective treatment.[1] On the other hand, *L. major* causing zoonotic CL requires no treatment, unless vital body parts such as the face, the ears, or joints are infected.[25]

Pteridine metabolism has long been studied in trypanosomatids, notably in *Leishmania* and associated species. *Leishmanial* parasites are auxotrophic, meaning they are able to synthesize neither folate nor pteridine metabolites required for their infectivity, proliferation, survival, and pathogenicity. These metabolites are salvaged from their vertebrate hosts and invertebrate vectors through multiple transporter mechanisms. Inside the parasite, folates and pteridines are reduced by a bifunctional DHFR-TS and also by a novel PTRl. PTR1 is able to serve as a metabolic bypass of DHFR inhibition and reduces the efficacy of existing anti-folate drugs.[26]

DHFR-TS and probably PTR1 represent an effective drug target, but one possibly compromised by the motivating the other; hence, it is necessary to prevent both of these enzymes simultaneously in order to advance active chemotherapy. The absence of an

efficacious and approved vaccine and safe and effective medication has made it imperative to search for safe, inexpensive, and efficient anti-leishmanial drugs.

According to recent reports, there is no entirely active compound for the treatment of leishmaniasis and as a result, the emergence of drug-resistant parasites is inevitable.[27] In a previous study, the PTR1 enzyme of *Leishmania*, with enzymatic activity similar to DHFR, decreased parasite susceptibility to methotrexate [20] and it was demonstrated that removal of the PTR1 gene in *Leishmania* treated with methotrexate caused the death of the parasite.[19] To inhibit gene expression, several modalities such as oligodeoxy nucleotides,[28] mRNA antisense,[29] and small interfering RNA (siRNA) molecules have been reported.[30]

In this study, two resistant and one standard isolates of *L. tropica* were used. The resistant isolates were collected from patients who received two courses of intralesional or systemic treatment with no recovery. The isolates were diagnosed by direct smears and identified by nested-PCR.

Antisense gene pteridine reductase after approval by the PCR method was transferred through the transformation into the desired plasmid. After confirmation of the construct, electroporation was carried out, and the construct transfected to the resistant and standard isolates. Isolates in form of promastigotes were treated with different concentrations of MA and read by an ELISA reader. The IC_{50} value was calculated. All three transfected isolates including two resistant and one standard strain showed a decrease in optical density compared with the promastigotes in respective resistant isolates, although with no significant difference.

Also, similar to promastigotes, amastigotes were grown in mouse macrophage, treated with various concentrations of MA. The culture glass slides were stained by Giemsa, and the mean number of amastigotes and infected macrophage were determined. In amastigote stage, there was a significant difference in sensitive strains as compared with the resistant isolates ($p < 0.05$).

There was no significant difference among non-healing *L. tropica* promastigotes compared with the healing one although the IC_{50} values were substantially different. As previously reported, promastigotes are a nonclinical stage and are clearly resistant to drugs.[31] This form is restricted to the female phlebotomine sand flies and also to culture media. In the amastigote stage (clinical form), there was a significant difference in responsive strains compared with the non-healing isolate ($p < 0.05$). Amastigotes are the pathogenic stage and basically susceptible to conventional drugs, although, intracellular unlike promastigotes, they readily absorb drugs and are capable of reducing SbV to trivalent antimony (SbIII).[32] Previous documents have shown that the clinical stage shows a marked susceptibility to drugs in comparison with the extracellular promastigotes as these two forms are physiologically different in their response to drugs.[33]

Drug resistance in *Leishmania* depends on several factors. Sometimes, several genes and proteins are involved in making a drug-resistant and proving the role of PTR 1 gene in resistance also needs further studies. It is necessary to measure the level of mRNA of the PTR-1 in the parasite from the responsive and unresponsive patients using RT-PCR assay; also, it is important to induce the expression of PTR-1 in the transfected parasites and show the return of resistance to SbV. We could not perform further experiments due to our limitations. More studies are needed on more samples to be able to draw better conclusions.

In a similar study, Kheirandish and colleagues have studied mRNA antisense isolates and were able to perform transcriptional regulation of the PTR1 gene. They introduced this method to be used in studying the pteridine salvage path in *Leishmania* or to measure the prospect of using gene expression inhibition in the treatment of leishmaniasis.[34]

The selection of unresponsive parasites carrying genetic mutations that reduce the organism's response to the assigned drug may emerge upon massive drug administration.[35] Even combination therapy may promote the emergence of resistance, which would be a potential threat to the WHO's recommendation of combinatory therapies in the absence of an effective vaccine.[3] It is vital to understand that unresponsiveness and resistance to a drug are not essentially synonymous although both have, on occasion, been used synonymously. The numerous risk factors influencing treatment outcomes include host behavior in receiving drugs and the background immune response, the parasite accessibility to the drug of choice (species and strain), and the environment (new foci and global warming). Nonetheless, response to drugs is a major determinant of treatment consequences. Therefore, understanding the underlying mechanisms whereby the organism becomes unresponsive to drugs is fundamental to monitoring drug resistance.

The results indicated that the PTR1 gene can reduce the efficacy of the drug and that its inhibition by antisense can improve the treatment of non-healing cases. We can pharmacologically inhibit the activity of this enzyme before prescribing anti-leishmanial drugs. These findings have implications in prophylactic and therapeutic modalities for non-healing *Leishmania* isolates.[36] Hence, the public health surveillance system could be a crucial tool for demanding case-detection methodologies to address serious concerns. As a considerable number of patients with ACL are still unresponsive to SbV, notably to MA, avoiding human-vector exposure, the advancement of novel alternative compounds and development of a new affordable and efficacious vaccine are also important measures against ACL.

The present findings demonstrated that the PTR1 gene reduced the efficacy of the drug, and its inhibition by antisense and could improve the treatment of non-healing cases. These findings have future implications in the prophylactic and therapeutic modality of *Leishmania* non-healing isolates to drug. Further studies are essential to measure the level of mRNA of the PTR1 in the parasite from the responsive and unresponsive patients.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

There is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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