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Role of Biopterin Transporter (BT1) Gene on Growth and Infectivity of *Leishmania*

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Abstract: *Leishmania* are known to be auxotrophic for pteridines that are known to play a critical role in the parasites survival. In the present work the role of biopterin transporter in the growth of the parasite and infectivity in to macrophages has been worked out. The role of biopterin transporter in the susceptibility of *Leishmania* to antimonial compounds has also been demonstrated. This role has been verified by using attenuated strains of *Leishmania* with single, double, and triple (null) biopterin (BT1) mutants made by targeted gene replacement with specific antibiotic markers. Growth analysis of these mutants revealed that wild type, single and double knock out cell lines maintained high growth rates in the medium supplemented with biopterin and folate, whereas the triple knock out or null BT1 mutants were unable to grow in the absence of supplemental biopterin. Using wild type and null BT1 mutants, we examined the role of BT1 gene in infectivity and parasite survival. The cell lines with amplified BT1 gene showed increased infectivity and survival in the macrophages where as the cell lines with disrupted BT1 gene showed reduced infectivity and survival in the macrophages. We also examined the interaction between pteridine and antimonial compounds using recombinant *Leishmania* strains with reduced or absent biopterin transporter gene (BT1) alleles. No difference in susceptibility to Pentostam or Glucantime was observed in both wild type and BT1-knock out strains. However, pterin or folate supplementation resulted in reversal of Glucantime but not Pentostam susceptibility in both wild type and BT1-knock out strains. The reversal of Glucantime susceptibility by pterins in BT1-knock out strains suggests that the effect may be exerted independently of biopterin transporter, possibly by blocking Glucantime uptake.

Key words: BT1 gene, biopterin transport, gene knock out, over-expression, growth, infectivity, *Leishmania*.

INTRODUCTION

Leishmania are flagellated protozoa of the order kinetoplastida. They cause diseases ranging from a mild cutaneous form to fatal visceral infection. Pentavalent antimonial compounds, Glucantime and Pentostam are the drugs of choice for this disease. Emergence of resistance to these commonly used antimonials has led to the need of alternative chemotherapeutic approaches to combat and prevent the disease.

The folate pathway has been the target of chemotherapeutic intervention in infectious diseases caused by phylogenetically related apicomplexan parasites as well as many bacterial pathogens. Several inhibitors, like sulfonamidies and sulfones, inhibitors of dihydropteroate synthetase (DHPS) and pyrimethamine, trimethoprim and methotrexate, inhibitors of dihydrofolate reductase (DHFR), have been used in the treatment of both malaria and toxoplasmosis[4, 13]. These inhibitors have been found to be ineffective against leishmaniasis[12, 22, 26]. *Leishmania* species are known to be auxotrophic for folate[19, 28-30]. However, *Leishmania* species have the ability to grow in a folate deficient medium supplemented with pterins thereby eliminating the folate requirement in the parasite[1]. Furthermore it has also been demonstrated that *L. donovani* promastigotes are capable of metabolizing biopterin to tetrahydrofolates unlike the mammalian host[2]. The precise pathway of conversion of pterins to folates is not known. It has been shown that pteridine reductase (PTR1) mediates the salvage of oxidized pteridines in *Leishmania*[3]. Purified PTR1 is capable of reducing both unconjugated (biopterin) and conjugated (folate pteridines from either the oxidized or the dihydrostate)[3]. The role of H_4-biopterin in *Leishmania* is not known.

Biopterin transporter gene (BT1) or ORFG is present in the LD1 common region of *Leishmania*. This gene is universally amplified in all strains of *Leishmania* showing LD1 amplification. It codes for a transmembrane protein with 10 –12 putative membrane spanning domains. These regions are predicted to form amphiphilic α-helix or β strands typical of type IV integral proteins[24]. Database searches of protein and nucleic acid homologies revealed that BT1 has 34% amino acid similarity with ESAG10 gene from the *Trypanosoma brucei* VSG expresion site. The 45-kDa BT1 protein in *Leishmania* is consistently and

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constitutively expressed in all the growth stages of the parasite [11]. Biopterin transport occurs exclusively via biopterin transporter 1 (BT1)[6, 20, 24]. The fact that parasite is capable of converting biopterin to tetrahydrofolates unlike the mammalian host provides a rational approach towards using this pathway as a drug target and further necessitates the need to characterize this drug target at the molecular level.

In order to study the possible importance of biopterin transporter, we have used attenuated *Leishmania* strains with decreased or null biopterin transporter. The role of over expression or disruption of BT1 in the growth and infectivity of *Leishmania* has been worked out.

The mechanism of action of pentavalent antimonials (Glucantime and Pentostam), the drugs of choice for the treatment of visceral leishmaniasis is poorly understood. Recently it has been reported that Glucantime susceptibility can be partially reversed by biopterin[23]. Although the metabolic role of biopterin and closely related compounds is not yet fully elucidated, they have been implicated in a number of reactions, such as formation of unsaturated fatty acids, as cofactors in the first desaturation of stearic acid to oleate[8, 14]or as precursors of folate[2, 29]. *Leishmania* species are auxotrophic for pteridines, but they have the ability to grow in a folate-deficient medium supplemented with pterins, thereby eliminating the folate requirement of the parasite[20]. In the present study the possible interaction between pteridine and glucantime susceptibility has been worked out by using recombinant *Leishmania* strains with reduced or absent biopterin transporter gene (BT1) alleles.

MATERIALS AND METHODS

Cell culture: All mutants of BT1 transporter gene were derived from *L. donovani* strain LSB51.1 (MHOM/SD/00/Khartoum). These attenuated strains were made by three rounds of targeted gene replacement [21] since this strain contains three copies of BT1, two copies within the LD1 locus on the chromosome 35 homologs and a single copy in the chromosome 27 rDNA locus. These knock out strains contain integrated puromycin, hygromycin and phleomycin drug resistance markers replacing the two BT1 alleles on 2.2-Mb chromosome 35 and one on 1.2-Mb chromosome 27 respectively [21]. Promastigotes of wild type 51.1, Single knock out mutant (SKO) of this strain that retained two intact BT1 copies, one on chromosome 35 and other on chromosome 27, Double knock out mutant (DKO) of the strain 51.1 that lacked both chromosome 35 BT1 copies but contained the intact BT1 copy on the chromosome 27, Triple knock out mutant or BT1 null mutant termed TKO were produced from DKO mutant by replacing the remaining BT1 copy, were used for the present study. Promastigotes were grown at 22-24°C in M199 media (Gibco BRL) with 10% heat inactivated fetal bovine serum (FBS, Biological Industries, Israel). The triple knock out mutant (TKO) was maintained in the above media with BF solution (7.5 μM each of biopterin (Sigma), dihydrobiopterin (BH2, Schircks Laboratories, Jona, Switzerland), folate (Sigma) and dihydrofolate (FH2, Sigma). Biopterin and BH2 solutions were made in 40mM Hepes, pH 7.4/5 mM DTT while folate and FH2 were made in distilled water. *L. donovani* AG83 (MHOM/IN/1983/AG83) strain was used for overexpression studies.

Leishmania strains were maintained in medium M199 (Gibco, BRL) with 10% foetal bovine serum (FBS) at 22-24°C and were sub-cultured when the cells reached the stationary phase.

Generation of BT1 overexpressing *Leishmania* strain: pSLαneoα, *Leishmania* expression vector was used to overexpress a 2.3 Kb BglII-NheI fragment of *L. donovani* BT1, (kindly provided by Marc Ouellette, Quebec, Canada). pSLαneoα-BT1 construct was transfected in the wild type *L.donovani* AG83 (MHOM/IN/1983/AG83) strain

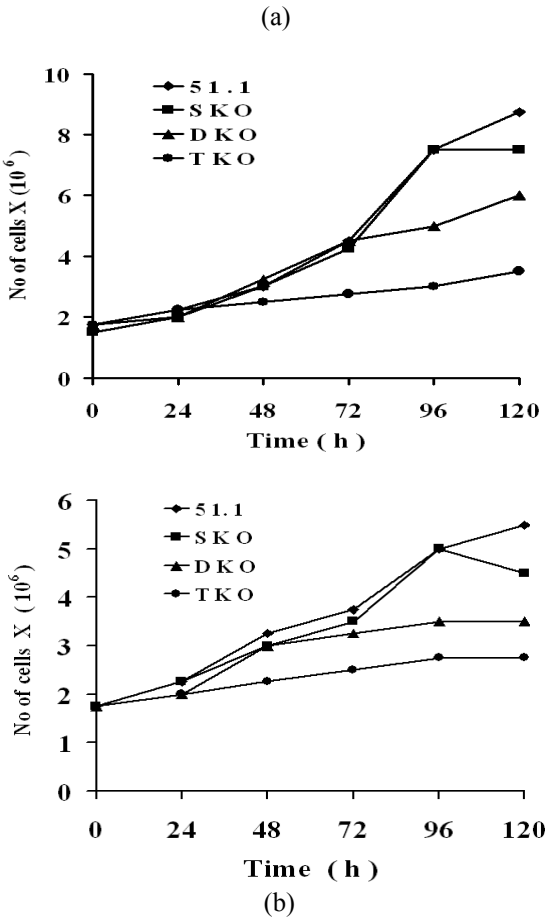

Fig 1: Growth curve of the wild type 51.1, single knockout (SKO), double knock out (DKO), and triple knock out (TKO) strain of *L. donovani* in the absence of (a) or in the presence of (b)

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7.5μM each of biopterin, dihydrobiopterin, folate and dihydrofolate. Approximately 2× 10^6 cells stationary phase promastigotes were inoculated into the medium and cell density was determined every 24h till 120h and plotted on a linear graph. Three replicate experiments were performed and similar results were obtained consistently.

via electroporation as reported previously [21]. AG83 strain contains two copies of BT1, within the LD1 locus on the chromosome 35 homologs. Transfected *Leishmania* was selected against 40 μg of G418. The strain was maintained in medium M199 (Gibco, BRL) with 10% FBS and 40 μg of G418 at 22-24ºC.

Growth studies: Growth profile of wild strain LSB51.1 and three transgenic strains with disrupted BT1 transporter gene, single (SKO), double (DKO) and triple knock out (TKO) was studied by inoculating stationary phase cells at a density of ~2×10^6 cells/ml of the minimum essential medium with α modification [24] supplemented with biopterin and folate (7.5 μM each of biopterin, dihydrobiopterin, folate and dihydrofolate) while the other was grown in un-supplemented medium. Growth rates of each of the cultures were determined at 24h interval. Growth studies with each individual cell line have been done at least three times and similar results were obtained consistently.

***In vitro* macrophage infection**: The mouse monocyte-macrophage cell line J774A.1 obtained from ATCC was cultured in RPMI-1640 medium with 10% FBS at 37°C in a CO_2 incubator with 5% O_2 and 95% humidity. Macrophages (10^6 cells/ml) were plated in 60 mm tissue culture plate. *In vitro* infection of J774A.1 macrophages was done with 1×10^7 stationary phase promastigotes of either wild type or transgenic strains. After 3 h of infection, the medium was removed and replaced with fresh RPMI 1640 medium with
10 % FBS in order to remove any free promastigotes. The cells were kept at 37°C for another 24 h. The plates in triplicate were taken out at the desired time intervals, washed with phosphate buffered saline (PBS), dried, fixed with methanol and stained with giemsa. The percentage infectivity was determined by counting 100 macrophages by examining microscopically. Number of infected macrophages and number of amastigotes per 100 macrophages was noted. Infectivity was also checked by infecting macrophages with radio-labelled *L. donovani*, labelled with tritiated thymidine. The results are mean ± SD of triplicate samples.

STATISTICAL ANALYSIS

All experiments were performed in triplicate. In the microscopic studies, a minimum of 200 cells was screened in each culture and the percentage of infected cells and the average number of amastigotes per 100 infected cells was recorded. The results represent mean ± SD of triplicates. Student's t-test was performed to determine the level of significance and $p < 0.05$ was considered to be significant.

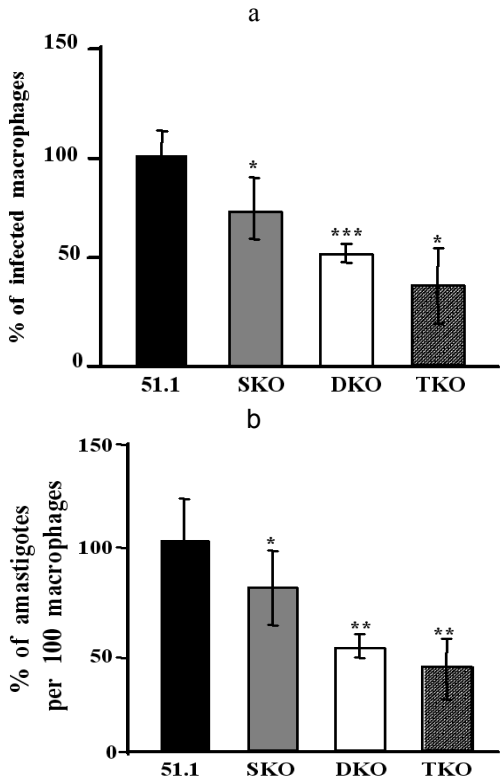

Fig 2: Comparison of infectivity of *L. donovani* LSB 51.1, wild type strain, single (SKO), double (DKO), and triple (TKO) knock out mutants of BT1 transporter gene in J774A.1 murine macrophage cell line. 10^6 J774A.1 murine macrophage cells in RPMI1640 medium with 10% heat inactivated foetal bovine serum (FBS) were infected with 10^7 stationary phase promastigotes of either wild type or transgenic leishmanial strains in a CO_2 incubator with 5% O_2 and 95% humidity. After 3h the medium was removed and replaced with fresh RPMI 1640 medium with 10% FBS in order to remove any free promastigotes. The cells were incubated at 37°C for another 24h. The plates were taken out at 24h interval, washed with PBS, dried, fixed with methanol and stained with Giemsa. The percentage infectivity was determined by counting 100 macrophages by microscopic examination. Percentage of infected macrophages and percentage of amastigotes per 100 macrophages was noted. The results represent mean ± SD of triplicates. * indicates p>0.01<0.05, ** indicates p>0.001<0.01, *** indicates p value < 0.001.

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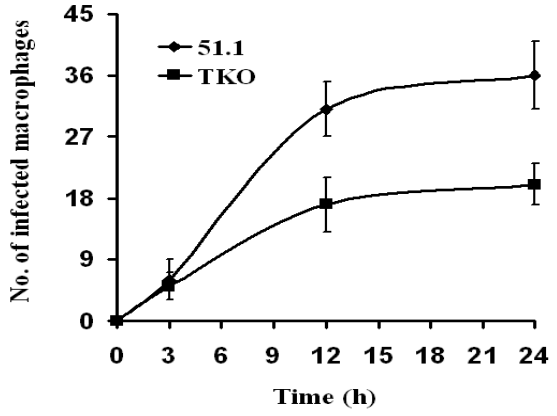
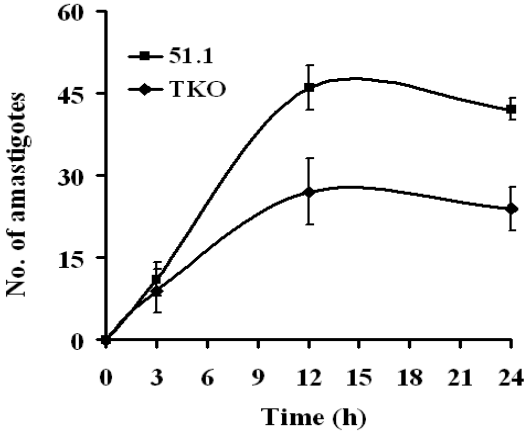
RESULTS

Growth rate analysis: A comparison of growth pattern of the wild type 51.1 and transgenic strains was done both in the medium supplemented with biopterin and folate (7.5μM each of biopterin, dihydrobiopterin, folate and dihydrofolate) and medium without any biopterin and folate supplement. Growth rate of each of the cell line was determined for a period of 120 h at an interval of 24 h. Figure1A shows the growth of all the strains in the absence of biopterin and folate and Fig1B shows the growth pattern of these *leishmania* strains in the presence of biopterin and folate. The doubling times of wild type, SKO, DKO and TKO were approximately 56, 48, 56 and 120 hour respectively in the presence of supplemental biopterin and folate. In the absence of supplemental biopterin and folate, the doubling times were 67, 72, 96 and >120 hour respectively. The wild type strain 51.1 and SKO transgenic showed a similar growth pattern in both the media till 72 h time interval, thereafter i.e. at 96 h time point the cell density was ~1.5 times more in media supplemented with biopterin and folate. Strain DKO, on the other hand showed a substantial difference in the growth of the cells in the presence and absence of the supplemented medium (Fig1A-B). As early as 72 h of growth there was a significant increase in the growth of these cells in the media supplemented with biopterin and folate. Each of the strain decreased in growth on removal of biopterin and folate. However the TKO strain, both in the presence and absence of the supplemented medium did not show any substantial change in the doubling time in both the media. The doubling time of TKO shifted to 120 h in the presence of biopterin and folate whereas in the absence of these supplements the TKO ceased to grow after this time interval. Comparison of growth rates of the wild type 51.1 and TKO in the presence of biopterin and folate showed that though the initial cell density of the two strains was same, at 96 h, the cell density of wild type had

(a)





(b)

Fig 3: Infection of J774A.1 murine macrophage cell line with wild type *L. donovani* strain LSB 51.1 and BT1 triple knock out (TKO). 10^6 J774A.1 macrophages were plated per 60mm tissue culture plate. After this the macrophages were infected with 10^7 *L. donovani* either wild type 51.1 strain or BT1 triple knock out (TKO) strain. After 3h of infection the medium in the plate was changed and the infection carried on till 12h and 24h. A: represents number of infected macrophages and B: represents number of amastigotes/100 macrophages at 3, 12 and 24h after infection. The results represent mean ± SD of triplicates.

reached 7.5×10^6 cells/ml whereas the cell density of TKO was only 3×10^6 cells /ml.

Co-relationship between BT1 targeted mutants and invasion and survival in macrophages: We tested the ability of these transgenic parasites with decreased expression of biopterin transporter to invade and survive in murine macrophages and compared this ability with that of the wild type strain 51.1. The effect of loss of one, two and all three BT1 copies on intracellular survival and invasion of macrophages is shown in Fig 2. A significant decrease in the number of parasitized cells was observed with the single ($p>0.01<0.05$), double ($p<0.001$) and triple ($p>0.01<0.05$) targeted BT1 mutants compared to wild type 51.1 following 24 h of infection. Similarly the number of amastigotes decreased drastically in SKO ($p>0.01<0.05$), DKO ($p>0.001<0.01$) and TKO ($p>0.001<0.01$) mutants. In case of triple targeted BT1 mutant, TKO, a significant decrease in the parasitized cells and infectivity levels was observed as early as 12

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h after infection. (Fig 3.A-B). Infectivity levels decreased steadily to reach 40% after 24 h.

In order to make sure that reduction of triple targeted BT1 mutants to infect macrophages arose specifically from the lack of biopterin transporter, the medium was supplemented with 7.5 μM each of biopterin, dihydrobiopterin, folate and dihydrofolate to check if it restored both infectivity and replication of the parasites. Addition of biopterin and folate to the medium restored both infectivity and replication of triple knock out (TKO) to the same levels as wild type strain 51.1 after 24 h of infection (Fig. 4). However addition of biopterin and folate supplemented media to the strain 51.1 did not alter its infectivity and replication (Fig 4).

Co-relationship between BT1 gene amplification and invasion and survival in macrophages: In order to find out a possible co-relationship, if any, exists between overexpression of BT1 gene and invasion and survival in macrophages, we used pSLαneoα-BT1 construct transfected AG83 strain. This overexpressing strain was then tested for its ability to infect macrophages by using tritiated thymidine labelled parasites. Fig. 5 shows that BT1 overexpressing strain exhibited a significantly higher infectivity ($p>0.01<0.05$) when compared to the wild type strain having the presence of BT1 gene on the 2.2 Mb chromosome only and not as amplified copies.

Interaction of pterins and pentavalent antimonials was examined in the knockout mutants: Stationary phase promastigotes at a density of ~2×10^6 cells were inoculated into α-MEM[16] either with, or without, supplementation

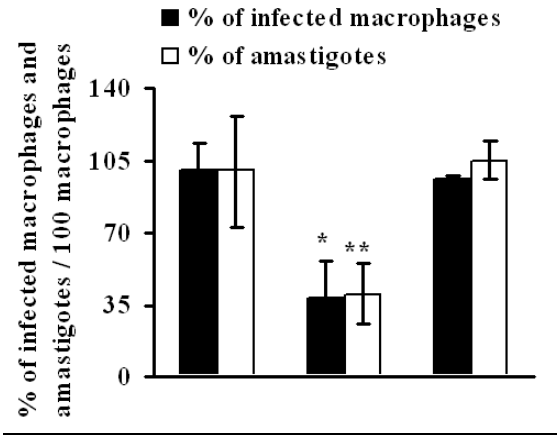

Fig 4: Infection of J774A.1 murine macrophage cell line with wild type L. donovani strain LSB 51.1 and BT1 triple knock out (TKO) in the presence and absence of biopterin and folate (+B+F) and absence of biopterin and folate supplemented medium (-B-F). 106 J774A.1 macrophages were plated per tissue culture plate. After 3h the macrophages were infected with 107 L. donovani either the wild type 51.1 strain or TKO strain. Three hours later the medium was removed and infection was carried on for another 24h. Percentage of infected macrophages and amastigotes/ 100 macrophages was determined by microscopic examination of the Giemsa stained slides. The results represent mean □ SD of triplicates. * indicates $p>0.01<0.05$, ** indicates $p>0.001<0.01$.

with 7.5 μM each of biopterin, dihydrobiopterin, folate and dihydrofolate. After 24 h, 200 μl of cell suspension was added to the wells of micro titer plates to which various concentrations of Pentostam (Welcome, UK) or Glucantime (Specia, France) were added. The cells were counted after 72 h incubation. The concentration that inhibited the growth of the promastigotes by 50% (IC_{50}) was determined from a plot of cell number versus drug concentration. The IC_{50} for Pentostam was found to be 2 mg/ml for the wild type and the BT1 knockout strains. Addition of 7.5 μM each of biopterin, dihydrobiopterin, folate, and dihydrofolate did not affect the IC_{50} for Pentostam of the wild type and knockout strains (data not shown). In contrast, pterin supplementation increased the IC_{50} of Glucantime from 2 mg/ml to >7.5 mg/ml in LSB-51.1 wild type, SKO, DKO and TKO strains (data not shown).

Biopterin and folate were tested individually to determine which of these pterins is responsible for the reduction in susceptibility to Glucantime in the LSB-51.1 wild type and TKO strain. The Glucantime IC_{50} values were >7.5 mg/ml for both the wild type and TKO strain with biopterin supplementation (7.5 μM each of biopterin and dihydrobiopterin) (data not shown). Similarly, addition of 7.5 μM each of folate and dihydrofolate resulted in a Glucantime IC_{50} value of >7.5 mg/ml for both the strains (data not shown).

DISCUSSION

The metabolic role of pterins in *Leishmania* is not known[26]. They have been implicated in a number of functions such as hydroxylation of phenylalanine and tyrosine in cleavage of ether lipids and biosynthesis of nitric oxide in other organisms[17]. However *Leishmania* does not have phenylalanine hydroxylase activity[18]. *Leishmania* are known to convert pterins to tetra

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hydrofolate[2]. In contrast to the mammalian hosts, *Leishmania* are pteridine auxotrophs and have an absolute requirement for an exogenous source[1, 19, 28-30]. *Leishmania* unlike the mammalian host they infect cannot synthesize the pteridine component of folate. Also, several species of *Leishmania* are capable of continual propagation in folate deficient media supplemented with biopterin[19, 28]. These studies were further supported by the fact that *Leishmania* is indeed capable of converting biopterin to tetrahydrofolate[2] and has evolved a complex pterdine salvage pathway that is capable of scavenging both conjugated and unconjugated pteridines[6]. Recent studies have shown that *Leishmania* require pteridines for survival besides folate[6, 21, 23]. Also the enzyme involved in the formation of H_4 biopterin from quinonoid H_4 biopterin in other organisms has a functional role in *Crithidia* and *Leishmania*[15, 3]. The existing data clearly indicates the critical importance of biopterin for the *Leishmanial* parasite. The disruption of BT1 gene has been achieved in *L tarentolae*[20], in *L. donovani* [21, 27] and in *L. major*[7]. In BT1 mutant strains biopterin uptake is completely abolished further suggesting that BT1 is likely the main transporter of *Leishmania* cells. Nonetheless pterin supplementation was essential

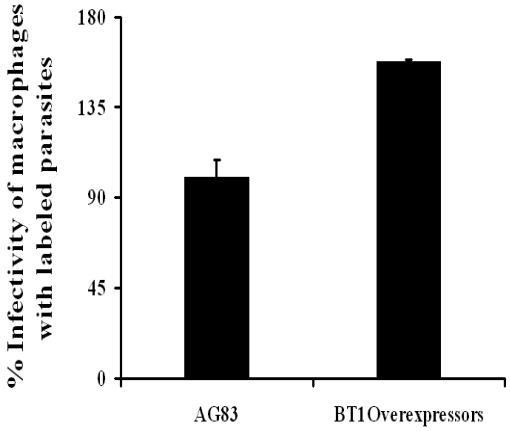

Fig 5: Infection of J774A.1 murine macrophage cell line with wild type L. donovani strain AG83 and BT1 overexpressing AG83. 106 J774A.1 macrophages were plated per 60mm tissue culture plate. The macrophages were infected with tritiated thymidine labelled, 107 L. donovani either wild type AG83 strain or BT1 overexpressing strain. After 3 h of infection, the free parasites were washed out with serum free RPMI followed by addition of complete media and further incubation of 24 h. After 24 h incubation, cells were lysed with 1% Triton X 100 and assayed for radioactivity counts using liquid scintillation counter. The result is a representative of three independent experiments done. * indicates $p>0.01<0.05$.

for the growth of BT1 mutants. We were unable to obtain null mutants by targeted gene replacement in standard growth medium without added biopterin and folate. All recombinants selected without pteridine supplementation contained amplified copies of BT1 gene in addition to knockout construct at the intended site[21]. Thus uptake of biopterin by BT1 appears critical for cell survival at physiological concentrations of biopterin.

The BT1 null mutant (TKO) showed significantly slower growth as promastigotes, even in the presence of supplemental biopterin and folate, suggesting that their ability to grow in this medium is due to uptake via passive diffusion or a secondary (low affinity) transporter[21, 27].

In order to further investigate the physiological role of BT1 transporter gene, BT1 knock out mutants were used. The physiological function of several *Leishmania* genes has been worked out using gene-targeting procedure[5, 11]. The present work shows the role of disruption of BT1 gene on the growth of *Leishmania* parasite. The role of over expression or disruption of biopterin transporter gene on the ability of the parasite to infect and survive in the macrophages was also worked out.

The transgenic parasites with double and triple BT1 disruption showed a slower growth in the absence of biopterin and folate. Growth rate was much slower in the TKO disrupted mutant when compared to DKO, in the absence of biopterin and folate. Thus the growth of the parasite correlates with the biopterin transport potential of the BT1 gene.

Interestingly, recombinant parasite possessing single, double and triple BT1 disruption showed a decrease in their ability to infect and survive in the macrophages, which correlates with the order of disruption. In case of TKO, there was a decrease in the infectivity and the number of amastigotes over a period of time with the number of amastigotes showing a 2.5 fold decrease at 24 h. Addition of biopterin and folate to the medium restored both survival and replication of BT1 triple knockout strain to approximately the wild type levels. It is reported in earlier studies also that in *L. major*, the growth of a BT1 null mutant can be rescued by adding biopterin[6].

In order to prove that the observed decreased intracellular survival is due to the BT1 gene, we used BT1overexpressing *Leishmania* strain. It was observed

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that the AG83 strain having overexpression of BT1 gene showed increased infectivity, attachment per *se* in the macrophages as compared to the wild type AG83 strain having no amplification of BT1 gene

BT1 gene, which appears to be required for parasite survival inside the macrophages, appears to be an attractive target, which can be exploited to incapacitate the parasite to generate a live vaccine. The studies in this laboratory have shown that recombinant BT1 gene product has a potential to be developed as a candidate vaccine for preventing experimental leishmaniasis[10]. BT1 mutants used in this study also showed attenuated capacity to survive in the macrophages. Recently, it has been shown that genetically manipulating the pterin transporter in *L. donovani*, it is possible to generate an attenuated organism that could be part of a vaccination strategy[27]. It would be interesting to use these stable BT1 disruption mutants in combination with other mutants for exploring the possibility of vaccine development for leishmaniasis.

The Glucantime IC_{50} values were >7.5 mg/ml for both the wild type and TKO strain with biopterin or folate supplementation (7.5 μM each of biopterin and dihydrobiopterin, 7.5 μM each of folate and dihydrofolate). This similar effect by biopterin and folate individually on the reversal of Glucantime susceptibility suggests that they may both act by a similar mechanism. Earlier studies have indicated that BT1 (ORFG) is not only a high affinity biopterin transporter but also transports folic acid, with a lower affinity[20]. A partial reversal of Glucantime susceptibility in *Leishmania* by addition of biopterin to the media has been observed previously[23]. In addition, strains that differed in fatty acid composition were found to differ with respect to Glucantime susceptibility[23]. Pterins have been implicated as cofactors in unsaturated fatty acids biosynthesis[8, 14]. Taken together, these data suggest a possible site of action for Glucantime within the fatty acids biosynthetic pathway. The reversal of Glucantime susceptibility by biopterin, even in strains that are deficient in biopterin transport, is somewhat surprising, suggesting that the effect may be exerted independently of biopterin transport, possibly by blocking Glucantime uptake. Alternatively, it is possible that a low intracellular level of pterins is sufficient for the reversal of the Glutamine susceptibility, and that even in the absence of the BT1-encoded biopterin transporter, sufficient intracellular pterins are present to exert an anti-Glucantime effect. Indeed, it appears that after prolonged growth in culture the TKO strain is able to partially compensate for its lack of BT1. Consequently, the role of biopterin and folate in Glucantime susceptibility needs to be investigated further.

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