Inactivation of the *Leishmania tarentolae* pterin transporter (BT1) and reductase (PTR1) genes leads to viable parasites with changes in folate metabolism and hypersensitivity to the antifolate methotrexate.

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Abstract

The protozoan parasite *Leishmania* is a folate and pterin auxotroph. The main biopterin transporter (BT1) and pterin reductase (PTR1) have already been characterized in *Leishmania*. In this study we have succeeded in generating a BT1 and PTR1 null mutant in the same *Leishmania tarentolae* strain. These cells are viable with growth properties indistinguishable from wild-type cells. However, in response to the inactivation of BT1 and PTR1, at least one of the folate transporter genes was deleted and the level of the folylpolyglutamate synthetase activity was increased, leading to increased polyglutamylation of both folate and methotrexate (MTX). Secondary events following gene inactivation should be considered when analyzing a phenotype in *Leishmania*. The BT1/PTR1 null mutant is hypersensitive to MTX but in a step by step fashion we could induce resistance to MTX in these cells. Several resistance mechanisms were found to co-exist including a reduced folate and MTX accumulation, demonstrating that cells with no measurable biopterin uptake but also greatly reduced folate uptake are viable, despite their auxotrophy for each of these substrates. The resistant cells have also amplified the gene coding for the MTX target dihydrofolate reductase. Finally, we found a marked reduction in MTX polyglutamylation in resistant cells. These studies further highlight the formidable ability of *Leishmania* cells to bypass the blockage of key metabolic pathways.
INTRODUCTION

The protozoan parasite *Leishmania* is responsible for a number of diseases with a wide range of clinical symptoms (1). Chemotherapy is currently the only effective way to control the infection but the emergence of drug resistant organisms, particularly against the first line antimonial containing drugs, is complicating the treatment (2). The discovery of new putative cellular targets is urgently required. *Leishmania* differs from its mammalian host in that it cannot synthesize pterins from guanosine triphosphate (GTP) and differs from several microorganisms in that it cannot synthesize folates *de novo*. Indeed, it lacks enzymes necessary for the conjugation of the three building blocks (pterin, para aminobenzoic acid, and glutamate) leading to folates (reviewed in (3)). This pterin and folate auxotrophy of *Leishmania* has resulted in a pterin (BT1) (4,5) and a multiplicity of folate (FT) transporters (6,7) that allow the transport of folate and pterin derivatives. These derivatives can be reduced into active molecules by the parasite dihydrofolate reductase-thymidylate synthetase (DHFR-TS) and by a novel pterin reductase PTR1 (3,8). *Leishmania* does have an active folylpolyglutamate synthetase (FPGS) (9) permitting *Leishmania* to synthesize folylpolyglutamates that are essential for cellular retention (10). Reduced folates are one carbon donors in various metabolic reactions including the synthesis of thymidine. Pterins are essential growth factors of *Leishmania* (11-14) and may play a number of other roles. Indeed, the almost completed *L. major* genome project has revealed homologues to aromatic amino acid hydroxylases and to products involved in the biosynthesis of the molybdopterin cofactor (3). Finally, experimental evidence has been provided to link pterin levels with parasite metacyclogenesis (15).

*Leishmania* cells often resist the antifolate drug methotrexate (MTX) by reducing the accumulation of the drug (16-18). This can be achieved by gene deletion of some of the folate transporter FT genes (7).
Since *Leishmania* is a folate auxotroph this gene deletion event needs to be compensated for, and we have found that in *L. tarentolae* strains, in which the main folate transporter is deleted, the biopterin transporter BT1, which can also transport some folates, is overexpressed (4). The *BT1* gene has been inactivated in a number of species (4,5,15) and while cells can grow in culture flasks, they are less virulent in animal models (19) demonstrating an important role for pterin transport in parasite biology.

The *PTR1* gene has also been inactivated in a number of species and these *PTR1* null mutants were more sensitive to MTX (20,21), had a decreased ability to reduce pterins and recently were shown in animal models to be more virulent, as low levels of reduced pterins induced metacyclogenesis (15).

While pterins cannot serve for the *de novo* biosynthesis of folates, there are several interconnections between folate and pterin metabolism and several *Leishmania* species can grow in folate deficient medium provided that pterins are present (12,14,20). It has been suggested that pterins could have a folate sparing effect, a phenomenon described 45 years ago in the related parasite *Crithidia fasciculata* (22), although the mechanism by which this is achieved is not understood. In an attempt to further increase our understanding of pterin metabolism in *Leishmania*, we succeeded, somewhat surprisingly, in generating mutants in which both the main pterin transporter (BT1) and pterin reductase (PTR1) were disrupted by homologous recombination. Cells were viable, but some of their folate metabolism was changed. Since BT1 overexpression appears to compensate for mutations in the folate/MTX transporter in *L. tarentolae* MTX resistant mutants (4), we assumed that selection of MTX resistance in a *BT1/PTR1* null mutant would leave folate transport intact. Selection of the *BT1/PTR1* cells for MTX resistance led to unexpected results that will be presented here.
Materials and Methods

Cell growth. The *L. tarentolae* cell line TarII WT and the TarII *BT1/PTR1* null mutants were grown in SDM-79 (23) or M199 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 5 µg/ml of hemin. The *BT1/PTR1* null mutant was selected for MTX resistance in a step by step fashion as reported previously (18) to lead to two mutants, MTX50.2 and MTX500.3, that were highly resistant to MTX. For HPLC analysis, cells were grown in M199 medium supplemented with 25 nM [³H]-folic acid (25.7 Ci/mmol) or 25 nM [³H]-MTX (26.62 Ci/mmol) (Moravek Biochemicals).

DNA manipulations. Total DNA for Southern blot analysis was isolated using DNAzol reagent (Gibco-BRL). Southern blots, hybridization and washing conditions were done following standard protocols (24). The *PTR1, BT1, DHFR-TS, FPGS, neomycin phosphotransferase (NEO), hygromycin phosphotransferase (HYG)* and *FT* probes were obtained by polymerase chain reaction (PCR). Wild type *L. tarentolae* promastigotes were transfected by electroporation as reported previously (25). Selections were done with hygromycin B (≥ 100 µg/ml) and G 418 (≥ 40 µg/ml).

DNA constructs. *PTR1* inactivation was carried out using a construct described previously (20). The linearized 3.3 kb *XhoI/XhoI* fragment containing *PTR1* interrupted by *NEO* was electroporated (25) in the TARII *BT1* null mutant previously described where the two *BT1* alleles were inactivated by the *HYG* gene (4). Selection was initially with 40 µg/ml G418 (Gibco-BRL) for disrupting one *PTR1* allele by homologous recombination. Disruption of the second *PTR1* allele was obtained by selection for loss of heterozygosity by increasing the G418 selection pressure to 200 µg/ml and cloning of the cell pool. TARII *BT1/PTR1* null mutants (TARII *BT1/PTR1* KO) were characterized by Southern blot analysis using PCR probes for the *BT1, PTR1, NEO* and *HYG* genes.
Enzymatic assays in crude extracts. FPGS activity of *Leishmania* cells was assayed using extracts prepared essentially as described previously (9). L-[\(^3\)H]-glutamate (5mCi/mmol) and 500 µM of either folic acid or MTX were added to the crude extracts of different strains. Separation of non incorporated L-[\(^3\)H]-glutamate from the folylpolyglutamates or the MTX-polyglutamates was done using a DEAE-cellulose column (Sigma).

Accumulation studies. Transport experiments of folic acid, biopterin and MTX were done as described previously (18). [\(^3\)H]-folic acid (25.7 Ci/mmol), [\(^3\)H]-biopterin (6Ci/mmol) and [\(^3\)H]-MTX (26.62 Ci/mmol) were purchased from Moravek Biochemicals and transport studies were done using 200 nM of substrate. Briefly, 1 x 10\(^6\) cells were layered over 100 µl of dibutylphthalate (Sigma) and put in the presence of radioactive pteridine. Accumulation of radioactive substrates was stopped at various time points (0, 0.5, 2, 5 and 20 minutes) by centrifugation through the inert dibutylphthalate layer. Unincorporated substrates were removed by aspiration, cells were washed once in HEPES-NaCl buffer and pellets were resuspended in scintillation liquid and counted. The amount of incorporated radioactivity was normalized with *Leishmania* cell number and values of uptake in cells incubated on ice were subtracted.

HPLC analysis. All HPLC reagents were obtained from US Bioscience and were of HPLC grade. HPLC standards were purchased from Dr. Schircks Laboratory, Jona, Switzerland. The intracellular folylpolyglutamates in *Leishmania* wild-type cells and mutants were determined as described previously (9) and the extent of MTX polyglutamylation was assayed by HPLC using essentially the same...
procedure as described previously (26). Cells were incubated with 25 nM of [H]-pteridines for 72 hours.
RESULTS

Generation of a BT1 and PTR1 null mutant of Leishmania tarentolae.

L. tarentolae cells becoming resistant to MTX by reducing folate/MTX uptake compensate by overexpressing the biopterin transporter BT1 gene (4). BT1 is a biopterin transporter that can also transport some folate. We reasoned that without BT1 we might select for mutations other than those affecting folate/MTX uptake. We first tried to select MTX resistant mutants starting from a BT1 null mutant, but in all mutants studied we found amplification of PTR1 (results not shown). We thus attempted to generate a BT1/PTR1 null mutant in the hope that after MTX selection a novel resistant mutation could be found that would provide new insights into folate metabolism.

Leishmania cells disrupted either in PTR1 (15,20,21) or BT1 (4,5) have been described but not BT1/PTR1 null mutants. The L. tarentolae BT1 null mutant (4) is presented briefly in Fig. 1A. The single copy BT1 gene was interrupted by the HYG marker and a BT1 HYG/HYG null mutant was obtained by loss of heterozygocity. Indeed, while the intact copy of BT1 is part of a 3.5 kb PstI-PstI fragment (Fig. 1B, lane 1), this fragment disappears in the BT1 null mutant and is replaced by two fragments of 2.7 kb and 1.8 kb consistent with the integration of the HYG marker in the BT1 gene (Fig. 1A, 1B, lane 2). In this BT1 null mutant we introduced a NEO construct that would enable us to target the PTR1 gene (Fig. 1C). Transfection of the BT1 null mutant with this construct led to G418 resistant parasites that indeed had integrated the NEO construct at the level of one PTR1 allele (not shown). By increasing the concentration of the selective drug G418 and subsequent cloning of the cell pool we were able to obtain by loss of heterozygocity a PTR1 null mutant in a BT1 null mutant background (Fig. 1). Indeed, while the intact PTR1 copy is part of an 2.3 kb XhoI-XhoI fragment (Fig. 1D, lane 1) this fragment increases to 3.2 kb in the null mutant and also hybridizes with a NEO probe (Fig. 1D, lane 2).
Due to the central role of pterins in *Leishmania* growth, we were surprised that we could obtain a *L. tarentolae* BT1/PTR1 null mutant. Even more surprisingly, the genetic mutant had no measurable growth defect in the folate rich (15 µM) medium SDM-79 (Fig. 2A) and had only a small growth defect in the low folate (20 nM) medium M199 (Fig. 2B). *L. tarentolae* cells disrupted in either *PTR1* or *BT1* were previously reported to be more sensitive to MTX while cells transfected with *PTR1* and *BT1* were found to be resistant to MTX (4,20). Indeed, the *BT1* null mutant was four times more sensitive to MTX while the *PTR1* null mutant was 50 times more sensitive than a wild-type cell (Fig. 3A). The *BT1/PTR1* null mutant was found to be 200 time more sensitive to MTX compared to wild-type cells in SDM79 medium (Fig. 3A). The roles of PTR1 and BT1 in MTX resistance were also confirmed by gene transfection. Transfectants overexpressing PTR1 become insensitive to MTX, while *BT1* transfectants were ~10 times more resistant when grown in SDM-79 medium (Fig. 3B). The susceptibility of *Leishmania* cells is highly dependent on the folate concentration of the medium and indeed the EC50 of wild-type cells to MTX is 100 nM in M-199 medium (compared to ~ 25 µM in SDM-79) and the *BT1/PTR1* null mutant was more sensitive to MTX with an EC50 of 15 nM (Fig. 3C). Cells in M199 medium overexpressing PTR1 were also highly resistant to MTX (result not shown), but surprisingly BT1 overexpressing cells were not much more resistant to MTX compared to wild-type cells in the M199 medium (Fig. 3C). We hypothesized that this discrepancy in BT1 mediated resistance between SDM-79 and M199 could be due to a difference in folate concentration. Indeed, when BT1 overexpressors were grown in M-199 medium, but with the addition of folic acid to concentrations found in SDM-79, the parasites were MTX resistant (Fig. 3C).

**Biological compensation following inactivation of the *BT1* and *PTR1* genes of *Leishmania*.**
Based on the central role of pterins in *Leishmania*, it could be suggested that *Leishmania* cells would not thrive well in the absence of PTR1 and BT1, two proteins key in providing active pterins to the cell. The *BT1/PTR1* mutant had minimal growth defects (Fig. 2) and cells have been propagated for over a year without problems. It is likely that several metabolic features are changed in this genetic mutant to compensate for the lack of BT1 and PTR1. As expected from a *BT1* null mutant, there is no measurable biopterin uptake in the *BT1/PTR1* null mutant (Fig. 4A). In *L. tarentolae* cells in which folate uptake is greatly impaired we have observed an increased uptake of biopterin (4) and because there are several interconnections between folate and pterin metabolism we tested here whether folate uptake was modulated in the *BT1/PTR1* null mutant. Surprisingly, we found that both folate and MTX uptake were slightly but repeatedly decreased in the *BT1/PTR1* null mutant (Fig 4B,C). Another aspect of folate metabolism that was recently studied in *Leishmania* cells concerned folate polyglutamylation, where folates are found mostly as pentaglutamates (9,27) while MTX is found predominantly in the form of triglutamates in *L. tarentolae* (26). We found that the glutamate chain lengths of both folate and most notably of MTX were increased significantly in the *BT1/PTR1* null mutant compared to wild-type cells (Tables 1, 2). The HPLC analysis of folates for the *BT1/PTR1* null mutant led to the observation of other radioactive peaks consistent with polyglutamates longer than Glu₆ (results not shown).

The alteration in folate transport and polyglutamylaction in the TarII *BT1/PTR1* null mutant compared to wild-type cells led us to test whether the copy number of genes coding for these activities was changed. Several genes are possibly implicated in folate transport (6,7) and we used a probe recognizing several members of this gene family. A rearrangement of some folate transporter genes was observed in the *BT1/PTR1* null mutant since a 2.5 kb *Pst*I-*Pst*I hybridizing fragment present in wild-type cells was absent in the genetic mutant (Fig. 5E, lane 1, 2). The copy number of *FPGS* was similar in the wild-type
and null mutant (Fig. 5F, lane 1, 2), but FPGS activity was significantly higher in the null mutant compared to wild-type cells (Fig 6).

Selection of MTX resistant mutants in the \textit{BT1/PTR1} null mutants

Despite the fact that \textit{BT1/PTR1} null mutant cells were hypersensitive to MTX, we were interested in generating MTX resistant mutants in the hope of finding novel resistance genes. We succeeded in generating two highly MTX resistant mutants TarII MTX 50.2 and TarII MTX 500.3 (Fig. 3). These highly resistant mutants had only a small growth defect compared to their parent genetic mutant (Fig. 2). We first tested whether folate/MTX uptake was impaired in these mutants that had no active BT1. Against our expectations, these mutants exhibited a marked decrease in folate and MTX uptake (Fig. 4B, C) with folate accumulating slightly more efficiently than MTX. Thus, \textit{Leishmania} cells can survive without a biopterin transporter and with a much reduced folate transport activity. Both the \textit{BT1} and \textit{PTR1} disrupted loci remained intact upon MTX selection (Fig. 5A, B). \textit{Leishmania} cells often resist drugs by gene amplification (28,29) and this can often be detected by simple examination of ethidium bromide stained gels (30). DNA amplification could easily be seen in the two independent mutants (Fig. 5C, lane 3 and 4). This amplified DNA codes for \textit{DHFR-TS} (Fig. 5D) the gene coding for the target of MTX but a locus usually not amplified in \textit{L. tarentolae} selected for MTX resistance (31). The transport defect of the MTX resistant mutant was due, particularly in mutant MTX 500.3 (Fig. 5E, lane 4), to a gross rearrangement and gene deletion in the folate transporter gene family. The \textit{FPGS} gene loci remained intact in the mutant (Fig. 5F) but the distribution of MTX polyglutamates changed drastically (Table 2). The distribution of folylpolyglutamates was decreased slightly in the mutants compared to the parent strain with a decrease in Glu$_6$ and Glu$_5$ and an increase in Glu$_4$ and Glu$_3$. The distribution of MTX polyglutamates in the mutant, however, was greatly perturbed with a switch from Glu$_4$ and Glu$_5$ in the
parent strain to the total absence of MTX-polyglutamates in MTX 500.3 and only a small proportion of Glu$_2$ with mostly MTX-monoglutamates in MTX 50.2 (Table 2). This drastic decrease in MTX polyglutamylation is not correlated with a decrease in FPGS activity (Fig. 6).
DISCUSSION

Pterins are important growth factors for *Leishmania* (11-14) and reduced pterins have been shown to be involved in parasite differentiation (15). We show here that cells without their main biopterin transporter and without their main pterin reductase are viable under the conditions tested (Fig. 2). The pterin requirement of the *BT1/PTR1* null mutant must be achieved by other means. For example, the catabolism of folic acid could lead to a pterin moiety for the cell. This catabolism could for example take place via a pteridine hydrolyzing enzyme described in many species of parasites related to *Leishmania* (32). Alternatively, the genome of *Leishmania* seems to have several homologues of the folate/pterin transporters (7,15), and alteration in the expression of one of these members could lead to sufficient biopterin accumulation to allow the cells to survive. In fact, we have observed something different where some of the *FT* members were deleted (Fig. 5E) and this or other defects led to a slightly decreased folate accumulation in the *BT1/PTR1* null mutant (Fig. 4B). The reason for transporter gene deletion in a *BT1/PTR1* null mutant is unclear. We could speculate that a pterin containing compound could be transported by one of the *FT* gene family members and that the absence of its reduction (by PTR1) could lead to the build up of a compound detrimental to growth, thus explaining the deletion of some transporters. The deleted gene(s) do(es) not correspond to the main folate transporter, however (Fig.4B). Further work will be required to try to test this speculative hypothesis, but in ongoing work, we sometimes find it difficult to overexpress some of the FT members in a wild-type background ((7) and D. Richard and MO, unpublished observations). It is possible also that in culture flasks there is sufficient biopterin that the biopterin requirement of the cell is met by diffusion, a phenomenon that has been suggested to occur in *Leishmania* (4,5). Since PTR1 is lacking in the same mutant, the reduction of pterins must also use another route. Since DHFR-TS cannot reduce pterins (33) it is likely that *Leishmania* has a second enzyme capable of reducing pterins and evidence for this activity has been
provided (21). Moreover, in Trypanosoma cruzi, a kinetoplastid parasite distantly related to Leishmania, two pteridine reductase enzymes have been characterized (34). In addition to these defects, the BT1/PTR1 null mutant had increased FPGS activity (Fig. 6) which leads to an increase in the level of long chain folylpolyglutamates (Table 2). This increased polyglutamylation may lead to increased cellular retention of folates and thus compensate for the loss of some folate transporters resulting from gene rearrangements.

An important anticipated but not yet demonstrated observation of this study is that gene disruption in a protozoan parasite can lead to an alteration in the expression of several other genes. This change in gene expression is possibly essential for enabling growth and this must always be taken into account, when looking at a phenotype following a gene disruption event. We were able to detect some of these changes (deletion of some putative transporters, increased FPGS activity) because they are involved in the same pathways as the gene under study, but it is possible that the expression of other gene products will also be altered. DNA arrays or proteomic studies (35-37) could pinpoint at the global level the genes/proteins whose expression are modified following gene inactivation.

While the BT1/PTR1 null mutant had no measurable growth defect it had a clear phenotype of hypersensitivity to MTX, which appears to consist of the additive contributions of the disruptions of PTR1 and BT1. PTR1 is an established MTX resistance determinant (25,38), while BT1 was isolated by functional cloning by selecting for MTX resistance (4). Amplification of BT1 has been observed in a number of Leishmania lines selected for MTX resistance (39), our unpublished observations). In contrast to SDM-79 medium (Fig. 3A, B), we found that BT1 overexpressors were not more resistant to MTX in M-199 medium (Fig. 3C). This may explain why BT1 was not isolated in an independent
functional cloning experiment using MTX selection in M-199 medium (40). We had hypothesized that BT1 may mediate MTX resistance by selectively transporting folate but not MTX in cells in which the folate transporters were deleted (4). Since folate concentration differs greatly between SDM-79 and M-199 we tested whether the difference in resistance profiles mediated by BT1 is due to a difference in folate concentration between the media. This seems to be indeed the case (Fig 3C). An increase in folate uptake mediated by BT1, measurable at high folate concentration (4), should confer MTX resistance, as folate should compete with MTX for targets.

We selected for two BT1/PTR1 null mutants highly resistant to MTX. We thought that since resistance to MTX mediated by reduced activity of the folate/MTX transporter in L. tarentolae is compensated by overexpressing BT1, cells deleted for BT1 would not resist MTX by reducing the activity of the folate transporter. This was not the case, however, where a markedly decreased uptake of both folate and MTX was noted in MTX resistant mutants (Fig. 4B, C). These mutants have a small growth defect (Fig.2) but less than one could have expected when both biopterin and folate uptake are decreased simultaneously (Fig.4). Since Leishmania is likely not able to synthesize folate and pterins de novo this suggests, at least in culture, that the small amount of pteridines taken up by these cells is sufficient to allow growth. Other mutations were also observed in these step by step selected resistant mutants, including amplification of the DHFR-TS gene (Fig.5). Amplification of the DHFR-TS gene has been observed on several occasions in MTX selected L. major (16,41), but never in L. donovani or L. tarentolae except when the PTR1 gene is deleted (31). We found previously that both the strength of the resistance gene and the environment of the genomic locus determine the efficiency with which a locus will be amplified in Leishmania (31).
In several *Leishmania* species, folates are found mainly as pentaglutamates (9,27). In contrast, the glutamate chain length of MTX is much shorter, with few MTX polyglutamates in *L. major* and predominantly MTX triglutamates in *L. tarentolae* (26,42). The difference between the two species is not due to a difference in FPGS activity but could be due to an α–MTX hydrolase that is highly active in *L. major* but not in *L. tarentolae* (17,26,42). Following the double inactivation of *BT1* and *PTR1*, cells have increased FPGS activity (Fig.6). This was observed for folate (Table1) but it was even more spectacular for MTX where the most abundant species became MTX-Glu$_5$. In the MTX resistant *BT1/PTR1* null mutants we found that MTX existed almost exclusively in the monoglutamate form (Table 2), while long chain folylpolyglutamates were still present. Reduced polyglutamylation of MTX is a well established resistance mechanism in mammalian cells (reviewed in (43)) and inactivation of a copy of *FPGS* in *L. tarentolae* can lead to MTX resistance (26). The absence of MTX polyglutamates in these mutants is not due to a decrease in FPGS activity (Fig.6) and is not due to an increased activity of the MTX α–hydrolase (result not shown). A number of hypotheses can be advanced to try to explain why MTX is not polyglutamylated. The level of polyglutamylation in mammalian cells is controlled by the dual activities of FPGS and a γ–glutamyl hydrolase (GGH) (reviewed in (44)). One could invoke a mutant form of GGH that could preferentially depolyglutamylate MTX. Being less polyglutamylated, MTX would be expelled more easily from cells. There is no evidence yet from the *Leishmania* genome project for the presence of a GGH. Other enzymatic activities such as ferritin and glutamate carboxypeptidase could also control the level of MTX polyglutamates (45), although again, no evidence for these activities have been found in *Leishmania*. Alternatively, since the level of pteridine entering these MTX resistant cells is low (Fig. 4), the little MTX that enters the cell may bind to the overexpressed DHFR-TS which could then prevent MTX polyglutamylation. However, in another MTX mutant, TarII MTX 1000.6, we observed decreased MTX polyglutamates but in this mutant the
DHFR-TS gene was not amplified (26). Further work will be required to pinpoint how exactly *Leishmania* manages to change the distribution of MTX polyglutamates while keeping the distribution of folylpolyglutamates relatively untouched.

In this study we have succeeded in generating a *L. tarentolae BT1/PTR1* null mutant. These cells, under the conditions tested, are viable but have modified part of their folate metabolism. The demonstration that the parasite responds to gene inactivation by altering various aspects of its metabolism has important consequences when one analyses the phenotype of a genetic mutant. Despite the *BT1/PTR1* null mutant being hypersensitive to MTX, we could still select for MTX resistant mutants. This led to the demonstration that cells with greatly diminished folate and pterin uptake can grow and led to the most interesting observation of a drastic redistribution of MTX polyglutamates. Folate and pterin metabolism has a number of unique features compared to their host cells that merit further studies to unravel putative new targets.
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Figure 1. Generation of a *Leishmania tarentolae BT1/PTR1* null mutant by gene targeting. **A.** A partial physical map of the *L. tarentolae BT1* locus and the expected null mutant is shown. Only one of the two alleles is shown. The expected size of the fragments obtained after *Pst*I digestion are depicted below the map. The *BT1* containing genomic 3.5 Kb *Pst*I-*Pst*I restriction fragment now changes in two *Pst*I-*Pst*I restriction fragments of 2.7 and 1.8 kb upon the integration of the *HYG* gene that contains a new *Pst*I site. **B.** Southern blot analysis of *Leishmania* cells. Total DNAs were digested with *Pst*I and hybridized to *BT1* and *HYG* probes independently. **C.** A partial physical map of the *L. tarentolae PTR1* locus and its relevant restriction sites. Only one of the two alleles is shown. Upon integration of the *NEO* gene at the homologous locus, the genomic 2.3 Kb *Xho*I-*Xho*I restriction fragment increases by the size of the *NEO* gene inserted. **D.** Southern blot analysis of *PTR1* inactivation in the *L. tarentolae BT1* null mutant. DNAs of wild-type cells and of a *PTR1* mutant obtained by loss of heterozygocity were digested with *Xho*I, electrophoresed on an agarose gel, transferred, and hybridized with *PTR1* and *NEO* probes. Lane 1, *L. tarentolae* wild-type (TarII WT); lane 2, *L. tarentolae BT1/PTR1* null mutant.

Figure 2. Growth properties of *Leishmania* cells. The growth of *L. tarentolae* TarII WT (●); of the BT1/PTR1 null mutant, TarII BT1/PTR1 KO (○); or of the two BT1/PTR1 null mutant clones selected for high level methotrexate resistance TarII MTX 50.2 (■); and TarII MTX 500.3 (□) were measured in SDM-79 medium (A) or in M-199 medium (B). Equal amounts of cells (5 x 10⁶) were inoculated in 5 ml of medium and grown at 29°C. Optical density at 600 nm was measured according to time. The average of triplicate independent measurements is shown.
Figure 3. Methotrexate hypersensitivity of the *L. tarentolae* *BT1/PTR1* null mutant. *Leishmania* cells were grown in SDM-79 medium (A and B) or M199 medium (± folic acid)(C), and their growth was measured at 68-72 hours while varying the concentration of methotrexate. Cells that were studied includes the wild type cell TarII WT, and several cells with genes inactivated or overexpressed. Cells in which *PTR1* was inactivated TarII PTR1 KO, where *BT1* was inactivated TarII BT1 KO, where both *BT1* and *PTR1* were inactivated TarII BT1/PTR1 KO, or MTX resistant cells derived from the latter, TarII MTX 50.2 and TarII MTX 500.3 were studied. Finally the susceptibility of cells in which PTR1 TarII[PTR1_{NEO}] or BT1 TarII[BT1_{NEO}] were overexpressed as part of episomal plasmids was also studied. The average of a minimum of triplicate measurements is shown.

Figure 4. Accumulation of radiolabeled pteridines in *Leishmania* cells. The transport studies in the *L. tarentolae* wild type cell (●), TarII *BT1/PTR1* null mutant (○), and in the *BT1/PTR1* null mutant cells selected for methotrexate resistance TarII MTX 50.2 (■) and TarII MTX 500.3 (□) were carried out as described in Materials and Methods. *Leishmania* cells were grown in SDM-79 and pteridine uptake was measured using 200 nM of substrate. The accumulated radioactivity was normalized with *Leishmania* cell numbers and background uptake in cells incubated on ice was subtracted. A. [³H]-bioppterin accumulation. B. [³H]-methotrexate accumulation and C. [³H]-folate accumulation. The result of one experiment done in duplicate is shown, which has been repeated several times with similar results.

Figure 5. Gene copy number determination of pterin/folate metabolic genes in *Leishmania* cells. Total DNAs were digested with the appropriate restriction enzymes *XhoI* (A, C and D), *PstI* (B and E) or *NcoI* (F), electrophoresed and stained with ethidium bromide (EtBr) (C) or transferred to
nitrocellulose and hybridized with the probes indicated below the diagrams. Lane 1, TarII WT; Lane 2, TarII BT1/PTR1 KO; Lane 3, Tar II MTX 50.2 and Lane 4, TarII MTX 500.3. Molecular weights were deduced from the 1 kb ladder.

**Figure 6. Folylpolyglutamate synthetase enzymatic activity in* Leishmania* cells.** The activity was measured from crude extracts of* Leishmania* cells grown in SDM-79 as detailed under the “Materials and Methods” section. TarII WT (white bar), TarII BT1/PTR1 KO (grey bar), TarII MTX 50.2 (black bar) and TarII MTX 500.3 (hatched bar). The average of three independent experiments are shown.
Table 1: Folylpolyglutamates in *Leishmania tarentolae* cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>% glutamate chain length¹</th>
<th>n=1</th>
<th>n=2</th>
<th>n=3</th>
<th>n=4</th>
<th>n=5</th>
<th>n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TarII WT</td>
<td></td>
<td>2.1 ± 0.7</td>
<td>1.2 ± 0.3</td>
<td>3.5 ± 0.4</td>
<td>25.7 ± 1.8</td>
<td>62.3 ± 1.0</td>
<td>5.1 ± 1.5</td>
</tr>
<tr>
<td>TarII <em>BT1/PTR1</em> KO</td>
<td></td>
<td>1.5 ± 0.5</td>
<td>0.8 ± 0.2</td>
<td>6.9 ± 4.1</td>
<td>19.3 ± 6.4</td>
<td>54.0 ± 4.7</td>
<td>17.7 ± 3.4</td>
</tr>
<tr>
<td>TarII MTX 50.2</td>
<td></td>
<td>2.2 ± 0.7</td>
<td>4.9 ± 4.0</td>
<td>12.8 ± 2.3</td>
<td>43.5 ± 10.0</td>
<td>29.3 ± 11.0</td>
<td>7.3 ± 2.0</td>
</tr>
<tr>
<td>TarII MTX 500.3</td>
<td></td>
<td>3.4 ± 1.7</td>
<td>5.4 ± 2.0</td>
<td>22.9 ± 10.0</td>
<td>22.6 ± 2.0</td>
<td>33.1 ± 7.0</td>
<td>12.5 ± 7.0</td>
</tr>
</tbody>
</table>

¹ The means and standard deviations of three experiments are shown.

Table 2: Distribution of methotrexate polyglutamates in *Leishmania tarentolae* cells.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>% glutamate chain length¹</th>
<th>n=1</th>
<th>n=2</th>
<th>n=3</th>
<th>n=4</th>
<th>n=5</th>
<th>n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TarII WT</td>
<td></td>
<td>5.3 ± 3.0</td>
<td>11.7 ± 5.0</td>
<td>74.2 ± 8.2</td>
<td>7.9 ± 2.1</td>
<td>0.5 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>TarII <em>BT1/PTR1</em> KO</td>
<td></td>
<td>0.2 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>8.6 ± 0.9</td>
<td>36.1 ± 2.5</td>
<td>47.2 ± 0.8</td>
<td>6.5 ± 1.4</td>
</tr>
<tr>
<td>TarII MTX 50.2</td>
<td></td>
<td>94.7 ± 0.3</td>
<td>5.3 ± 0.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TarII MTX 500.3</td>
<td></td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ The means and standard deviations of three experiments are shown.
Fig. 1
Fig. 2
Fig 3
Fig. 4

**A**

- pmol biopterin/10^9 cells
- Symbols: 
  - • TarII WT
  - ○ TarII B71/PTRI KO
  - □ TarII MTX 50.2
  - □ TarII MTX 500.3

**B**

- pmol methotrexate/10^9 cells

**C**

- pmol folate/10^9 cells

Time (minutes)
Fig. 5
Fig. 6
Inactivation of the Leishmania tarentolae pterin transporter (BT1) and reductase (PTR1) genes leads to viable parasites with changes in folate metabolism and hypersensitivity to the antifolate methotrexate

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