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Effect of polyglutamylation of methotrexate on its accumulation and the development of resistance in the protozoan parasite *Leishmania*

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Abstract

Folates are polyglutamylated in most organisms, and in cancer cells the polyglutamylation of folates and of the antifolate methotrexate (MTX) is an important determinant of MTX susceptibility. The folylpolyglutamate synthetase (FPGS) responsible for polyglutamylation of folates was recently characterized in the parasite *Leishmania*. We show here that MTX is polyglutamylated in *Leishmania tarentolae* and that triglutamates are the predominant form. The glutamate chain length of MTX increases significantly in *Leishmania* cells transfected with the *FPGS* gene and decreases in cells with one *FPGS* allele disrupted. Modulation in the expression of the *FPGS* gene also has a profound effect on MTX susceptibility and this effect was found to be dependent on the folate concentration of the medium. In the folate-rich medium SDM-79, overexpression of *FPGS* will confer MTX resistance while in M-199 medium, which has much less folates, FPGS transfectants are more sensitive to MTX. Cells with one allele of *FPGS* disrupted are more resistant to MTX in low folate medium. The modulation of *FPGS* expression affects both the short-term and long-term accumulation of folate and MTX, showing a marked decrease in accumulation in the *FPGS* haploid mutant. This differential accumulation was mediated by decreased influx of the drug into the cell. Finally, the analysis of MTX-resistant *Leishmania* mutants indicated that the presence of shorter glutamate chains on MTX is correlated with MTX resistance.

Keywords: Drug accumulation; Drug resistance; Folate metabolism; Folylpolyglutamate synthetase; *Leishmania*; Methotrexate

1. Introduction

Reduced folates have important metabolic functions in reactions requiring the transfer of one carbon group, notably in the conversion of dUMP to dTMP (reviewed in ref. [1]). Folates exist mainly as polyglutamate derivatives where a glutamate tail is linked to the γ-carboxyl group of the side chain of folates [2,3]. The polyglutamylation reaction is catalyzed by the enzyme FPGS. The polyglutamylation of folates increases their cellular retention and makes them better substrates or cofactors for some of the enzymes involved in folate metabolism [2,3]. Folate analogs, such as MTX, are also polyglutamylated within the cell and the level of polyglutamylation of these antifolate drugs can modulate their retention and cytotoxicity [4,5].

Currently available antifolates are effective in the treatment of some protozoal infections, such as malaria, but they are not effective against *Leishmania* [1,6]. Indeed, they are poor inhibitors of the *Leishmania* dihydrofolate reductase (DHFR), the target of antifolates [7], and the inhibition of the *Leishmania* DHFR can be by-passed by the amplification of a gene coding for a pterin reductase (PTR1) that has folate reductase activity [8,9]. Nonetheless, recent work has significantly increased our understanding of pterin and folate metabolism in *Leishmania* and has pinpointed a number of interesting putative cellular targets [1,6]. Indeed, novel antifolate inhibitors were synthesized and were shown to be active against *Leishmania* [10,11].

Leishmania are auxotrophic for pterins and folates and thus rely on the environment to meet their pteridine requirements [1,6]. Most of our understanding of pteridine metabolism in *Leishmania* is derived from studies on the

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Abbreviations: BT1, biopterin transporter; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; GGH, γ-glutamyl hydrolase; MAPA, *N*-10-methyl-4-deoxy-4-aminopteorate; MTX, methotrexate.

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mechanism of resistance to the model antifolate drug MTX. One biopterin transporter (BT1) and one high affinity folate transporter (FT5) have recently been characterized in *Leishmania* [12–14], and several other members of this family of transporters are present in *Leishmania* [14,15]. The activity of these transporters is modulated in MTX-resistant *Leishmania* with BT1 overexpressed and FT5 and other folate transporter members being deleted [12,14,16–18]. In *Leishmania*, resistance to MTX can also be mediated by amplification of the *DHFR-TS* gene [16,19,20] and by amplification of the *PTR1* gene coding for a short-chain dehydrogenase [21,22] that can reduce both folate and pterins [8,9]. The *Leishmania FPGS* gene was recently isolated [23] and the folates of *Leishmania* were found to be polyglutamylated, with pentaglutamates being the predominant forms in a number of species and throughout the life cycle, hence resembling folylpolyglutamates of mammalian cells [23,24]. In contrast to folates, MTX polyglutamates were not observed in *Leishmania major* even after 72 hr of incubation [25]. The availability of the *Leishmania FPGS* gene has permitted us to revisit the polyglutamylation of MTX in *Leishmania* and to test whether it could play a role in susceptibility to antifolates.

2. Materials and methods

2.1. Strains and cultures

The *Leishmania tarentolae* cell line TarII WT and the *L. major* Friedlin line were grown in SDM-79 medium [26] or in M-199 medium each supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5 mg/mL hemin. The MTX-resistant mutants used in this study as well as the *L. tarentolae* cells overexpressing FPGS (TarII + FPGS) or in which one allele of *FPGS* was disrupted by homologous recombination (TarII *FPGS/HYG*) have been described previously [23,26]. For HPLC analysis, cells were grown in M-199 medium supplemented with [^{3}H]folic acid (25.7 Ci/mM) or with [^{3}H]MTX (26.6 Ci/mM) (Moravek Biochemicals).

2.2. Enzymatic assays in crude extracts

The FPGS activity of *Leishmania* cells was measured in crude extracts prepared essentially as described previously [23] to which L-[^{3}H]glutamate (5 mCi/mM, Moravek Biochemicals) and 500 μM folic acid or MTX were added. Folate/MTX polyglutamates were separated from unincorporated [^{3}H]glutamate using a DEAE–cellulose column (Sigma). For the MTX α-hydrolase activity, *Leishmania* cells (6×10^8) in log phase were washed twice with HEPES–NaCl and resuspended in 50 mM Tris, pH 7.3, in a final volume of 0.2 mL. Cells were sonicated and centrifuged at 16,000 *g* for 15 min at 4°. The hydrolase activity was determined in the resulting supernatant essentially as described previously [25]. The rate of conversion of MTX to *N*-10-methyl-4-deoxy-4-aminopteorate (MAPA) in all strains was determined from the absorbance change monitored at 320 nm. For each strain, 25 μL of crude extracts was added to 800 μL of 50 mM Tris, pH 7.3, containing 30 μM MTX. Absorbance changes of the crude extracts without MTX and of MTX without crude extracts in the same buffer were subtracted.

2.3. HPLC analysis of intracellular folylpolyglutamate and MTX polyglutamates

PABAglu$_N$ and MTX-glu$_N$ standards (N = 1–6) were purchased from Dr. Shircks Laboratories. All the HPLC reagents were obtained from US Bioscience and were of HPLC grade. The extent of folylpolyglutamylation in *Leishmania* cells was measured as described previously [23]. For the analysis of MTX polyglutamates, cells were grown in M-199 medium for 72 hr with 25 nM [^{3}H]MTX (26.6 Ci/mM) and intracellular MTX metabolites were processed essentially as described [25]. The MTX polyglutamates were separated according to the glutamate chain length by HPLC as previously described [27] on a C_{18} Bondapack column using a 20–40% acetonitrile gradient in a Schimadzu HPLC system at a flow rate of 1 mL/min. MTX-glu$_{1–6}$ standards were chromatographed with the *Leishmania* metabolites. Their elution positions were detected at $A_{280\ nm}$. The elution times of the MTX polyglutamate standards were glu$_1$ = 12.6 min, glu$_2$ = 17.6 min, glu$_3$ = 21.6 min, glu$_4$ = 26.2 min, glu$_5$ = 32.4 min, glu$_6$ = 41.2 min, and were consistent with the elution observed by others while using similar conditions [27,28].

2.4. Pteridine accumulation and transport experiments

Cells were grown in either M-199 or SDM-79 medium, washed, and resuspended at a density of 5×10^6 cells/mL in the folate deficient medium fdDMEL [29] in the presence of 200 nM [^{3}H]folic acid or [^{3}H]MTX. Uptake experiments were done essentially as described previously [26]. Briefly, 1×10^6 cells were layered over 100 μL of dibutylphtalate (Sigma) and put in the presence of one radioactive pteridine. Accumulation of radioactive substrates was stopped at various times (0, 0.5, 2, 5, and 20 min) by centrifugation through the inert dibutylphtalate layer. Unincorporated substrates were removed by aspiration, cells were washed once in HEPES–NaCl buffer, and pellets were resuspended in scintillation liquid and counted by liquid scintillation counter. The amount of incorporated radioactivity was normalized with *Leishmania* cell number and values of uptake in cells incubated on ice were subtracted. For the determination of the kinetic parameters presented in Table 4, we measured initial uptake rates using short time points (0, 1, and 2 min) that led to straight lines that crossed the origin.

3. Results

3.1. MTX is polyglutamylated in Leishmania tarentolae

Folates are found mainly as pentaglutamates in *L. major*, *L. tarentolae*, and *Leishmania donovani infantum* promastigotes and amastigotes [23,24]. In contrast, MTX is not polyglutamylated in *L. major* [25]. One possible explanation for the lack of MTX polyglutamylates in *L. major* could be a reduced FPGS activity towards MTX in *Leishmania*. Recently, we have cloned the *L. tarentolae FPGS* gene and set up an enzymatic assay to look at FPGS activity in *Leishmania* (Table 1; [23]). We show here that the *L. tarentolae* FPGS is active with MTX as a substrate (Table 1). *Leishmania major* has also a basal FPGS activity towards folic acid, which is even higher than in *L. tarentolae* (Table 1), and somewhat surprisingly, extracts derived from *L. major* had also FPGS activity towards MTX (Table 1).

The activity of the *Leishmania* FPGS towards MTX has prompted us to reassess the level of MTX polyglutamates in *Leishmania* cells. The pools of MTX polyglutamates were studied in *Leishmania* cells using HPLC analyses. In contrast to what reported for *L. major* [25], and consistent with the enzymatic activities shown in Table 1, we found that MTX is polyglutamylated in *L. tarentolae* (Fig. 1) with mostly triglutamates (Table 2). The chain length of MTX polyglutamates was lower than the chain length for folates, which are mostly found as pentaglutamates (Table 3; [23]). Overexpression of *FPGS* in *Leishmania* cells increases FPGS activity [23] and was found to increase by 3-fold the accumulation of MTX (Table 2). This higher accumulation is correlated with a significant increase in the glutamate chain length of MTX in the FPGS transfectant (TarII+FPGS) with higher glu_4, glu_5, and glu_6 while the percentage of shorter chain lengths decreases (Table 2). *Leishmania tarentolae* cells with one *FPGS* allele disrupted (TarII *FPGS/HYG*) accumulated less MTX and have a higher proportion of MTX short-chain glutamates (Table 2). Transfecting back an episomal copy of *FPGS* in that mutant (TarII *FPGS/HYG* + FPGS) restored MTX accumulation which was associated with long glutamate chain length (Table 2).

Table 1
Enzymatic activities toward folate and MTX in *Leishmania*

	FPGS activity[a] (pmol/mg/hr)		MTX hydrolase[a] activity (nmol/mg/hr)
	Folic acid	MTX	
L. tarentolae WT	19.8 ± 1.7	21.9 ± 1.8	<4
L. tarentolae MTX 1000.5 rev	10.6 ± 2.2	12.3 ± 2.9	<4
L. tarentolae MTX 1000.6	42.4 ± 7.1	35.3 ± 10.5	<4
L. major WT	107.4 ± 1.7	101.9 ± 12.6	310 ± 43

[a] The means and standard deviations of three independent experiments are shown. The activity was measured from crude extracts as detailed in Section 2.

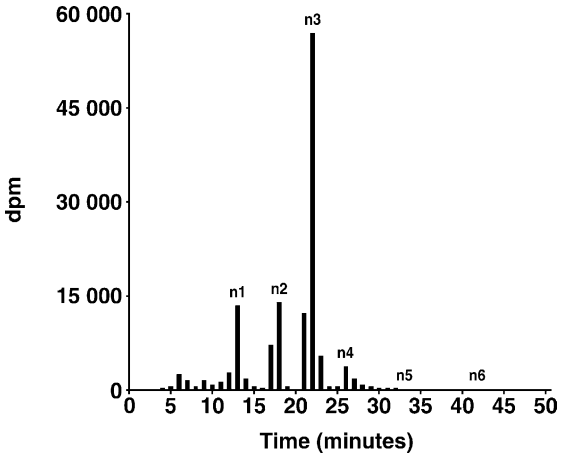

Fig. 1. Separation of MTX polyglutamates by reverse phase HPLC in *Leishmania* cells. Analysis of radioactive [^{3}H]MTX polyglutamates as determined by HPLC separation of MTX polyglutamates in *L. tarentolae* wild-type cells. The profile illustrates the distribution of radioactivity in *L. tarentolae* incubated for 72 hr with 25 nM [^{3}H]MTX. The numbers n1–n6 above the peaks represent the numbers of glutamates in the corresponding HPLC elution profile of MTX-$glu_{1–6}$ standards chromatographed on a C_{18} column. This elution is consistent with the elution observed by others using similar conditions [27,28].

We did not work with an *FPGS* null mutant because all our attempts to generate such a mutant failed [23].[1] The polyglutamylation of MTX in *L. tarentolae* prompted us to reassess whether MTX was polyglutamylated in *L. major*. We found that MTX accumulated less in *L. major* and the level of polyglutamylation is less with monoglutamates predominating but we could detect some MTX-glu_2 and minimal amounts of longer glutamate chain length (Table 2). The lower level of polyglutamylation of MTX in *L. major* is not due to a lack of activity of the *L. major* FPGS against MTX, however, which is in fact five times higher than the FPGS activity towards MTX found in *L. tarentolae* (Table 1).

One reason invoked for the absence, or in our study, lower levels of MTX polyglutamates in *Leishmania* is the presence of a highly active MTX hydrolase leading to the production of MAPA in *L. major* [25], *L. donovani* [17], and *Crithidia fasciculata* [30]. This preferential hydrolysis of MTX in these species could limit its availability as a substrate for polyglutamylation. In our hands, this hydrolase activity was determined to be 310 ± 43 nM/mg protein/hr in *L. major* (Table 1) in good agreement with activity measured by others [25]. Under identical conditions, the activity of this hydrolase derived from *L. tarentolae* extracts was lower than background values (Table 1), providing a plausible explanation for the presence of higher levels of polyglutamates of MTX in *L. tarentolae*.

3.2. MTX sensitivity and FPGS expression

In mammalian cells, the level of polyglutamylation modulates the activity of MTX, and a decrease in *FPGS*

[1] Unpublished observations.

Table 2
Levels of MTX polyglutamates in *Leishmania* species with various levels of *FPGS* expression

Cell lines	pmol MTX-glu$_N$/10^9 cells[a]					
	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6
TarII WT	3.3 ± 0.1	3.7 ± 0.1	13.1 ± 0.2	1.0 ± 0.2	0.1	–
TarII WT + FPGS	0.5	1.1	10.7 ± 0.3	32.7 ± 0.2	11.0 ± 0.2	0.7
TarII WT *FPGS/HYG*	2.7 ± 0.3	1.2 ± 0.1	0.7 ± 0.1	–	–	–
TarII WT *FPGS/HYG* + FPGS	0.3 ± 0.1	1.5 ± 0.4	18.7 ± 3.3	22.1 ± 1.7	7.2 ± 1.9	0.4 ± 0.2
L. major	3.1 ± 0.1	0.9	0.2	0.1	0.1	–

[a] The means and standards deviations (when applicable) of three independent experiments are shown. Cells were incubated for 72 hr in M-199 medium in the presence of 25 nM [^{3}H]MTX.

expression is often correlated with increased MTX resistance [31–35]. Since the FPGS of all *Leishmania* species tested can polyglutamylate both folate and MTX (Table 1; [23]), it is possible that a modulation in FPGS activity could contribute to MTX resistance. Indeed, *L. tarentolae* FPGS overexpressors were found to be 2-fold more resistant to MTX than control cells in SDM-79 medium (Fig. 2A), a medium rich in folates (15 μM). In contrast, the same cell lines were four times more sensitive than wild-type cells in M-199, a medium poorer in folates (22 nM) (Fig. 2B). As reported previously [6,14], the EC_{50} of MTX in *Leishmania* cells is highly dependent on the concentration of folates in the medium (Fig. 2). Indeed, while the EC_{50} is close to 25 μM in SDM-79, it is only 0.25 μM in M-199 medium (Fig. 2A and B). We have also studied the susceptibility properties of TarII *FPGS/HYG*, in which one allele of *FPGS* was inactivated. *Leishmania tarentolae* TarII *FPGS/HYG* cells were four times more resistant to MTX in M-199 medium than wild-type cells (Fig. 2B). This phenotype is specific to a defect in FPGS since transfecting back an *FPGS* construct in the mutant restores sensitivity to MTX (Fig. 2B). To test whether the difference in resistance to MTX mediated by FPGS between SDM-79 and M-199 medium was due to a difference in folate concentration, we repeated the growth curves in a version of the SDM-79 media in which folate concentration was adjusted to that found in M-199 media. As expected, when folate levels were decreased in this modified SDM-79 medium, the EC_{50} of the wild-type cell to MTX decreases by ~200-fold (Fig. 2C). In fact, the sensitivity to MTX is even lower in this medium compared to M-199 suggesting that in addition to the folate effect, there is another component of the media that possibly influences MTX susceptibility. Significantly, however, the MTX resistance patterns of cells with various copy numbers of FPGS grown in SDM-79 medium with a low folate concentration mirrored those of cells grown in M-199 medium as opposed to the pattern seen in high folate SDM-79 medium.

3.3. FPGS expression alters folate and MTX accumulation

The *FPGS* copy number appears to change the accumulation of MTX in *Leishmania* cells (Table 2). This was confirmed in another independent experiment (Fig. 3) where the long-term MTX accumulation in M-199 medium was increased in the FPGS transfectant and decreased in TarII *FPGS/HYG* (Fig. 3A). A similar accumulation kinetic was noted for folate with higher and lower accumulation in TarII + FPGS and TarII *FPGS/HYG*, respectively, compared to wild-type cells (Fig. 3A). A similar trend was also seen in the long-term accumulation of pteridines in cells grown in the folate-rich SDM-79 medium (Fig. 3B) but

Table 3
Distribution of polyglutamates of folic acid and MTX in *Leishmania tarentolae* MTX-resistant cells

Cell lines	pmol folic acid-glu$_N$/10^9 cells[a]					
	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6
TarII WT	1.2 ± 0.3	0.2	0.4 ± 0.1	4.0 ± 0.3	12.1 ± 0.5	1.4 ± 0.1
TarII MTX 1000.3	0.3	0.6	1.5	6.9 ± 0.4	9.8 ± 0.6	0.3
TarII MTX 1000.5 rev	0.8 ± 0.1	1.1 ± 0.1	2.1 ± 0.1	12 ± 0.4	2.9 ± 0.2	0.6
TarII MTX 1000.6	0.3 ± 0.1	0.3	0.4	6.7 ± 0.4	11.1 ± 0.4	0.6
	pmol MTX-glu$_N$/10^9 cells[a]					
	N = 1	N = 2	N = 3	N = 4	N = 5	N = 6
TarII WT	3.3 ± 0.1	3.7 ± 0.1	13.1 ± 0.2	1.0 ± 0.2	0.1	–
TarII MTX 1000.3	12.2 ± 0.2	5.3 ± 0.6	3.9 ± 1.1	0.3	–	–
TarII MTX 1000.5 rev	8 ± 0.1	4.1 ± 0.1	8.9 ± 0.1	0.7 ± 0.1	0.2	–
TarII MTX 1000.6	18.2 ± 0.2	2.3 ± 0.1	0.3	–	–	–

[a] The means and standards deviations (when applicable) of three independent experiments are shown.

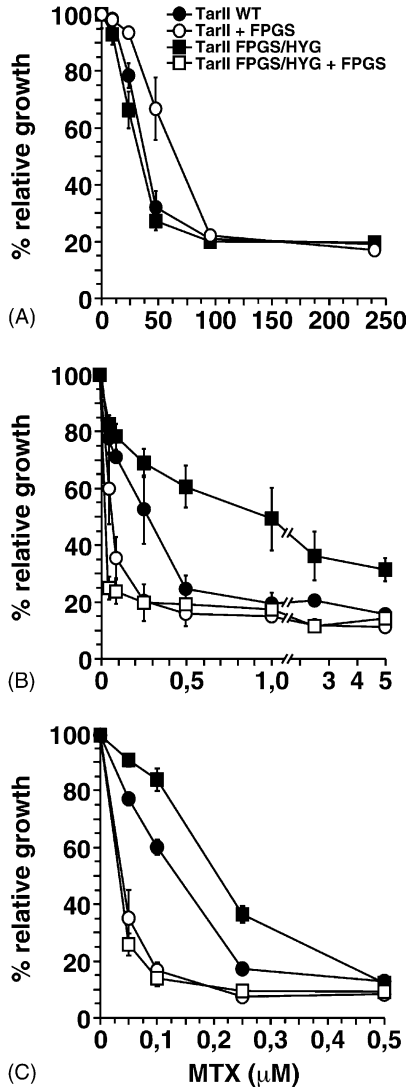

Fig. 2. FPGS copy number and MTX susceptibility of *Leishmania* cells. Susceptibility to MTX of *L. tarentolae* cells grown in the folate-rich medium SDM-79 (A), in M-199 medium (B), and in low folate SDM-79 medium (C) was studied. Growth was measured by optical density at 600 nm. (●), *L. tarentolae* wild-type cells (TarII WT); (○) *L. tarentolae* transfected with an episomal FPGS construct (TarII + FPGS); (■) *L. tarentolae* with one *FPGS* allele disrupted (TarII *FPGS/HYG*); (□) *L. tarentolae FPGS* haploid mutant transfected with an FPGS episomal construct (TarII *FPGS/HYG* + FPGS).

much less MTX entered those cells as it was competed by the folic acid present in the medium. We also show here that the copy number of FPGS has important implications for the short-term accumulation of pteridines. Indeed, while the short-term accumulation of both folate and MTX is similar for the first 5 min in M-199 medium in the TarII + FPGS cells and the wild-type cells, after 20 min a reproducible increase is observed in the *FPGS* transfectant (Fig. 4A and B). The accumulation of folate and MTX was greatly impaired in TarII *FPGS/HYG* and this was specific to the loss of one copy of *FPGS* since rescue mutants with an episomal copy of FPGS restored the transport phenotype (Fig. 4A and B). The same reduction in the accumulation of folate and MTX was observed in the TarII *FPGS/HYG* cells grown in SDM-79 medium (Fig. 4C and D). However, in contrast to TarII + FPGS cells grown in M-199 medium, the same cells grown in SDM-79 medium had a lower steady-state accumulation of MTX and folate compared to wild-type cells (Fig. 4C and D). While FPGS copy number clearly influences folate/MTX accumulation, the folate concentration in which cells were grown seem to have an even greater effect. Indeed, a 10-fold reduction in folate/MTX accumulation was observed in cells grown on high folate compared to cells grown in M-199 (Fig. 4). Under all conditions tested, a specific and marked decrease of folate/MTX was observed in TarII *FPGS/HYG*.

The accumulation of folate and MTX is known to be related to the *FPGS* copy number [33,36] (Figs. 3 and 4). Accumulation is likely to be the result of a complex interplay between polyglutamylation, the rate of entry and the rate of efflux. A preliminary characterization of an MTX efflux system has been done previously in *L. major* [37]. We have also demonstrated the presence of a rapid active efflux system of MTX in wild-type *L. tarentolae* cells loaded for 30 min with [^{3}H]MTX but were not able to show conclusively that efflux was altered in TarII + FPGS or TarII *FPGS/HYG* compared to wild-type cells (results not shown). We hypothesize that if efflux is not changed drastically, it could be the rate of influx that is altered. We have carried out double reciprocal plot analysis of uptake (as measured from 0 to 2 min) and showed that the rate of influx (V_{max}) was decreased significantly in TarII *FPGS/HYG* compared to wild-type cells (Table 4). The V_{max} was similar between TarII + FPGS and wild-type cells (Table 4) and this is consistent with the similar accumulation kinetic at short time point (Fig. 4B).

3.4. Folate and MTX polyglutamates in MTX-resistant *Leishmania*

FPGS expression and hence polyglutamylation can modulate the activity of antifolates in *Leishmania* (Fig. 2) as well as their accumulation (Figs. 3 and 4). We therefore studied folate and MTX polyglutamylation in *L. tarentolae* MTX-resistant mutants. These strains have numerous mutations, including transport defects, for folic acid and MTX. In one class of mutants, including MTX

Table 4
Kinetic parameters of MTX influx in *Leishmania tarentolae* cells with varying copy numbers of FPGS

Strain	V_{max} (pmol/10^9 cells/min)[a]	K_m (nM)[a]
TarII WT	39.5	224
TarII + FPGS	39.5	236
TarII *FPGS/HYG*	4.9	123

[a] Average of three independent determinations with a substrate range between 10 and 1000 nM in cells grown in M-199 medium. All values were derived in the first 2 min where uptake was linear.

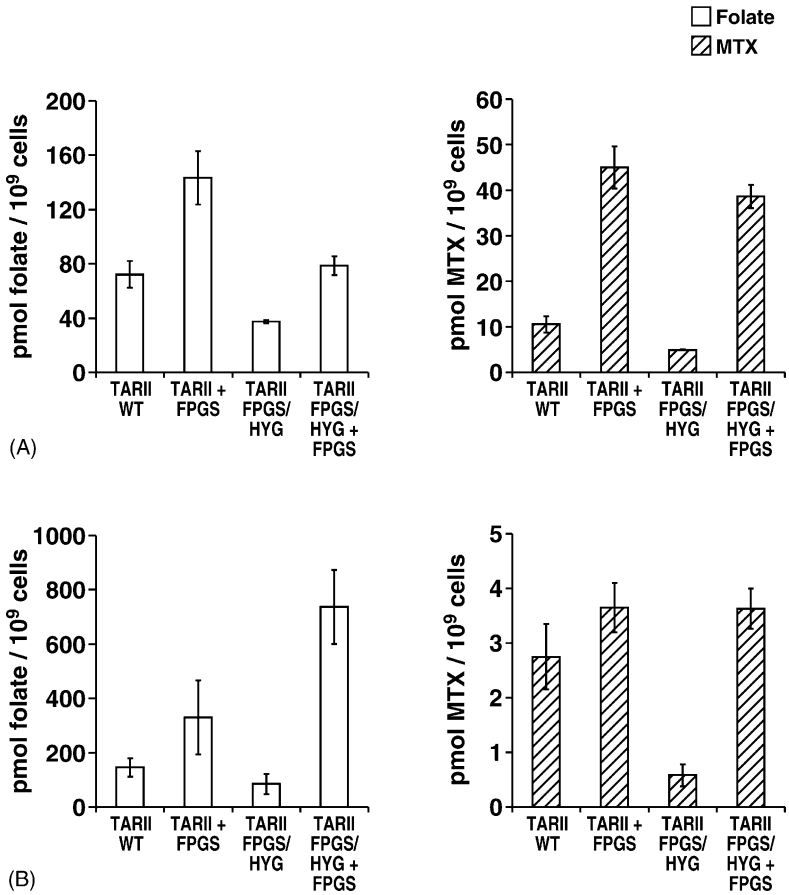

Fig. 3. FPGS copy number and long-term accumulation of folate/MTX in *Leishmania* cells. The long-term accumulation of 50 nM [^{3}H]folate and of [^{3}H]MTX in M-199 (A) and SDM-79 (B) medium was measured at 72 hr. The quantity of accumulated radioactivity was normalized with *Leishmania* cell numbers. The mean and standard deviation of three independent experiments is shown.

1000.5 rev, MTX accumulation was reduced by 5-fold while in MTX 1000.3 and MTX 1000.6 accumulation was decreased by more than 50-fold, as measured after 20 min [26]. Nonetheless, in long-term accumulation experiments (72 hr) similar amounts of pteridines were accumulated in the mutants compare to wild-type cells (Table 3). In all the mutants that we have analyzed, the distribution of folyl-polyglutamates was similar to that of wild-type cells with pentaglutamates prevailing except in MTX 1000.5 rev, which contained mostly glu_4 folates (Table 3). In contrast, although MTX is polyglutamylated in wild-type cells the level of polyglutamylation is reduced greatly in all the resistant mutants studied, most notably in TarII MTX 1000.6 (Table 3). This is remarkable since a higher FPGS activity was noted for this mutant with both folate and MTX compared to wild-type cells (Table 1). Southern blot analysis of genomic DNAs using an *FPGS* probe suggested that this genomic locus is intact in the MTX-resistant mutants that have lower chain length of MTX polyglutamates (results not shown). Since FPGS has equal activity for folate and MTX, it is possible that in these mutants, the expression of the specific MTX hydrolase activity leading to MAPA is increased. However, similarly to *L. tarentolae* wild-type cells, only background levels of this hydrolase activity could be measured in TarII MTX 1000.6 (Table 1). Thus, the reduction in polyglutamylation of MTX in *L. tarentolae* MTX-resistant mutants must occur by another mechanism.

4. Discussion

While folates are found as polyglutamates in *Leishmania* [23,24], MTX was not found to be polyglutamylated in *L. major* [25], a striking difference compared to other type of cells. The reason for this differential polyglutamylation between folate and MTX was unknown. We showed that MTX is polyglutamylated in *L. tarentolae* (Fig. 1, Table 2), while we confirmed that MTX is only minimally polyglutamylated in *L. major* (Table 2). This reduction in MTX polyglutamates is not due to the FPGS enzyme itself since the *L. major* FPGS can add glutamates equally well to either folate or MTX (Table 1). It is therefore possible that our inability to detect MTX polyglutamates in *L. major* is due to the highly active MTX α-hydrolase that was previously described [25] and which could reduce substrate availability for FPGS. Since this activity is much lower in *L. tarentolae* it could explain why MTX is found polyglutamylated in this

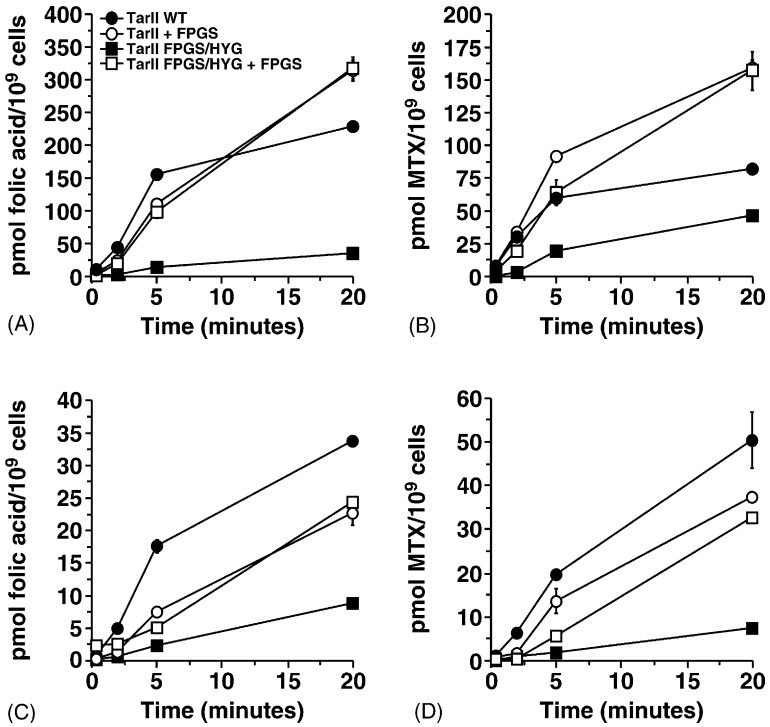

Fig. 4. FPGS copy number and transport of [^{3}H]folate and [^{3}H]MTX by *Leishmania* cells. The uptake of 200 nM of both [^{3}H]folate and [^{3}H]MTX in *Leishmania* cells was evaluated in cells grown in M-199 medium (A and B) and in folate-rich medium SDM-79 (C and D). The results of one experiment in triplicate are shown, which has been repeated several times with similar results. The quantity of accumulated radioactivity was normalized with *Leishmania* cell numbers and uptake in cells incubated on ice was subtracted. (●) *L. tarentolae* wild-type cells (TarII WT); (○) *L. tarentolae* transfected with an episomal FPGS construct (TarII + FPGS); (■) *L. tarentolae* with one *FPGS* allele disrupted (TarII *FPGS/HYG*); (□) *L. tarentolae FPGS* haploid mutant transfected with an FPGS episomal construct (TarII *FPGS/HYG* + FPGS).

species. Alternatively, a number of folate/MTX metabolizing enzymes could also modulate the level of polyglutamation (see below).

Overexpression of *FPGS* leads to MTX resistance in SDM-79 medium and susceptibility in M-199 medium (Fig. 2). Inactivation of one allele of *FPGS* had the opposite effect on resistance with increased resistance in M-199 medium and slightly increased susceptibility in SDM-79 (Fig. 2). In mammalian cells, a reduction of FPGS activity is associated with MTX resistance [32–35]. Mutations in FPGS were also linked to resistance to antifolates in murine cell lines [38]. One important difference between SDM-79 and M-199 is the folate concentration. This folate effect was tested by preparing SDM-79 medium with the folate concentration found in M-199. In this case, TarII *FPGS/HYG* was resistant while TarII + FPGS was more sensitive to MTX. Folate concentration in the medium can greatly modulate antifolate susceptibility in *Leishmania* (see Fig. 2), and this effect is likely due to the marked difference in MTX accumulation in both medium (Fig. 3). We also show that the external folate concentration can have a profound effect on the resistance mediated by FPGS (Fig. 2). This effect has also to be related, at least in part, to the differential long-term accumulation of folate and MTX into cells. In M-199 medium, there is significantly less MTX that accumulates at 72 hr in TarII *FPGS/HYG* compared to wild-type cells and there is more folic acid accumulated, thus providing an explanation for the resistance of these cells. TarII + FPGS cells accumulate more MTX compared to wild-type cells at 72 hr and thus could explain why these cells become more sensitive, specially in folate poor medium. In SDM-79 medium, the FPGS transfectant is 2-fold more resistant than wild-type cells (Fig. 2A). Since SDM-79 medium is rich in folic acid, little [^{3}H]MTX enters the cells under these conditions (Fig. 3B) and the fold increase in folic acid accumulation appears greater than for MTX (Fig. 3B) possibly explaining why these cells are resistant to MTX. Recently, folate levels in the medium were also found to modulate the activity of some antifolates in mammalian cells [39,40]. This study clearly establishes FPGS as an MTX susceptibility/resistance marker in *Leishmania*.

Inactivation of one *FPGS* allele had a drastic effect on accumulation of both folate and MTX (Figs. 3 and 4). Our inability to generate an *FPGS* null mutant [23] may be due to the strong correlation between folate accumulation and *FPGS* copy number. *Leishmania* could presumably not survive in the absence of folate accumulation. *Leishmania* has an MTX efflux system which is extremely rapid [37] but we had no evidence that it could contribute to the reduced accumulation described here. Instead, we show

that the influx of MTX was significantly diminished in TarII *FPGS/HYG* (Table 4). Thus, a reduction in FPGS has an effect on the rate of uptake of folate. A number of possibilities could explain this observation. For example, one cannot exclude that FPGS in *Leishmania* could interact with one of the FT member to control the rate of entry and that low FPGS levels could be rate limiting. Another possibility is that during inactivation of one *FPGS* allele, cells compensate by downregulating the expression of one *FT* gene. Further work will be required to test those hypotheses. In M-199 media, more folate and MTX accumulate in the TarII + FPGS cells. These cells contain a higher proportion of long-chain glutamates of both MTX (Table 2) and folate [23] and thus can increase the long-term accumulation of pteridine (Fig. 3). An increase is also seen in short-term uptake experiment since after 20 min there is a significant difference in accumulation of pteridines in TarII + FPGS cells (Fig. 4B). Since polyglutamylation is thought to be a relatively slow process—the polyglutamate forms in *Leishmania* do not reach a steady-state distribution by 24 hr [24]—it suggests that the addition of at least one or two glutamates to pteridines contribute to the increased cellular retention observed after 10 min (Fig. 4A and B). In SDM-79 (which contains nearly 1000-fold more folate), we observed that there is a decreased accumulation of both folate and MTX by an FPGS transfectant compared to wild-type cells (Fig. 4C and D). A similar observation has been made with cells transfected with a mammalian *FPGS* gene in the presence of high folate concentration. This was explained by a competition between mono- and polyglutamylated substrates for FPGS where not enough long-chain glutamate folates would be formed and thus retained [36].

The increased prevalence of shorter MTX-glutamate chain length in either *L. major* (Table 2) or in the *L. tarentolae* MTX-resistant mutants (Table 3) cannot be explained by a reduced efficacy of FPGS (Tables 1 and 3). *Leishmania major* has an active MTX α-hydrolase, which may explain the lack of MTX polyglutamates, but this cannot be the case for the *L. tarentolae* MTX-resistant mutants (see Table 1). Thus, the presence of shorter glutamate chains in the MTX-resistant mutants must be explained by another mechanism. In other *Leishmania* cells (*BT1/PTR1* null mutants) selected for MTX resistance, we observed an even more spectacular correlation between reduction of MTX polyglutamylation and resistance.[2] Steady-state levels of folate and antifolate polyglutamates in mammalian cells are likely to depend on the balance of FPGS and of a γ-glutamyl hydrolase (GGH), a peptidase cleaving the glutamate residues of folylpolyglutamates [41]. Increased activity of GGH has been reported in MTX-resistant mammalian cells [42] although it is not known whether *Leishmania* has a GGH activity. Increased activity of GGH could be one mechanism explaining the presence of shorter glutamate chains of MTX in resistant mutants. Recent work, however, has suggested that overexpression of GGH alone would not be sufficient to confer MTX resistance in mammalian cells [43]. New folate catabolizing enzymes, such as ferritin or glutamate carboxypeptidase [44], have recently been isolated from mammalian cells. Thus, alterations in other enzymatic activities could also explain the increase in short-chain glutamate MTX in resistant *Leishmania* cells.

In this study, we have identified a novel mechanism by which *Leishmania* can resist MTX. Indeed, resistance to MTX can be achieved by decreased uptake of the drug, by overexpression of the biopterin transporter BT1, and by amplification of the genes coding for either the target DHFR-TS or the pterin reductase PTR1. Alteration of *FPGS* expression, as demonstrated by gene transfection or gene inactivation, can now be added to this list. Reduced FPGS activity has a major influence on the accumulation of MTX and thus also on susceptibility. The occurrence of shorter MTX-glutamate chains in resistant mutants further demonstrates the role of MTX modification in MTX resistance in *Leishmania*. Further work will be required to understand how these shorter MTX-glutamate chains are being formed. Our inability to inactivate the *FPGS* gene and its key role in folate accumulation (Figs. 3 and 4) make the *Leishmania* FPGS an interesting drug target. Inhibitors of FPGS could even be used in combination with nonpolyglutamylatable antifolates. Indeed, inhibition of FPGS would likely increase the activity of these antifolates as less folates would be present in the cell. Since the activity of antifolates is highly dependent on folate concentration, it may also be helpful that these antifolates are lipophilic so they do not accumulate through the folate transporters. In that manner this could circumvent one main resistance mechanism consisting in reduced drug uptake and would diminish the role of folate concentration on antifolate activity.

[2] A.E. and M.O., unpublished observations.

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