Increased Glycolytic ATP Synthesis Is Associated with Tafenoquine Resistance in *Leishmania major*\(^\dag\)

José Ignacio Manzano, Luis Carvalho, José M. Pérez-Victoria, Santiago Castanys,\(^*\) and Francisco Gamarro\(^*\)

Instituto de Parasitología y Biomedicina López-Neyra, CSIC, Parque Tecnológico de Ciencias de la Salud, Avda. del Conocimiento s/n, 18100 Armilla, Granada, Spain

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Tafenoquine (TFQ), an 8-aminoquinoline used to treat and prevent *Plasmodium* infections, could represent an alternative therapy for leishmaniasis. Indeed, TFQ has shown significant leishmanicidal activity both in vitro and in vivo, where it targets *Leishmania* mitochondria and activates a final apoptosis-like process. In order not to jeopardize the life span of this potential antileishmania drug, it is important to determine the likelihood that *Leishmania* will develop resistance to TFQ and the mechanisms of resistance induced. To address this issue, a TFQ-resistant *Leishmania major* promastigote line (R4) was selected. This resistance, which is unstable in a drug-free medium (revertant line), was maintained in intramacrophage amastigote forms, and R4 promastigotes were found to be cross-resistant to other 8-aminoquinolines. A decreased TFQ uptake, which is probably associated with an alkalization of the intracellular pH rather than drug efflux, was observed for both the R4 and revertant lines. TFQ induces a decrease in ATP synthesis in all *Leishmania* lines, although total ATP levels were maintained at higher values in R4 parasites. In contrast, ATP synthesis by glycolysis was significantly increased in R4 parasites, whereas mitochondrial ATP synthesis was similar to that in wild-type parasites. We therefore conclude that increased glycolytic ATP synthesis is the main mechanism underlying TFQ resistance in *Leishmania*.

Treatment for leishmaniasis currently relies on a reduced arsenal of drugs, including pentavalent antimonials (which cannot be given in areas where drug resistance is endemic), amphotericin B deoxycholate, lipid formulations of amphotericin B, miltefosine, and paromomycin, all of which have drawbacks in terms of toxicity, efficacy, price, and inconvenient treatment schedules (6, 17). To increase the therapeutic life span of these drugs and delay the emergence of resistance, the World Health Organization has recommended combination therapy as a strategy to be implemented in clinical trials. Of the new drugs under development, 8-aminoquinolines such as sitamaquine (WR6026; GlaxoSmithKline), which is currently in phase 2b clinical trials (12, 34), represent a promising new oral leishmanicidal treatment. Although the mechanism of action of sitamaquine is still unknown, it has been reported that acido-calcosomes play a key role in the accumulation of sitamaquine, although they do not determine the leishmanicidal potency of the drug (14). Other 8-aminoquinolines chemically related to primaquine have been synthesized and evaluated for their antiparasitic activity (1, 25). Thus, tafenoquine (TFQ), formerly known as WR238605, is a primaquine analogue which is being developed jointly by the Walter Reed Army Institute of Research and GlaxoSmithKline pharmaceuticals for the treatment of relapsing malaria (31). Phase I, II, and III clinical studies with this drug have shown that TFQ is a safe, well-tolerated, and highly effective oral chemoprophylactic agent for the treatment of plasmodial infections (8, 24, 30). Recently, we have proposed that TFQ could be used as a new leishmanicidal drug (33) and have determined that TFQ targets *Leishmania* mitochondria by specifically inhibiting mitochondrial cytochrome c reductase, thus leading to a final apoptosis-like process (3). However, to ensure the future long life of this promising leishmanicidal drug, it is important to determine how easy it is to induce TFQ resistance experimentally, as this information can then be extrapolated to the possible emergence of drug resistance in the field. The mechanism of resistance to other aminoquinoline derivatives, such as the 4-aminoquinoline derivative chloroquine, in *Plasmodium*, for example, has been associated with a reduction in drug accumulation (13, 27).

In the present study, we have determined the mechanism of resistance to TFQ in *Leishmania* parasites and have found that TFQ resistance is unstable. TFQ accumulation was lower in resistant parasites than in sensitive parasites, although reduced drug accumulation was found not to be a mechanism of resistance, as it was present in revertant (i.e., nonresistant) lines. However, the mechanism of TFQ resistance does appear to be linked to increased ATP synthesis from glycolysis.

**MATERIALS AND METHODS**

**Chemical compounds.** TFQ [2-methoxy-4-methyl-5-(3-trifluoromethylphenox)-primaquine succinate], sitamaquine [N,N-diethyl-N’-(6-methoxy-4-methylquinolin-8-yl)hexane-1,6-diamine] dihydrochloride and [benzene ring-U-\(^1\)C]sitamaquine [(\(^1\)C)sitamaquine; 2.07 GBq/nmol] were provided by GlaxoSmithKline (Greenford, United Kingdom). [\(^1\)C]Glucose (11.8 GBq/nmol) was purchased from PerkinElmer. Amplex Red, LysoTracker Green DND-26, LysoTracker Red DND-99, BCECF-AM [2’,7’-bis-(2-carboxyethyl)-5- (and-6)-carboxyfluorescein, acetoxymethyl ester], and BCECF (free acid) were purchased from Invitrogen. Dicyclohexylcarbodiimide (DCCD), trivalent anti-
mony, pentamidine, paromomycin, amphotericin B, ketoconazole, chloroquine, quinine, mefloquine, primaquine, glucose, sodium azide, ammonium chloride, nigericin sodium salt, 2-deoxy-D-glucose, DAPI (4′,6-diamidino-2-phenylindole diacetate), phosphonopyruvate, propidium iodide, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], phenylmethanesulfonyl fluoride (PMSF), flavin adenine dinucleotide (FAD), thiamine pyrophosphate (TPP), horseradish peroxidase (HRP), pyruvate oxidase, and sodium pyruvate were purchased from Sigma-Aldrich (St. Louis, MO). Lactate dehydrogenase was purchased from Roche Applied Science, and miltefosine was purchased from Astrea Zentaris (Frankfurt, Germany). All chemicals were of the highest quality available.

Leishmania culture conditions. Promastigotes of Leishmania major (MHOM/ JL/80/Friedlin) and derivative lines used in this study were cultured at 28°C in RPMI 1640 modified medium (Invitrogen, Carlsbad, CA) supplemented with 20% heat-inactivated fetal bovine serum (JBSF, Irvine) and 10% newborn calf serum (JBSF, Irvine), described previously (10). All parasite lines were collected from culture by centrifugation after 48 h of growth and washed in phosphate-buffered saline (PBS; 1.2 mM KH2PO4, 8.1 mM Na2HPO4, 130 mM NaCl, and 2.6 mM KCl adjusted to pH 7). The final parasite concentration was determined using a Coulter Z1 counter.

Generation of a TFQ-resistant L. major line. A TFQ-resistant L. major line was obtained by following a previously described stepwise selection process (4, 23) with a starting concentration of 2.5 μM TFQ increasing to 4 μM TFQ over 10 days (14). The TFQ-resistant (R4) line was grown in the continued presence of 4 μM TFQ. To determine the stability of the resistant phenotype, the R4 line was grown in a drug-free medium for 1 month (revertant; revR4). The sensitivity of wild-type (WT), R4 and revR4 promastigotes to TFQ and the cross-resistance profile of the R4 line to different compounds were determined after incubation for 72 h at 28°C in the presence of increasing concentrations of the drugs. The concentration of TFQ required to inhibit parasite growth by 50% (IC50) and the resistance indices (IC50 for resistant and WT parasites) were calculated using an MTT colorimetric assay, as described previously (11).

TFQ sensitivity in intracellular amastigotes of Leishmania. Late-stage promastigotes from the WT and R4 lines were used to infect mouse peritoneal macrophages from BALB/c mice (Charles River, Ltd.) at a macrophage:parasite ratio of 1:10, as described previously (14). After infection for 6 h, extracellular parasites were removed by washing twice with ice-cold PBS, and the resistance indices (IC50 for resistant and WT parasites) were calculated using an MTT colorimetric assay, as described previously (11).

pHi measurement. The intracellular pH (pHi) of L. major promastigotes was determined fluorimetrically using a BCECF-AM probe, as described previously (28). Briefly, promastigotes (2 × 107 per ml) were resuspended in standard buffer (136.89 mM NaCl, 2.68 mM KCl, 1.47 mM KH2PO4, 8.46 mM Na2HPO4, 11.1 mM glucose, 1 mM CaCl2, 0.8 mM MgSO4, and 20 mM HEPES [pH 7.4]), reincubated for 30 min at 28°C in the presence of BCECF-AM (5 μg/ml), the parasites were washed twice. The fluorescence ratios (excitation ratio, 490 nm/440 nm; emission, 535 nm) were monitored continuously using an Aminco Bowman series 2 spectrometer. For calibration curves, the BCECF fluorescence as a function of pH was obtained using a suspended parasite suspension incubated with BCECF-AM and treated with the ionophore nigericin (5 μg/ml). Different pH values were obtained by the addition of 1 M MES (morpholineethanesulfonic acid; pH 5.0) or 1.3 M Tris-Cl (pH 8.8) followed by measurement of the pHi. The intracellular alkalization of parasites was observed after pretreatment of L. major promastigotes at 28°C with 20 mM NaHCl for 1 min, followed by incubation with 5 μM TFQ in the presence of NH4Cl for 15 min. For the studies involving parasite acidification, the pHi was lowered using the NH4Cl prepulse technique, as described previously (15). Thus, promastigotes (2 × 107) were resuspended in 50 μl of standard buffer (described above) containing 40 mM NH4Cl at 28°C for 15 min. The parasites were then centrifuged and resuspended in standard buffer with or without the H+-ATPase inhibitor DCCD and in chloride-free buffer (135 mM sodium glucose, 5 mM potassium glutamate, 5 mM glucose, 1 mM calcium chloride, 1 mM MgSO4, 5 mM HEPES-Tris for pH 7.4) for 10 min. To determine the acidification produced by TFQ and sodium azide, BCECF-loaded promastigotes (2 × 107 per ml) were incubated in culture medium with 5 μM TFQ or 20 mM sodium azide for different times (1, 3, 5, 10, and 30 min).

Proton efflux measurements for Leishmania parasites. Extracellular pH, which reflects the total proton influx of parasites, was determined as described previously (15). Thus, parasites (1 × 107) were washed and resuspended in 2 ml of weakly buffered (0.1 mM HEPES-Tris; pH 7.4) standard buffer containing 0.38 μM BCECF (free acid). The fluorescence excitation ratio (490 nm/440 nm), emission, 535 nm) was then recorded and translated into nM of H+ released/min on the basis of ratios obtained at various extracellular pHs by the addition of known HCI equivalents.

ATP measurement for L. major lines. ATP was measured using a CellTiter-Glo luminescent assay (Promega), which generates a luminescent signal proportional to the amount of ATP present. Promastigotes (4 × 108 per ml) were incubated in culture medium at different TFQ concentrations (1, 2, 5, 10, and 20 μM) for 60 min. For glycolytic and mitochondrial ATP determination, parasites were incubated separately in HBS buffer plus 20 mM sodium azide to inhibit mitochondrial oxidative ATP generation, and glucose-free HBS buffer plus 5 mM 2-deoxy-D-glucose to inhibit glycolytic ATP generation for 1 h at 28°C. For the study of ATP levels at different pH values, promastigotes (2 × 107 per ml) were incubated in distinct buffers to obtain a different pH, as described previously (15). The buffers used were regular buffer (135 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM CaCl2, 1 mM MgSO4 and 10 mM HEPES-Tris for pH 7.4 or 10 mM MTris for pH 5.5), regular buffer containing 25 mM HCO3−, sodium-free buffer (135 mM choline Cl, 5 mM KCl, 5 mM glucose, 1 mM CaCl2, 1 mM MgSO4 and 10 mM HEPES-Tris [pH 7.4]) and chloride-free buffer (described above). The pH of parasites was measured using BCECF-AM as the pH-sensitive fluorescent probe, as described above. A 25-μl aliquot of parasites was then transferred to a 96-well plate, mixed with the same volume of CellTiter-Glo, and incubated in the dark for 10 min, and the bioluminescence was measured using an Infinite F200 microplate reader (Tecan Austria GmbH, Austria).

Determination of intracellular pyruvate levels in Leishmania lines. Pyruvate was extracted as described by Zhu et al. (35). Briefly, 5 × 107 parasites were incubated in HBS buffer or in glucose-free HBS buffer plus 5 mM 2-deoxy-D-glucose for 1 h at 28°C. The parasites were then collected and resuspended in 0.1 ml of ice-cold 0.25 M HClO4 and incubated on ice for 5 min. The resulting mixture was neutralized with 2.8 μl of 5 M K2CO3 (pH = 6.5). The supernatant was collected after centrifugation at 10,000 × g for 5 min. Pyruvate was measured using a fluorometric assay (35) based on the oxidation of pyruvate by pyruvate oxidase. The hydrogen peroxide generated reacts with nonfluorescent Amplex Red at a 1:1 stoichiometry to form the red fluorescent product resoruvin. This fluorescence is proportional to the initial pyruvate concentration in the solution. Briefly, 20 μl of intracellular pyruvate extract was pipetted into a 96-well plate, and 180 μl of reaction solution (final concentration, 100 mM potassium phosphate with 1 mM EDTA [pH 6.7], 1 mM MgCl2, 0.1 mM FAD, 0.2 mM TPP, 0.2 U/ml pyruvate oxidase, 50 μM Amplex Red, and 0.2 U/ml HRP) was then
and 0.95 ± 0.02 μM for intracellular WT and R4 amastigotes, respectively) and that R4 amastigotes were 2.5-fold more resistant, similar to the value obtained for R4 promastigotes. Additionally, the R4 line profile showed significant cross-resistance to the 8-aminoquinolines sitamaquine (2-fold more resistant) and primaquine (1.9-fold; Table 1); no cross-resistance to the 4-aminoquinolines chloroquine and mefloquine and the quinoline derivative quinine was observed. As can be seen from Table 1, the R4 parasites did not show any significant cross-resistance to other antileishmanial drugs, such as trivalent antimony, pentamidine, miltefosine, paromomycin, and ketoconazole.

**TFQ uptake and accumulation in acidic vesicles.** To determine whether TFQ resistance in R4 parasites was associated with a failure to accumulate the drug, TFQ uptake experiments were therefore carried out using spectrofluorometric techniques. The time course uptake of TFQ in WT, R4, and revR4 lines showed that TFQ uptake became saturated after 5 min (data not shown), with a 24% lower uptake in R4 parasites with respect to their WT counterparts and intermediate values for the revR4 line (data not shown). Additionally, no significant differences in TFQ efflux between the R4 and WT parasites were observed (data not shown). A decrease in R4 and revR4 drug uptake was also observed for [14C]sitamaquine, which present a 55% lower uptake in R4 parasites than in WT parasites, and the revR4 line presents accumulation values of 25% (data not shown). Since TFQ and sitamaquine accumulate in acidic vesicles, such as acidocalcisomes (3, 14), the differences in the size of such organelles could determine the TFQ accumulation. In fact, with the acidic organelles in WT parasites being larger than those in the R4 and revR4 lines, as can be seen from the significantly lower accumulation of LysoTracker Green (data not shown), a fluorescent acidotropic probe was used to label acidic organelles (mainly acidocalcisomes) in *Leishmania* (18).

**RESULTS**

**Generation of a TFQ-resistant *L. major* line.** A TFQ-resistant *L. major* line was selected in *vitro* by a stepwise adaptation process up to a maximum of 4 μM TFQ. Subsequent attempts to increase the level of TFQ resistance were unsuccessful. This TFQ-resistant line (R4) has an EC50 of 5.45 ± 0.35 μM, a 2.5-fold-higher concentration than for the WT line (Fig. 1 and Table 1). The R4 parasites showed a growth rate similar to that of the WT line. Additionally, no morphological differences were observed between the two lines, as determined by light microscopy and flow cytometry analyses (data not shown). The resistance phenotype was unstable in drug-free medium for up to 4 weeks; the revertant line (revR4) presented a sensitivity (2.26 ± 0.02 μM) that was similar to that of the WT parasites (Fig. 1). Additionally, we found that TFQ resistance in the promastigote forms was maintained in intracellular amastigotes obtained after infection of mouse peritoneal macrophages with WT and R4 promastigotes (EC50 of 0.38 ± 0.03 μM).

**Statistical analysis.** Statistical significance was calculated by using Student’s *t* test. Differences were considered significant at a *P* value of <0.05.

**TABLE 1. Drug sensitivity profile for *L. major* promastigote lines**

<table>
<thead>
<tr>
<th>Drug</th>
<th>EC50 (μM)</th>
<th>RI</th>
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<tr>
<td></td>
<td>WT R4</td>
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<tr>
<td>Tafenoquine</td>
<td>2.24 ± 0.15</td>
<td>5.49 ± 0.35</td>
</tr>
<tr>
<td>Sitamaquine</td>
<td>12.02 ± 0.38</td>
<td>24.49 ± 1.92</td>
</tr>
<tr>
<td>Primaquine</td>
<td>27.73 ± 0.24</td>
<td>53.04 ± 1.14</td>
</tr>
<tr>
<td>Quinine</td>
<td>9.41 ± 0.11</td>
<td>9.53 ± 0.06</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>18.75 ± 1.25</td>
<td>23.82 ± 0.81</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>12.48 ± 0.55</td>
<td>13.92 ± 0.86</td>
</tr>
<tr>
<td>SbIII</td>
<td>15.43 ± 0.73</td>
<td>21.12 ± 0.88</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>1.87 ± 0.12</td>
<td>3.16 ± 0.23</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>19.98 ± 0.56</td>
<td>28.05 ± 0.71</td>
</tr>
<tr>
<td>Pentamidine</td>
<td>0.28 ± 0.02</td>
<td>0.45 ± 0.04</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>17.69 ± 0.51</td>
<td>25.69 ± 1.12</td>
</tr>
<tr>
<td>Paromomycin</td>
<td>4.29 ± 0.04</td>
<td>3.98 ± 0.14</td>
</tr>
<tr>
<td>Daunomycin</td>
<td>0.22 ± 0.01</td>
<td>0.38 ± 0.01</td>
</tr>
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</table>

* *L. major* WT and R4 lines were grown as described in Materials and Methods for 72 h at 28°C in the presence of increasing concentrations of drugs. Subsequently, cell viability was determined using an MTT-based assay.

* Data are means of EC50 ± SD from three independent experiments.

* Resistance index (RI) was calculated by dividing the EC50 for R4 parasites by that for WT parasites. Significant differences were determined by Student’s *t* test (*, *P* < 0.05).

* *EC50* for SbIII is expressed in μg/ml.
An increase in pHi reduces TFQ accumulation. The accumulation of weakly basic aminoquinolines, such as amodiaquine, chloroquine, and more recently, sitamaquine, seems to be pH dependent (9, 14). Initially, we determined the pHi of Leishmania lines using BCECF as a fluorescent-dependent pH indicator (28). Under physiological culture conditions, WT promastigotes maintained a steady-state pHi of 6.75 ± 0.01 (n = 5) (Fig. 2), a value similar to that reported by other authors (28), whereas R4 parasites presented a pHi of 7.21 ± 0.02 (n = 5) (Fig. 2), which is 0.46 pH units higher than that observed for WT parasites. Interestingly, a similar pHi alkalinization was observed in revR4 parasites, which presented a pHi of 7.11 ± 0.01 (Fig. 2), 0.36 units higher than that for WT parasites. These findings suggest that an increased pHi could be associated, in part, with decreased TFQ accumulation in R4 parasites. To validate this hypothesis, we induced an intracellular alkalization of parasites with NH4Cl and then incubated the parasites with TFQ. The pHi obtained for WT parasites pretreated with NH4Cl increased by 0.94 units (0.01 versus the corresponding control without NH4Cl). Similar results were obtained for revR4 parasites, which presented a recovery rate intermediate between those for WT and R4 parasites (0.28 ± 0.02; Table 3). The recovery rates for the different Leishmania lines were slightly lower when using the specific H+ -ATPase inhibitor DCCD, and a significant decrease in the final pHi was also observed. These results support the presence of a plasma membrane H+ -ATPase as a major pHi regulator (15). The pHi recovery was also determined by using chloride-free buffer, as the chloride ion is essential for acid extrusion in Leishmania promastigotes (29). The recovery rates for the R4 and revR4 Leishmania lines were found to be higher than that for WT parasites under these conditions (Table 3).

**Activation of a plasma membrane P-type H+ -ATPase in R4 parasites.** One proposed mechanism for pHi regulation in Leishmania promastigotes involves a plasma membrane P-type H+ -ATPase (15) identified for other unicellular organisms (15, 28). In light of the possibility that the increased pHi observed with the R4 and revR4 Leishmania lines could be due to an increased P-type H+ -ATPase activity, we determined the proton efflux activity as described previously (15). The fluorescence ratios obtained (excitation at 505/440 nm and emission detected at 530 nm) were transformed into the amount of H+ released min−1. The results confirmed that the proton efflux in R4 parasites was significantly higher, with a value of 24.58 ± 0.05 nmol H+ /min per 108 cells (7.65 ± 0.44 nmol H+ /min per 108 WT parasites). The R4 parasites therefore presented a higher proton efflux activity (3.21-fold) than WT parasites as a mechanism of pHi alkalinization. Additionally, revR4 parasites presented a proton efflux activity similar to that of R4 parasites (22.63 ± 0.37 nmol H+ /min per 108 cells). Experiments to determine the recovery capacity of pHi following acidification support these findings. Thus, as can be seen from Table 3, R4 parasites recovered their pHi significantly faster than WT parasites (recovery rates of 0.32 ± 0.04 and 0.17 ± 0.02, respectively). Similar results were obtained for revR4 parasites, which presented a recovery rate intermediate between those for WT and R4 parasites (0.28 ± 0.02; Table 3). The recovery rates for the different Leishmania lines were slightly lower when using the specific H+ -ATPase inhibitor DCCD, and a significant decrease in the final pHi was also observed. These results support the presence of a plasma membrane H+ -ATPase as a major pHi regulator (15). The pHi recovery was also determined by using chloride-free buffer, as the chloride ion is essential for acid extrusion in Leishmania promastigotes (29). The recovery rates for the R4 and revR4 Leishmania lines were found to be higher than that for WT parasites under these conditions (Table 3).
chain activity involved in the generation of ATP. Under our experimental conditions, sodium azide decreased the ATP levels in all Leishmania lines studied, although the ATP levels in R4 parasites were significantly higher than those observed for the WT and revR4 lines (Fig. 3B), thus suggesting that glycolytically generated ATP is higher in R4 parasites. We therefore inhibited glycolysis in Leishmania lines deprived of glucose but provided with 2-deoxy-D-glucose, a competing substrate for hexokinase, and pyruvate to ensure that the majority of ATP generation was dependent on mitochondrial electron transport and the function of complex V. This inhibition of glycolytic ATP synthesis significantly reduced ATP levels to reach similar values in all Leishmania lines (Fig. 3B).

Increased ATP synthesis by glycolysis in R4 parasites. The TFQ-resistant Leishmania line upregulates glycolysis, whose final step involves the conversion of phosphoenolpyruvate into pyruvate by the enzyme pyruvate kinase. The change in intracellular pyruvate concentration is therefore proportional to the glycolytic activity. We compared the levels of pyruvate between Leishmania lines using a highly sensitive Amplex Red-based fluorescent assay. As shown in Fig. 4A, the pyruvate concentration in R4 parasites was higher (approximately 3-fold) than that observed for the WT line, in agreement with the higher levels of glycolytically generated ATP. The revR4 parasites presented pyruvate levels intermediate between those for the WT and R4 lines. Furthermore, when parasites were pretreated with the glycolysis inhibitor 2-deoxy-D-glucose, the amount of pyruvate in the three lines dropped significantly (Fig. 4A), thereby supporting the hypothesis that the higher pyruvate levels present in the R4 line result from glycolysis. As pyruvate kinase activity is known to be critical in glycolysis, we determined this activity in Leishmania lines; no significant differences between parasite lines were observed (data not shown). Furthermore, as trypanosomatids normally use glucose as their main carbon source, we tested whether R4 parasites increased their glucose uptake as the starting product for glycolysis. Thus, the parasites were cultured in the presence of 0.5 mM [14C]glucose for 10 min, and analysis of the uptake of [14C]glucose showed no significant differences between the different Leishmania lines (data not shown). Additionally, to discard the possibility that the observed higher level of ATP produced by glycolysis in the R4 line could be due to a different pH, which could influence the activity of key enzymes involved in glycolysis, we determined the ATP levels for the Leishmania WT line with different pH values. The results showed that total ATP values were similar at the different Leishmania pH values studied (Fig. 4B).

**TABLE 3. pH recovery in L. major lines**

<table>
<thead>
<tr>
<th>Buffer (pH 7.4)</th>
<th>Final pH</th>
<th>Recovery rate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>R4</td>
</tr>
<tr>
<td>Standard</td>
<td>6.73 ± 0.03</td>
<td>7.13 ± 0.02*</td>
</tr>
<tr>
<td>DCCD (50 μM)</td>
<td>6.52 ± 0.04†</td>
<td>7.03 ± 0.03*†</td>
</tr>
<tr>
<td>Cl−-free</td>
<td>6.37 ± 0.03†</td>
<td>6.87 ± 0.01†+</td>
</tr>
</tbody>
</table>

* BCECF-loaded (5 mg/ml, 30 min) WT, R4, and revR4 promastigotes were acidified by NH4Cl pretreatment (40 mM) for 15 min and resuspended in standard or Cl−-free buffers at pH 7.4. The H+ -ATPase inhibitor DCCD (dicyclohexylcarbodiimide) was added to the standard buffer at 50 μM. Rate of recovery from acidification was determined from the slope of the initial 100 s of recovery, and final intracellular pH (pHi) was determined after 10 min as described by Marchesini and Dacampo (15). Data are the means ± SD from three independent experiments. Significant differences (*, P < 0.05 versus WT parasites; †, P < 0.05 versus standard buffer) were determined by Student’s t test.

[FIG. 3. Effect of TFQ on ATP levels in L. major promastigotes. Promastigotes (4 × 10⁶ per ml) of WT (black histograms), R4 (gray histograms), and revR4 (white histograms) L. major lines were incubated (A) in culture medium with different concentrations (1, 2, 5, 10, and 20 μM) of TFQ for 60 min or (B) in HBS buffer (control), HBS buffer plus 20 mM sodium azide (AZ) to inhibit mitochondrial oxidative ATP generation, glucose-free HBS buffer plus 5 mM 2-deoxy-D-glucose (DEO) and 5 mM sodium pyruvate (PYR) to inhibit glycolytic ATP generation (DEO + PYR), and glucose-free HBS buffer plus 5 mM DEO and 20 mM AZ to inhibit both glycolytic and oxidative ATP generation (AZ + DEO). Afterward, 25-μl aliquots of the parasites were transferred to a 96-well plate, mixed with the same volume of CellTiter-Glo (Promega), and incubated for 10 min in the dark, and the sample bioluminescence was measured. ATP levels were calculated relative to 100% of the control WT. Data are the means ± SD from three independent experiments. Significant differences were determined using Student’s t test (*, P < 0.05 versus WT parasites; †, P < 0.05 versus R4 parasites).]
The results of this study showed that TFQ produces a significant and rapid acidification of pHi after 1 min of treatment (Fig. 5A). Indeed, after 30 min, the pHi values for WT and revR4 parasites were 6.50 and 6.49, respectively, whereas the acidification induced by TFQ in R4 parasites was significantly lower (pHi 6.94) (Fig. 5A). These results support our hypothesis that R4 parasites increase their ATP synthesis in order to maintain the functionality of a plasma membrane \( H^+ / H_1 \)-ATPase involved in the regulation and recovery of pHi as a defensive strategy against the TFQ toxicity. Similar results were observed after treatment with sodium azide (Fig. 5B), which produces a reduction in pHi as a consequence of ATP depletion and nonoptimal functioning of the plasma membrane \( H^+ / H_1 \)-ATPase in \textit{Leishmania}.

DISCUSSION

8-Aminoquinolines, such as sitamaquine and TFQ, have recently been reported to be promising antileishmania drugs (12, 33, 34). Furthermore, we have shown that TFQ induces mitochondrial dysfunction in \textit{Leishmania}, with the resulting decreased oxygen consumption and depolarization of the mitochondrial membrane potential leading to a final apoptosis-like process (3). In this study, we induced experimental resistance to TFQ in order to determine the ability of \textit{Leishmania} to generate resistance to this 8-aminoquinoline and as a strategy to validate the mechanism of action of these compounds in this protozoan parasite. Thus, we selected for a TFQ-resistant \textit{L. major} (R4) line that presents 2.5-fold higher EC50s for TFQ in both the promastigote and intracellular amastigote stages, with an unstable resistant phenotype after 1 month without drug pressure. Experiments to increase the TFQ resistance level further were unsuccessful, although it should be noted that other authors have obtained 5- and 3-fold-higher resistance levels for sitamaquine in the promastigote and intracellular amastigote forms of \textit{Leishmania}, respectively (2). None of the standard leishmanicidal drugs (SbIII, amphotericin B, miltefosine, and paromomycin) displayed a cross-resistance profile with TFQ in the R4 line; cross-resistance was limited to other 8-aminoquinolines, such as sitamaquine and primaquine. The cross-resistance to sitamaquine detected is interesting, as this is a promising oral drug against leishmaniasis.

Reduced drug uptake is one of the main mechanisms of resistance in \textit{Leishmania} (2, 5, 19); however, this mechanism was not relevant for the resistance to TFQ, as decreased TFQ uptake levels were similar for the R4 and revR4 lines. Similarly to sitamaquine (14) and other aminoquinolines (9), TFQ appears to cross the plasma membrane by a pH gradient-driven diffusion process. The R4 parasites present a higher pHi than WT parasites, as does the revR4 line, thus suggesting that these parasites maintain a phenotype with characteristics similar to that of the resistant line even though the TFQ sensitivity sites. The results of this study showed that TFQ produces a significant and rapid acidification of pHi after 1 min of treatment (Fig. 5A). Indeed, after 30 min, the pHi values for WT and revR4 parasites were 6.50 and 6.49, respectively, whereas the acidification induced by TFQ in R4 parasites was significantly lower (pHi 6.94) (Fig. 5A). These results support our hypothesis that R4 parasites increase their ATP synthesis in order to maintain the functionality of a plasma membrane \( H^+ / H_1 \)-ATPase involved in the regulation and recovery of pHi as a defensive strategy against the TFQ toxicity. Similar results were observed after treatment with sodium azide (Fig. 5B), which produces a reduction in pHi as a consequence of ATP depletion and nonoptimal functioning of the plasma membrane \( H^+ / H_1 \)-ATPase in \textit{Leishmania}.
was identical to that for WT parasites. The increase of pH in R4 and revR4 *Leishmania* lines could contribute to the lower TFO accumulation observed for these parasites. Indeed, the change in pH observed as a result of pretreatment with NH₄Cl resulted in a significant reduction in TFO accumulation. Additionally, we observed that R4 and revR4 *Leishmania* lines have a higher capacity to regulate the pH than the WT line, although this regulation is not sufficient to prevent the toxicity of this drug in revR4.

Under physiological conditions, approximately 70% of the total bioenergetic requirements of *Leishmania* are fulfilled by oxidative phosphorylation. This metabolic process produces more ATP molecules from a given amount of glucose than glycolysis. However, when the ability of parasites to generate ATP through mitochondrial oxidative phosphorylation is compromised, parasites are able to adapt alternative metabolic pathways, such as increasing their glycolytic activity, to maintain their energy supply. We have demonstrated that an increase in glycolytic metabolism observed for R4 parasites is associated with increased ATP delivery to essential ATP-consuming cell processes, such as the maintenance of ion-motive ATPases required to retain their pH values. A similar situation has been described for cancer cells, where mitochondrial metabolic defects due, in part, to mutations in mitochondrial DNA, dysfunction of the electron transport chain, aberrant expression of enzymes involved in energy metabolism, and insufficient oxygen available in the cellular microenvironment contribute to an increased dependency on glycolysis (32). This results in increased expression of enzymes required for glycolysis, such as hexokinase II, the enzyme catalyzing the first step of the glycolytic pathway (16). *Leishmania* R4 parasites can increase glycolytic ATP synthesis in several different ways, including upregulation of glycolytic enzymes or an increased uptake of glucose as a carbon source. Additionally, the modification of pH in the R4 line can also change the enzymatic activities of glycolytic enzymes. We have observed that *Leishmania* R4 parasites present an increased accumulation of pyruvate as the end product of glycolysis but with no modification of their pyruvate kinase activity, this kinase being one of the key enzymes involved in glycolysis in trypanosomatids (26). Additionally, R4 parasites do not modify the accumulation of glucose as a carbon source. Thus, *Leishmania* R4 parasites can increase glycolytic ATP either through upregulation of the metabolism using other substrates as a carbon and energy source or by upregulation of other glycolytic enzymes. The first option can be ruled out in light of the fact that the ATP levels after treatment with sodium azide and deoxyglucose, which inhibits both mitochondrial synthesis and glycolysis, are significantly diminished. This indicates that R4 parasites cannot make use of other carbon sources, such as β-oxidation of fatty acids or the catabolism of certain amino acids (22).

Experiments are under way to determine which glycolytic enzymes are upregulated and could therefore be used as molecular markers of TFO resistance in *Leishmania*. Additionally, further studies using a metabolomics-based approach will be undertaken to identify the metabolic pathways associated with TFO resistance.

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7. Reference deleted.


