Identification of new leishmanicidal peptide lead structures by automated real-time monitoring of changes in intracellular ATP

J. Román LUQUE-ORTEGA*, José M. SAUGAR*, Cristina CHIVA†, David ANDREU† and Luis RIVAS*[1]

*Centro de Investigaciones Biológicas (CSIC), Velázquez 144, 28006 Madrid, Spain, and †Universitat Pompeu Fabra, Department of Experimental and Health Sciences, Doctor Aiguader 80, 08003 Barcelona, Spain

Leishmanicidal drugs interacting stoichiometrically with parasite plasma membrane lipids, thus promoting permeability, have raised significant expectations for *Leishmania* chemotherapy due to their nil or very low induction of resistance. Inherent in this process is a decrease in intracellular ATP, either wasted by ionic pumps to restore membrane potential or directly leaked through larger membrane lesions caused by the drug. We have adapted a luminescence method for fast automated real-time monitoring of this process, using *Leishmania donovani* promastigotes transfected with a cytoplasmic luciferase form, previously tested for anti-mitochondrial drugs. The system was first assayed against a set of well-known membrane-active drugs [amphotericin B, nystatin, cecropin A-melittin peptide CA(1-8)M(1-18)], plus two ionophoric polyethers (narasin and salinomycin) not previously tested on *Leishmania*, then used to screen seven new cecropin A–melittin hybrid peptides. All membrane-active compounds showed a good correlation between inhibition of luminescence and leishmanicidal activity. Induction of membrane permeability was demonstrated by dissipation of membrane potential, SYTOX™ Green influx and membrane damage assessed by electron microscopy, except for the polyethers, where ATP decrease was due to inhibition of its mitochondrial synthesis. Five of the test peptides showed an ED_{50} around 1 μM on promastigotes. These peptides, with equal or better activity than 26-residue-long CA(1-8)M(1-18), are the shortest leishmanicidal peptides described so far, and validate our luminescence assay as a fast and cheap screening tool for membrane-active compounds.

Key words: ATP level, *Leishmania*, luminescence, membrane permeabilization, peptide screening.

INTRODUCTION

The intracellular parasite *Leishmania* is the causative agent of leishmaniasis, a disease that threatens 350 million people worldwide, with an annual incidence of 2 million cases (http://www.who.int/emc/diseases/leish/leisdis1.html). It acts as an opportunistic pathogen in immunocompromised patients, with special relevance as a co-infection with HIV [1]. Presently, chemotherapy is the only available treatment, with pentavalent organic antimonials as first-line drugs [2]. However, their efficacy is severely impaired by the increasing incidence of drug-resistant clinical isolates, as well as the frequent severe side effects associated with this treatment [3]. AmB (amphotericin B), allopurinol, paromomycin and, more recently, miltefosine, are either used as alternative drugs in clinics or under advanced stages of clinical trials [2]. Nevertheless, most of them are not without secondary effects; new formulations with lower toxicity have been developed, but their high price precludes their widespread use in developing countries [4]. In order to tackle this problem, definition of new targets and improvement in new drugs with a low probability of developing resistance are urgently needed.

In contrast with the loss of drug efficacy by single point mutation of enzymic targets, drugs causing membrane permeability through physical interaction with lipid components are unlikely to induce resistance, since this would require drastic changes in lipid composition, presumably affecting many membrane-based transport and enzymic systems. AmB and cationic eukaryotic antibiotic peptides are typical prototypes of this group of drugs, interacting respectively with ergosterol [5,6] or acidic phospholipids from the outer leaflet of the plasma membrane [7–9]. These drugs cause dissipation of ionic gradients or large lesions in the membrane leading to a rapid drop in intracellular ATP levels, either wasted by ionic pumps in a futile attempt to recover ionic gradients or by release of ATP into the external medium. In a recent study we described a system to monitor *in vivo* changes in intracellular ATP levels on *Leishmania donovani* promastigotes transfected with a C-terminal mutated form of *Photinus pyralis* luciferase. In contrast to the native form, which is imported into glycosomes, the mutant enzyme is confined in the cytoplasm [10], where a luciferin ester [11], capable of overcoming the poor membrane permeability of luciferin at neutral pH, can react with it. This method was successfully employed to screen for drugs affecting mitochondrial ATP production, oxidative phosphorylation being the major source for ATP in *Leishmania* [12,13].

We have now evaluated this method as a tool to screen for potential new membrane-active leishmanicidal compounds. Our trial set consists of two ionophoric anticoccidial polyethers, salinomycin and narasin [14,15], as well as seven short cecropin A–melittin hybrid peptides [16]. Results are validated both by evaluating membrane permeabilization by alternative techniques and by comparison with well-established membrane-active drugs such as AmB and nystatin [5,17], two antifungal and leishmanicidal polyenes, and the 26-residue cecropin A–melittin hybrid peptide CA(1-8)M(1-18) [8].

Abbreviations used: $\Delta\Psi_m$, mitochondrial membrane potential; AmB, amphotericin B; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein triacetoxymethyl ester; bisoxonol, bis-(1,3-diethylthiobarbituric) trimethine oxonol; DMNPE-luciferin, D-luciferin 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hanks + Glc, Hanks' balanced salt medium plus 10 mM D-glucose; HIFCS, heat-inactivated foetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; rhodamine 123, xanthylium 3,6-diamino-9-[2-(methoxycarbonyl)-phenyl] chloride.

[1] To whom correspondence should be addressed (e-mail luis_rivas@cib.csic.es).

MATERIALS AND METHODS

Parasites

The *L. donovani* 3-Luc strain has been described previously [11]. Briefly, *L. donovani* promastigotes (MHOM/SD/00/1S-2D) were transfected with the expression vector pX63NEO-3Luc, encoding for a *P. pyralis* luciferase form mutated in its C-terminal tripeptide. Parasites were grown at 25 °C in RPMI 1640 medium (Gibco, Paisley, U.K.) supplemented with 10 % HIFCS (heat-inactivated foetal calf serum), 24 mM $NaHCO_3$, 25 mM Hepes, 2 mM L-glutamine, 100 units/ml uniciline, 48 μg/ml gentamycin and 30 μg/ml geneticin (G-418; Gibco) at pH 7.2 (RPMI + HIFCS).

Chemicals

All peptides (Table 1) were prepared in C-terminal carboxamide form by solid-phase synthesis methods using Boc chemistry on *p*-methylbenzhydrylamine resin in a model 430A synthesizer (Applied Biosystems, Foster City, CA, U.S.A.) as described in [18]. AmB, nystatin, salinomycin, narasin and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma (St. Louis, MO, U.S.A.); structures are shown in Table 1. Bisoxonol [bis-(1,3-diethylthiobarbituric) trimethine oxonol], SYTOX™ Green, rhodamine 123 {xanthylium 3,6-diamino-9-[2-(methoxycarbonyl)phenyl] chloride}, DMNPE-luciferin [D-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester] and BCECF-AM [2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein, triacetoxymethyl ester] were obtained from Molecular Probes (Leiden, The Netherlands).

Cell-proliferation measurements

Promastigotes were harvested at late exponential phase, washed twice in Hanks' buffer supplemented with 10 mM D-glucose (pH 7.2; Hanks + Glc) at 4 °C, and resuspended in the same buffer at 2×10^7 cells/ml. These conditions were maintained for the rest of the experiments unless otherwise stated. Parasites (100 μl) were incubated with the drugs for 2 h at 25 °C. Afterwards, 20-μl aliquots of this suspension were taken, added to 1 ml of Hanks + Glc and centrifuged to eliminate unbound reagent. Washed parasites were resuspended in 100 μl of RPMI + HIFCS devoid of Phenol Red, transferred into another 96-well microplate, and incubated for 48 h at 25 °C. Finally, 100 μl of MTT solution (1 mg/ml) in Hanks + Glc was added, and substrate reduction was allowed to proceed for 2 h at 25 °C. Precipitated formazan was solubilized by addition of 100 μl of 10 % (w/v) SDS solution and read in a 450 Bio-Rad ELISA microplate reader equipped with a 600-nm filter [19]. To measure cell viability, the remaining 80 μl (1.6×10^6 promastigotes) of initial parasite suspension were washed with 1 ml of Hanks + Glc, resuspended and assayed for MTT reduction as above. These conditions had earlier been shown to provide a linear correlation with the number of viable cells, plus a very good signal-to-noise ratio [8].

Bioluminescence assays

A 5 mM stock solution of DMNPE-luciferin was made in DMSO. The substrate was added to the promastigote suspension (2×10^7 cells/ml) in Hanks + Glc at a final concentration of 25 μM, and immediately distributed into a 96-well microplate (100 μl/well). Drugs were added to the corresponding wells once the luminescence reached a plateau. This point was considered as time zero, and its luminescence value as 100 %. Changes in luminescence were recorded in a Fluorostat Galaxy microplate reader fitted with luminescence optics (BMG Labotechnologies, Offenburg, Germany), with measurements averaged every 4 s.

Measurement of total ATP

ATP was extracted by addition of 100 μl of 0.9 M HCl to 2×10^7 promastigotes previously incubated with drugs for 60 min. Supernatant was neutralized by addition of 40 μl of 0.8 M Na_2HAsO_4 and 14 μl of 0.4 M NaOH. Then 50 μl of this solution were added to 200 μl of a firefly lantern extract reagent (Sigma) plus 750 μl of water. Luminescence was read in an LKB Bio-Orbit 1250 luminometer at 1 and 3 min after mixing and compared with a standard ATP curve [8]. Protein was estimated by the Bradford assay (Bio-Rad, Munich, Germany).

Promastigote membrane permeabilization

The procedure described by Thevissen et al. [20] was adapted to *Leishmania* promastigotes. Briefly, parasites (2×10^7 cells/ml) were incubated with SYTOX™ Green in Hanks + Glc (1 μM final concentration) for 5 min in the dark and 100-μl aliquots from this suspension were transferred into 96-well microplates. After fluorescence stabilization, drugs were added at the concentrations indicated in the corresponding Tables and Figures. Fluorescence increase, due to binding of dye to intracellular nucleic acids, was measured in a Fluorostat Galaxy microplate reader using 485- and 520-nm filters for excitation and emission wavelengths respectively.

Monitoring of changes in membrane potential

Changes in membrane potential were assessed by the increase in bisoxonol fluorescence, a potential-sensitive anionic dye, after its insertion into the parasite membrane once the cell becomes depolarized. Assays were performed under standard conditions, except for the inclusion of 0.2 μM bisoxonol. Fluorescence changes were monitored in a Fluorostat Galaxy microplate reader. Excitation and emission wavelengths were 544 and 584 nm respectively [8].

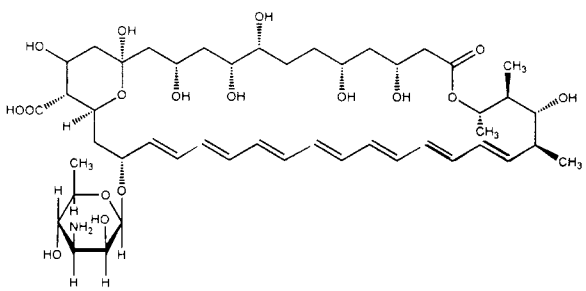
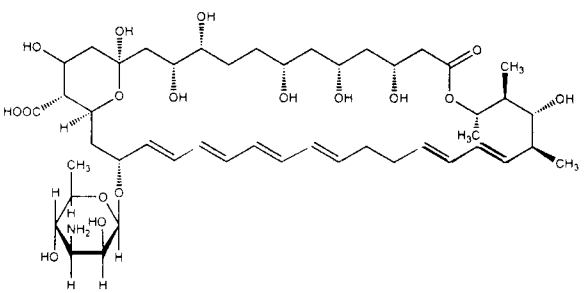
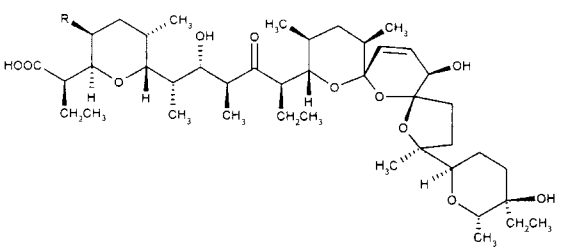
Measurement of intracellular pH changes

Dissipation of H^+/OH^- across the plasma membrane in *Leishmania* was monitored in parasites loaded with the pH-sensitive fluorescence dye BCECF-AM. Briefly, parasites in Hanks + Glc (pH 7.0; 10^9 promastigotes/ml) were incubated for 30 min at 25 °C in the dark with BCECF-AM at a final concentration of 6 μM with gentle stirring. Afterwards, they were washed twice and resuspended at 2×10^7 cells/ml in Hanks + Glc previously adjusted at the corresponding final pH (5.5, 7.0 or 8.0). Fluorescence was measured in a Hitachi F-2000 fluorescence spectrophotometer at 500- and 525-nm excitation and emission wavelengths, respectively. Drugs were added after fluorescence stabilization. Complete permeabilization was defined as that obtained after addition of 0.1 % Triton X-100.

Evaluation of $\Delta\Psi_m$ (mitochondrial membrane potential)

$\Delta\Psi_m$ in intact promastigotes was estimated by rhodamine 123 accumulation [8]. Parasites (2×10^7 promastigotes/ml) were resuspended in Hanks + Glc and incubated (100 μl final volume)

Table 1 Sequences and structures of the assayed compounds

Name	Sequence/Formula
CA(1-8)M(1-18)*	KWKLFKKIGIGAVLKVLTTGLPALIS-NH_2
CM1	KWKLLKKIGAVLKVL-NH_2
CM2	KWKLFKKVLKVL-NH_2
CM3	KWKLFKKILKVL-NH_2
CM4	WKKLFKKLKIL-NH_2
CM5	WKLFKKILKVL-NH_2
CM6	KWKLLLLLLKWK-NH_2
CM7	KLKLLLLLKLK-NH_2
Amphothericin B	
Nystatin	
Salinomycin (R = H) Narasin (R = CH_3)	

* Residues underlined with solid and dotted lines correspond to the sequences of cecropin A and melittin, respectively.

with the respective drugs at 25 °C for 15 or 30 min. Unbound drug was washed by addition of 1 ml of the same medium at 4 °C, and parasites loaded with rhodamine 123 (0.3 μg/ml, 5 min, 37 °C), washed by centrifugation and resuspended in Hanks + Glc at 2×10^6 cells/ml. Rhodamine 123 accumulation was monitored in a Coulter XL EPICS cytofluorimeter (excitation and emission wavelengths of 488 and 525 nm respectively). Parasites depolarized with 10 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) were taken as negative controls.

Oxygen-consumption rates

Oxygen-consumption rates were measured in a Clark oxygen electrode (Hansatech, Kings Lynn, Norfolk, U.K.) at 25 °C, using 0.8 ml of a parasite suspension (10^8 cells/ml) as described in [8]. For salinomycin and narasin, promastigotes were preincubated with the drug for 15 and 30 min before measuring the oxygen-consumption rate.

Electron microscopy

After incubation with drugs for 1 h, promastigotes were washed twice in PBS, fixed in 5 % (w/v) glutaraldehyde in PBS, included with 2.5 % (w/v) OsO_4 for 1 h, gradually dehydrated in ethanol [30, 50, 70, 90 and 100 % (v/v); 30 min each] and propylene oxide (1 h), embedded in Epon 812 resin and observed using a Philips 2200 electron microscope.

Statistical analysis

Data represent the means ± S.D. from triplicate samples. ED_{50} values were calculated by the Litchfield and Wilcoxon procedure and 95 % confidence intervals are included in parentheses. Comparison between cell proliferation and luminescence was carried out by simple linear-regression test, with a 95 % confidence interval.

RESULTS

Inhibition of promastigote proliferation

Compounds to be tested (Table 1) were initially classified into two structural groups: peptides and other drugs. The peptide set contained several peptides derived from the well-known cecropin A–melittin hybrid CA(1-7)M(2-9) [18,21], such as CM1 (a $Phe^5 \rightarrow Leu$ analogue), CM2 [CA(1-7)M(5-9)], CM3 (an analogue of CM2 with a conservative $Val^8 \rightarrow Ile$ replacement), CM4 and CM5, plus two peptides (CM6 and CM7) differing more substantially from the parent structures. Each group included as a positive control one structure known to cause plasma-membrane permeabilization in *Leishmania* promastigotes: the 26-residue cecropin A–melittin hybrid CA(1-8)M(1-18) [8] and AmB [5] respectively.

As shown in Table 2, all compounds inhibited promastigote proliferation at or below the micromolar range. Among the non-peptide drugs, AmB showed the highest leishmanicidal activity (ED_{50}, 0.08 μM), whereas salinomycin (ED_{50}, 4.0 μM) and narasin (ED_{50}, 2.0 μM), differing only in one methyl group at position 4 (Table 1), displayed the lowest.

Within the peptide set, 26-residue CA(1-8)M(1-18) had an ED_{50} of 1.5 μM, whereas the shorter (11–15 residue) analogues were more active, with ED_{50} values within a narrow range (0.5–1.4 μM). Since CM3 showed the highest reproducibility and minimal deviation within this group, and given its low cytotoxicity for eukaryotic cells (6 % haemolytic activity at ED_{90}, 2.5 μM; results not shown), it was chosen for the complete set of experiments, whereas the remaining peptides were only assayed for bioluminescence. CM6 and CM7, structurally less related to the cecropin A–melittin hybrid pattern, did not inhibit parasite proliferation below 5 μM. Nevertheless, the peptides were leishmanicidal, since their abilities to inhibit cell viability and cell proliferation were coincident (Table 2).

Bioluminescence and total ATP decrease

In order to discard false positives (i.e. direct action on luciferase), compounds were tested for inhibition of purified luciferase prior to their assay on promastigotes. No inhibition beyond 5 % was observed (results not shown).

Table 2 Effect of the different compounds on some viability parameters of 3-Luc *L. donovani* promastigotes

ED_{50} values were estimated by the Litchfield and Wilcoxon procedure. The 95 % confidence interval (CI) values are shown in parentheses. Assays were carried out as described in the Materials and methods section. For ATP extraction, cells were treated with the drugs at their respective ED_{50} values. ATP was measured after acidic extraction with 0.9 M HCl as described in the Materials and methods section.

	ED_{50} (μM)			
Drug	Proliferation	Viability	Luminescence	ATP extracted (%)
CA(1-8)M(1-18)	1.5 (0.8–2.1)	1.3 (1.1–1.4)	0.8 (0.4–1.8)	48
CM1	0.5 (0.3–0.9)	0.6 (0.4–0.9)	0.4 (0.2–0.9)	–
CM2	1.4 (1.1–1.6)	1.4 (1.2–1.6)	0.7 (0.5–0.8)	–
CM3	0.7 (0.6–0.9)	0.8 (0.7–0.9)	0.4 (0.2–0.9)	54.8
CM4	0.9 (0.7–1.1)	0.9 (0.8–1.0)	0.2 (0.2–0.3)	–
CM5	1.2 (0.7–2.1)	1.1 (0.8–1.5)	0.5 (0.4–0.6)	–
CM6	> 5	> 5	> 2.5	–
CM7	> 5	> 5	> 2.5	–
AmB	0.08 (0.06–0.1)	0.09 (0.07–0.1)	0.09 (0.02–0.3)	53.5
Nystatin	1.4 (0.4–4)	1.1 (0.6–2.5)	0.6 (0.3–1.0)	47.5
Salinomycin	1.9 (1.7–2.1)	2.1 (1.8–2.6)	4.3 (1.5–12.0)	54
Narasin	0.5 (0.4–0.6)	0.5 (0.4–0.6)	2 (1.5–2.5)	67.6

Inhibition of luminescence on transfected promastigotes was dose-dependent for all drugs (Figure 1). With peptides, a fast decrease in luminescence was observed, reaching final values in less than 10 min. Polyene and polyether antibiotics displayed slower time courses. Within the assay concentration range, the decrease in luminescence systematically exceeded that in proliferation; sole exceptions were salinomycin and narasin, suggesting different mechanisms of action for these polyethers (Table 2).

We next tried to correlate this inhibition of luminescence with the decrease in ATP levels caused by the compounds acting on the membrane. The intracellular ATP content of promastigotes treated at ED_{50} with the compounds was measured by standard ATP extraction. After 1 h incubation, initial ATP levels (22.5 nmol/mg of protein) were reduced by roughly one-half, in good agreement with the dose used and with previous results using inhibitors of oxidative phosphorylation [11]. The only exception was narasin, with an ATP reduction of only 30 % (Table 2).

In order to gain insight into the molecular mechanisms underlying these results, the two more likely hypotheses, membrane permeabilization and inhibition of oxidative phosphorylation, were tested by a variety of techniques as summarized in Scheme 1.

Membrane-permeabilization assays

Verification of the first hypothesis listed above involved analysis of membrane damage by three different methods (Scheme 1C). The first one, i.e. increase in SYTOX fluorescence, required a large enough membrane lesion to allow the dye to cross the plasma membrane and bind to intracellular nucleic acids. This was the case with CA(1-8)M(1-18), CM3, AmB and nystatin (Figure 2), but not with polyether compounds, even at 13 μM, a concentration far exceeding complete inhibition of proliferation. CA(1-8)M(1-18) and CM3 at 2.5 and 2.0 μM, respectively, inhibited parasite viability by more than 90 %, with fluorescence values close to maximal, i.e. those obtained by parasite permeabilization with Triton X-100. In contrast, fluorescence at 0.5 μM AmB, a concentration causing complete lethality, only reached 70 % (Figure 2).

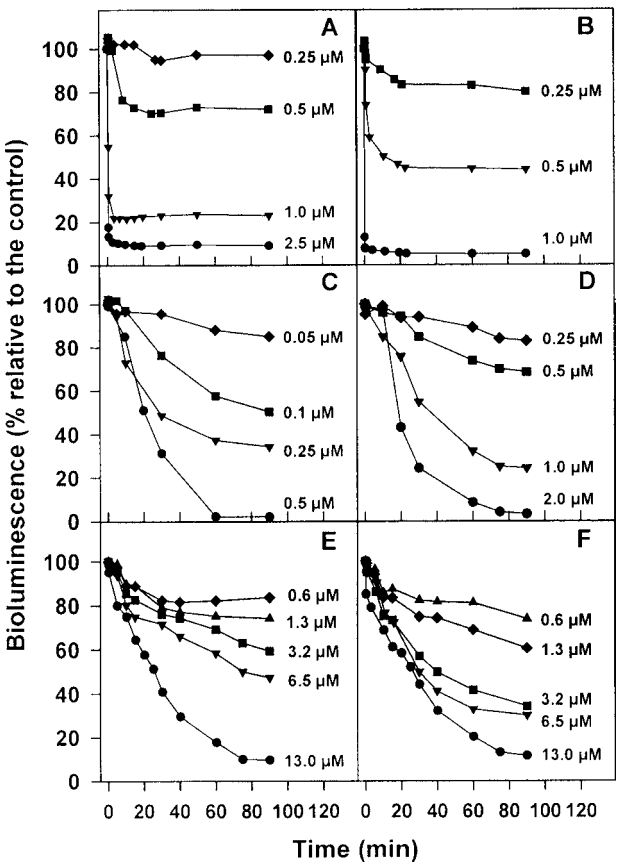


Figure 1 Intracellular ATP decrease after drug addition assessed by luminescence *in vivo*

Promastigotes (2×10^7 cells/ml) from *L. donovani* 3-Luc strain were incubated with cell-permeable ester DMNPE-luciferin (25 μM) and luminescence was monitored until it reached a constant value, at which point ($t = 0$) the corresponding drug was added. Luminescence decay was measured and corrected for that of control parasites. (**A**) CA(1-8)M(1-18); (**B**) CM3; (**C**) AmB; (**D**) nystatin; (**E**) salinomycin; (**F**) narasin.

The second method measured dissipation of membrane potential caused by the collapse of ionic gradients across the membrane. This process was monitored by the increase in bisoxonol fluorescence, which proceeded in a dose-dependent manner for AmB, nystatin, CM3 and CA(1-8)M(1-18) (Figure 3). Full depolarization was defined as the fluorescence value for 2.5 μM CA(1-8)M(1-18) [8]. AmB at 0.25 μM matched this value, though a slight rebound was observed after 10 min, an effect absent for CA(1-8)M(1-18), possibly reflecting a limited capacity of the cell to repair initial damage (Figure 3). In contrast, neither salinomycin nor narasin increased bisoxonol fluorescence substantially.

Thirdly, dissipation of H^+/OH^- gradients across the plasma membrane was also assayed. *Leishmania* promastigotes are known to maintain an intracellular pH of 6.8 over a wide range of external pHs (5.5–8.0) [22]. For parasites loaded with the pH-sensitive dye BCECF-AM, treatment with salinomycin or narasin caused only a slow and partial equilibration between internal and external pHs. In contrast, nystatin and AmB induced a fast, almost complete pH equilibration across the membrane. The effect was even more pronounced for CM3 (Figure 4), whose pattern closely resembled the one previously reported for the longer hybrid CA(1-8)M(1-18) [8].

Taken together, these results confirm membrane depolarization as the basis for the leishmanicidal mechanism of peptides and polyenes. For both types of antibiotic, a high correlation between variation in bisoxonol fluorescence and inhibition of proliferation was found (Table 3). On the other hand, variations in SYTOX™ Green uptake reveal different levels of plasma-membrane damage by the antibiotics: peptides induce large disruptions whereas polyenes form small, discrete pores and polyethers do not have the membrane as a main target.

Modification of *L. donovani* bioenergetic parameters

As shown above, the decrease in intracellular ATP levels was conclusively linked to plasma-membrane permeabilization for peptides and polyenes, but not for polyethers (Scheme 1C). This prompted us to test the other alternative, namely the inhibition of ATP production, as assessed by changes in oxygen-consumption rate and $\Delta\Psi_m$.

At short incubation times (up to 30 min), CM3 did not cause a significant change in rhodamine 123 accumulation (Figure 5), as expected for fast plasma-membrane permeabilization and reported for CA(1-8)M(1-18) [8]. In contrast, the typical mitochondrial uncoupler FCCP caused a $>50\%$ decrease in the initial fluorescence intensity. On the other hand, both salinomycin and narasin decreased rhodamine 123 fluorescence by 34 and 51%, respectively, after 15 min incubation despite their poor performance in membrane-permeabilization assays (Figure 5).

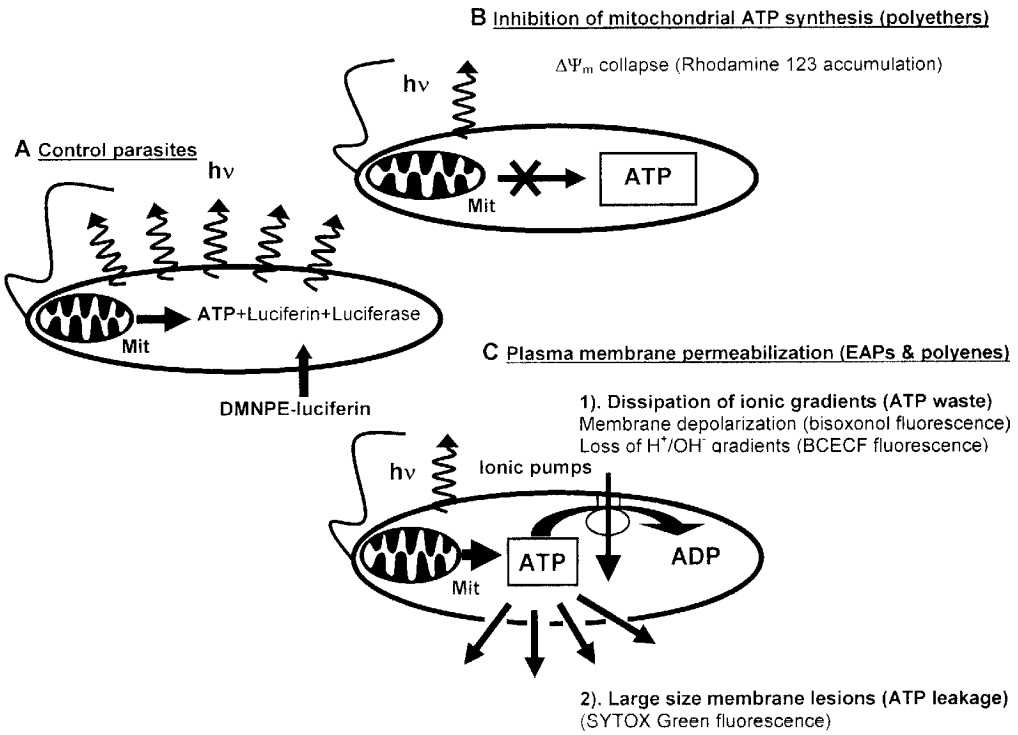
In order to correlate $\Delta\Psi_m$ decrease with a possible misfunction of the oxidative phosphorylation, oxygen-consumption rates were measured. Parasites incubated with polyethers at their respective ED_{50} reduced the oxygen-consumption rate (14 nmol of $O_2 \cdot min^{-1} \cdot 10^8$ cells^{-1} for control cells) by 25%. Nevertheless, this process required a minimal incubation time of around 15 min. This suggests an intracellular re-equilibration of the drug to reach its intracellular target, most likely mitochondria, the main source for ATP production in *Leishmania* [12]. For CM3 and CA(1-8)M(1-18), the oxygen-consumption rate was also inhibited but $\Delta\Psi_m$ was maintained, possibly a consequence of a loss of cellular homeostasis, as reported previously for CA(1-8)M(1-18) [8]. These results are compared with those for membrane permeabilization in Table 3.

Electron microscopy

The extent and frequency of parasite damage were dose-dependent for all assayed compounds. For concentrations below the ED_{50}, only slight effects and/or a low number of injured cells were observed, whereas at or beyond their ED_{50} most parasites showed severe structural damage. Nevertheless, clear differences among drug types were found: for parasites treated with peptides or polyenes, plasma-membrane disruption and substantial detachment from the subpellicular layer of microtubules (Figures 6B, 6E and 6F) was observed. In contrast, for parasites treated with salinomycin or narasin, the membrane appeared only slightly ruffled but extensive cytoplasm vacuolization was found (Figures 6C and 6D).

DISCUSSION

The physical matrix of *Leishmania* membranes is considered a promising pharmacological target, since resistance against drugs disrupting membrane structure through stoichiometrical interaction with lipid components has not yet been reported clinically [23], only in the laboratory and under increasing AmB pressure [24]. Resistant *Leishmania tarentolae* phenotypes against this drug, involving amplification and simultaneous



Scheme 1 Diagram of the luminescence assay and cell processes affected by the drugs

(**A**) Control parasites. Luminescence in luciferase-transfected parasites is produced in the presence of the cell-permeable caged substrate DMNPE-luciferin, converted to luciferin by cytoplasmic esterases. (**B**) Inhibition of mitochondrial ATP synthesis. Decrease of ATP-dependent luminescence correlated with loss of $\Delta\Psi_m$. (**C**) Plasma-membrane permeabilization. Luminescence decreases by ATP loss caused by (1) dissipation of ionic gradients, whereupon ionic pumps hydrolyse ATP to recover the gradients, or (2) ATP leakage through lesions in the membrane. Oxygen-consumption rate and electron microscopy, two non-specific techniques, are applied in both (**B**) and (**C**) and were not shown. Mit, mitochondrion.

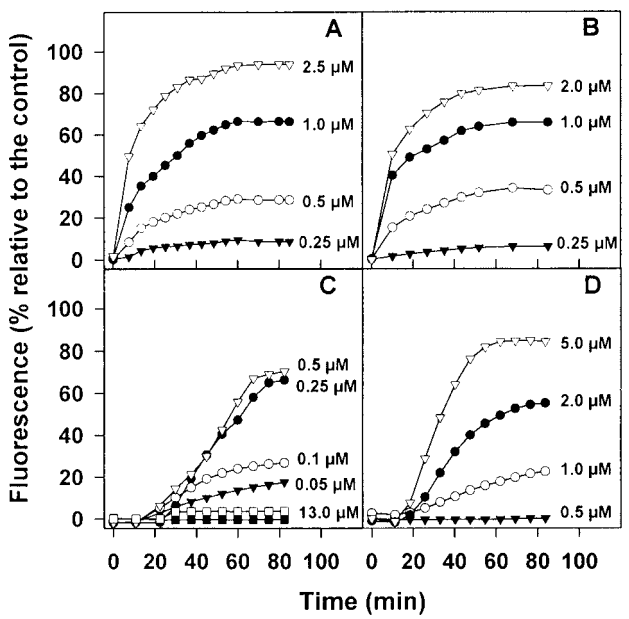


Figure 2 SYTOX™ Green influx into *L. donovani* 3-Luc promastigotes after drug addition

Parasites (2×10^7 cells/ml) were incubated with 1 μM SYTOX™ Green in Hanks + Glc. Once basal fluorescence reached a constant value ($t = 0$), the corresponding drug was added, and the increase in fluorescence (485 nm excitation, 520 nm emission) due to dye binding to intracellular nucleic acids was plotted as a percentage of the fluorescence relative to that of parasites fully permeabilized by 0.1 % Triton X-100. (**A**) CA(1-8)M(1-18); (**B**) CM3; (**C**) AmB (0.05–0.5 μM) and 13 μM narasin (□) and 13 μM salinomycin (■); (**D**) nystatin.

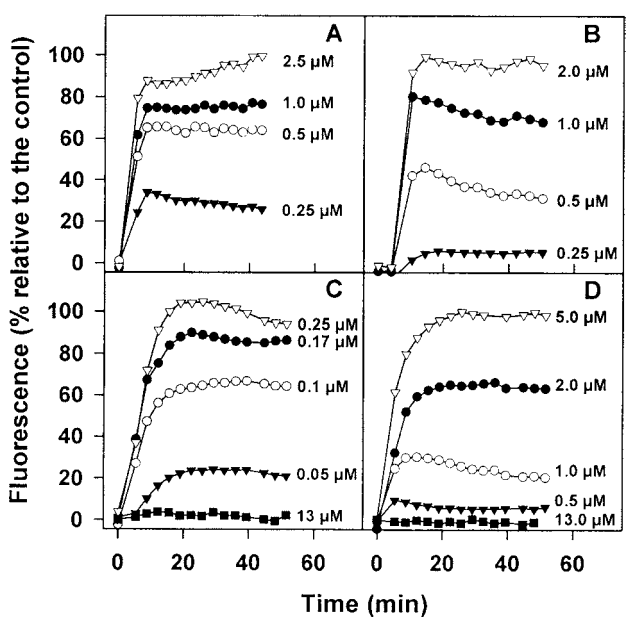


Figure 3 Changes in plasma-membrane potential in *L. donovani* 3-Luc promastigotes after treatment with different reagents

Variation in plasma-membrane potential was monitored by changes in the fluorescence of the potential-dependent dye bisoxonol (544 nm excitation, 584 nm emission) after drug addition ($t = 0$) to the parasite suspension (2×10^7 promastigotes/ml), and plotted as a percentage of the fluorescence relative to maximal parasite depolarization [as achieved by 2.5 μM CA(1-8)M(1-18)]. (**A**) CA(1-8)M(1-18); (**B**) CM3; (**C**) AmB (0.05–0.25 μM) or 13 μM salinomycin (■); (**D**) nystatin (0.5–5 μM) or 13 μM narasin (■).

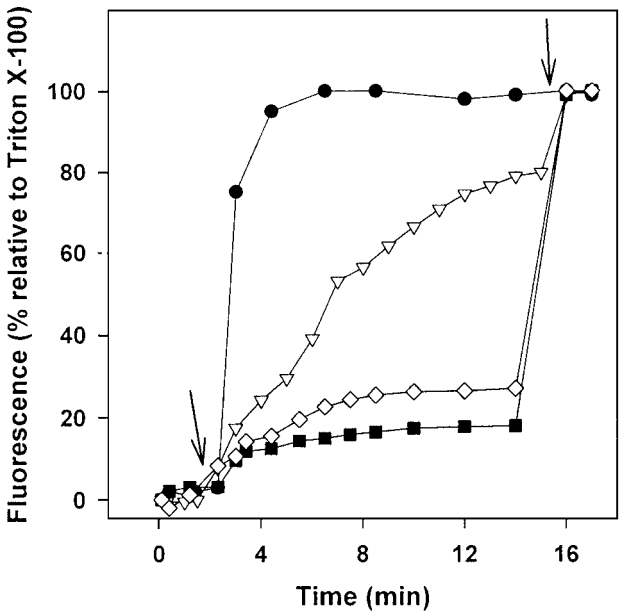


Figure 4 Dissipation of H^+/OH^- gradients across the plasma membrane of *L. donovani* promastigotes after addition of different drugs

Internal pH changes in *L. donovani* 3-Luc as assessed by the variation of fluorescence of parasites (2×10^7 promastigotes/ml) previously loaded with BCECF (500 nm excitation, 525 nm emission), and resuspended in Hanks + Glc, pH 8.0. 100 % fluorescence is taken as the value after parasite permeabilization by 0.1 % Triton X-100. ●, 2.5 μM CM3; ▽, 0.5 μM AmB; ◇, 13 μM narasin; ■, 13 μM salinomycin. The first and second arrows indicate drug and Triton X-100 addition, respectively.

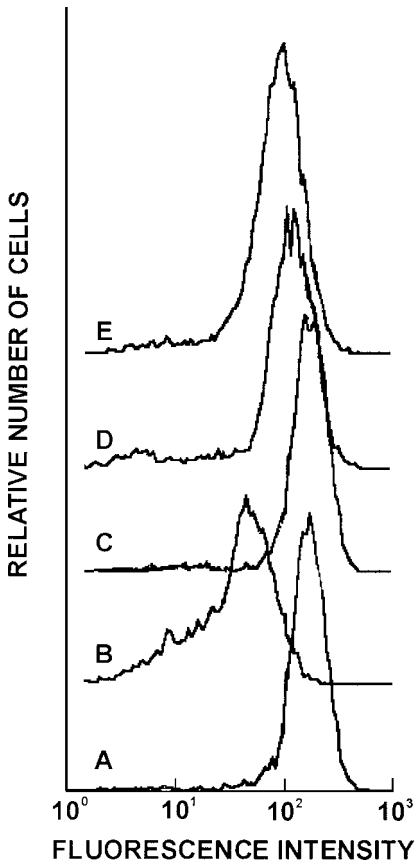


Figure 5 $\Delta\Psi_m$ of *L. donovani* measured by rhodamine 123 accumulation after treatment with different drugs

3-Luc parasites were loaded with rhodamine 123 as described in the Materials and methods section; dye accumulation was measured by cytofluorimetry (488 nm excitation, 525 nm emission). (**A**) Control parasites; (**B**) 10 μM FCCP; (**C**) 5 μM CM3; (**D**) 4 μM salinomycin; (**E**) 2 μM narasin.

mutation of several genes, have also been obtained under similarly severe conditions [25].

In tune with the demand for new membrane-active leishmanicidal agents, we have designed an efficient luminescence method, based on monitoring the decrease of intracellular ATP levels that allows fast, real time, *in vivo* screening of candidate compounds. The method had been successfully validated earlier for mitochondrial drugs affecting ATP production [11].

According to the luminescence/proliferation ED_{50} ratio, our set of compounds can be broadly classified into two different groups. The first group is composed of salinomycin and narasin, two ionophoric polyether antibiotics for which the ED_{50} values for proliferation are lower than the ED_{50} values for luminescence. Both of them have been used previously in poultry coccidiosis or to improve cattle feed [26,27]. The mechanism of action for these polyethers is based on their antiporter activity: they can form lipophilic complexes with either protons or alkaline ions, each with different diffusibility across the membrane [14,28–30], creating an osmotic unbalance across organelle membranes that results in their subsequent swelling [31]. Salinomycin and narasin have higher affinity for K^+ than for Na^+ [14,28,29], contrary to monensin, a perturbing agent for protein traffic in *Leishmania* promastigotes [32,33]. Salinomycin and narasin cause extensive damage to intracellular structures of *Eimeria* sporozoites, including mitochondria [15,34,35], and for the former compound a strong inhibition of coupled and uncoupled oxidative phosphorylation has been reported [36]. Leishmanicidal

Table 3 Variation in 3-Luc *L. donovani* promastigote bioenergetic and membrane-permeabilization parameters upon drug treatment at their respective ED_{50} for proliferation

	Luminescence inhibition*	SYTOX Green uptake†	Increase in bisoxonol fluorescence‡	Inhibition of O_2 consumption*	Inhibition of rhodamine 123 accumulation*
CA(1-8)M(1-18)	81.7 ± 5.1	73.0 ± 4.1	84.2 ± 6.3	41.8 ± 3.5	0.2 ± 0.1
CM3	71.6 ± 4.2	46.7 ± 8.2	45.0 ± 4.2	32.6 ± 4.8	0.3 ± 0.1
AmB	49.0 ± 10.1	27.0 ± 3.4	60.0 ± 10.1	3.2 ± 1.2	6.2 ± 0.2
Nystatin	82.0 ± 3.3	37.0 ± 5.1	40.1 ± 6.1	2.2 ± 0.7	5.4 ± 0.5
Salinomycin	25.0 ± 3.8	1.5 ± 0.8	2.8 ± 1.3	23.4 ± 2.1	34.1 ± 2.6
Narasin	27.0 ± 2.1	2.6 ± 1.2	3.6 ± 1.9	25.0 ± 4.3	51.0 ± 1.4

* Percentage relative to untreated parasites.
† Percentage relative to maximal permeabilization (0.1% Triton X-100).
‡ Percentage relative to highest depolarization [2.5 μM CA(1-8)M(1-18)].

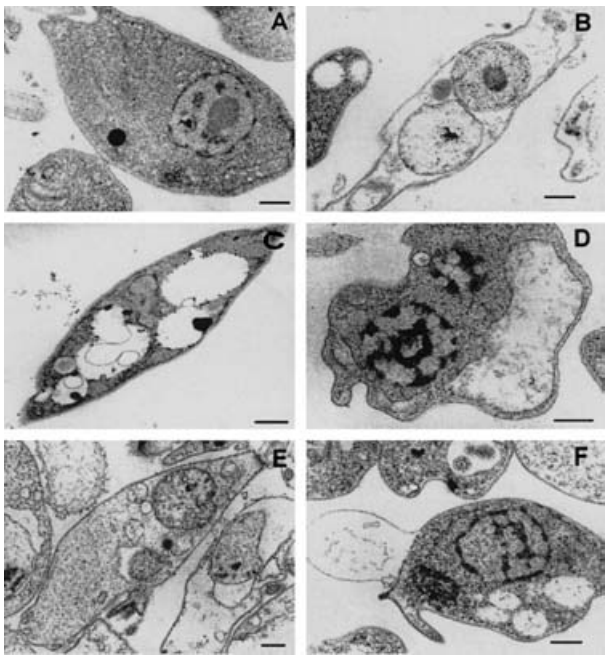


Figure 6 Electron microscopy of *L. donovani* 3-Luc promastigotes treated with different drugs

(**A**) Control parasites; (**B**) 2 μM CM3; (**C**) 5 μM narasin; (**D**) 10 μM salinomycin; (**E**) 0.5 μM AmB; (**F**) 1.5 μM nystatin. Scale bars, 0.5 μm.

activity for these compounds is in agreement with their inhibition of both oxidative phosphorylation and oxygen-consumption rate. Hence the drop in ATP levels and associated luminescence, together with the longer incubation time required to achieve full inhibition, since the polyether must equilibrate among the internal structures of the parasite. In fact, a greater decrease in luminescence (70 %) was obtained if the parasites are incubated with polyethers for 6 h prior to the luminescence assay (results not shown). During at least the first minute after salinomycin addition to *Eimeria* sporozoites, the plasma membrane appeared almost unharmed, even at the lethal concentration for this compound [15]. This is in full agreement with our results on *Leishmania* promastigotes, where the plasma membrane did not suffer extensive modifications and preservation of membrane potential, H^+/OH^- gradients and impermeability to vital dyes was observed at concentrations higher than ED_{90}. Preferential activity on mitochondria rather than plasma membrane was reported for nigericin [37], although additional intracellular targets, such as acidocalcisomes, cannot be excluded completely [38]. Nevertheless, the therapeutic index of salinomycin for *Leishmania* is much lower than for *Plasmodium*, precluding its use as a leishmanicidal reagent in clinics [14].

The second group of compounds is characterized by a lower ED_{50} for luminescence than for proliferation. Within this group, we have studied two structurally unrelated compounds, polyenes and cecropin A–melittin peptides. AmB and nystatin are known for their fungicidal and leishmanicidal activity, with a killing mechanism directly related to plasma membrane permeabilization [5,6,17,39,40]. Membrane depolarization was the parameter most sensitive to AmB concentration, followed by luminescence, SYTOX entrance and parasite proliferation. This fits the model proposed by Cohen [6] to account for *Leishmania* killing by AmB: at low concentration, a non-aqueous AmB channel is formed without the concourse of sterols; this causes K^+ efflux, as reflected by plasma-membrane depolarization. Nevertheless, this channel does not cause *Leishmania* killing, and despite the loss of K^+ the cell is capable of maintaining its viability, at least for a certain time; hence its capacity to synthesize ATP, preventing a large decrease in luminescence. After a certain threshold concentration, an aqueous pore is formed, inducing cell swelling and formation of larger lesions, which cause *Leishmania* killing, inhibition of proliferation, influx of impermeable vital dyes and extensive membrane damage, as described previously [6].

The other compounds in this group are short cecropin A–melittin peptides [16]. An important goal in antibiotic peptide design is to combine activity with minimal size. In cecropin A–melittin analogues, the original CA(1-8)M(1-18) prototype was shortened to 11–15 amino acid residues without a substantial decrease in antibacterial activity [18]. In *Leishmania*, similar results have been observed, with N-terminal fatty acid acylation preserving or even increasing the activity of the peptide [21].

In the present study, CA(1-8)M(1-18), which we consider a standard for this type of compound, produced a parallel decrease in both luminescence and membrane potential, together with an increase in SYTOX influx. This agrees with previous results showing a loose size discrimination in the release of encapsulated FITC–dextrans from permeabilized liposomes [41]. On the other hand, inhibition of promastigote proliferation was consistently less susceptible than the aforementioned parameters. A partial recovery of the parasite from the initial hit, either by peptide degradation or by translocation into the membrane inner leaflet, as proposed by the two-state model [42], can account for these results. Luminescence inhibition thus turned out to be a more sensitive parameter than proliferation, in terms of identifying candidates for further optimization, i.e. peptides performing poorly as proliferation inhibitors but causing some membrane damage.

The potential of this method to screen for new lead compounds was tested on a set of new cecropin A–melittin analogues of 11–15 residues long. For five of them, showing a higher leishmanicidal activity than CA(1-8)M(1-18), ED_{50} for inhibition of luminescence was 2–3 times lower than for proliferation. On the other hand, cytotoxicity for higher eukaryotic cells was much lower, as expected from the removal of the haemolytic cationic region at the C-terminus of melittin. Haemolysis of human erythrocytes by CM3 at its ED_{90} for *Leishmania* was only 6 % (results not shown).

Despite the length reduction relative to the CA(1-8)M(1-18) prototype, all five peptides preserved a membrane-permeabilization ability, causing significant enough lesions to allow luciferase leakage (results not shown). This was demonstrated conclusively for CM3, which caused complete depolarization of the membrane, in tune with SYTOX influx and with membrane damage assessed by electron microscopy. Action on other intracellular targets may be partially compatible with this scenario, as translocation of the peptide across the membrane will cause a transient perturbation of its structure [42]. Nevertheless, recovery of the initial plasma membrane potential was never observed. Action of CM3 on mitochondria, a likely target considering that ATP production in *Leishmania* is mainly the product of oxidative phosphorylation [13], was discarded, since rhodamine 123 accumulation did not change relative to control parasites, even at peptide concentrations far exceeding ED_{90}. The inhibition of oxygen consumption observed with CM3 is a consequence of the irreversible deterioration of the parasite after loss of internal homoeostasis, as described previously for CA(1-8)M(1-18) [8].

Other membrane-active eukaryotic peptides shorter than 20 amino acids, such as indolicidin and the temporins, are also able to promote membrane permeabilization by massive

accumulation at the outer leaflet of the membrane, without requiring the formation of a membrane-spanning channel, too short for a helical peptide of only 12 residues [43,44].

In conclusion, we have described a useful tool with which to screen and analyse membrane-active leishmanicidal compounds in a fast, sensitive and automatable manner. Furthermore, the method discriminates between membrane-damaging agents, causing a fast drop in ATP levels, and other compounds causing the same effect but either acting at the mitochondria or producing membrane damage by other internal processes, with much slower kinetics. This has allowed us to identify new leishmanicidal cecropin A–melittin peptides acting on promastigotes. In recent years, new expression vectors for reporter proteins in amastigotes have been developed [45,46]. This allows adaptation of our technique to this parasite form, the obvious target for leishmanicidal drugs in the vertebrate host. Work along this direction is in progress in our laboratories.

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