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Defeating *Leishmania* resistance to Miltefosine (hexadecylphosphocholine) by peptide-mediated drug smuggling: A proof of mechanism for trypanosomatid chemotherapy ☆

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ABSTRACT

Miltefosine (hexadecylphosphocholine, HePC), the first orally active drug successful against leishmaniasis, is especially active on the visceral form of the disease. Resistance mechanisms are almost exclusively associated to dysfunction in HePC uptake systems. In order to evade the requirements of its cognate receptor/translocator, HePC-resistant *Leishmania donovani* parasites (R40 strain) were challenged with constructs consisting of an ω-thiol-functionalized HePC analogue conjugated to the cell-penetrating peptide (CPP) Tat(48–60), either through a disulfide or a thioether bond. The conjugates enter and kill both promastigote and intracellular amastigote forms of the R40 strain. Intracellular release of HePC by reduction of the disulfide-based conjugate was confirmed by means of double tagging at both the CPP (Quasar 670) and HePC (BODIPY) moieties. Scission of the conjugate, however, is not mandatory, as the metabolically more stable thioether conjugate retained substantial activity. The disulfide conjugate is highly active on the bloodstream form of *Trypanosoma b. brucei*, naturally resistant to HePC. Our results provide proof-of-mechanism for the use of CPP conjugates to avert drug resistance by faulty drug accumulation in parasites, as well as the possibility to extend chemotherapy into other parasites intrinsically devoid of membrane translocation systems.

1. Introduction

Among human protozoan diseases, leishmaniasis ranks second only to malaria in terms of mortality and morbidity, with an incidence of 2 million cases a year in 2010, of which 0.5 million correspond to visceral leishmaniasis (VL), fatal if untreated [1]. A reliable human vaccine remains elusive, and chemotherapy has traditionally relied on pentavalent antimonials, dating back over 50 years and imperiled by resistance phenomena in endemic foci [2]. More recently miltefosine (hexadecylphosphocholine, HePC), liposomal amphotericin B and paromomycin [3] have been added as first-line drugs. Clearly, novel therapeutic approaches, particularly those aimed at bypassing or inhibiting resistance mechanisms and currently limited to combination therapies [4], are required to curb this negative scene.

HePC, the only leishmanicidal clinical drug orally active on VL, is currently administered as an alternative to antimonials in areas of India where resistance to these drugs is rampant. Although no HePC-resistant clinical isolates have yet been reported, they can somehow be anticipated given that (i) resistant strains are relatively easy to generate in the laboratory [5]; (ii) in animal models, resistant parasites retain the virulence of the wild type [6]; (iii) partial cross-resistance with antimonials has been observed in both laboratory [7] and field [8] isolates, and (iv) irregular treatment compliance and poor outpatient surveillance (hospitalization is only required in extreme cases) may eventually succeed in generating resistance in the field.

HePC resistance is easily induced by growing *Leishmania* parasites under increasing drug concentrations [5]. Although substantial (low-

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[1] This work is dedicated to the memory of Prof. Francisco Amat-Guerri, who passed away during the preparation of this article.

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mM) intracellular HePC concentrations are rapidly achieved in susceptible promastigotes, a resistant phenotype can soon be observed [9], characterized by a severely diminished intracellular HePC concentration, due to overexpressed efflux pumps [10] and, to a much larger extent, to the failure of the HePC-dedicated transporter, the aminophospholipid translocase LdMT (*Leishmania donovani* miltefosine transporter). Variations in subunit expression of this uptake system underlie *Leishmania* HePC susceptibility, at both inter- and intra-species level [11]. This is yet another example of how the selective permeability of plasma membrane to various bioactive molecules poses a serious obstacle for effective drug targeting [12]. In such a scenario, the development of molecular tools for bypassing the resistance posed by faulty uptake mechanisms becomes a highly desirable goal.

Cell-penetrating peptides (CPPs) are ideal candidates in this regard, due to their ability to transport various bioactive cargo molecules – complexed or covalently conjugated – across plasma or endosomal membranes into target cells [13–15]. The substantial literature on CPPs focuses mostly on the release into mammalian [16,17], much less on protozoan cells [18–20]. For trypanosomatids like *Leishmania* in particular, a plasma membrane structure and functionality quite unlike that of mammalian cells may underlie the relative paucity and lack of conclusiveness of reports on CPP applications for these parasites.

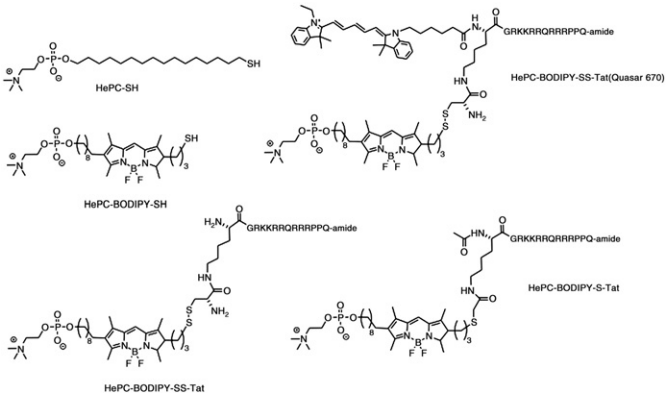
In the present work, we have used Tat(48–60) (GRKKRRQRRRPPQ-amide), a CPP prototypical for mammalian cells, to redress the failure of HePC-resistant R40 *L. donovani* strain to uptake HePC, and to expand the range of HePC-susceptible parasites into the naturally HePC-resistant African trypanosome *Trypanosoma brucei brucei*. Our approach uses conjugates with either a redox-scissile (disulfide) or stable (thioether) linkage of HePC to the CPP, which enter and kill R40 parasites. For the disulfide-linked conjugate, intracellular reductive release of HePC from the CPP was established, by double tagging of both HePC and Tat moieties with fluorescent dyes. Thus, by disregarding the non-functional LdMT transporter, CPP vector-based delivery overcomes HePC resistance and facilitates the killing of R40 promastigotes. Our construct also penetrates and reduces the parasite load of macrophages infected with the R40 strain, predictably by accumulation within the intracellular amastigotes followed by reductive cleavage and HePC release.

This first account – to the best of our knowledge – of reversing the therapeutic failure of a leishmanicidal drug by CPP-mediated delivery constitutes proof-of-mechanism of the potential of CPP vehicles in trypanosomatid chemotherapy.

2. Materials and methods

2.1. HePC-Tat conjugates

The synthesis of HePC analogues with ω-thiol-functionalization (HePC-SH) and with an internal BODIPY tag has been previously described [21,22]. For the present work, an analogue featuring both thiol and BODIPY groups (HePC-BODIPY-SH) was synthesized. Both HePC-SH and HePC-BODIPY-SH building blocks were subsequently conjugated to a Tat(48–60) sequence (hereafter named Tat) bearing (or not) a Quasar 670 fluorescent moiety and appropriate modifications to allow conjugation. Tat and its variants were conveniently made by Fmoc-based solid phase peptide synthesis methods and were conjugated to HePC-BODIPY either via disulfide (HePC-BODIPY-SS-Tat, reduction-sensitive) or thioether (HePC-BODIPY-S-Tat, reduction-stable) linkages (see Scheme 1 for structures). In the first case, the disulfide conjugate resulted from reaction of HePC-BODIPY-SH with the Npys (3-nitro-2-pyridylsulfenyl)-activated thiol group of a Cys residue added to the N-terminus of Tat. For the thioether conjugate, Tat was N-terminally elongated – while still on the solid phase – with an ε-Mmt (4-methoxytrityl)-protected Lys residue, followed by selective deprotection of the ε-amino group, acylation with chloroacetic acid and deprotection/ cleavage. After purification, the resulting chloroacetyl-functionalized Tat underwent nucleophilic substitution by HePC-BODIPY-SH to give the target thioether conjugate. A detailed description of the synthesis of these constructs exceeds the scope of this paper and will be given elsewhere. For Tat and all conjugates, purities of 95% or higher were established by HPLC, and identities satisfactorily confirmed by MALDI-TOF MS.



Scheme 1. Structures of the HePC surrogates.

2.2. Cell culture

L. donovani promastigotes (strain MHOM/ET/67/L82) (WT), and the HePC resistant strain MHOM/ET/67/L82R40 (R40), kindly provided by Prof. S. L. Croft (London School of Hygiene and Tropical Medicine), were grown at 26 °C in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, gentamycin, penicillin, and 2 mM glutamine. For the resistant strain, 40 μM HePC was added to the growth medium to maintain the resistant phenotype. As standard procedure, prior to each assay, *Leishmania* parasites were harvested at late exponential phase and washed twice in Hanks balanced salt solution buffer supplemented with 10 mM D-glucose (pH 7.2) (HBSS-Glc).

Peritoneal macrophages from BALB/c mice were obtained by sodium thioglycolate elicitation of mice three days before extraction according to the protocol approved by the animal welfare committee of CIB-CSIC; cells were harvested by peritoneal washing of the intraperitoneal cavity, and maintained in RPMI + HIFCS under 5% CO_2 atmosphere [23].

The bloodstream forms (BSF) of *T. b. brucei* 221 were cultured at 37 °C in 5% CO_2 in HMI-9 medium supplemented with 10% fetal bovine serum and 10% Serum Plus (SAFC Biosciences, Spain,) [24]. Unless otherwise stated, samples were assayed by triplicate, and experiments were repeated at least twice regardless of the parasite assayed.

2.3. Measurement of cytotoxic effects

For inhibition of proliferation, R40 promastigotes were seeded in full growth medium devoid of phenol red at 2×10^6 cells/ml. Tat, HePC-BODIPY, HePC-BODIPY-SS-Tat, and HePC-BODIPY-S-Tat, were dissolved in the same buffer, and added at their corresponding concentrations to the parasite suspension. Parasites were allowed to proliferate for 72 h at 26 °C. Inhibition of proliferation was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction (0.5 mg/ml final concentration) solubilized with 5% (w/v) SDS and detected at 600 nm with a Bio-Rad microplate reader 640 [25].

T. b. brucei BSF were harvested at exponential phase, resuspended in growth medium (2×10^6 cells/ml) into 96-well microplates and incubated with the different reagents. After 24 h, resazurin (440 μM final

concentration) was added to each well. Fluorescence was read in a Varioskan Flash (Thermo Scientific) microplate reader ($\lambda_{exc} = 528$ nm; $\lambda_{em} = 590$ nm).

For intracellular amastigote killing measurements, the protocol described in [23] was followed, except that Giemsa staining was substituted by Hoechst 342, as detailed below. BALB/c peritoneal macrophages were seeded in a Lab-Tek™ 8-well Permanox™ chamber slide system, at 10^5 cells/well in RPMI 1640 + 10% HIFCS, and incubated for 6 h with R40 *L. donovani* promastigotes at a 1:10 macrophage:promastigote ratio. After removal of unbound parasites, infection was allowed to progress for 72 h at 37 °C, followed by treatment with HePC-SS-Tat or HePC for 48 h in the same culture medium. Cells were finally stained with 10 μg/ml of the vital dye Hoechst 342 for 45 min, fixed in 4% (w/v) paraformaldehyde, mounted with Mowiol and observed in a Leitz Dialux 20 fluorescence microscope coupled to a Leica DFC340FX CCD camera, using a standard DAPI filter. The infection index, taken as the amastigote: macrophage ratio, averaged over 200 macrophages regardless of whether they were infected or not, was measured in triplicate.

2.4. Assessment of plasma membrane permeabilization

Alterations in the plasma membrane permeability were evaluated by vital dye entrance [26]. Briefly, R40 promastigotes in complete growth medium (2×10^7 cells/ml) were treated with the reagents and after 4 h propidium iodide (PI) (5 μg/ml, final concentration) was added. After 15 min incubation, fluorescence was measured in a Polarstar Galaxy microplate reader ($\lambda_{exc} = 544$ nm; $\lambda_{em} = 612$ nm). Results are expressed as the percentage of fluorescence relative to parasites fully permeated with 0.1% Triton X-100 (TX-100).

2.5. Intracellular accumulation of fluorescent analogues in R40 promastigotes

Promastigotes were resuspended in complete growth medium (2×10^7 cells/ml) devoid of phenol red and incubated with the corresponding reagent for 3 h at 26 °C. Next, parasites were washed twice with 10 mg/ml fatty acid-free bovine serum albumin (BSA) in HBSS-Glc at 4 °C, resuspended in 100 μl of the same buffer, transferred into a 96-well black microplate and lysed with SDS (1% final concentration). Fluorescence was then measured in a Varioskan microplate reader ($\lambda_{exc} = 480$ nm; $\lambda_{em} = 520$ nm). Results were compared to a standard curve for each of the different reagents.

2.6. Intracellular distribution of fluorescent analogues

R40 promastigotes were resuspended at 2×10^7 cells/ml in HBSS-Glc plus Hoechst 342 (10 μg/ml final concentration). After 1 h, the different HePC reagents were added at 2.5 μM final concentration and fluorescence observed up to 3 h on a Leica TCS-SP2-AOBS-UV ultraspectral confocal microscope (Leica Microsystems, Heidelberg, Germany) without fixation. Excitation/emission wavelengths were respectively 350/460 nm for Hoechst 342, 488/520 nm for BODIPY-labeled compounds, and 644/670 nm for the HePC-BODIPY-SS-Tat (Quasar 670) conjugate.

BALB/c peritoneal macrophages, seeded on 35 mm glass bottom culture dishes (MatTek), were infected with SNARF®-1 (carboxylic acid, acetate, succinimidyl ester)-pre-stained R40 promastigotes (10 μM, 30 min) at a 1:10 macrophage:parasite ratio for 4 h in growth medium. Infection was allowed to progress for 48 h at 37 °C, followed by treatment with HePC-BODIPY or HePC-BODIPY-SS-Tat(Quasar 670) at 2.5 μM for different time periods. Cells were washed and freshly observed on a Leica TCS SP2 confocal microscope. Excitation and emission wavelengths were as above; for SNARF-1 visualization they were set at 514 and 578 nm, respectively.

For short-term observation of *T. b. brucei* BSFs, parasites were resuspended in trypanosome dilution buffer (TDB) [27] at 10^7 cells/ml, supplemented with 1 μg/ml DAPI. 40-μl aliquots of this suspension were incubated for 2 min with the corresponding reagents at 5 μM concentration. Parasites were then harvested by centrifugation, resuspended in 10 μl of TDB plus 40 μl of 0.8% low melting point agarose at 37 °C, and freshly observed in an Olympus R IX81in vivo fluorescence microscope. At least 100 parasites in different fields were observed; pictures are representative of the whole population.

3. Results

3.1. HePC conjugation to Tat bypasses Leishmania HePC resistance

As a first step, we tested whether the conjugation of HePC to Tat was able to abrogate resistance in R40 promastigotes. This is characterized by a faulty HePC uptake, as previously evidenced using radioactive [9] or fluorescent HePC analogues [21,28]. As shown in Fig. 1 the HePC-BODIPY-SS-Tat conjugate inhibited proliferation of resistant R40 promastigotes in a dose-dependent manner with an IC_{50} of 2.4 ± 0.8 μM, while incubation with either HePC-BODIPY or Tat, added separately or together (molar ratio 1:1), was innocuous (<10% inhibition of proliferation) up to 40 μM. In comparison, the IC_{50} for the WT strain was slightly higher, 3.1 ± 0.9 μM.

To discard that the lethal effect might result from membrane permeabilization by the different reagents, entrance of the vital dye propidium iodide (PI) in R40 promastigotes was assayed. After 4 h incubation, the percentage of PI uptake (referred to cells fully permeabilized with 0.1% TX-100) was 6.9 ± 3.0, 7.9 ± 2.8, 3.2 ± 2.3, 3.8 ± 2.5, and 2.6 ± 1.2, for HePC-BODIPY-SS-Tat, HePC-BODIPY-S-Tat, HePC-BODIPY, unconjugated Tat + HePC-BODIPY (1:1 ratio), and Tat, respectively, while for untreated promastigotes it was 1.3 ± 0.9. Thus, the lethal effect was clearly due to the action of the drug once inside the parasite.

3.2. Tat promotes HePC-BODIPY uptake on HePC-resistant promastigotes

Having discarded membrane permeabilization by HePC analogues as the cause of R40 promastigote killing, we proceeded to quantify uptake in both WT and R40 strains, with the accumulation endpoint set up at 3 h to prevent massive parasite death. From the results on Fig. 2 we conclude: (i) HePC conjugation to Tat is required for entrance in R40 promastigotes; (ii) uptake of Tat conjugates (both disulfide and thioether) was greater for R40 than for WT parasites, with a slightly larger accumulation for the thioether over the disulfide; (iii) in the WT strain, none of the conjugates challenged HePC uptake by its cognate receptor, as evidenced by the higher IC_{50} of the disulfide conjugate (see previous heading).

We next calculated HePC-BODIPY concentration inside R40 promastigotes. Using the intracellular volume reported in the literature [29], a 0.8 ± 0.3 mM value was determined, in the same range than other fluorescent [21] and radioactive HePC analogues [30].

The uptake process is highly dependent on temperature; at 4 °C it is only 18.9 ± 2.5% of that at standard 26 °C conditions. This is not unusual for CPPs such as Tat, for which temperature-sensitive and – insensitive uptake mechanism e.g., endocytosis and membrane translocation, respectively – have been shown to coexist in mammalian cells, their respective contribution depending on multiple factors [31,32].

3.3. A scissile HePC moiety is not mandatory for leishmanicidal activity

Our rationale for choosing a disulfide conjugate was to ensure payload (HePC) release into the cytoplasm by the redox system of the parasite. In order to confirm this point, a doubly tagged conjugate, HePC-BODIPY-SS-Tat(Quasar 670), was synthesized, where the green-emitting HePC-BODIPY moiety was linked to a non-overlapping, far red-emitting Tat derivative bearing a Quasar 670 fluorophore (Scheme 1). Parasites incubated with 2.5 μM conjugate in HBSS-Glc showed after 5 min a rather homogenous fluorescence pattern, mostly associated to the plasma

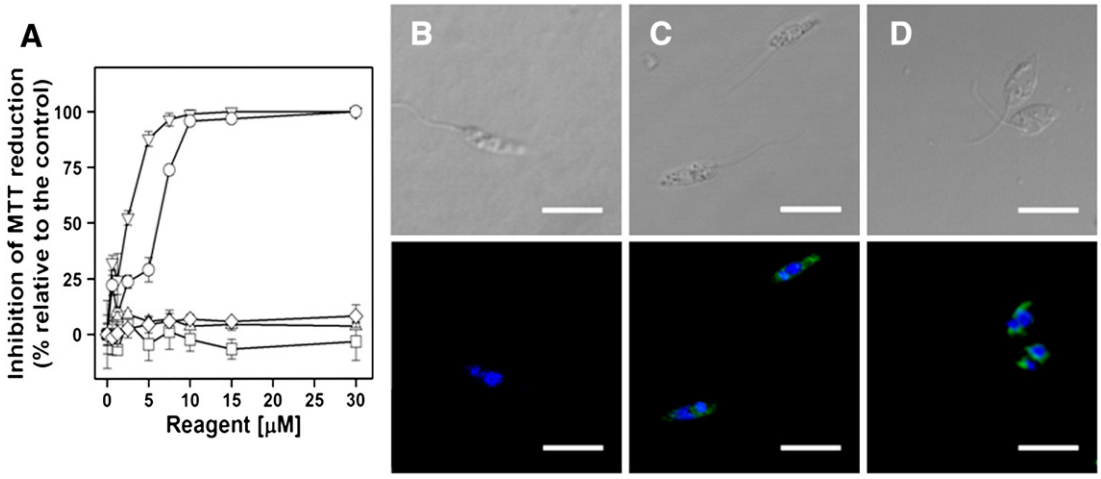


Fig. 1. HePC resistance in R40 *Leishmania donovani* promastigotes is averted by conjugation to Tat. Panel A: Inhibition of R40 proliferation by (□) HePC-BODIPY, (▽) HePC-BODIPY-SS-Tat, (○) HePC-BODIPY-S-Tat, (△) Tat and (◊) Tat + HePC-BODIPY (molar ratio 1:1). Panels B–D: Uptake of HePC-BODIPY, HePC-BODIPY-SS-Tat and HePC-BODIPY-S-Tat, respectively, by R40 promastigotes. Parasites were pre-stained with Hoechst 342 (blue fluorescence), then incubated for 3 h with 2.5 μM BODIPY-labelled reagents. Excitation/emission wavelengths were 350/460 nm and 488/520 nm for Hoechst 342 and BODIPY, respectively. Bar = 10 μm.

membrane and the flagellar pocket. After 3 h incubation, substantial changes were noted: fluorescence spread to most intracellular space of the parasite except the nucleus and the kinetoplast and, most importantly, areas where a given fluorophore (green or red) prevailed could be observed (Fig. 3) and assigned to HePC-BODIPY-SH or HS-Tat(Quasar 670) enrichment, respectively, over either unsplit conjugate or other byproducts. Reductive intracellular HePC release, however, is not required for leishmanicidal activity, as shown by HePC-BODIPY-S-Tat, whose HePC and Tat moieties are connected by a metabolically stable thioether bond, yet displays significant activity ($IC_{50} = 5.9 \pm 1.4$ μM), not much unlike its disulfide counterpart ($IC_{50} = 2.4 \pm 0.8$ μM, Fig. 1).

3.4. The HePC-SS-Tat conjugate abrogates HePC resistance in intracellular R40 amastigotes

In a further step, we assessed the efficacy of HePC-Tat conjugates on intracellular amastigotes, as the intracellular form fully retained the HePC resistance phenotype [6]. To this end, peritoneal macrophages were infected with R40 promastigotes and, three days later, were challenged with either HePC-BODIPY-SS-Tat(Quasar 670) or HePC-BODIPY and analyzed by confocal microscopy in unfixed form. As shown in Fig. 4, live infected macrophages incubated overnight with 2.5 μM HePC-BODIPY displayed only a faint, widely distributed fluorescence, with no privileged localization and with nil accumulation inside intracellular R40 amastigotes. In contrast, incubation with 2.5 μM HePC-BODIPY-SS-Tat(Quasar 670) under identical conditions produced far more intense fluorescence, with both HePC-BODIPY and Quasar 670 fluorophores co-localizing almost exclusively inside the intracellular amastigotes (Fig. 4). Shorter incubation times (1 h) produced a similar pattern, albeit with lower intensity (not shown). Most importantly, infected macrophages treated with 2.5 μM HePC-BODIPY-SS-Tat showed a sharp decrease in parasite:macrophage ratio; thus, whereas for untreated and HePC-BODIPY-treated macrophages averaged ratios were 4.2 ± 0.7 and 3.9 ± 0.4, respectively (Supplemental material, Fig S1), for conjugate-treated macrophages the ratio was 0.3 ± 0.2. To discard artifacts due to fluorophore inclusion, non-fluorescent HePC-SH and HePC-SS-Tat [21] controls were assayed and gave ratios of 5.1 ± 0.2 and 0.6 ± 0.4, respectively.

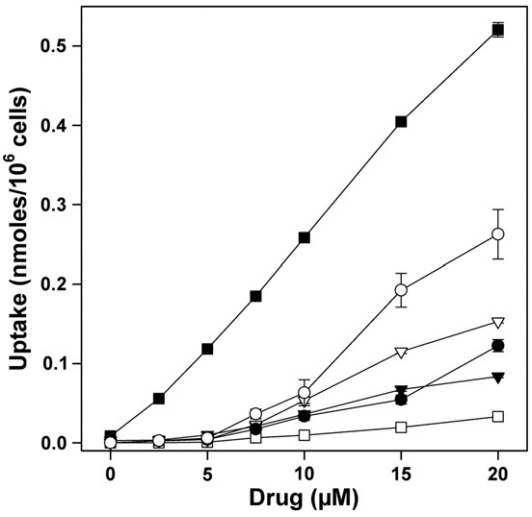


Fig. 2. Intracellular accumulation of HePC fluorescent analogues in WT and R40 *L. donovani* promastigotes. Parasites were incubated with either HePC-BODIPY (square), HePC-BODIPY-SS-Tat (triangle), or HePC-BODIPY-S-Tat (circle) for 3 h in complete growth medium, as described in the Materials and methods. Afterwards, cells were lysed and dye was measured by fluorometry at $\lambda_{EXC} = 480$ nm and $\lambda_{EM} = 520$ nm excitation and emission wavelengths. Filled and empty symbols stand for WT and R40 promastigotes, respectively.

3.5. Conjugation to Tat broadens HePC activity spectrum into the bloodstream forms of African trypanosomes

Bloodstream trypomastigotes of *T.b. brucei* are much less susceptible to HePC than *L. donovani* promastigotes [33]. Upon 15 min incubation of BSF with 5 μM HePC-BODIPY-SS-Tat, these parasites showed an initial fluorescence pattern, mostly accumulated at a defined spot, most likely the lysosome (Fig. 5, column A). After 90 min, fluorescence had spread throughout the cell body (Fig. 5, column B) and the parasite was devoid of movement and with severe structural damage leading to death (Fig. 5)

4. Discussion

Translocation across trypanosomatid membranes has been documented for some antimicrobial peptides in *Leishmania* [34–36], as well as for standard CPPs such as TP10 in *Trypanosoma brucei* [37], or Tat in *Leishmania* [38]. Nevertheless, to the best of our knowledge the use of CPPs for release of chemotherapeutic reagents into parasites is confined to the work of Corradin et al. [38], who conjugated a leishmanolysin substrate peptide to Tat to inhibit the proteolytic

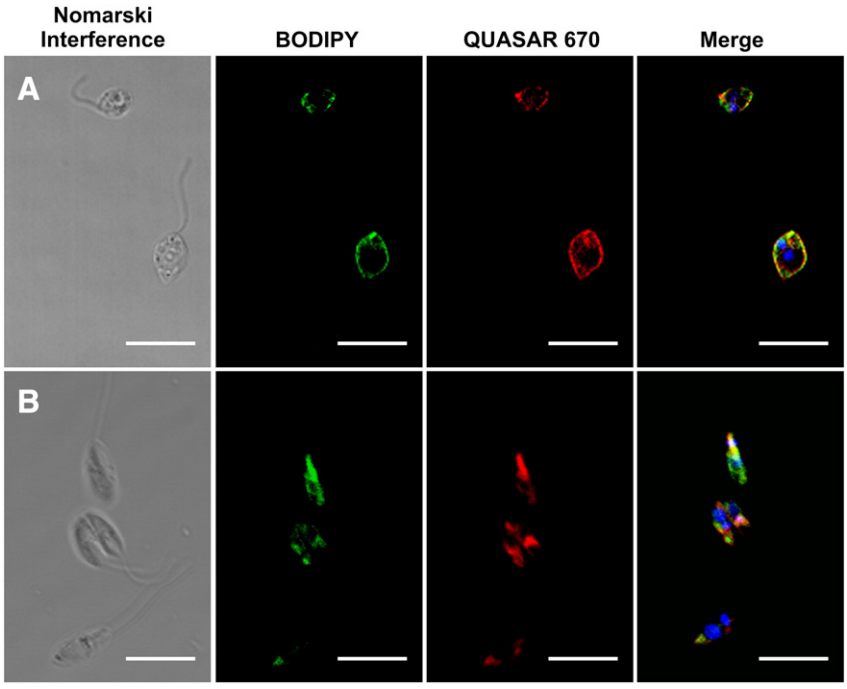


Fig. 3. Time-dependence of HePC-BODIPY-SS-Tat(Quasar 670) intracellular distribution in R40 parasites. Cells were pre-stained with Hoechst 342 and incubated with 2.5 μM HePC-BODIPY-SS-Tat(Quasar 670) for 5 min (A) or 3 h (B), then observed unfixed by confocal microscopy. Excitation/emission wavelengths were: BODIPY fluorescence (green fluorescence), 488/520 nm; QUASAR 670 (red fluorescence), 644/670 nm. Fluorescence settings for Hoechst 342 were 350/460 nm. Bar = 10 μm.

activity of the enzyme; while the construct was successfully translocated, it did not decrease parasite viability. In this work, we demonstrate the efficacy of HePC-CPP conjugates in averting resistance in *Leishmania*. This model constitutes an excellent proof-of-mechanism because, in the R40 strain we have used, HePC resistance is almost exclusively associated to deficient drug uptake, hence recovered lethality is univocally linked to efficient drug delivery by Tat. Identical CPP-drug conjugate strategy was used to overcome resistance to daunomycin [39], methotrexate [40] or doxorubicin [41,42] in cancer cells.

To visualize drug entrance and localization we have used HePC-BODIPY, a new analogue that (i) preserves key structural features of HePC such as a phosphocholine polar head group or a long (18 carbon atoms, vs. 16 in HePC) alkyl chain, (ii) has improved photostability over previous fluorescent HePC derivatives [28], and (iii) retains the leishmanicidal activity of HePC as well as the recognition by its cognate transporter in *Leishmania*.

Conjugation to Tat is required to restore the leishmanicidal activity of HePC on the resistant strain; the HePC-BODIPY-SS-Tat conjugate entered R40 promastigotes while unconjugated drug showed only a faint fluorescence associated to parasite membrane. Although Tat has some activity on bacteria and fungi [43–45], its IC_{50} values in the present system, much above those of the conjugate, discard any determining effect in the leishmanicidal activity.

Endocytosis accounts for most Tat uptake in *Leishmania*. The special features of its plasma membrane exclude macropinocytosis, as for other trypanosomatids [46], and genome mining appears to rule out also

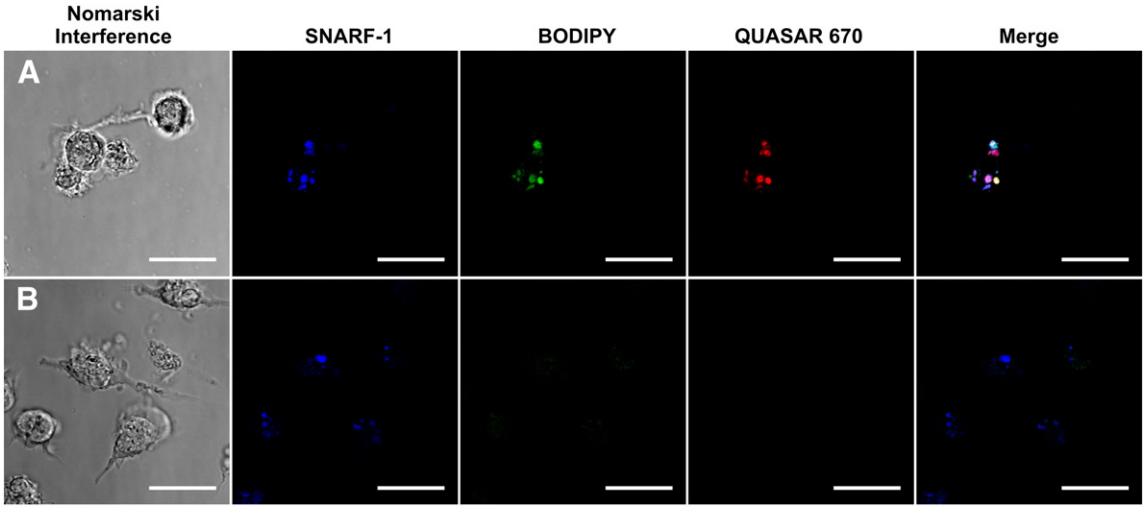


Fig. 4. Effect of Tat conjugation on uptake by live R40-infected murine peritoneal macrophages. BALB/c mouse peritoneal macrophages were infected with SNARF-1-prelabeled R40 promastigotes and treated overnight with either HePC-BODIPY-SS-Tat(Quasar 670) (A) or HePC-BODIPY (B), both 2.5 μM. Excitation/emission wavelengths were: 514 /578 nm for SNARF-1 (blue fluorescence, false colored to highlight differences with QUASAR 670), 488/520 nm for BODIPY (green fluorescence), and 644/670 for QUASAR 670. Bar = 25 μm.

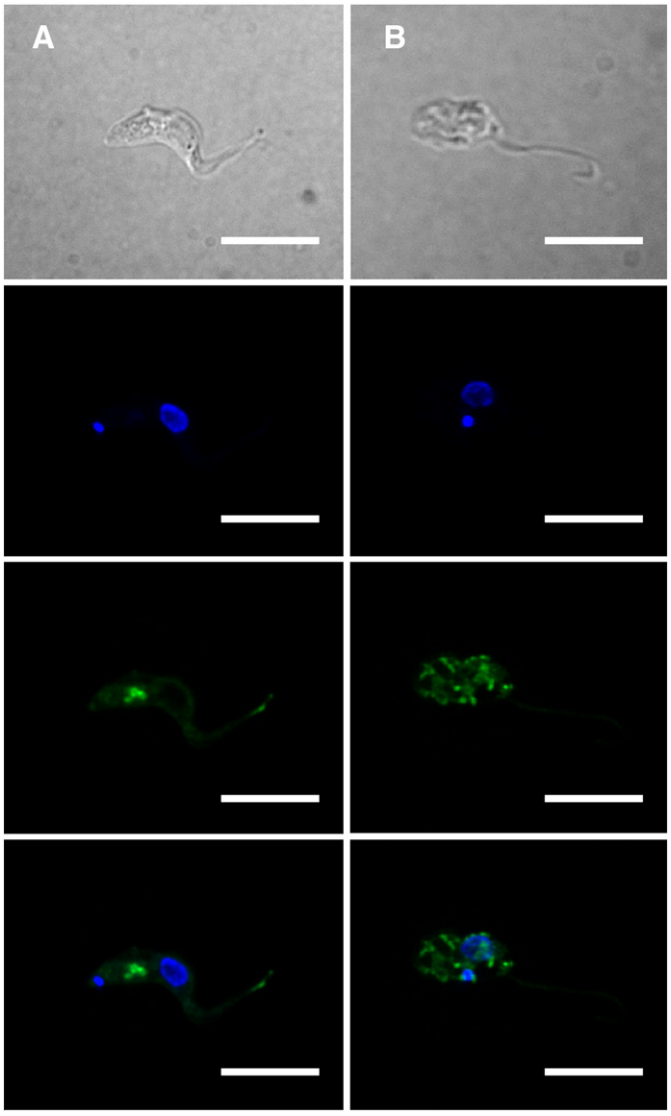


Fig. 5. HePC-BODIPY-SS-Tat uptake by *T. b. brucei* trypomastigotes. Parasites resuspended in trypanosome dilution buffer (10^7 cells/ml) were prelabeled with 1 μg/ml DAPI (blue) (350 nm/460 nm and incubated with 5 μM HePC-BODIPY-SS-Tat (green) for 15 (column A) or 90 min (column B). Excitation/emission wavelengths: 350/460 nm and 488/520 nm for DAPI and BODIPY respectively. Bar = 10 μm.

caveolin-mediated endocytosis from the feasible uptake pathways. Clathrin-mediated endocytosis, on the other hand, is essential for *T.b. brucei* [47,48] and may be in effect in the present case. The uptake mechanism involves a receptor, which for Tat in mammalian cells is an anionic glycosaminoglycan such as heparan sulfate [49,50], although a direct role for it in CPP membrane translocation has been questioned [51]. On the other hand, about one fifth of the total uptake was still taking place at low temperature, suggesting a simple crossover of the plasma membrane by the conjugate, aside from energy-demanding mechanisms. Indeed, Tat translocation to the lumen of giant plasma membrane vesicles has been reported [52], and histatin 5, a leishmanicidal peptide targeting mitochondria, is known to translocate across the plasma membrane of the parasite [36]. Also, in CHO mammalian cells direct translocation has been found to coexist with endocytosis, and to be favored for conjugates with low molecular weight cargoes [53]. In the present case, this pathway might be additionally facilitated by the considerable hydrophobicity of the HePC moiety of the conjugate, which would increase partition into the membrane [31]. Direct interaction between the membrane and the conjugate may also explain the small increase in uptake of R40 vs. WT promastigotes. The higher phosphatidylethanolamine content of the plasma membrane external leaflet [54] would favor negative membrane curvature, hence Tat translocation by formation of an inverted micelle [55]

In trypanosomatids, particularly African BSFs, endocytic membrane turnover rates are high enough to make a specific receptor unnecessary [48], a situation that may explain the results found for *T. brucei* in this work; hence, simple electrostatic binding of Tat to the external anionic phospholipids of the plasma membrane will suffice for engulfment through endocytic entrance [48].

The type of linkage between a CPP and its cargo molecule may affect the intracellular fate of the conjugate, particularly the interaction of the payload with its target [56]. Our choice of a disulfide bond [57] for HePC-Tat conjugation aimed to obtain efficient interaction of HePC with its targets upon intracellular reductive cleavage of the disulfide, a process that in trypanosomatids is enhanced by the high redox potential of trypanothione. That this was indeed the case was confirmed by the time-dependent changes in intracellular fluorescence of parasites challenged with the dual tagged conjugate HePC-BODIPY-SS-Tat(Quasar 670). The structure of the conjugate ruled out fluorophore separation by proteolysis instead of disulfide reduction. Even so, cleavage is not mandatory for HePC activity, as the non-cleavable thioether conjugate HePC-BODIPY-S-Tat is also active. Although metabolically stable drug-CPP linkages may severely affect the activity of the cargo molecule [58], a thioether is advantageous over the disulfide in that it prevents promiscuous exchange with surface thiol groups [59]. It is worth noting that accumulation of the thioether conjugate is slightly higher than the disulfide, albeit with lower toxicity for the parasite. Given the variety of targets reported for HePC in *Leishmania* [60,61] and the intracellular concentrations in the low-mM range achieved by the conjugates (see above), interaction with even low affinity targets must be expected. While the non-cleavable thioether bond may hamper some of these interactions, the substantial fraction remaining is likely to inflict damage to the parasite.

We have also explored the activity of HePC conjugates on intracellular amastigotes, the pathological form of the parasite in vertebrate hosts. As R40 parasites showed close-to-nil uptake of non-conjugated drug, accumulation in intracellular amastigotes requires that the conjugate, in full or in part, arrive intact to the parasitophorous vacuole where amastigotes reside. Since, to the best of our knowledge, *Leishmania* does not induce a preferential permeability of this parasitophorous vacuole which might account for the privileged accumulation of both Tat and HePC, the more plausible explanation is that, similar to other mammalian cells [31], the typical pathway is followed of (i) interaction of conjugate with the macrophage membrane, presumably via glucosaminoglycans, and (ii) subsequent inclusion into an endosomal vacuole that fuses with the parasitophorous vacuole. The alternative, direct translocation across the macrophage membrane and further traffic into the parasitophorous vacuole, is most unlikely, since to reach the parasite the conjugate would have to cross both plasma and parasitophorous vacuole membranes and, additionally, risks reductive cleavage by glutathione in the macrophage cytoplasm. A dedicated transporter for cytoplasmic HePC into the parasitophorous vacuole remains to be described, even less for the Tat moiety. Nonetheless, the faint fluorescence pattern observed in the cytoplasm of the macrophage might be likely ascribed to this secondary pathway.

The HePC-BODIPY-SS-Tat conjugate was also tested on the BSFs of *T. b. brucei*, an extracellular parasite endowed with a natural higher resistance to HePC and other alkyl lysophospholipids [33]. The conjugate accumulated first in the flagellar pocket and then spread on the entire parasite, with ensuing death. Bloodstream trypomastigotes have an extremely high endocytic activity, and may accumulate HePC non-specifically adsorbed in the membrane; toxic effects,

however, are much lower than for the conjugate. It is worth noting that, after conjugate incubation, an internal giant vesicle, phenotypically similar to the big-eye phenotype, was formed. This is typical of blocked endocytosis, but has also described for parasite knockouts for neutral sphingomyelinase [62], an endoplasmic reticulum enzyme inhibited in vitro by HePC, hence it is tempting to speculate that HePC-Tat conjugation may afford intracellular access to this enzyme.

There may be additional advantages to the metabolically stable (thioether-linked) HePC-Tat conjugates. For instance, they may attenuate the effect of transporters promoting the efflux of intracellular drug [10,63,64], a mechanism of HePC resistance that in *Leishmania* is only second in importance to faulty uptake. The impact of this pathway in the present model is currently under investigation. A second benefit might reside in the reported preferential accumulation of CPPs in the liver and the spleen [65], both main reservoirs for visceral leishmaniasis.

To summarize, we have provided proof-of-mechanism for the fitness of Tat and other CPPs as vectors for otherwise hard-to-deliver leishmanicidal drugs, using Tat-conjugated miltefosine to avert resistance in *Leishmania* parasites. Secondly, release of the drug by the parasite redox system can be visualized by a doubly tagged conjugate. Finally, we have shown how this strategy can broaden the range of susceptible pathogens. Nonetheless, several issues remain to be solved before the potential of this approach in trypanosomatid chemotherapy is fully realized. Organelle specificity, for one thing, must be addressed, possibly by inclusion of structural motifs whose exclusive recognition by macrophage receptors improves selective uptake, as reported for branched poly-lysine carriers conjugated to methotrexate [66,67]. Particulate presentation of conjugates, a strategy exploiting the high endocytosis rate of macrophages to increase drug concentration in the parasitophorous vacuole [68–70], also needs to be further explored. Finally, CPPs with intrinsic leishmanicidal properties [e.g.,VIP [34] or histatin 5 [36]] appear to be promising in that the synergy between payload and carrier may reduce the amount of drug required. In conclusion, while open questions remain about the practical application of CPPs, it is fair to say that workable routes for circumventing such problems also exist, opening new chemotherapeutic prospects into a field where they are sorely needed.

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