

Peptidomimetic and Organometallic Derivatives of Primaquine Active against *Leishmania infantum*

Sílvia Vale-Costa, Nuno Vale, Joana Matos, Ana Tomás, Rui
Moreira, Paula Gomes and Maria Salomé Gomes
Antimicrob. Agents Chemother. 2012, 56(11):5774. DOI:
10.1128/AAC.00873-12.
Published Ahead of Print 27 August 2012.

Updated information and services can be found at:
<http://aac.asm.org/content/56/11/5774>

These include:

REFERENCES

This article cites 44 articles, 13 of which can be accessed free
at: <http://aac.asm.org/content/56/11/5774#ref-list-1>

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new
articles cite this article), [more»](#)

Information about commercial reprint orders: <http://journals.asm.org/site/misc/reprints.xhtml>
To subscribe to to another ASM Journal go to: <http://journals.asm.org/site/subscriptions/>

Peptidomimetic and Organometallic Derivatives of Primaquine Active against *Leishmania infantum*

Sílvia Vale-Costa,^{a,b} Nuno Vale,^{c,d} Joana Matos,^{c,d} Ana Tomás,^{a,b} Rui Moreira,^e Paula Gomes,^{c,d} and Maria Salomé Gomes^{a,b}

Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal^a; Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal^b; Centro de Investigação em Química, Universidade do Porto, Porto, Portugal^c; Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Porto, Portugal^d; and iMed.UL, CECF, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal^e

The current treatment of visceral leishmaniasis is made difficult by the low efficacy, elevated costs, low bioavailability, and high toxicity of many of the available drugs. Primaquine, an antimalarial 8-aminoquinoline, displays activity against *Leishmania* spp., and several of its derivatives have been developed as potential antileishmanial drugs. However, primaquine exhibits low oral bioavailability due to oxidative deamination of its aliphatic chain. We previously developed peptidomimetic and organometallic derivatives of primaquine, with higher resistance to proteolytic degradation and oxidative deamination, which presented significant activity against primaquine-sensitive pathogens such as *Plasmodium* or *Pneumocystis*. In light of these relevant findings, we decided to evaluate these compounds against both the promastigote and intramacrophagic amastigote forms of *Leishmania infantum*, the agent of Mediterranean visceral leishmaniasis. We found that several of these compounds had significant activity against *L. infantum*. One of the peptidomimetic (3c) and one of the organometallic (7a) derivatives of primaquine were active against the clinically relevant intramacrophagic amastigote form of the parasite, causing >96% reductions in the number of amastigotes per 100 macrophages at 60 and 40 μ M, respectively, while being less cytotoxic for host cells than the reference drugs sitamaquine and miltefosine. Hence, compounds 3c and 7a represent new entries toward the development of new antileishmanial leads.

Leishmania species are digenetic protozoa that alternate between motile promastigotes in the gut of the sand fly and non-motile amastigotes inside macrophage phagolysosomes of the mammalian host. These parasites are the causative agents of leishmaniasis, a disease with clinical symptoms that range in severity from self-healing cutaneous lesions to serious mucocutaneous disfigurement and fatal visceralizing infection. The World Health Organization estimates that more than 12 million people are currently infected, with 2 million new cases occurring every year and 350 million people, in 98 different countries, at risk of acquiring the infection (43).

Visceral leishmaniasis (VL) is the most severe form of the disease, being fatal if left untreated, with an annual incidence estimated at 0.5 million cases, causing around 50,000 deaths annually (a rate exceeded only by malaria among protozoan diseases) (43). VL is caused by *Leishmania donovani* in East Africa and the Indian subcontinent and by *L. infantum* in Europe, North Africa, and Latin America. Active VL is characterized by weight loss, fever, weakness, and hepatosplenomegaly, among other symptoms (23). The increasing number of HIV-*Leishmania* coinfections has raised the incidence of the disease, reduced the likelihood of a therapeutic response, and greatly contributed to the probability of relapse (1).

Since there are currently no effective vaccines to prevent *Leishmania* infections, management of VL relies on chemotherapy with first-line drugs, i.e., pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), and second-line drugs, i.e., pentamidine, paromomycin, amphotericin B or its lipid formulations, and miltefosine (5). However, these drugs present several problems, such as specific toxicities, elevated costs, prolonged treatment regimens, low patient compliance, and parasite resistance (18). Therefore, alternative drugs and combination regi-

mens with improved therapeutic effectiveness are urgently needed to treat VL (41).

8-Aminoquinolines (8AQ) have been established as a promising class of drugs for the oral treatment of malaria, *Pneumocystis jirovecii* pneumonia, leishmaniasis, and trypanosomiasis (35, 40). Primaquine (PQ) (compound 1 in Fig. 1), an antimalarial 8AQ, is known to exhibit activity against visceral leishmaniasis (3, 12, 13, 15, 25, 29). Since it leads to some adverse side effects and has a lower therapeutic index than those of VL reference drugs, PQ currently has no applicability in the VL clinical setting. The optimization of the PQ structure has already led to the discovery of three promising 8AQ: NPC1161B (24), tafenoquine (44), and sitamaquine (24, 25), which demonstrated high activity against experimental VL. Sitamaquine has completed phase IIb clinical trials by GlaxoSmithKline, although it has shown variable results and unexpected cases of toxicity (10, 14, 34, 42).

Interestingly, earlier works suggested that PQ encapsulation in liposomes or nanoparticles enhances its leishmanicidal activity either in infected macrophages (29) or in animal models of infection (3, 29). This enhanced effect may be due to the fact that encapsulation of PQ prevents it from undergoing metabolic inactivation to carboxyprimaquine (compound 2 in Fig. 1) (39), thus increasing the drug's bioavailability. Therefore, it is reasonable to

Received 3 May 2012 Returned for modification 11 July 2012

Accepted 19 August 2012

Published ahead of print 27 August 2012

Address correspondence to Maria Salomé Gomes, sgomes@ibmc.up.pt.

P.G. and M.S.G. contributed equally to this article.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.00873-12

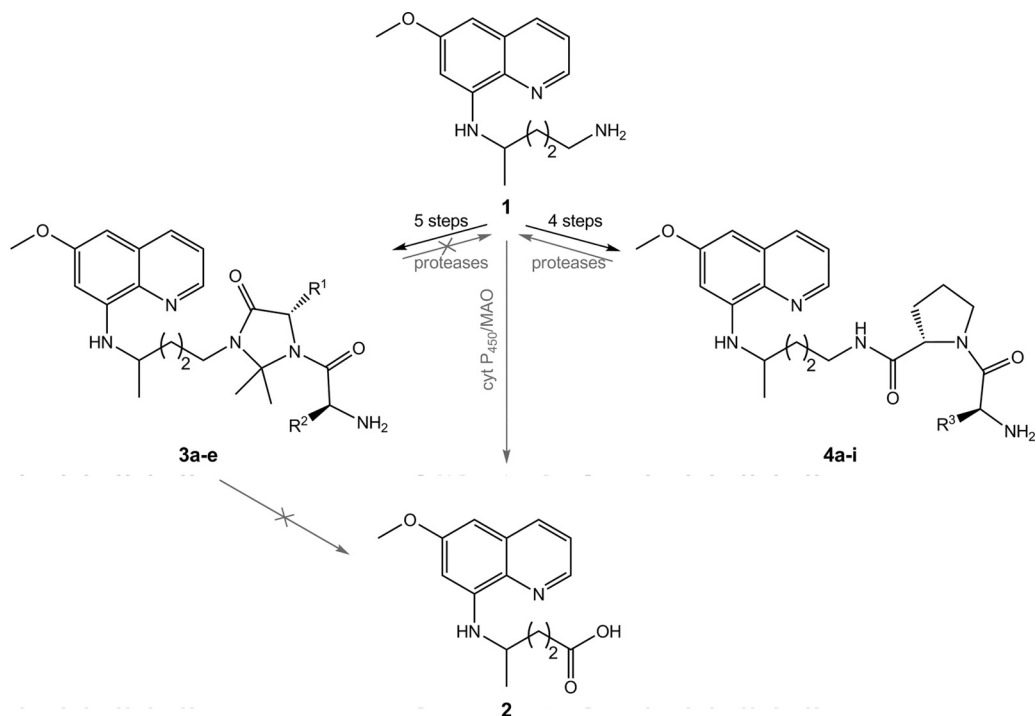


FIG 1 Chemical structures of primaquine (1), its main metabolite, carboxyprimaquine (2), and its peptidomimetic (3a-e) and dipeptidic (4a-i) derivatives. Cyt P₄₅₀, cytochrome P450; MAO, monoamine oxidase.

expect that alternative strategies that prevent PQ conversion into its inactive metabolite, compound 2, will possibly contribute to improvement of the drug's activity against VL.

We have been working on the synthesis and biological evaluation of PQ derivatives in which the aliphatic amine of the parent

drug has been masked by acylation with a peptidomimetic (compound 3) (Fig. 1) or organometallic (compounds 5 to 10) (Fig. 2) moiety as a strategy to (i) avoid premature oxidative deamination of PQ to compound 2 and (ii) confer resistance to proteolytic degradation, which affects PQ dipeptide derivatives such as com-

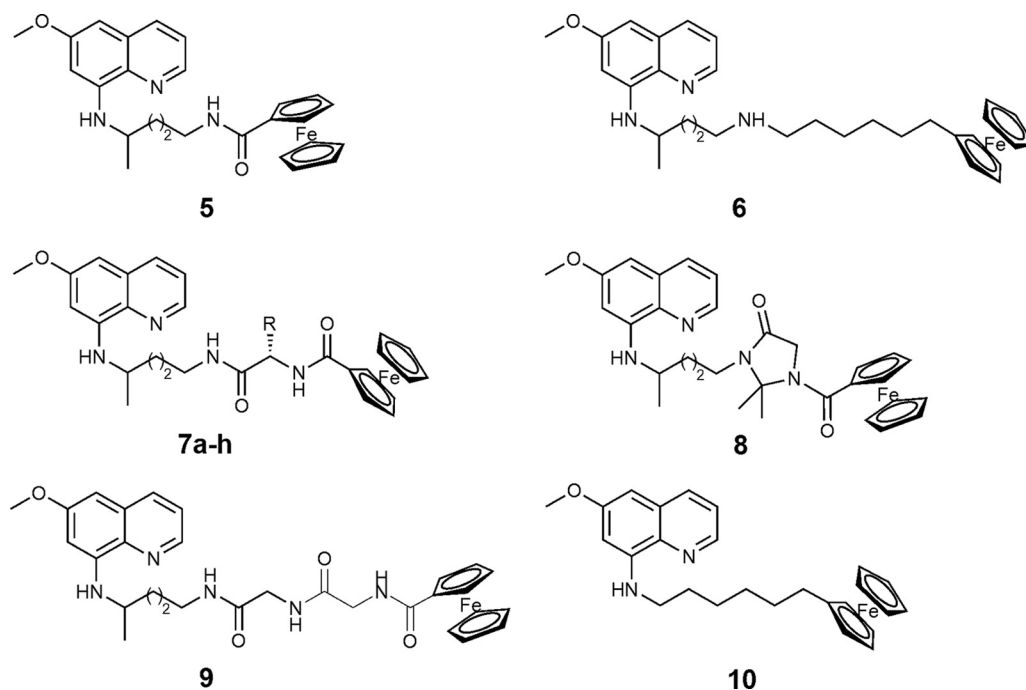


FIG 2 Chemical structures of primaquine's organometallic derivatives 5 to 10.

pound 4 (Fig. 1) (2, 26, 38, 40). Compounds 3 and 5 to 10 have previously revealed remarkable activity against PQ-sensitive pathogens, namely, *Plasmodium* (malaria) and *Pneumocystis* (pneumocystic pneumonia), in some cases with better performances than that of PQ at the activity and/or toxicity level (19, 20, 36, 40). In view of this and the known antileishmanial properties of 8AQ analogues of PQ, such as sitamaquine, the present work aimed to explore the activity of the peptidomimetic and organometallic PQ derivatives against *L. infantum*, the causative agent of Mediterranean VL. As presented here, some of these compounds exhibited antileishmanial activity which was stronger than that of PQ and comparable to that of the reference antileishmanial drugs sitamaquine and miltefosine, while having lower cytotoxicity than the latter. These findings emphasize the importance of scrutinizing the activity of PQ derivatives or analogues against protozoan pathogens other than *Plasmodium*.

MATERIALS AND METHODS

Chemical synthesis. Synthetic procedures and structural data have been reported elsewhere for compounds 3a to e (40) and 4b (38). Other compounds of the PQ-Pro-Xaa series, i.e., 4a and c to i, were synthesized as previously described for compound 4b (38), and spectroscopic data as well as high-pressure liquid chromatography (HPLC) traces are available upon request. Synthetic procedures and chromatographic/spectroscopic data were reported elsewhere for compounds 5 to 10 (19, 20), except for compound 7h, for which relevant procedures and data are available upon request.

Reagents. Sitamaquine was supplied by GlaxoSmithKline (Brentford, Middlesex, United Kingdom). Miltefosine was purchased from Cayman Chemical (Ann Arbor, MI). All compounds used in this study were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C .

Parasites. Promastigotes of the *L. infantum* strain MHOM/MA/67/ITMAP-263 were differentiated at 25°C in complete Schneider's medium (Sigma-Aldrich Co., St. Louis, MO) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 U ml^{-1} penicillin, $100\text{ }\mu\text{g ml}^{-1}$ streptomycin (all from Gibco, Life Technologies, Carlsbad, CA), 2% human urine, $5\text{ }\mu\text{g ml}^{-1}$ phenol red (Sigma), and 5 mM HEPES sodium salt (Sigma), pH 7.4, from amastigotes present in the spleens of infected mice. Promastigote cultures were expanded at 25°C , for a maximum of 5 passages, in RPMI 1640 GlutaMAX-I medium (Gibco, Life Technologies) containing 20% FBS, 50 U ml^{-1} penicillin, $50\text{ }\mu\text{g ml}^{-1}$ streptomycin, and 25 mM HEPES sodium salt, pH 7.4. Promastigote differentiation from the exponential to the stationary phase was promoted by culture at 25°C , without medium renovation, for 4 to 5 days.

Drug screening assay on promastigotes. Promastigotes (1×10^6 /well) were cultured at 25°C in complete RPMI medium supplemented with the various compounds at concentrations between 2.5 and $160\text{ }\mu\text{M}$ ($200\text{-}\mu\text{l}$ total volume). After 24 h of culture, $20\text{ }\mu\text{l}$ of a 2.5 mM resazurin solution (freshly prepared and filtered in phosphate-buffered saline, pH 7.4 [Sigma]) was added to each well. The fluorescence intensity, corresponding to resazurin conversion to the fluorescent compound resorufin, was determined 48 h after resazurin addition (excitation wavelength of 560 nm and emission wavelength of 590 nm on a SpectraMAX GeminiXS fluorometer [Molecular Devices LLC, Sunnyvale, CA]). All experimental conditions were carried out in triplicate. The 50% inhibitory concentration (IC_{50}) (μM) values were determined with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA) from plots of percentages of parasite growth in relation to control versus inhibitor concentrations.

Bone marrow-derived macrophages. Macrophages were derived from the bone marrow of BALB/c mice as follows. Each femur was flushed with 5 ml of Hanks' balanced salt solution (HBSS; Gibco). The resulting cell suspension was centrifuged and the cells resuspended in complete Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U ml^{-1} penicillin, 50

$\mu\text{g ml}^{-1}$ streptomycin, 10 mM HEPES, pH 7.4, and 10% L929 cell-conditioned medium (LCCM), as a source of macrophage colony-stimulating factor (M-CSF). The cells were distributed in 24- or 96-well plates with or without sterile and lipopolysaccharide (LPS)-free microscopy slides and were incubated at 37°C in a 7% CO_2 atmosphere. Three days after seeding, $100\text{ }\mu\text{l}$ of LCCM was added. On the 7th day of culture, the medium was renewed.

Infection of bone marrow-derived macrophages. On the 10th day of culture, completely differentiated macrophages adhered to microscopy slides (4×10^5 macrophages/well) were infected with stationary promastigotes of *L. infantum* at an approximate macrophage/parasite ratio of 1:10. After 4 h at 37°C in a 7% CO_2 atmosphere, cells were washed with warm HBSS to remove noninternalized promastigotes and reincubated in complete DMEM. Compounds were added to the desired concentrations after 24 h of culture. Cells were fixed and stained with HemaColor (Merck KGaA, Darmstadt, Germany) 24 or 72 h later. The slides were visually inspected under a light microscope to determine the number of parasites present inside macrophages. The "parasite index" was calculated as the number of amastigotes per 100 macrophages. Each experimental condition was tested in triplicate wells. A minimum of 400 macrophages was counted per slide. Representative pictures of stained cells were obtained with an Olympus CX31 light microscope equipped with a DP-25 camera (CellB imaging software; Olympus, Center Valley, PA), at a magnification of $\times 400$.

Drug cytotoxicity assay on bone marrow-derived macrophages. On the 10th day of culture, macrophages (8×10^4 /well) were treated with the various compounds at the desired concentrations ($200\text{-}\mu\text{l}$ total volume). Twenty-four or 72 h later, $20\text{ }\mu\text{l}$ of a 1.25 mM resazurin solution was added to each well, and the plates were reincubated at 37°C for another 3 h. The fluorescence intensity was determined as described above. All experimental conditions were carried out in triplicate. The 50% cytotoxic concentration (CC_{50}) (μM) values were determined with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA) from plots of percentages of viability in relation to control relative to inhibitor concentrations.

Statistical analysis. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple-comparison *post hoc* test was performed to determine the statistical significance of differences between groups treated with vehicle and groups treated with the different compounds.

RESULTS

In vitro activity against *L. infantum* promastigotes. Our first approach was to test PQ and its peptidomimetic, dipeptidic, and organometallic derivatives on axenic cultures of *L. infantum* promastigotes. The promastigote stage of *Leishmania* is best suited to this purpose due to its simplicity of cultivation, allowing a fast and easy way to screen a large number of drugs.

Results obtained for compounds of the 3 and 4 series (Fig. 1) are depicted in Table 1. Two of these compounds, 3c and 4c, were as active as PQ (compound 1) against *L. infantum* promastigotes, whereas all other derivatives from these series displayed lower or no activity. Although compounds 3c and 4c are among the PQ derivatives with the highest lipophilicity values (clogP values) (compound 3c ranks 1st), there was no direct correlation between clogP values and IC_{50} s (Table 1). This is clearly shown by the significantly different IC_{50} s obtained with compounds 4d to 4f, whose calculated clogP values are identical. It is interesting, though, that β -ramification of the amino acid (Xaa) residue may have a role in antimicrobial activity, as all PQ derivatives which displayed IC_{50} s of $<35\text{ }\mu\text{M}$ bore a β -ramified Xaa residue. Other structural features in Xaa, such as the presence/absence of ionizable (as in compound 4h) or hydrogen-bonding (as in compound 4g or 4h) groups, as well as the bulkiness of the hydrocarbon

TABLE 1 Antiparasitic activities and macrophage toxicities of primaquine (1), its peptidomimetic (3a to e) and dipeptidic (4a to i) derivatives, and the reference drugs sitamaquine (11) and miltefosine (12), with estimated clogP values and Charton's steric parameter (ν) values for relevant hydrocarbon amino acid side chains

| Compound | R ^{1a} | R ^{2a} | R ^{3a} | IC ₅₀ (μ M) on <i>L. infantum</i> promastigotes at 72 h (mean \pm SD) | CC ₅₀ (μ M) on mouse bone marrow-derived macrophages at 24 h (mean) | clogP value ^b | ν (for R ² or R ³) ^c |
|-----------------|-----------------|-------------------------------------|---|---|---|--------------------------|--|
| 1 | | | | 32.2 \pm 1.0 | >60 | 2.04 | |
| 3a | H | H | | 74.8 \pm 1.7 | | 1.82 | 0 |
| 3b | H | Me | | 64.4 \pm 1.2 | | 2.22 | 0.52 |
| 3c ^e | H | ⁱ Pr | | 31.8 \pm 1.0 | >60 | 3.03 | 0.76 |
| 3d | H | (CH ₂) ₂ SMe | | >80 | | 2.40 | |
| 3e | Me | H | | >80 | | 2.10 | |
| 4a | | | H | >80 | | 1.05 | 0 |
| 4b | | | Me | >80 | | 1.45 | 0.52 |
| 4c ^e | | | ⁱ Pr | 31.6 \pm 1.0 | >60 | 2.25 | 0.76 |
| 4d | | | sBu | 37.6 \pm 1.0 | | 2.72 | 1.02 |
| 4e | | | ⁱ Bu | >80 | | 2.72 | 0.98 |
| 4f | | | Bzl | 52.0 \pm 1.4 | | 2.72 | 0.70 |
| 4g | | | <i>p</i> -(OH)Bzl | >80 | | 2.43 | |
| 4h | | | (CH ₂) ₄ NH ₂ | 62.9 \pm 1.5 | | 1.35 | |
| 4i | | | (CH ₂) ₂ SMe | 43.3 \pm 1.0 | | 1.62 | |
| 11 | | | | 7.4 \pm 1.0 | <60 | 5.84 ^d | |
| 12 | | | | 14.4 \pm 1.1 | >60 | 3.30 | |

^a R stands for the amino acid side chain. H, hydrogen; Me, methyl; ⁱPr, isopropyl; ⁱBu, isobutyl; ^sBu, *sec*-butyl; Bzl, benzyl.

^b Calculated using the OSIRIS Property Explorer (<http://www.organic-chemistry.org/prog/peo/>).

^c Taken from reference 6.

^d Experimental value taken from reference 16.

^e β -Ramified amino acid.

amino acid side chains in subsets 3a to c and 4a to f (expressed by Charton's steric factor ν [6] [Table 1]), did not seem to have any particular effect on a compound's activity against *L. infantum*.

Since several reports indicate that iron potentiates the leishmanicidal activity of several drugs (21, 33), and since we have

TABLE 2 Antiparasitic activities and macrophage toxicities of primaquine (1), its organometallic derivatives 5 to 10, and the reference drugs sitamaquine (11) and miltefosine (12)

| Compound | R ^a | IC ₅₀ (μ M) on <i>L. infantum</i> promastigotes at 72 h (mean \pm SD) | CC ₅₀ (μ M) on mouse bone marrow-derived macrophages at 72 h (mean \pm SD) |
|-----------------|---|---|--|
| 1 | | 26.5 \pm 1.2 | >80 |
| 5 | | 22.7 \pm 1.1 | >80 |
| 6 | | 4.9 \pm 2.3 | 6.8 \pm 1.2 |
| 7a | H | >80 | >80 |
| 7b | Me | 44.7 \pm 1.1 | |
| 7c ^b | ⁱ Pr | >80 | |
| 7d | ⁱ Bu | >80 | |
| 7e | Bzl | >80 | |
| 7f | (CH ₂) ₄ NH ₂ | 40.3 \pm 2.5 | |
| 7g | CH ₂ NH ₂ | 39.2 \pm 6.2 | |
| 7h | CH(CH ₃)OH | 63.3 \pm 1.3 | |
| 8 | | 11.5 \pm 1.0 | >80 |
| 9 | | >80 | |
| 10 | | >80 | |
| 11 | | 7.4 \pm 1.0 | |
| 12 | | 14.4 \pm 1.1 | 24.7 \pm 0.5 |

^a R stands for the amino acid side chain. H, hydrogen; Me, methyl; ⁱPr, isopropyl; ⁱBu, isobutyl; Bzl, benzyl.

^b β -Ramified amino acid.

previously shown that ferrocene (Fc) derivatives of PQ are active against *Plasmodium* (19) and *Pneumocystis* (20), we next tested a series of Fc derivatives of PQ for activity against *L. infantum* promastigotes. The results obtained with compounds 5 to 10 (Fig. 2) are shown in Table 2. The compounds of series 7, in which PQ is linked to Fc through a variable amino acid spacer, were generally inactive or performed worse than PQ and the reference drugs sitamaquine (compound 11) and miltefosine (compound 12) (Fig. 3). Relevantly, removal of the amino acid spacer between PQ and Fc, giving compound 5, led

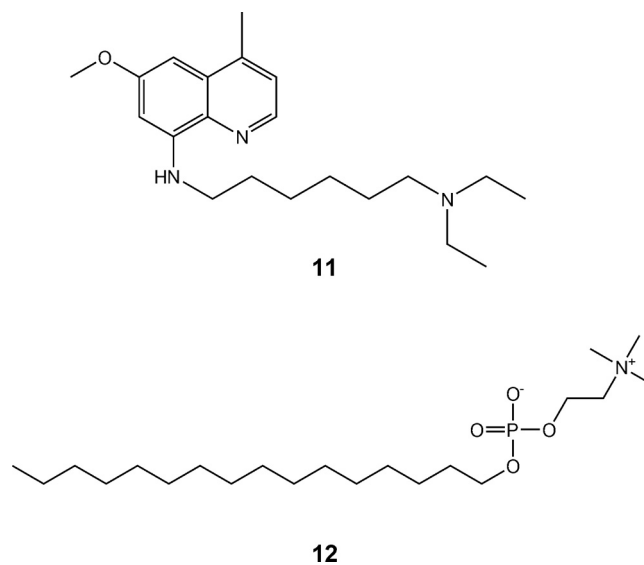


FIG 3 Chemical structures of sitamaquine (11) and miltefosine (12).

TABLE 3 Activities of PQ (1), 3c, 4c, and sitamaquine (11) against intramacrophagic *L. infantum*

| Compound | Concentration (μM) | Mean no. of amastigotes/100 macrophages ± SD (24 h) | % Reduction in amastigotes |
|----------|--------------------|---|----------------------------|
| Vehicle | | 313.0 ± 18.3 | |
| 1 | 60 | 221.4 ± 29.5 | 29.3 |
| | 30 | 235.0 ± 53.7 | 24.9 |
| 3c | 60 | 7.3 ± 1.8 | 97.7 ^a |
| | 30 | 168.5 ± 35.9 | 46.2 ^a |
| 4c | 60 | 322.3 ± 57.1 | -3.0 |
| | 30 | 320.5 ± 54.4 | -2.4 |
| 11 | 60 | 8.7 ± 6.9 | 97.2 ^a |
| | 30 | 84.1 ± 27.1 | 73.1 ^a |

^a *P* < 0.001.

to an increase in activity, which became higher than that of PQ but lower than those of the reference drugs sitamaquine (compound 11) and miltefosine (compound 12). Compounds 9 and 10 were inactive, although the latter had shown the highest activity against *Plasmodium* (19). Compound 10 was obtained by directly binding a hexylferrocene moiety to the PQ heteroaromatic core, 8-amino-6-methoxyquinoline. Interestingly, inserting the same hexylferrocene moiety of compound 10 into PQ itself led to compound 6, which exhibited a remarkably higher activity against promastigotes and was more potent than both reference drugs (compounds 11 and 12). Finally, compound 8, whose structure encompasses an imidazolidin-4-one and an Fc moiety, also showed significant activity against *L. infantum* promastigotes, comparable to that of miltefosine (compound 12). With the exception of compound 6, no other PQ derivative ranked higher than sitamaquine (compound 11). Nonetheless, toxicity issues with this drug, the fact that peptidomimetic derivatives of PQ are promising alternatives to PQ due to enhanced chemical and metabolic resistance (37, 40), and the good activities demonstrated by the organometallic series of compounds motivated us to further explore the potential of some of them against the amastigote form of *Leishmania* parasites.

In vitro activity against intramacrophagic *L. infantum* amastigotes. In light of the observations made in the previous section, compounds 3c and 4c (Fig. 1), as well as compounds 5 and 8 (Fig. 2), were selected for evaluation on intramacrophagic *L. infantum* amastigotes, the clinically relevant parasite stage. The high toxicity of compound 6 toward mouse bone marrow-derived macrophages (Table 2) excluded it from further testing. Compound 7a was included in these studies for comparison with compound 8, for which it is the synthetic acyclic precursor. Moreover, PQ (compound 1), sitamaquine (compound 11), and miltefosine (compound 12) were included as reference drugs.

The antileishmanial activity of the peptidomimetic PQ derivative 3c was confirmed in this assay, as this compound was found to be more effective than PQ at eliminating intramacrophagic *L. infantum* (Table 3). Conversely, the dipeptide derivative 4c had no effect on the elimination of intramacrophagic *L. infantum* amastigotes (Table 3). As shown in Table 4, among the organometallic derivatives, compound 5 showed no activity against amastigotes, while the activity of compound 8 was confirmed on this intramac-

TABLE 4 Activities of PQ (1), 5, 7a, 8, and miltefosine (12) against intramacrophagic *L. infantum*

| Compound | Concentration (μM) | Mean no. of amastigotes/100 macrophages ± SD (72 h) | % Reduction in amastigotes |
|----------|--------------------|---|----------------------------|
| Vehicle | | 135.8 ± 27.5 | |
| 1 | 80 | 10.6 ± 5.1 | 92.2 ^b |
| | 40 | 66.8 ± 23.6 | 50.8 ^a |
| 5 | 80 | 127.8 ± 33.5 | 5.9 |
| | 40 | 99.0 ± 32.2 | 27.1 |
| 7a | 80 | 3.7 ± 1.1 | 97.3 ^b |
| | 40 | 5.3 ± 1.7 | 96.1 ^b |
| 8 | 80 | 29.0 ± 5.4 | 78.6 ^b |
| | 40 | 30.5 ± 3.3 | 77.6 ^b |
| 12 | 20 | 5.0 ± 1.0 | 96.3 ^b |

^a *P* < 0.05.^b *P* < 0.001.

rophagic form of the parasite (Table 4). Surprisingly, compound 7a also showed potent activity against amastigotes, revealing an efficacy of 40 μM, which is comparable to that of miltefosine (compound 12), at 20 μM (Table 4). Due to its high toxicity toward macrophages (Table 2 and Fig. 4C), miltefosine (compound 12) could not be used at concentrations higher than 20 μM in these assays. Lastly, both compounds 3c (Fig. 4E and Table 1) and 7a (Fig. 4F and Table 2) caused no significant toxicity toward macrophages, in contrast to reference drugs 11 and 12 (Fig. 4B and C and Tables 1 and 2). Reference drugs 11 (60 μM) and 12 (20 μM) clearly decreased the confluence and altered the morphology of infected macrophages compared to those with vehicle, whereas compounds 1 (60 μM), 3c (60 μM), and 7a (80 μM) did not significantly affect any of these parameters at the highest concentrations tested (Fig. 4).

DISCUSSION

Chemotherapy is currently the only option for the management of the life-threatening disease visceral leishmaniasis. However, the limited number of available drugs and their serious drawbacks reinforce the urgent need for adequate therapies (5). The ideal drug should be nontoxic, affordable, extremely effective, and easily administered during a short period in the outpatient setting, with no associated parasite resistance. To conform to these criteria, the rational modification of known antiparasitic molecules such as PQ is a valuable strategy. The modification of the PQ scaffold has already led to the discovery of new 8AQ with potent antileishmanial activities, such as NPC1161B (24), tafenoquine (44), and sitamaquine (25). However, the first two drugs have no applicability in the clinical setting at present, and the latter has been correlated with adverse side effects (10, 14, 34, 42). This highlights the importance for the continuous design of new primaquine-derived compounds as antileishmanial agents.

Structural modifications that involve the inclusion of imidazolidin-4-one (peptidomimetics; compound 3) and ferrocene (organometallics; compounds 5 to 10) moieties into PQ improve its activity against *Plasmodium* and *Pneumocystis* and/or its safety (19, 20, 36, 40). The results disclosed in this article show that the peptidomimetic 3c and organometallic 7a derivatives of PQ are more effective than the parent drug at eliminating intramacrophagic amastigotes of *L. infantum* and show decreased toxicity

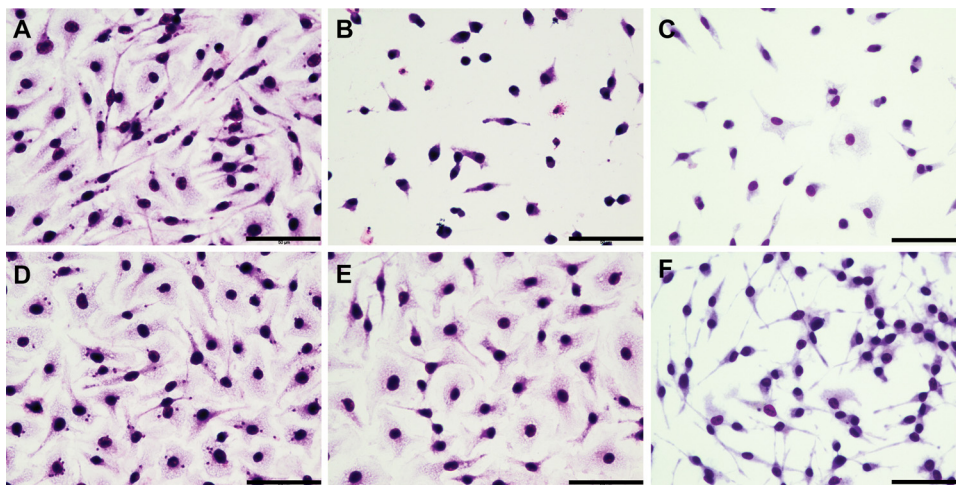


FIG 4 Micrographs of *L. infantum*-infected bone marrow-derived macrophages exposed to vehicle (A), compound 11 at 60 μ M (B), compound 12 at 20 μ M (C), compound 1 at 60 μ M (D), compound 3c at 60 μ M (E), or compound 7a at 80 μ M (F). Bars, 50 μ m.

and similar activity compared to those of sitamaquine and miltefosine.

We tested 5 peptidomimetic and 9 dipeptidic derivatives of PQ for activity against *L. infantum* promastigotes. Among these, compounds 3c and 4c were the most active, with IC_{50} s close to that of PQ (compound 1). It is remarkable that compounds 3c and 4c are closely related, as compound 3c is the imidazolidin-4-one peptidomimetic surrogate of PQ's dipeptide derivative 4c. This is hardly coincidental and shows that the valine residue common to the two is beneficial for *in vitro* antileishmanial activity against promastigotes. Interestingly, when the compounds were tested for antileishmanial activity inside macrophages, compound 3c was much more active than compound 4c. This is not surprising, since compound 4c is more susceptible than compound 3c to proteolytic degradation (37), which could be aggravated by the fact that protease activity is increased in *Leishmania*-infected macrophages (27). Proteolytic degradation would cause cleavage of the dipeptide moiety in compound 4c, leading to PQ release. However, if this were the case, compound 4c would be expected to display an activity similar to that of the parent drug, which did not occur. Another possible explanation for the discrepant behaviors of compounds 1, 3c, and 4c against either promastigotes or intracellular amastigotes could be the fact that the macrophage metabolizes compound 3c, but not compound 1 or 4c, to originate a compound which is more toxic to *L. infantum*. This lack of activity of compound 4c may also be due to the action of a macrophage efflux pump able to recognize dipeptide substrates, e.g., a multisubstrate transporter from the ubiquitous ATP-binding cassette (ABC) superfamily (4) or peptide/histidine transporters PHT1 and PHT2, which are expressed in macrophages (22). It is likely that such transporters would not recognize the peptidomimetic imidazolidin-4-one moiety in compound 3c. The most remarkable finding on compound 3c, however, is the fact that this compound was as potent as sitamaquine (compound 11) at the highest concentration tested (60 μ M) (Table 3), with the further advantage that compound 3c was not cytotoxic at this concentration, whereas sitamaquine was (Table 1; Fig. 4). Though sitamaquine (compound 11) performed better than compound 3c at the lowest concentration tested (30 μ M), its high toxicity hampers its applica-

tion in clinics, which urges the discovery of safer antileishmanial leads such as compound 3c. Given this situation, we have begun an evaluation of the efficacy of compound 3c in a mouse model of VL. Preliminary data indicate that low doses (116.5 nmol/mouse/day for 21 days) of compound 3c are as effective as PQ (compound 1) at reducing hepatic parasite loads (51.6% versus 41.8%) (data not shown), with no evidence of negative side effects. Moreover, we have experimentally confirmed that peptidomimetic derivatives of PQ are less prone than PQ (compound 1) to undergo metabolic conversion mediated by rat liver enzymes (37). Thus, our peptidomimetic derivatives display higher *in vivo* antileishmanial activity, lower toxicity, and higher stability than those of PQ.

This study allowed us to draw some conclusions regarding structure-activity relationships. β -Ramification of the amino acid (Xaa) residue may have a role in antimicrobial activity, as all PQ derivatives which displayed IC_{50} s of <35 μ M bore a β -ramified Xaa residue (Table 1). It is possible that this particular feature favors uptake by *L. infantum* parasites, mediated by specific amino acid permeases or transmembrane peptide transporters (28) parallel to, e.g., the Arg-specific transporter previously reported for *L. donovani* (9). Curiously, enhanced activity and uptake when β -ramified amino acids were present were previously described by us, though with other compounds and pathogens (30, 31). Although the most active compounds had high lipophilicity, we found no direct correlation between this parameter and antiparasitic activity (Table 1). Other structural features in Xaa do not seem to have any particular effect on a compound's activity against *L. infantum*.

Concerning the Fc derivatives of PQ, i.e., compounds 5 to 10 (Fig. 2), the results obtained on promastigotes (Table 2) were very variable, reflecting the high structural diversity of these organometallic compounds. Series 7a to h, in which PQ is linked to Fc through a variable amino acid spacer, was generally not very promising, while the removal of the amino acid spacer between PQ and Fc, giving compound 5, led to a substantial increase in activity. Also somewhat surprising was the fact that compound 10, the most promising antimalarial compound (19), was inactive against *L. infantum* promastigotes. Interestingly, compound 6, which has the same hexylferrocene moiety as that in compound

10, but linked to the PQ's aliphatic amine group rather than directly bound to the aryl-amine group in 8-amino-6-methoxyquinoline (the heteroaromatic core of PQ), appeared remarkably active against promastigotes, being more potent than both sitamaquine (compound 11) and miltefosine (compound 12). The second most active compound against *L. infantum* promastigotes was compound 8, with an activity comparable to that of miltefosine (compound 12). This is an interesting finding, as compound 8 includes both structural modifications pursued by our group over the past few years in order to improve PQ's therapeutic properties (2, 19, 20, 40): it encompasses an imidazolidin-4-one and an Fc moiety, i.e., it can be seen as an organometallic surrogate of peptidomimetic compound 3 (Fig. 1).

Since the intramacrophagic amastigote form of *Leishmania* is the most biologically relevant form, the most promising organometallic PQ derivatives were also tested in this model. Unfortunately, compound 6, which showed the highest activity against promastigotes, revealed a high toxicity toward bone marrow-derived macrophages, which excluded it from further studies. The CC_{50} on macrophages was about 7 μ M, in agreement with the cytotoxicity previously displayed on Huh-7 human hepatoma cells (19). In contrast, compounds 5 and 8 (similar to the peptidomimetics of the 3 and 4 series tested previously) had very low cytotoxicity and were selected for subsequent analysis on intramacrophagic amastigotes. Compound 7a, though inactive against promastigotes, was also used further for such studies, as it is representative of the noncytotoxic (Table 2) acyclic precursor of compound 8. In fact, compounds 5, 7a, and 8 can be seen as sequential analogues of each other, i.e., compound 7a is an analogue of compound 5 in which the simplest amino acid (glycine) has been introduced as a spacer between PQ and the Fc moiety, and in turn, compound 8 is a derivative of compound 7a in which an additional cyclization step has introduced an imidazolidin-4-one ring. While compound 5 lost its activity inside macrophages, compound 8 revealed a significant activity in this setting. Yet the most striking observation was that compound 7a had potent activity against amastigotes that was not far from that of miltefosine (compound 12) (Table 4). Thus, in contrast to what was observed with the peptidomimetic series, the presence of an imidazolidin-4-one ring was not necessary for intramacrophagic activity in the case of the organometallic derivatives. Eventually, the joining of the constrained cyclic imidazolidin-4-one motif with the bicyclic ferrocene moiety in compound 8 represents a structure too large or too stiff to easily enter the macrophage and/or the amastigote. By removing the imidazolidin-4-one ring, as in compound 7a, such a problem might be minimized or eliminated, explaining the higher activity of compound 7a than that of compound 8. Another clear fact is the key role of the amino acid residue: compound 7a differs from compound 5 only in the fact that the latter is missing the glycine residue present in the former, yet this difference was enough to remove antileishmanial activity for compound 5. Curiously, both compounds 7a and 5 were previously found to be inactive either against blood-stage *Plasmodium* or as malaria transmission-blocking agents, while presenting very good activity against liver-stage malaria parasites (19, 20). This suggests a stage-specific antiparasitic activity, which might deserve further investigation in the future. Our results also clearly show that the assessment of potential antileishmanial drugs should be performed at the intramacrophagic amastigote stage, not the promastigote stage, of *Leishmania* parasites.

The mechanisms of antileishmanial activity are not known for most of the drugs available. In the case of sitamaquine, it is known that the weakly basic and lipophilic characteristics are key factors for crossing parasite membranes (8, 11). This agrees with the fact that compound 3c, being the most active peptidomimetic derivative of PQ, is also the most lipophilic of the series 3 compounds. It was also proposed, but subsequently questioned (17), that sitamaquine is targeted to the parasite's acidocalcisomes, which are acidic vesicles where accumulation of weak bases such as sitamaquine would be favored. One thing is certain and common to observations with other 8AQ drugs, such as PQ (39): sitamaquine causes swelling and impairs the function of parasitic mitochondria, possibly affecting the electron transport chain and favoring the generation of reactive oxygen species (ROS), which are probably related to its high antileishmanial activity (32). In this connection, metallocene derivatives of PQ such as compounds 5 to 10 may also prove useful against *Leishmania*, as the ferrocene moiety is known to undergo a Fenton-like redox reaction leading to highly reactive oxygen species that both are pernicious for membrane-unsaturated fatty acids and promote chain reactions through peroxidation products (7, 19). Moreover, several reports point to the pivotal role of iron, which is present in the Fc moiety, in mediating drug toxicity. Iron potentiates the leishmanicidal activity of the antimalarial drug artemisinin (33) or the metalloids arsenic(III) and antimony(III) (21) by enhancing the generation of ROS that consequently lead to cell death in *L. donovani* promastigotes. Thus, strategies intended to augment the iron status of *Leishmania*, such as inclusion of an Fc moiety in a drug, could increase the sensitivity of the parasite to treatment.

Factors underlying the different activities described in this study remain to be determined. However, it is clear that the screening of a variety of PQ derivatives for antileishmanial activity revealed two compounds with significant effects on the clinically relevant intramacrophagic stage of *L. infantum*. These compounds join together interesting (compound 3c) or highly potent (compound 7a) activities with very low toxicity for host cells. This makes them worthy candidates as leads for the development of novel and safer PQ-based antileishmanials and emphasizes the relevance of exploring the potential of PQ-based structures against protozoan pathogens other than *Plasmodium*.

ACKNOWLEDGMENTS

This research project was jointly supported by the Fundação para a Ciência e Tecnologia (FCT; grant PTDC/QUI/65142/2006) and the European Union-FEDER (FCOMP-01-0124-FEDER-007418). S.V.-C. thanks the FCT for doctoral grant SFRH/BD/36661/2007. N.V. thanks the FCT for postdoctoral grant SFRH/BPD/48345/2008.

We are grateful to GlaxoSmithKline for kindly providing the sitamaquine used throughout this study.

REFERENCES

- Alvar J, et al. 2008. The relationship between leishmaniasis and AIDS: the second 10 years. *Clin. Microbiol. Rev.* 21:334–359.
- Araujo MJ, et al. 2005. Imidazolidin-4-one derivatives of primaquine as novel transmission-blocking antimalarials. *J. Med. Chem.* 48:888–892.
- Banerjee G, Medda S, Basu MK. 1998. A novel peptide-grafted liposomal delivery system targeted to macrophages. *Antimicrob. Agents Chemother.* 42:348–351.
- Borges-Walmsley MI, McKeegan KS, Walmsley AR. 2003. Structure and function of efflux pumps that confer resistance to drugs. *Biochem. J.* 376: 313–338.
- Chappuis F, et al. 2007. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat. Rev. Microbiol.* 5:873–882.

6. Charton M. 1975. Steric effects. I. Esterification and acid-catalyzed hydrolysis of esters. *J. Am. Chem. Soc.* 97:1552–1556.
7. Chavain N, et al. 2008. Investigation of the redox behavior of ferroquine, a new antimalarial. *Mol. Pharm.* 5:710–716.
8. Coimbra ES, et al. 2010. Mechanism of interaction of sitamaquine with *Leishmania donovani*. *J. Antimicrob. Chemother.* 65:2548–2555.
9. Darlyuk I, et al. 2009. Arginine homeostasis and transport in the human pathogen *Leishmania donovani*. *J. Biol. Chem.* 284:19800–19807.
10. Dietze R, et al. 2001. Phase 2 trial of WR6026, an orally administered 8-aminoquinoline, in the treatment of visceral leishmaniasis caused by *Leishmania chagasi*. *Am. J. Trop. Med. Hyg.* 65:685–689.
11. Duenas-Romero AM, Loiseau PM, Saint-Pierre-Chazalet M. 2007. Interaction of sitamaquine with membrane lipids of *Leishmania donovani* promastigotes. *Biochim. Biophys. Acta* 1768:246–252.
12. Gaspar R, Opperdoes FR, Preat V, Roland M. 1992. Drug targeting with polyalkylcyanoacrylate nanoparticles: in vitro activity of primaquine-loaded nanoparticles against intracellular *Leishmania donovani*. *Ann. Trop. Med. Parasitol.* 86:41–49.
13. Heurtault B, et al. 2001. The antileishmanial properties of surface-modified, primaquine-loaded nanocapsules tested against intramacrophagic *Leishmania donovani* amastigotes in vitro. *Ann. Trop. Med. Parasitol.* 95:529–533.
14. Jha TK, et al. 2005. A phase II dose-ranging study of sitamaquine for the treatment of visceral leishmaniasis in India. *Am. J. Trop. Med. Hyg.* 73:1005–1011.
15. Kaur K, et al. 2007. Synthesis, antimalarial, antileishmanial, antimicrobial, cytotoxicity, and methemoglobin (MetHB) formation activities of new 8-quinolinamines. *Bioorg. Med. Chem.* 15:915–930.
16. Loiseau PM, Cojean S, Schrével J. 2011. Sitamaquine as a putative antileishmanial drug candidate: from the mechanism of action to the risk of drug resistance. *Parasite* 18:115–119.
17. Lopez-Martin C, Perez-Victoria JM, Carvalho L, Castanys S, Gamarro F. 2008. Sitamaquine sensitivity in *Leishmania* species is not mediated by drug accumulation in acidocalcisomes. *Antimicrob. Agents Chemother.* 52:4030–4036.
18. Maltezou HC. 2010. Drug resistance in visceral leishmaniasis. *J. Biomed. Biotechnol.* 2010:617521.
19. Matos J, et al. 2012. Novel potent metallocenes against liver stage malaria. *Antimicrob. Agents Chemother.* 56:1564–1570.
20. Matos J, et al. 2010. Primacenes: novel non-cytotoxic primaquine-ferrocene conjugates with anti-*Pneumocystis carinii* activity. *MedChemComm* 1:199–201.
21. Mehta A, Saha C. 2006. Mechanism of metalloid-induced death in *Leishmania* spp.: role of iron, reactive oxygen species, Ca²⁺, and glutathione. *Free Radic. Biol. Med.* 40:1857–1868.
22. Moreau A, Le Vee M, Jouan E, Parmentier Y, Fardel O. 2011. Drug transporter expression in human macrophages. *Fundam. Clin. Pharmacol.* 25:1–10.
23. Murray HW, Berman JD, Davies CR, Saravia NG. 2005. Advances in leishmaniasis. *Lancet* 366:1561–1577.
24. Nanayakkara NP, et al. 2008. Antiparasitic activities and toxicities of individual enantiomers of the 8-aminoquinoline 8-[(4-amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3,4-dichlorophenoxy]quinoline succinate. *Antimicrob. Agents Chemother.* 52:2130–2137.
25. Peters W, Trotter ER, Robinson BL. 1980. The experimental chemotherapy of leishmaniasis. V. The activity of potential leishmanicides against “*L. infantum* LV9” in NMRI mice. *Ann. Trop. Med. Parasitol.* 74:289–297.
26. Portela MJ, et al. 1999. Dipeptide derivatives of primaquine as transmission-blocking antimalarials: effect of aliphatic side-chain acylation on the gametocytocidal activity and on the formation of carboxyprimaquine in rat liver homogenates. *Pharm. Res.* 16:949–955.
27. Prina E, Antoine JC, Wiederanders B, Kirschke H. 1990. Localization and activity of various lysosomal proteases in *Leishmania amazonensis*-infected macrophages. *Infect. Immun.* 58:1730–1737.
28. Rochette A, et al. 2008. Genome-wide gene expression profiling analysis of *Leishmania major* and *Leishmania infantum* developmental stages reveals substantial differences between the two species. *BMC Genomics* 9:255–280.
29. Rodrigues JM, Jr, Croft SL, Fessi H, Bories C, Devissaguet JP. 1994. The activity and ultrastructural localization of primaquine-loaded poly(D,L-lactide) nanoparticles in *Leishmania donovani* infected mice. *Trop. Med. Parasitol.* 45:223–228.
30. Santos C, et al. 2008. Dipeptide derivatives of AZT: synthesis, chemical stability, activation in human plasma, hPEPT1 affinity, and antiviral activity. *ChemMedChem* 3:970–978.
31. Santos CR, et al. 2009. Structure-activity relationships for dipeptide prodrugs of acyclovir: implications for prodrug design. *Eur. J. Med. Chem.* 44:2339–2346.
32. Seifert K. 2011. Structures, targets and recent approaches in antileishmanial drug discovery and development. *Open Med. Chem. J.* 5:31–39.
33. Sen R, Saha P, Sarkar A, Ganguly S, Chatterjee M. 2010. Iron enhances generation of free radicals by artemisinin causing a caspase-independent, apoptotic death in *Leishmania donovani* promastigotes. *Free Radic. Res.* 44:1289–1295.
34. Sundar S, et al. 2011. Pharmacokinetics of oral sitamaquine taken with or without food and safety and efficacy for treatment of visceral leishmaniasis: a randomized study in Bihar, India. *Am. J. Trop. Med. Hyg.* 84:892–900.
35. Tekwani BL, Walker LA. 2006. 8-Aminoquinolines: future role as anti-protozoal drugs. *Curr. Opin. Infect. Dis.* 19:623–631.
36. Vale N, et al. 2008. Anti-*Pneumocystis carinii* and antiplasmodial activities of primaquine-derived imidazolidin-4-ones. *Bioorg. Med. Chem. Lett.* 18:485–488.
37. Vale N, Fernandes I, Moreira R, Mateus N, Gomes P. 2012. Comparative analysis of in vitro rat liver metabolism of the antimalarial primaquine and a derived imidazoquinone. *Drug Metab. Lett.* 6:15–25.
38. Vale N, Matos J, Moreira R, Gomes P. 2008. Electrospray ionization-ion trap mass spectrometry study of PQAAPro and PQProAA mimetic derivatives of the antimalarial primaquine. *J. Am. Soc. Mass Spectrom.* 19:1476–1490.
39. Vale N, Moreira R, Gomes P. 2009. Primaquine revisited six decades after its discovery. *Eur. J. Med. Chem.* 44:937–953.
40. Vale N, et al. 2009. Imidazoquinones as antimalarial and antipneumocystis agents. *J. Med. Chem.* 52:7800–7807.
41. van Griensven J, et al. 2010. Combination therapy for visceral leishmaniasis. *Lancet Infect. Dis.* 10:184–194.
42. Wasunna MK, et al. 2005. A phase II dose-increasing study of sitamaquine for the treatment of visceral leishmaniasis in Kenya. *Am. J. Trop. Med. Hyg.* 73:871–876.
43. WHO. 2010. Control of the leishmaniasis. World Health Organ. Tech. Rep. Ser. 2010:1–186.
44. Yardley V, Gamarro F, Croft SL. 2010. Antileishmanial and antitrypanosomal activities of the 8-aminoquinoline tafenoquine. *Antimicrob. Agents Chemother.* 54:5356–5358.