

## Synthesis, in Vitro Evaluation, and Antileishmanial Activity of Water-Soluble Prodrugs of Buparvaquone

Antti Mäntylä,<sup>\*,†</sup> Tracy Garnier,<sup>‡</sup> Jarkko Rautio,<sup>†</sup> Tapio Nevalainen,<sup>†</sup> Jouko Vepsäläinen,<sup>§</sup> Ari Koskinen,<sup>||</sup> Simon L. Croft,<sup>‡</sup> and Tomi Järvinen<sup>†</sup>

Department of Pharmaceutical Chemistry, University of Kuopio, PO Box, 1627, FIN-70211 Kuopio, Finland, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT, U.K., Department of Chemistry, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland, and Laboratory of Organic Chemistry, Helsinki University of Technology, PO Box, 6100, FIN-70211 HUT, Finland

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Water-soluble phosphate prodrugs of buparvaquone (**1**), containing a hydroxynaphthoquinone structure, were synthesized and evaluated in vitro for improved topical and oral drug delivery against cutaneous and visceral leishmaniasis. The successful prodrug synthesis involved a strong base; e.g., sodium hydride. Buparvaquone-3-phosphate (**4a**) and 3-phosphonoxymethyl-buparvaquone (**4b**) prodrugs possessed significantly higher aqueous solubilities (>3.5 mg/mL) than the parent drug ( $\leq 0.03 \mu\text{g/mL}$ ) over a pH range of 3.0–7.4. Moreover, **4a** and **4b** maintained adequate lipophilicity as indicated by distribution coefficients ( $\log D$ ) between 0.5 and 3.0 over this pH range. Both **4a** and **4b** were also shown to be substrates for alkaline phosphatase in vitro and thus are promising bioreversible prodrugs for the improved topical and oral bioavailability of **1**. Buparvaquone and its prodrugs showed nanomolar or low-micromolar  $\text{ED}_{50}$  activity values against species that cause cutaneous leishmaniasis, e.g., *L. major*, *L. amazonensis*, *L. aethiopica*, *L. mexicana*, and *L. panamensis* and also *L. donovani*, which is the causative agent of visceral leishmaniasis. From these results, the human skin permeation of the prodrugs **4a** and **4b** were studied in vitro. While no buparvaquone permeated across post mortem skin in vitro during 72 h of experiments, both prodrugs **4a** and **4b** permeated readily through the skin. In addition, **4b** easily released the parent drug in human skin homogenate and, therefore, is a promising prodrug candidate to deliver buparvaquone through the skin for the treatment of cutaneous leishmaniasis.

### Introduction

Leishmaniasis is a widespread parasitic disease caused by protozoan parasites of the genus *Leishmania*.<sup>1</sup> This disease occurs in two major forms: cutaneous (CL) and visceral leishmaniasis (VL) as well as rarer forms such as diffuse cutaneous (DCL) and mucocutaneous (MCL).<sup>2</sup> The most severe form, VL, known as kala-azar, is nearly always fatal if not treated. Leishmaniasis is endemic in tropical and subtropical areas in both the Old and New Worlds. It is estimated that about 1.5 million new cases of CL and 0.5 million VL occur annually, and over 350 million people live at risk of infection.<sup>3</sup> Over 20 species of *Leishmania* have been identified, and the most common species that cause CL in the Old World are *L. major*, *L. tropica*, and *L. aethiopica* and in the New World *L. braziliensis*, *L. panamensis*, *L. amazonensis*, and *L. mexicana*.<sup>4</sup> Typically, CL presents as a nodule at the site of an insect bite, that gradually develops into an open lesion before self-cure within 3 to 18 months. VL is mainly caused by *L. donovani*,<sup>4</sup> in which the parasite spreads from the site of inoculation to multiply as an amastigote in macrophages of the

spleen, liver, lymph nodes, and bone marrow. Since the 1940s, pentavalent antimony compounds have been the main drugs used in antileishmanial chemotherapy.<sup>3,4</sup> Although these drugs are effective, they have several disadvantages such as the need for parenteral administration at high dosages, long duration of therapy (several weeks), toxic side-effects, and variable efficacy. Thus, a real need exists for improved antileishmanials, particularly for oral administration in the treatment of VL and for topical administration in the treatment of CL.

The antiprotozoal activity of hydroxynaphthoquinones was already reported over 10 years ago.<sup>5,6</sup> Many naphthoquinones have been tested against *L. donovani*, and other species both in vitro and in vivo, as oral, subcutaneous, and topical administrations.<sup>7,8</sup> Buparvaquone has shown high activity in vitro against *L. donovani* amastigotes.<sup>8</sup> However, the in vivo activity of buparvaquone against *L. donovani* was low after subcutaneous administration.<sup>8</sup> This might be due to its poor distribution from the site of injection and subsequent lack of distribution of the drug to the intracellular target. In addition, the oral bioavailability of buparvaquone is low. These properties are most probably due to a combination of the low aqueous solubility and high lipophilicity of buparvaquone.

The prodrug approach is an effective means of improving the oral bioavailability of poorly water-soluble drugs by chemical derivatization to more water-soluble

\* To whom correspondence should be addressed. Tel: +358-17-163658. Fax: +358-17-162456. E-mail: Antti.Mantyla@uku.fi.

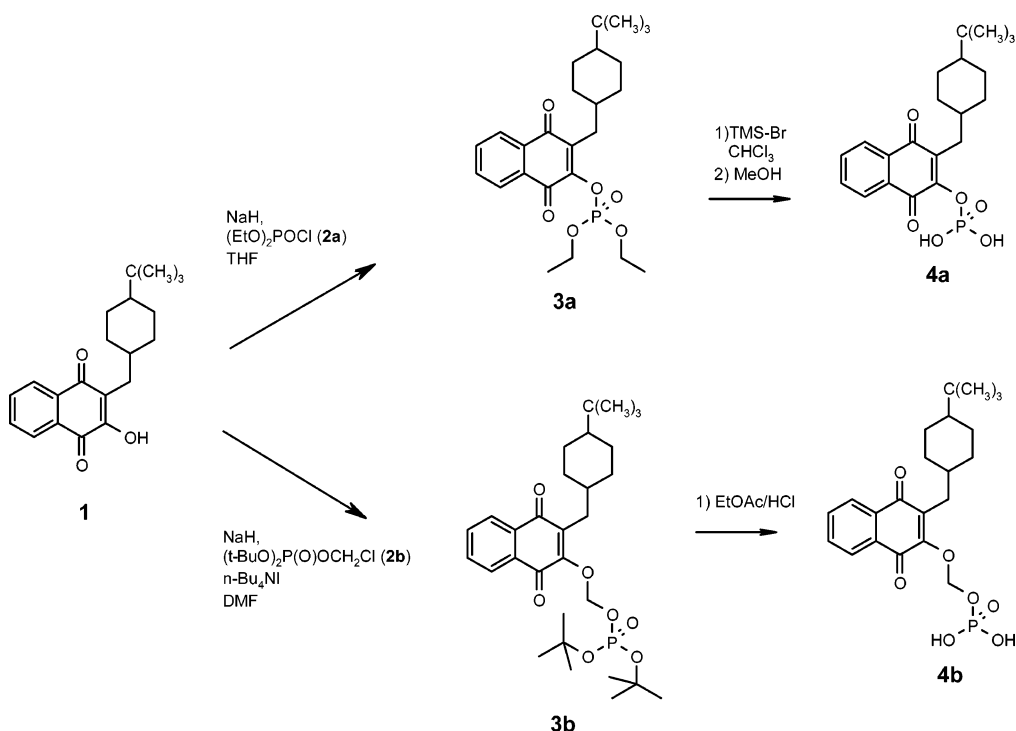
<sup>†</sup> Department of Pharmaceutical Chemistry, University of Kuopio.

<sup>‡</sup> Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine.

<sup>§</sup> Department of Chemistry, University of Kuopio.

<sup>||</sup> Laboratory of Organic Chemistry, Helsinki University of Technology.

Scheme 1



compounds. The most commonly used prodrug strategy for increasing aqueous solubility of drugs containing a hydroxyl group is to produce esters containing an ionizable group; e.g., phosphate group.<sup>9–11</sup> Phosphate promoiety can be bound either directly or via a spacer group to molecules having an hydroxyl functionality.<sup>12,13</sup> A prodrug is typically designed to release the active drug in the body during or after absorption by enzymatic and/or chemical hydrolysis. However, a phosphate group that is directly attached to a hydroxyl group is not always cleaved enzymatically due to steric hindrance. Therefore, much interest has been focused on designing enzymatically labile phosphonoxymethyl prodrugs.<sup>14–16</sup> Such prodrugs release the parent drug via a two-step hydrolysis reaction; the first step is enzymatic cleavage of the ester group to give the hydroxymethyl intermediate, which then spontaneously releases the parent drug.

The prodrug approach is also widely used to improve topical drug delivery of various drugs by masking hydroxyl or amino groups via esterification.<sup>16–18</sup> Topical formulations offer significant advantages over systemic therapy (e.g., antimonials) such as ease of administration, patient compliance, lower adverse effects, and low cost. Local treatment remains an attractive approach for the simple localized forms of CL, not at risk of MCL or involving critical organs. Treatment aims to accelerate healing and minimize scarring. A successful dermal prodrug should have an optimum lipophilicity (log *D* 2–3) and adequate aqueous solubility.<sup>19</sup> In this study, phosphate prodrugs to improve buparvaquone skin permeation for topical drug delivery were explored. In CL, intracellular amastigotes survive and multiply within the parasitophorous vacuole of macrophages in the lower dermis. It was reasoned that phosphate prodrugs will release buparvaquone during skin permeation and lipophilic buparvaquone will become “locked” in the epidermis or dermis and produce a local effect in lesions of CL.

In the present study, buparvaquone-3-phosphate (4a) and 3-phosphonoxymethyl-buparvaquone (4b) prodrugs were synthesized to increase the aqueous solubility of buparvaquone and thus improve both oral and topical drug delivery properties. The hydrolytic properties of the prodrugs in aqueous buffers, specific enzyme solution, and human skin homogenate were determined in addition to in vitro permeation across post mortem human skin. The in vitro activities of buparvaquone and its phosphate prodrugs were also determined against the promastigote and amastigote forms of several *Leishmania* species causing both CL and VL.

## Results and Discussion

**Synthesis.** The prodrugs 4a and 4b were prepared by the route described in Scheme 1. Typical phosphorylation methods such as using phosphorus oxychloride<sup>12,20</sup> in dry pyridine or concentrated phosphoric acid and phosphorus pentoxide<sup>21</sup> or synthesis with di-*tert*-butyl-*N,N*-diisopropylphosphoramidite in the presence of 1*H*-tetrazole<sup>22</sup> were unsuccessful. However, when 1 was first treated with sodium hydride followed by protected chlorophosphate (2a), 3a was produced in good yield. The ethyl protecting groups were removed by treatment with trimethylsilyl bromide in dry dichloromethane to yield the prodrug 4a. In the case of 4b, the protected chloromethyl phosphate 2b, prepared by an earlier described method,<sup>23</sup> was condensed with 1 to produce 3b in moderate yield. After removal of the di-*tert*-butyl protecting group by a saturated EtOAc/HCl solution, the resulting prodrug 4b was isolated. The preparation of the –O–CH<sub>2</sub>–O–P– functionality through a methylthiomethyl ether intermediate<sup>24,25</sup> was not successful.

**Aqueous Solubility, Lipophilicity, and Ionization Constant.** The aqueous solubilities, distribution coefficients (log *D*) and ionization coefficients of 1 and the phosphate prodrugs 4a,b are summarized in Table

**Table 1.** Aqueous Solubility, Distribution Coefficients, and  $pK_a$  Values of Buparvaquone (BPQ) and Phosphate Prodrugs **4a,b**

compd	aqueous solubility (mg/mL)			log <i>D</i>			$pK_a$
	pH 7.4	pH 5.0	pH 3.0	pH 7.4	pH 5.0	pH 3.0	
BPQ	$0.03 \pm 0.01 \mu\text{g/mL}^a$	$<0.03 \mu\text{g/mL}$	$<0.03 \mu\text{g/mL}$			$7.02^b$	$5.70^c$
<b>4a</b>	$>3.5^d$	$>3.5^d$	$>3.5^d$	$0.47^c$	$1.27^c$	$1.87^c$	$2.68^c$
<b>4b</b>	$>3.5^d$	$>3.5^d$	$>3.5^d$	$1.16^c$	$1.83^c$	$2.96^c$	$6.74^c$ $2.28^c$ $6.76^c$

<sup>a</sup> Mean  $\pm$  SD;  $n = 3$ . <sup>b</sup> Determined using the capacity factors of RP-HPLC. <sup>c</sup> Determined by Sirius PCA 200. <sup>d</sup> Limited amount of the compound precluded further analyses.

**Table 2.** Rates of Hydrolysis of Buparvaquone Phosphate Prodrugs **4a,b** in Buffer Solutions (pH 7.4, pH 5.0, and pH 3.0) at 37 °C

compd	$t_{1/2}$ (h) (borate buffer pH 7.4)	$t_{1/2}$ (h) (acetate buffer pH 5.0)	$t_{1/2}$ (h) (citric acid buffer pH 3.0)
<b>4a</b>	17	50	72
<b>4b</b>	0.5	8	97

1. The aqueous solubility of **1** is low in both acidic aqueous solutions ( $<0.03 \mu\text{g/mL}$  at pH 3.0 and 5.0) and in neutral aqueous solution ( $0.03 \mu\text{g/mL}$  at pH 7.4). The log *D* value of **1** was determined by using the capacity factors of RP-HPLC, because the pH-metric method (Sirius PCA200) was limited by the high lipophilicity and low aqueous solubility of **1**. These results are in agreement with an earlier study<sup>8</sup> that reported buparvaquone to possess poor distribution from the site of injection and inefficient absorption in oral drug delivery, which is the most probable reason for the low in vivo activity of **1** against *L. donovani*. Ideally, the drug molecule should possess adequate solubility in acidic aqueous solution in order to dissolve in the small intestine, and thus be available for absorption. On the other hand, an optimal drug molecule should also be lipophilic enough for the effective absorption via passive transcellular diffusion. A log *D* value of 2–3 is considered to be optimal for gastrointestinal absorption.<sup>26</sup> Compared to buparvaquone, the aqueous solubilities of the phosphate prodrugs **4a,b** were significantly greater in both acidic and neutral aqueous solutions and, moreover, they maintained log *D* values between 0.5 and 3.0 over the examined pH range. The maximum aqueous solubilities of **4a** and **4b** could not be determined due to the small amounts of each prodrug available, and thus the difference in aqueous solubilities between compounds **4a** and **4b** were not compared. However, compound **4b** had a higher log *D* value than **4a** over the studied pH range, which was probably due to the presence of the methyl linker in compared **4a**. The results show that both prodrugs **4a** and **4b** have excellent aqueous solubilities over a pH range of 3.0 to 7.4 and also adequate lipophilicity for efficient absorption across biological membranes.

**Chemical Hydrolysis.** The kinetics of chemical hydrolysis for **4a,b** followed first-order kinetics over several half-lives are listed in Table 2. In the present study, only the hydrolysis of prodrugs was determined and the amount of degradation product (buparvaquone) was not quantified due to its low aqueous solubility. However, the subsequent precipitate in aqueous medium was verified to be buparvaquone. The aim of chemical hydrolysis studies was to determine whether the phosphate prodrugs would be sufficiently stable in

**Table 3.** Rates of Hydrolysis of Buparvaquone Phosphate Prodrugs **4a,b** in Alkaline Phosphatase Solution (pH 7.4), and in Human Skin Homogenate (pH 7.4) at 37 °C (mean;  $n = 2$ )

compd	$t_{1/2}$ (min) alkaline phosphatase solution	$t_{1/2}$ (min) human skin homogenate
<b>4a</b>	1.2	522
<b>4b</b>	3.8	10.5

the GI-tract before their absorption and also to find the most suitable pH (i.e., maximum stability) for topical formulations. The phosphate prodrugs **4a** and **4b** were considered to be stable enough in acidic aqueous solutions ( $t_{1/2} = 72$  and  $97$  h, respectively) for oral drug delivery (Table 2). For topical drug delivery, stabilities in aqueous solutions at pH 3–5 were low, and thus further formulation studies are needed to stabilize prodrugs **4a** and **4b**.

**Enzymatic Hydrolysis. Alkaline Phosphatase Solution.** The phosphate esters **4a** and **4b** must undergo a chemical or enzymatic bioconversion to release the parent drug in order to fulfill the prodrug criteria. In addition to red blood cells<sup>27</sup> alkaline phosphatases have been found in various human tissues; e.g., placenta, intestine, liver, bone, and kidney,<sup>28</sup> and they have been reported to be the responsible enzymes for the hydrolysis of phosphate esters.<sup>16,29</sup> The susceptibility of buparvaquone phosphate esters (**4a,b**) to enzymatic hydrolysis was studied in alkaline phosphatase enzyme solution (Table 3). Both phosphate prodrugs **4a** and **4b** hydrolyzed rapidly ( $t_{1/2} = 1.2$  and  $3.8$  min, respectively) in alkaline phosphatase solution and released the parent drug, and thus showed these compounds to be good substrates for this enzyme.

**Enzymatic Hydrolysis. Skin Homogenate.** The half-lives of **4a** and **4b** in human skin homogenate are summarized in Table 3. Similarly to chemical hydrolysis studies only hydrolysis of prodrugs was determined. The degradation product was confirmed to be buparvaquone. The rates of hydrolysis of **4a** and **4b** in human skin homogenate (pH 7.4) were from 2 to 3 times faster, respectively, than the rates of chemical hydrolysis in 0.185 M borate buffer (pH 7.4). These results suggest that the hydrolysis of the prodrugs in human skin homogenate is at least partly enzymatic hydrolysis. Because **4b** hydrolyzed to buparvaquone rapidly in skin homogenate, it could be considered useful as a topical prodrug.

**In Vitro Skin Permeation Study.** The steady-state fluxes ( $J_{ss}$ ) of buparvaquone and prodrugs **4a** and **4b** are shown in Table 4. The results show that the post mortem skin permeation of buparvaquone can be improved by the phosphate prodrug strategy. While buparvaquone permeation in aqueous buffers or in buffer–ethanol mixtures was not observed at all, the prodrugs



**Table 4.** Steady-State Fluxes ( $J_{ss}$ ) for Delivery of Total Buparvaquone Species through Human Skin in Aqueous Buffers (pH 3.0 and pH 5.0) and Ethanol-Buffer (pH 5.0) Mixture at 37 °C

compd	flux (nmol/cm <sup>2</sup> /h) (mean $\pm$ SD, $n = 2-4$ )		
	isotonic citric acid buffer (40 mM, pH 3.0)	isotonic sodium acetate buffer (50 mM, pH 5.0)	50% isotonic sodium acetate buffer (50 mM, pH 5.0) and 50% ethanol
BPQ	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
<b>4a</b>	0.051 $\pm$ 0.02	0.133 $\pm$ 0.40	0.106 $\pm$ 0.08
<b>4b</b>	0.199 $\pm$ 0.01	1.500 $\pm$ 0.12	0.135 $\pm$ 0.05

<sup>a</sup> No drug permeation observed at 72 h.

**4a** and **4b** permeated across the post mortem skin in all studied media. The prodrug **4b** afforded a 11-fold and a 4-fold flux compared to **4a** at pH 5.0 and pH 3.0, respectively, most probably due to its higher lipophilicity. A high drug concentration drives passive diffusion, and thus the prodrugs **4a** and **4b** themselves resulted in a 2.6-fold and a 7.5-fold higher fluxes at pH 5.0 than at pH 3.0, respectively. Moreover, buparvaquone and the phosphate prodrugs **4a** and **4b** have shown high activities in vitro against species that cause CL and thus may be good candidates for topical drug administration.

**In Vitro Parasite Sensitivity.** Assays involving both the extracellular promastigotes and intracellular amastigotes were carried out to confirm the antileishmanial activity of the compounds. The amastigote is the clinically relevant form of the parasite.

**(a) Extracellular Promastigotes.** Activity against *L. major* promastigotes has recently been determined using AlamarBlue.<sup>30</sup> However, cultures should ideally be left to incubate for 48–72 h before final addition of the dye. In this assay, fluorescent readings were taken within 8 h post addition of the dye since fluorescent response did not increase proportionally with time during long incubation periods (> 24 h). Fluorescence measurement, being more sensitive than the colometric assessment used in this previous paper, allowed shorter incubation with the dye.

The ED<sub>50</sub> values for promastigotes indicated that buparvaquone and its prodrugs **4a** and **4b** have potent in vitro activity (2 to 124 nM), in many cases having greater activity than the amphotericin B control drug (Table 5). Against the majority of species investigated, the activity in order of decreasing potency is: buparvaquone > **4b** > **4a** > amphotericin B. The activity of prodrugs (**4a** and **4b**) is probably due to their hydrolysis to buparvaquone during the experiment. Comparison of species sensitivity (Table 5) using the AlamarBlue method should be interpreted with caution. Differences in fluorescent response seen among species may result from rate/extent of AlamarBlue uptake or metabolism and cell number/size (reflected in sensitivity), rather than intrinsic sensitivity to drugs. After the 72-h incubation, none of the compounds (in media alone) were found to reduce the dye (data not shown).

**(b) Intracellular Amastigotes.** Drug sensitivity against the intracellular amastigotes demonstrated similar interspecies sensitivity to the promastigote form (Table 6). The activity in order of decreasing potency is buparvaquone > **4b** > **4a** > Sb<sup>v</sup>. The activity of buparvaquone was in the range of 0.37 to 5.50  $\mu$ M. Here again, the activity of prodrugs (**4a** and **4b**) is probably

caused by the release of buparvaquone. Inter-species comparisons should take into consideration the differences in infection rate, both % of infected macrophages (%M $\phi$ ) and mean number of amastigotes per macrophage (mean amastigote/M $\phi$ ). Buparvaquone activity against in vitro *L. donovani* amastigotes has already been shown.<sup>8</sup> For example, *L. mexicana* infected macrophages (Table 6) contained a high number of amastigotes in the untreated control and as a result an ED<sub>50</sub> could not be calculated for the Pentostam control. Poor response rates to antimonials have been reported for *L. mexicana*, *L. major*, and *L. aethiopica*.<sup>4</sup> It is important to consider that many previously reported results use longer incubation periods and because of this efficacy may appear lower in the 72-hour assay. Also the % infection (often not stated) may vary significantly depending on the infection ratio and whether amastigotes or promastigotes were used. The pharmacodynamics of different antileishmanial drugs may result in different rates of amastigote clearance from macrophages and higher ED<sub>50</sub> values.

## Conclusion

Buparvaquone-3-phosphate (**4a**) and 3-phosphonooxymethyl-buparvaquone (**4b**) were obtained in high yields by using sodium hydride. The aqueous solubility of buparvaquone was significantly increased by phosphorylation of the hydroxyl group and lipophilicity was maintained within an optimal range (log *D* 2–3) for absorption across biological membranes. Prodrugs **4a** and **4b** rapidly release buparvaquone in alkaline phosphate solution.

The present study reports potent in vitro activity (nanomolar to low micromolar range) for buparvaquone and its phosphate prodrugs against the *Leishmania* species that cause CL and VL. Against the promastigote and amastigote form of most species investigated, the activity in order of decreasing potency was buparvaquone > **4b** > **4a** > control drug. It cannot be excluded that the activity caused by prodrugs (**4a** and **4b**) is due to their conversion to buparvaquone. The study also shows that buparvaquone permeation through human skin can be significantly improved by using phosphate prodrugs. Furthermore, the prodrug **4b** was rapidly converted to the parent drug in human skin homogenate. Thus, 3-phosphonooxymethyl-buparvaquone (**4b**) is a promising compound for topical treatment of CL. These phosphate prodrugs (**4a** and **4b**) will be further investigated for both oral and topical drug administration in vivo.

## Experimental Section

**General Procedures.** <sup>1</sup>H, <sup>31</sup>P, and <sup>13</sup>C NMR spectra were recorded on a Bruker Avance DRX500 spectrometer (Bruker, Rheinstetter, Germany) operating at 500.13, 202.45, and 125.76 MHz, respectively. TMS was used as an internal reference for <sup>1</sup>H and <sup>13</sup>C, and 85% H<sub>3</sub>PO<sub>4</sub> as an external standard for <sup>31</sup>P. The splitting pattern abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dt = doublet of triples, bs = broad singlet, bm = broad multiplet. Mass spectra were acquired by a LCQ quadrupole ion trap mass spectrometer with an electrospray ionization source (Finnigan, San Jose, CA). The samples were dissolved in methanol (100  $\mu$ g/mL) and 5  $\mu$ L of samples were injected. The mobile phase consisted of acetonitrile/0.1% formic acid mixture (50:50 v/v), and the flow was set to 200  $\mu$ L/min.

**Table 5.** ED<sub>50</sub> Values in μM (95% confidence intervals) at 72 h for Promastigotes

species	BPQ	4a	4b	Amp B (control)	sensitivity
old world					
<i>L. donovani</i> L82	0.006 (0.004–0.011)	0.009 (0.006–0.012)	0.003 (0.003–0.004)	0.038 (0.037–0.038)	14–16
<i>L. aethiopica</i> Khartoum	0.013 (0.008–0.017)	0.122 (0.072–0.171)	0.057 (0.027–0.087)	0.022 (0.013–0.031)	20–27
<i>L. major</i> JISH118	0.001 (0.001–0.002)	0.064 (0.055–0.073)	0.012 (0.008–0.016)	0.104 (0.098–0.110)	11–18
new world					
<i>L. amazonensis</i> LV81	0.004 (0.003–0.006)	0.024 (0.020–0.027)	0.007 (0.006–0.008)	0.104 (0.097–0.111)	32–36
<i>L. mexicana</i> LV4	0.004 (0.003–0.004)	0.039 (0.026–0.051)	0.010 (0.008–0.012)	0.123 (0.091–0.156)	29–35
<i>L. mexicana</i> BEL21	0.002 (0.000–0.005)	0.024 (0.018–0.029)	0.005 (0.004–0.007)	0.043 (0.036–0.051)	25–27
<i>L. panamensis</i> BOYNTON	0.042 (0.037–0.046)	0.124 (0.089–0.168)	0.120 (0.084–0.156)	0.083 (0.076–0.089)	14–17

**Table 6.** ED<sub>50</sub> Values in μM (95% confidence intervals) at 72 h for CL Amastigotes in Mφs at 34°C and *L. donovani* L82 Amastigotes in Mφs at 37 °C

species	<i>n</i>	BPQ	4a	4b	Sb <sup>v</sup> (control)	% Mφs (0 h)	% Mφs (72 h)	amastigotes/Mφ
old world								
<i>L. donovani</i> L82	1	0.48 (0.42–0.54)	4.12 (3.83–4.41)	1.25 (1.23–1.26)	35.24 (13.41–57.06)	94.75 (SD = 2.22)	98.50 (SD = 0.58)	45.55 (SD = 29.51)
	2	0.43 (0.40–0.45)	4.31 (3.60–5.02)	1.22 (1.17–1.26)	23.04 (15.41–30.67)	99.00 (SD = 0.82)	99.50 (SD = 1.00)	82.50 (SD = 20.70)
<i>L. aethiopica</i> Khartoum	1	0.633 (0.473–0.793)	2.334 (1.454–3.213)	1.659 (1.449–1.869)	34.010 (27.143–40.877)	78.25 (SD = 7.14)	88.25 (SD = 5.68)	12.75 (SD = 12.55)
	2	3.643 (3.325–3.962)	7.351 (6.426–8.276)	3.990 (3.892–4.087)	***	86.00 (SD = 7.79)	98.75 (SD = 1.26)	60.00 (SD = 34.22)
<i>L. major</i> JISH118	1	0.434 (0.390–0.478)	1.374 (1.020–1.728)	0.810 (0.660–0.959)	136.569 (23.786–249.353)	76.75 (SD = 4.79)	82.00 (SD = 8.41)	23.15 (SD = 17.41)
	2	1.804 (1.594–2.014)	6.274 (4.574–7.975)	1.893 (1.803–1.983)	***	94.50 (SD = 2.13)	98.00 (SD = 1.83)	23.85 (SD = 11.28)
new world								
<i>L. amazonensis</i> LV81	1	5.495 (5.196–5.794)	15.731 (14.580–16.882)	8.814 (8.553–9.076)	73.847 (46.652–101.042)	95.5 (SD = 0.71)	96.75 (SD = 3.77)	84.85 (SD = 21.99)
	2	1.436 (1.316–1.556)	7.030 (5.469–8.591)	2.583 (2.380–2.785)	***	90.00 (SD = 2.83)	97.25 (SD = 1.71)	71.25 (SD = 23.00)
<i>L. mexicana</i> LV4	1	1.253 (1.203–1.304)	4.027 (3.872–4.183)	2.508 (2.042–2.975)	***	95.25 (SD = 4.86)	100.00 (SD = 0.00)	76.20 (SD = 25.90)
	2	0.370 (0.393–0.348)	0.555 (0.941–0.170)	<1.12	0.794 (0.301–1.287)	89.50 (SD = 3.87)	93.50 (SD = 3.70)	19.25 (SD = 19.53)
<i>L. panamensis</i> BOYNTON	1	0.923 (0.781–1.074)	2.057 (1.932–2.183)	1.188 (1.096–1.280)	25.844 (0.066–51.622)	89.00 (SD = 1.49)	91.75 (SD = 1.71)	25.50 (SD = 11.82)

<sup>a</sup> \*\*\* ED<sub>50</sub> values could not be calculated within the concentration ranges tested. Concentrations for Sb<sup>v</sup> (Pentostam) are expressed as μg of Sb<sup>v</sup> per liter as the MWT of sodium stibogluconate is unknown. *n* = experiment number. % Mφs = percentage of infected macrophages. Amastigotes/Mφ = mean number of amastigotes per macrophage.

TLC analyses were run on silica gel 60 F<sub>254</sub> plates (Merck), and column chromatography was performed with silica gel 60 (Merck) (0.063–0.200 mm). Elemental analyses were carried out on a ThermoQuest CE Instruments EA 110-CHNS-O elemental analyzer. All reagents were from commercial suppliers and used without further purification.

**3-(trans-4-tert-butyl-cyclohexylmethyl)-1,4-dioxo-1,4-dihydro-naphthalen-2-yl Diethyl Phosphate (3a).** Compound **1** (2.0 g, 6.13 mmol) and 80% sodium hydride in mineral oil (0.37 g, 12.2 mmol) were dissolved in dry tetrahydrofuran (80 mL) under N<sub>2</sub> atmosphere. The mixture was vigorously stirred at 0 °C for 30 min, followed by the addition of diethyl chlorophosphate (**2a**) (1.59 g, 9.2 mmol) in dry tetrahydrofuran (20 mL) with continuous vigorous stirring overnight at room temperature. The mixture was poured to ice (200 mL) and extracted three times with ethyl acetate (50 mL). The combined organic phase was dried with sodium sulfate and filtered, the solvent was evaporated, and the residue was dried under high vacuum. The product was purified by flash chromatography using petroleum ether/ethyl acetate (2:1) as eluent affording **3a** as a yellow wax (2.36 g, 83.2%): TLC *R*<sub>f</sub> 0.47 (petroleum ether/ethyl acetate 2:1). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 0.80 (9H, s, CMe<sub>3</sub>), 0.82–1.04 (5H, m, c-HexH), 1.44 (6H, t, *J* = 7.1 Hz, CH<sub>2</sub>CH<sub>3</sub>), 1.53 (1H, bm, c-HexH), 1.75 (4H, bm, c-HexH), 2.61 (2H, d, <sup>3</sup>J<sub>HH</sub> = 7.5 Hz, =CCH<sub>2</sub>C), 4.40 (4H, m, OCH<sub>2</sub>), 7.73 (2H, m, ArH), 8.08–8.12 (2H, m, ArH).

**Mono-[3-(trans-4-tert-butyl-cyclohexylmethyl)-1,4-dioxo-1,4-dihydro-naphthalen-2-yl] Phosphate (4a).** **3a** (2.36 g, 5.10 mmol) was dissolved in dry dichloromethane (15 mL), and the solution was cooled to 0 °C. Excess trimethylsilyl

bromide (5 mL) was added dropwise, and the mixture was allowed to warm to room temperature with continuous stirring for 4 h, then evaporated to dryness under high vacuum. The product was recrystallized from methanol to yield **4a** as a yellow solid (1.01 g, 40.6%): mp 121 °C. <sup>1</sup>H NMR (d<sub>6</sub>-DMSO) δ 0.80 (9H, s, CMe<sub>3</sub>), 0.82–1.02 (5H, m, c-HexH), 1.51 (1H, m, c-HexH), 1.67–1.73 (4H, bm, c-HexH), 2.54 (2H, d, *J* = 7.0 Hz, =CCH<sub>2</sub>C), 7.87 (2H, m, ArH), 8.02 (2H, m, ArH). <sup>13</sup>C NMR (d<sub>6</sub>-DMSO) δ 26.7, 27.3, 31.0, 32.0, 33.2, 37.2 (d, <sup>4</sup>J<sub>CP</sub> = 1.4 Hz), 47.3, 125.8, 126.0, 130.5, 131.3, 134.0, 134.2, 135.7 (d, <sup>3</sup>J<sub>CP</sub> = 6.0 Hz), 151.6 (d, <sup>2</sup>J<sub>CP</sub> = 7.8 Hz), 178.8 (d, <sup>3</sup>J<sub>CP</sub> = 0.9 Hz), 185.0 (d, <sup>4</sup>J<sub>CP</sub> = 0.9 Hz). <sup>31</sup>P NMR (CDCl<sub>3</sub>) δ -4.51 (s) ESI-MS: 405.6 (M - 1). Anal. (C<sub>21</sub>H<sub>27</sub>O<sub>6</sub>P·1.0H<sub>2</sub>O) C, H.

**Di-tert-butyl-3-(trans-4-tert-butyl-cyclohexylmethyl)-1,4-dioxo-1,4-dihydro-naphthalen-2-ylxymethyl Phosphate (3b).** Compound **1** (1.89 g, 5.80 mmol), 80% sodium hydride in mineral oil (0.28 g, 11.60 mmol), and *n*-Bu<sub>4</sub>NI (0.43 g, 1.16 mmol) were dissolved in dry dimethylformamide (DMF) (20 mL), and di-tert-butyl chloromethyl phosphate (**2b**) (1.50 g, 5.80 mmol) was added in DMF (10 mL) while stirring at 0 °C under N<sub>2</sub> atmosphere. The mixture was allowed to warm to room temperature and stirred at 50 °C for 3 days. The reaction mixture was washed with saturated NaHCO<sub>3</sub> solution and extracted three times with ethyl acetate. The combined organic layers were dried with sodium sulfate. The resulting residue was purified by flash column chromatography using petroleum ether/ethyl acetate (2:1) as eluent, affording **3b** as a yellow oil (1.09 g, 34.3%): TLC *R*<sub>f</sub> 0.37 (petroleum ether/ethyl acetate 2:1) <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 0.80 (9H, s, CMe<sub>3</sub>), 0.82–1.06 (5H, m, c-HexH), 1.44 (18H, s, OMe<sub>3</sub>), 1.53 (1H, m,

c-HexH), 1.73 (4H, bm, c-HexH), 2.62 (2H, d,  $^3J_{\text{HH}} = 7.3$  Hz, =CCH<sub>2</sub>C), 5.82 (2H, d,  $^3J_{\text{HP}} = 11.6$  Hz, OCH<sub>2</sub>O), 7.70 (2H, m, ArH), 8.07 (2H, m, ArH).

**Mono-[3-(trans-4-tert-butyl-cyclohexylmethyl)-1,4-dioxo-1,4-dihydro-naphthalen-2-yloxymethyl] Phosphate (4b).** A solution of **3b** (0.5 g, 0.9 mmol) in ethyl acetate (2 mL) was added dropwise to saturated hydrochloric acid/ethyl acetate solution (10 mL) at 0 °C. The mixture was stirred at room temperature for 1 h and evaporated in vacuo. The residue was washed with ethyl acetate to yield **4b** as a yellow solid (0.17 mg, 43.3%): mp 54 °C. <sup>1</sup>H NMR (*d*<sub>6</sub>-DMSO)  $\delta$  0.79 (9H, s, CMe<sub>3</sub>), 0.82–1.02 (5H, m, c-HexH), 1.48 (1H, bm, c-HexH), 1.68 (4H, bm, c-HexH), 2.51 (2H, d,  $^3J_{\text{HH}} = 7.0$  Hz, =CCH<sub>2</sub>C), 5.70 (2H, d,  $^3J_{\text{HP}} = 13.5$  Hz, OCH<sub>2</sub>O), 7.84 (2H, m, ArH), 8.00 (2H, m, ArH). <sup>13</sup>C NMR (*d*<sub>6</sub>-DMSO)  $\delta$ : 26.7, 27.3, 30.6, 32.0, 33.2, 37.2, 47.3, 89.9 (d,  $^2J_{\text{CP}} = 4.6$  Hz), 125.8, 125.9, 131.0, 131.4, 133.7, 134.1, 135.7, 154.7, 180.2, 184.9. <sup>31</sup>P NMR (CDCl<sub>3</sub>)  $\delta$  -1.32 (s) ESI-MS: 435.5 (M - 1). Anal. (C<sub>22</sub>H<sub>29</sub>O<sub>7</sub>P·1.0H<sub>2</sub>O) C, H.

**HPLC Analysis.** HPLC system consisted of a Beckman System Gold Programmable Solvent Module 126, a Beckman System Gold Detector Module 166 with variable wavelength (set at 250 nm for buparvaquone and 278 nm for its prodrugs) and a Beckman System Gold Autosampler 507e. Separations were performed with a Purospher RP-C8 column (125 × 4 mm, 5  $\mu$ m). Gradient elution was carried out using mobile phase consisting of 90% (v/v) acetonitrile and 0.02 M phosphate buffer solution (pH 2.5) at a flow rate of 1.0 mL/min and at 30 °C.

**Aqueous Solubility.** Buparvaquone aqueous solubility was determined in phosphate buffer (0.16 M) at pH 3.0, pH 5.0 and pH 7.4 at room temperature. An excess of the compound was added to buffer solutions, and the suspensions were shaken for 72 h and filtered (Millipore 0.45  $\mu$ m). The filtered solutions were analyzed by HPLC. The pH of the suspensions was held constant during the study. The aqueous solubilities of buparvaquone phosphate prodrugs **4a,b** were determined in citric acid buffer (0.04 M) at pH 3.0, in sodium acetate buffer (0.05 M) at pH 5.0, and in borate buffer (0.185 M) at pH 7.4. The solutions were shaken for 1 h and filtered solutions were analyzed by HPLC.

**Ionization Constant.** Ionization constant ( $pK_a$ ) titrations of the prodrugs were carried out over a pH range of 1.5 to 10.5 under argon atmosphere at 25.2 °C (average temperature). A known amount of the compound (5.0–10.0 mg) was dissolved in three different methanol mixtures of 30, 40, and 50 (vol %) in 0.15 M KCl and consequently three separate titrations were performed. The cosolvent, methanol, which normally is used to increase the solubility of compounds, was now used to stabilize the phosphate prodrugs during the measurements. The pH was adjusted automatically to pH 10.5, the starting pH of the titration. The maximum titrant volume increment for one titration step was limited to 0.25 mL. The pH change per titrant addition was limited to 0.2 pH units. A total of 42 pH readings were collected from each titration. The pH values were recorded when the pH-drift was lower than 0.002 pH per minute. Processing of the three separate titration data and the extrapolation of the aqueous  $pK_a$  based on the Yasuda-Shedlovsky approach<sup>31</sup> were carried out using the Sirius Refine200 Revision 1.000 (Sirius Analytical Instrument Ltd., Forest Row, UK). The used pH-metric method is described more detailed elsewhere.<sup>31,32</sup>

**Distribution Coefficient.** Distribution coefficients of the prodrugs were determined by the pH-metric technique using a Sirius PCA200 titrator. For the determination of distribution coefficient, 1-octanol was saturated with 0.15 M KCl solution by vigorously shaking for 24 h. A known amount of the compound (5.0–10.0 mg) was dissolved in 7.5 mL of 0.15 M KCl solution, and three separate titrations were performed with different amounts of 1-octanol (0.25 mL, 1.25 mL, 11.25 mL). The three separate measurements were combined in accordance with the MultiTitrations procedure.<sup>33</sup> Processing of titration data was carried out using the Sirius Refine200 Revision 1.000. The used pH-metric log *D* method has been

described elsewhere.<sup>33</sup> The log *D* value of buparvaquone was estimated from the capacity factors (*k*) of reversed-phase liquid chromatography.<sup>34</sup> A Purospher RP-C8 column (125 × 4 mm, 5  $\mu$ m) was used as the stationary phase, and the mobile phase consisted of 0.02 M phosphate buffer (pH 2.5) and methanol (30:70 v/v). The flow rate was 1.0 mL/min and the compounds were detected at 254 nm. The calibration curve was determined using nine standard compounds which are described elsewhere.<sup>34,35</sup> The log *D* values of the standard compounds were plotted against the measured log *k*' values, and a linear relationship ( $r^2 = 0.9469$ ) was found between these two parameters. The log *D* value of buparvaquone was determined from this linear relationship, because the traditional shake-flask method was not useful due to the high lipophilicity and low aqueous solubility of **1**.

**Hydrolysis in Aqueous Solution.** The chemical hydrolysis rates of **4a** and **4b** were determined in 0.04 M citric acid buffer (pH 3.0), 0.05 M sodium acetate buffer (pH 5.0), and 0.185 M borate buffer (pH 7.4) at 37 °C. An appropriate amount of prodrug was dissolved in 5 mL of preheated buffer, the filtered solutions (0.45  $\mu$ m Millipore) were placed in a water bath at 37 °C, and at suitable intervals samples were withdrawn. The samples were then analyzed by HPLC. Pseudo-first-order half-life ( $t_{1/2}$ ) for the hydrolysis of prodrug was calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug versus time.

**Hydrolysis in Alkaline Phosphatase Solution.** The rate of hydrolysis of prodrugs **4a** and **4b** in alkaline phosphatase solution was determined in 0.185 M borate buffer (pH 7.4) at 37 °C. An appropriate amount of **4a** and **4b** were dissolved in 2 mL of preheated buffer solution and then filtered (0.45  $\mu$ m Millipore). The solutions were placed in a water bath at 37 °C, and 2  $\mu$ L (57.1 units) of alkaline phosphatase EC 3.1.3.1 (Type VII-S; from bovine intestinal mucosa, Sigma, St. Louis, MO) was added to the solution. Samples (200  $\mu$ L) were withdrawn at determined intervals and 200  $\mu$ L of cold acetonitrile was added to stop enzymatic hydrolysis. The samples were kept on ice for 1 h and centrifuged for 10 min at 14 000 rpm. The clear supernatant was analyzed for remaining prodrug by HPLC. Pseudo-first-order half-life ( $t_{1/2}$ ) for the hydrolysis of prodrug was calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug against time.

**Hydrolysis in Skin Homogenate.** The rates of hydrolysis in post mortem human skin homogenate were studied by weighting an appropriate amount of the prodrug in 5.0 mL borate buffer (0.185 M, pH 7.4). The solutions were filtered (0.45  $\mu$ m Millipore), and 0.5 mL of the preheated (37 °C) skin homogenate was added to 4.5 mL of the prodrug solution. The solutions were placed in a water bath and, at suitable intervals, 200  $\mu$ L samples were withdrawn and 400  $\mu$ L of cold acetonitrile was added to precipitate protein from the skin homogenate. After immediate mixing, the samples were kept on ice for 1 h, and then centrifuged for 10 min at 14 000 rpm. The supernatant was analyzed for remaining prodrug by HPLC using the method described earlier. Pseudo-first-order half-life ( $t_{1/2}$ ) for the hydrolysis of prodrug was calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug against time. For the skin homogenate, a required amount of dried, post mortem human skin was homogenized (Ystral 10/25, dispensing tool 10G, Dottingen, Germany) in 0.185 M borate buffer (pH 7.4) at 22,000 rpm, to obtain a final concentration of 360 mg/mL. The supernatant was separated after centrifugation at 14,000 rpm (Eppendorf 5415C, Germany), and lipid drops were extracted from the surface of the clear mixture and then stored at -20 °C.

**In Vitro Skin Permeation Study.** Human skin was obtained from the abdominal region of adult cadavers from the Kuopio University Hospital (Kuopio, Finland). Epidermal membranes was isolated from the dermis by heating at 60 °C in distilled water for 2 min, after which the skin specimens were dried 2–4 days at room temperature and frozen. The permeation studies were carried out in Franz-type diffusion cells (PermeGear, Inc., Riegelsville, PA). Skin specimens were



dehydrated before placing them onto the top of the acceptor chamber. The acceptor chamber medium (0.05 M isotonic phosphate buffer pH 7.4 in 5% hydroxypropyl- $\beta$ -cyclodextrin) was stirred and kept at 37 °C throughout the study. HP- $\beta$ -CD was used to maintain 'sink' conditions for penetrated/released lipophilic buparvaquone. The compounds (buparvaquone, **4a** and **4b**) were applied (200  $\mu$ L) as suspensions in 0.05 M sodium acetate buffer at pH 5.0, 0.04 M citric acid buffer at pH 3.0, and ethanol–0.05 M sodium acetate buffer mixture (50% v/v) at pH 5.0. At appropriate time intervals, 250  $\mu$ L samples were taken and the volume was replaced with the acceptor medium. The drug concentrations were determined by HPLC by the method described earlier. The skin flux values of buparvaquone and its prodrugs were determined from Fick's law of diffusion;  $J_{ss} = V(dC/dt)/A$ , where  $J_{ss}$  is the steady-state skin flux (nmol/cm<sup>2</sup>/h),  $V$  is the receptor volume (mL),  $A$  is the surface area of the diffusion cell (cm<sup>2</sup>),  $C$  is the receptor concentration (nmol/mL), and  $t$  is time (h). The steady-state skin flux was determined from the slope of the linear portion of the cumulative amounts (in nmol) of the parent drug and prodrug as a sum which were measured in the receptor phase versus time plot and dividing the slope of the steady-state position by the surface area of the diffusion cell (0.71 cm<sup>2</sup>).

**In Vitro Leishmania Models.** (a) **Parasites.** *L. major* (MHOM/SA/85/JISH118), *L. panamensis* (MHOM/PA/67/BOYNTON), *L. mexicana* (MHOM/BZ/82/BEL21), *L. mexicana* (LV4); *L. aethiopia* (Khartoum), *L. amazonensis* (LV81), and *L. donovani* (MHOM/ET/67/L82) promastigotes were cultured in Schneider's *Drosophila* medium (GibcoBRL, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (HI-FCS) (Harlan Sera-Lab., Crawley, UK) at 26 °C. Amastigotes for *L. donovani* (MHOM/ET/67/L82) were freshly obtained from the spleen of the female golden hamster (*Mesocricetus auratus*).

(b) **Drugs.** Stock drug solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma, UK) 24 h prior to use and left on a 'ball and mill' to aid solution or to ensure a fine suspension. Sodium stibogluconate (Pentostam) (GSK, UK) concentrations are expressed as  $\mu$ g Sb<sup>v</sup> per liter as the MW is unknown. Amphotericin B deoxycholate was obtained as Fungizone from Bristol Myers Squibb. Drugs were then diluted in either Schneider's *Drosophila* (Gibco, UK) for promastigote or RPMI 1640 medium (Sigma, UK) for amastigote studies supplemented with 10% heat inactivated fetal calf serum (HI-FCS) (Harlan Sera-Lab, Crawley, UK).

(c) **Promastigote Drug Sensitivity Assay.** A modified method of the one presented by Mikus et al. was used.<sup>30</sup> Promastigotes were plated at  $1 \times 10^6$ /mL in 96-well plates (Becton Dickinson, Franklin Lakes) and incubated at 26 °C in the presence of serial drug dilutions over eight concentrations each in triplicate. Untreated controls were included in each plate with amphotericin B was used as the standard positive control. After 72 h incubation at 26 °C, additions of AlamarBlue (Biosource International, Belgium) were made at 10% (v/v) of the final well volume. Fluorescent readings were taken within 8 h post addition of the dye (previously shown to be optimal) using a Molecular Devices Spectramax plate reader. The settings used 544 nm excitation wavelength and 590 nm emission wavelength, the optimum for Schneider's medium in this method. Normalized fluorescence standard units (FSU) were calculated to correct for background noise and serum quenching (fluorescence of blank media was subtracted from each reading).<sup>36</sup> The limit of detection was estimated as three times the background noise (media alone and dye) and sensitivity calculated as the ratio of fluorescent response to background noise. Results were expressed as % of untreated control for each drug concentration.

(d) **Amastigote Drug Sensitivity Assay.** Peritoneal macrophages (PEM) were obtained by lavage with 10 mL of RPMI 1640 supplemented with gentamicin 50  $\mu$ g/mL<sup>37</sup> from outbred CD1 mice (Charles Rivers Ltd, Margate, UK) 24 h after induction<sup>38</sup> with 2 mL of 2% (w/v) soluble starch (BDH, UK). Pooled macrophages from several mice were counted by a Neubauer haemocytometer and the concentration was adjusted

to  $5 \times 10^5$  macrophages/mL in RPMI 1640 supplemented with 10% HI-FCS; 100  $\mu$ L added to each well of a 16-well tissue culture chamber slides (Lab-Tek, Life Technologies, Paisley, UK). After 24 h at 37 °C in 5% CO<sub>2</sub>/95% airadherent macrophages were infected with 'stationary phase' *Leishmania* promastigotes at a ratio of 10 promastigotes: 1 macrophage and incubated at 34 °C. For *L. donovani*, amastigotes were used for infection at a ratio of 7 amastigotes: 1 macrophage and incubated at 37 °C. After 24 h, cultures were washed prior to addition of medium containing serial dilutions of the test compounds. Control slides were taken at this point to determine % infection. The infected cultures were exposed for 72 h to compounds in a 3-fold dilution series over four concentrations in quadruplicate at each concentration. After 72 h slides were fixed with methanol and stained with 10% (v/v) Giemsa's stain (BDH, UK). The % of infected macrophages was determined microscopically at each drug concentration, compared to the untreated 72-h control.

(e) **Statistical Analysis.** ED<sub>50</sub> values were estimated using Microsoft XLfit (version 2.0.2). Data were fitted using the nonlinear sigmoidal curve-fitting Levenburg Marquardt algorithm.

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