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THE ACTIVITY OF ALKYL PHOSPHORYLCHOLINES AND RELATED DERIVATIVES AGAINST *LEISHMANIA DONOVANI*

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Abstract—Several alkyl phosphorylcholines and related derivatives were tested against *Leishmania donovani* amastigotes in mouse peritoneal macrophages *in vitro* and ED_{50} values were determined in the range of 1–12 μM. The three alkyl phosphorylcholines tested against *L. donovani* in BALB/c mice were active, an ED_{50} of 12.8 mg/kg/day × 5 was ascertained for one compound, but an alkyl phosphorylethanolamine was inactive.

Leishmaniasis is a complex of diseases, with visceral and cutaneous forms caused by haemoflagellate parasites which survive and multiply as intracellular amastigotes in various macrophage populations in mammalian hosts. *Leishmania donovani* is the causative organism of the potentially fatal visceral leishmaniasis (kala-azar). The clinical treatment of leishmaniasis is primarily dependent upon pentavalent antimonial drugs, which require the use of high doses, long courses of treatment and parenteral administration [1].

As part of a programme to find alternative drugs for the treatment of visceral and cutaneous leishmaniasis we have recently examined the activity of a range of clinically established and experimental compounds against *Leishmania* spp. in *in vitro* and *in vivo* models [2–4]. We report here the novel activity of several alkyl phosphorylcholines (APCs) and related compounds against *Leishmania donovani*.

MATERIALS AND METHODS

Parasites. Leishmania donovani (MHOM/ET/67/L82; LV9) was routinely maintained in male golden hamsters (Wright's strain) which weighed about 50 g at the time of infection. Amastigotes were isolated from the spleen of infected hamsters after 6–8 weeks for experimental *in vitro* and *in vivo* studies.

In vitro *techniques*. The procedure follows that described in detail elsewhere [2]. Briefly, mouse peritoneal macrophages were isolated from the peritoneal cavity of outbred CD1 mice (Charles Rivers Ltd.) and were cultured in tissue chamber slides in RPMI 1640 medium plus 10% heat-inactivated foetal calf serum (FCS). They were incubated at 37° in a 5% CO_2–air mixture. Macrophages were infected with freshly isolated amastigotes 24 hr before the start of drug treatment. Following the initial addition of medium containing experimental compounds to the cultures, two further changes of medium with drug were carried out during the seven day test. Compounds were tested in a threefold dilution series from a maximum level of 27 mg/l, with four replicates at each concentration. The proportion of infected macrophages in Giemsa-stained preparations was determined after the 7-day exposure to drugs and ED_{50} values were calculated by linear regression analysis. Activities were confirmed in repeat experiments; results are given (Table 1) for the most representative experiment.

To examine activity against promastigotes of *L. donovani* these forms were cultivated in Schneider's medium plus 20% heat-inactivated FCS at 26°. The parasites were diluted to 2×10^6/ml in the same medium in a microtitre dish. Experimental compounds were added to give a final concentration of 50, 10 and 2 mg/l and their motility and viability monitored through an inverted microscope for 48 hr.

In vivo *techniques*. Male BALB/c mice (Olac Ltd.) were infected with 5×10^6 freshly isolated *L. donovani* amastigotes by the tail vein. After one week the infected mice were divided into groups of five mice and treatment commenced. In the first experiment groups of mice, other than untreated controls, were treated with 100 mg/kg body weight of the experimental compounds (I, III, VII and VIII) by the subcutaneous route. In the second experiment, groups of mice were treated with doses of 45, 15 or 5 mg/kg body weight of compound I or 45, 15 or 5 mg Sb^v/kg body weight of sodium stibogluconate (Pentostam, Wellcome Foundation Ltd), both drugs administered subcutaneously. In both experiments mice were dosed once a day for five consecutive days. Four days after the completion of treatment the mice were sacrificed, livers removed, weighed and impression smears prepared from a cut surface of this organ. The smears were fixed with methanol and stained with Giemsa's stain. Drug activity was evaluated by comparing the number of amastigotes/

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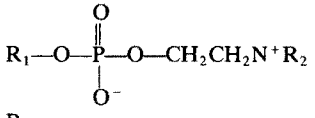
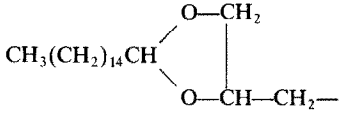
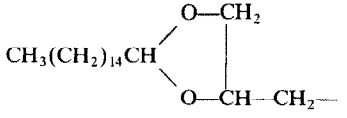
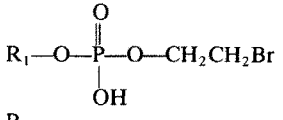
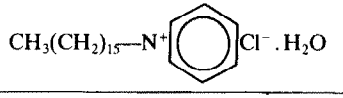
Table 1. Activity of compounds against *Leishmania donovani* amastigotes *in vitro*

Compound number	Compound structure		Max. conc^{n} tolerated by macrophages (mg/l)	ED_{50} mg/l (P_{95} fiducial limits)	[μM]
Group 1 alkyl phosphorylcholines					
	R_1	R_2			
I	$CH_3(CH_2)_{15}$—	$(CH_3)_3$	27	5.0 (5.4–4.7)	[11.39]
II	$CH_3(CH_2)_{19}$—	$(CH_3)_3$	9	1.5 (1.4–1.6)	[3.08]
III	$CH_3(CH_2)_{13}CH(CH_3)$—	$(CH_3)_3$	9	1.2 (1.4–0.9)	[2.95]
IV	$CH_3(CH_2)_{15}CH(CH_3)$—	$(CH_3)_3$	9	5.3 (6.0–4.7)	[11.55]
V	$CH_3(CH_2)_{17}CH(C_2H_5)CH_2$—	$(CH_3)_3$	9	0.5 (0.6–0.4)	[0.97]
VI	$CH_3(CH_2)_{17}CH(C_3H_7)CH_2$—	$(CH_3)_3$	9	1.4 (1.6–1.2)	[2.63]
VII		$(CH_3)_3$	9	4.1 (4.8–3.5)	[8.25]
VIII		$(H_3)_3$	9	2.3 (2.6–2.1)	[5.25]
Group 2 alkyl phosphorylbromoethanol derivatives					
	R_1				
IX	$CH_3(CH_2)_{13}CH(CH_3)$—		27	no activity at 27 mg/l	
X	$CH_3(CH_2)_{18}CH_2$—		27	no activity at 27 mg/l	
Group 3 detergents					
XI	$CH_3(CH_2)_{15}$—$N^+(CH_3)_3Br^-$		0·3	0.16 (0.3–0.1)	[0.44]
XII			0.1	0.02 (0.02–0.015)	[0.056]

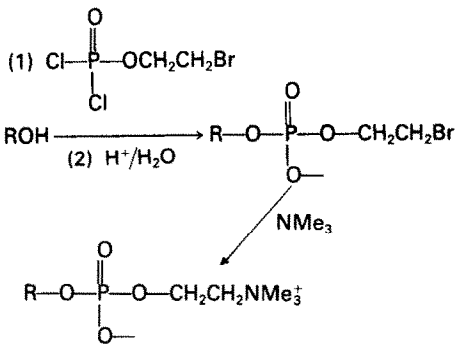


Fig. 1. Synthesis of alkyl phosphorylcholines.

500 liver cells in mice from untreated and treated groups. In the second experiment ED_{50}s were determined by linear regression analysis.

Compounds. The alkyl phosphorylcholines were synthesised by phosphorylation of the appropriate fatty alcohol with 2-bromoethylphosphoryl bromide, hydrolysis, and subsequent amination with trimethylamine, by a modication of the method of Hirt and Berchtold [5] (see Fig. 1). Structures were confirmed by elemental analysis and ^{1}H-NMR spectroscopy and are shown in Table 1. The further compounds, 1-hexadecylpyridinium chloride (XII) and hexadecyltrimethylammonium bromide (XI) were obtained from Aldrich Chemical Co.

RESULTS

The synthetic phospholipid analogues which showed activity against *L. donovani* amastigotes in macrophages at 37° (see Table 1) contained either a terminal trimethyl ammonium group (phosphorylcholines I to VII) or a protonated primary amine (phosphorylethanolamine VIII). Compounds lacking these groups (IX and X) were inactive at the concentrations tested. The ED_{50} values of active APCs were within the range of 0.5–5.3 mg/l (1–12 μM), compound V possessing an ethyl side group being the most active. In the same *in vitro* model the ED_{50} of sodium stibogluconate was 4.2 mg Sb^v/l [2]. However, no clear structure–activity relationships within the alkyl moiety, for example with respect to chain length or degree of branching, were evident due to the limited number of compounds available. The APCs showed clear selective toxicity to the amastigotes compared with their mammalian host cells. The most active antileishmanial compound (V) showed at least a 15-fold difference in toxicity between the two cell types. The presence of a quaternary or primary ammonio-group also seemed to be a determinant of the toxicity to the macrophages. The two detergents (compounds XI and XII) also showed selective toxicity to the amastigotes although they were active against both cell types at much lower concentrations than the APCs.

The phospholipids showed a similar pattern of activity against cultured extracellular promastigotes at 26° when monitored over 48 hr. Only compounds with choline or ethanolamine head groups were active, although clear activity as indicated by flagellate motility and numbers, was seen only at the highest concentration of 50 mg/l. Only compounds V and VII completely eliminated promastigotes at this level, with compounds I and II also showing moderate activity.

Four of the compounds were tested further against *L. donovani in vivo* in BALB/c mice. Results from an initial experiment in which compounds were tested at a single dose level (100 mg/kg body weight) showed that the phosphorylcholines (I, III and VII) were active whereas the phosphorylethanolamine (VIII) showed no activity (Table 2). Some of the compounds, particularly I, showed toxicity to mice at this dose level. In a subsequent experiment the ED_{50} of compound I was determined as 12.8 (14.3–11.4) mg/kg body weight. At the top dose in this experiment (45 mg/kg body weight) a 10% weight loss in mice following treatment was recorded. In the same experiment the ED_{50} of the standard antileishmanial, sodium stibogluconate, was 14.9 mg Sb^v/kg body weight.

DISCUSSION

Alkyl phospholipid analogs possessing a phosphorylcholine or phosphorylethanolamine head group show significant selective toxicity to *L. donovani* amastigotes in macrophages *in vitro*. The small number of compounds tested limits further comments on structure–activity relationships. This activity of phosphorylcholines *in vitro* was translated into activity in an *in vivo* model whereas the phosphorylethanolamine (VIII) was not active. In both *in vitro* and *in vivo* models activities were comparable with that of the standard antileishmanial sodium stibogluconate, although APCs were more toxic to mammalian macrophages used in the *in vitro*

Table 2. Activity of compounds against *L. donovani* in BALB/c mice at 100 mg/kg body wt/day × 5 (s.c. route)

Compound	% inhibition (± standard error)	% Wt change of surviving mice after treatment*
I	100	−15.2†
III	98 ± 0.8	+1.9
VII	95 ± 1.7	−3.5
VIII	−1 ± 6.5	−1.2

* Untreated mice showed a +3.6% wt change over same time period.
† One mouse died on day 4 of treatment; surviving mice not treated on day 5.

system. The results suggest that further studies with similar compounds, if they were available, would be of interest. Platelet-activating factor, which is a phosphatidylcholine, was found to be inactive against *L. donovani* amastigotes *in vitro* (Croft, unpublished). APCs have also been shown to be active against the protozoan *Tetrahymena pyriformis* and a variety of fungi [6]. The mode of action of these compounds against protozoa has not been determined. The cell membrane seems to be a possible site of action and may involve a surfactant action as well as interaction with other components, e.g. phospholipid metabolism enzymes, of the membrane [6]. Although the *in vitro* test systems for amastigotes and promastigotes are not directly comparable, APCs did appear to be more active against the intracellular amastigote. Indirect antileishmanial activity of the APCs through their ability to activate macrophages [7] could help to explain any difference in this respect.

This is not the first report of the use of potentially surface active agents in the treatment of leishmaniasis or trypanosomiasis. Fulton [8] described the activity of polyoxyethylene ethers "Macrocyclon" and "Triton W.R. 1339" against *L. donovani* in hamsters and Goble *et al.* [9] against *Trypanosoma congolense* in mice. These compounds are also immunomodulators and stimulants of the reticulo-endothelial system. More recently El-On and colleagues have shown the activity of the quaternary ammonium compound methyl benzethonium chloride against *L. major* amastigotes *in vitro* [10], and the synergistic activity of this compound with the aminoglycoside paromomycin against cutaneous leishmaniasis in mice [11] and man [12]. Other quaternary ammonium compounds have shown activity against *Plasmodium falciparum in vitro* through the inhibition of phosphatidylcholine biosynthesis [13, 14]. The potential of selective surface-active compounds in the treatment of protozoal diseases indicates an area worthy of further research.

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