Antileishmanial Structure-Activity Relationships of Synthetic Phospholipids: In Vitro and In Vivo Activities of Selected Derivatives^{▽†}

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Received 4 April 2007/Returned for modification 26 June 2007/Accepted 23 September 2007

Antileishmanial activities of 91 synthetic phospholipids against *Leishmania donovani* were evaluated in vitro and cytotoxicity assessed against two mammalian cell lines. Promising compounds were tested further in vivo. In vitro structure-activity relationships showed a positive contribution of head groups bearing ring systems (*N*-methylpiperidino and *N*-methyldmorpholino) to antileishmanial activity.

Leishmaniasis is a disease complex comprising visceral leishmaniasis (VL), cutaneous leishmaniasis, and mucocutaneous leishmaniasis. VL is a severe form of the disease, with fatality rates as high as 100% within 2 years if left untreated (<http://www.who.int/leishmaniasis/en/>). *Leishmania donovani* is the causative species on the Indian subcontinent and in eastern Africa and is endemic in 62 countries, with a total of 200 million people at risk (9). The annual incidence is estimated at 500,000 cases (6).

Miltefosine (Impavido; Zentaris GmbH, Germany) was registered as the first oral drug for treatment of VL in India in 2002 and in Germany in 2004 and for treatment of cutaneous leishmaniasis in Colombia in 2005.

Miltefosine is an alkylphosphocholine (APC) (hexadecylphosphocholine), originally developed as an anticancer drug (7). It was one of several phospholipid analogues that reached clinical trials for treatment of cancer (3). Several groups of synthetic phospholipids have also shown activity against *Leishmania* and other protozoa (4).

Miltefosine is an effective drug, but there are side effects and drawbacks related to treatment. These include gastrointestinal reactions and teratogenic potential (12). The latter was observed in one species (rat). Elevated creatinine levels are frequently observed for VL patients but usually normalize during therapy. An increase in serum creatinine was often observed in association with dehydration (14). As part of a search for new analogues with improved antileishmanial drug properties, we screened 91 phospholipid derivatives (both alkylglycerophosphocholines and APCs) in vitro against both life cycle stages (extracellular and intracellular) of *L. donovani* to evaluate antileishmanial activity and against two mammalian cell lines to determine selectivity. Some compounds were tested further in vivo.

Miltefosine and a chemical library of phospholipids were kindly provided by Zentaris GmbH (Frankfurt am Main, Germany). For in vitro testing, stock solutions or suspensions of compounds were prepared in 100% dimethyl sulfoxide. Suspension of compounds with poor solubility was obtained by ultrasonication. *L. donovani* strain MHOM/ET/67/HU3 was used throughout the study, and amastigotes were harvested from the spleen of an infected hamster (*Mesocricetus auratus*). *L. donovani* promastigotes were maintained in culture, and drug sensitivity assays were carried out in vitro, as described elsewhere (13). Assays of drug activity against intracellular amastigotes in peritoneal macrophages in vitro were carried out as described previously, at infection ratios of five amastigotes to one macrophage (8). To assess cytotoxicity in vitro, phospholipid analogues were screened against human carcinoma KB and human monocytic leukemia THP-1 cells. Briefly, cells were exposed to threefold serial dilutions of compounds and incubated at 37°C, 5% CO₂ for 72 h. Alamar blue was added for the last 6 h, and 50% effective concentration (EC₅₀) values were calculated by sigmoidal analysis. Therapeutic indices (TIs) (EC₅₀ towards cell line/EC₅₀ towards *L. donovani*) were calculated to guide the selection of phospholipids for further in vivo testing. In some experiments, hexadecylphosphocholine was tested blind as an internal control. In vivo experiments were conducted adhering to the United Kingdom Government Animals (Scientific Procedures) Act of 1986, as described elsewhere (5). In two experiments, compounds were administered orally (p.o.) at 30, 10, and 3.3 mg per kg of body weight in a standard suspension vehicle (0.5% carboxymethylcellulose, 0.5% benzyl alcohol, 0.4% Tween 80 in 0.9% NaCl).

To assess structure-activity relationships, compounds were divided into three activity groups for both life cycle stages: high activity against *L. donovani* (EC₅₀ between 0.1 μM and 5 μM), moderate activity (EC₅₀ between 5 μM and 12 μM), and low activity (EC₅₀ over 12 μM up to 30 μM). Volumes of alkyl chains of phospholipids were calculated using Hyperchem and plotted against activity. This showed a preferred volume for antipromastigote activity of approximately 1,100 Å³, corresponding to a chain length of 18 carbon atoms. For anti-amastigote activity, two main populations were found, one at a volume of approximately 951 Å³, representing a chain length

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† Supplemental material for this article may be found at <http://aac.asm.org/>.

[▽] Published ahead of print on 1 October 2007.

TABLE 1. Phospholipid derivatives that display higher activity than miltefosine against *L. donovani* amastigotes in vitro

Phospholipid designation ^a	Structure	EC ₅₀ (μM) phospholipid ^b	EC ₅₀ (μM) miltefosine ^b	Ratio ^c
D-23261*		0.25 (0.33–0.17)	3.34 (4.26–2.43)	0.07
D-20317		0.30 (0.59–0.02)	3.34 (4.26–2.43)	0.09
D-21573*		0.58 (0.81–0.35)	5.30 (6.05–4.55)	0.11
D-22829*		0.40 (0.44–0.36)	3.34 (4.26–2.43)	0.12
D-22883**		0.40 (0.71–0.09)	3.34 (4.26–2.43)	0.12
D-22830		0.41 (0.57–0.25)	3.34 (4.26–2.43)	0.12
D-21720		0.43 (0.76–0.19)	3.34 (4.26–2.43)	0.13
D-21636		0.71 (1.11–0.31)	5.30 (6.05–4.55)	0.13
D-21298*		0.55 (0.69–0.42)	3.34 (4.26–2.43)	0.16
D-22207		0.94 (1.30–0.58)	5.30 (6.05–4.55)	0.18
D-21719*		1.19 (1.48–0.90)	5.30 (6.05–4.55)	0.22
D-22455*		1.22 (1.32–1.11)	5.30 (6.05–4.55)	0.23
D-23167*		0.76 (0.85–0.68)	3.34 (4.26–2.43)	0.23
D-22827**		0.82 (0.97–0.68)	3.34 (4.26–2.43)	0.25
D-22642		1.68 (2.17–1.18)	5.30 (6.05–4.55)	0.32
D-23322		1.32 (1.98–0.66)	3.34 (4.26–2.43)	0.40
D-21381		1.37 (1.64–1.10)	3.34 (4.26–2.43)	0.41

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TABLE 1—Continued

Phospholipid designation ^a	Structure	EC ₅₀ (μM) phospholipid ^b	EC ₅₀ (μM) miltefosine ^b	Ratio ^c
D-20681*		2.46 (3.01–1.90)	5.30 (6.05–4.55)	0.46
D-24062*		2.62 (3.54–1.70)	5.30 (6.05–4.55)	0.49
D-23156**		0.99 (1.34–0.64)	2.04 (2.38–1.70)	0.49
D-22084*		2.64 (3.54–1.74)	5.30 (6.05–4.55)	0.50

^a *, monohydrate structure; **, dihydrate structure.^b Data are given for a representative experiment. Values in parentheses are 95% confidence intervals.^c The ratio indicates EC₅₀ of phospholipid/EC₅₀ of miltefosine, with a cutoff of ≤0.5.

of 16 carbon atoms, and one at a volume of around 1,060 Å³, representing a chain length of 18 carbon atoms. Due to the high structural diversity of the library, head group analysis was limited to compounds with an alkyl chain of 16 or 18 methylene groups (C₁₆ and C₁₈) and activities of representative compounds were plotted against polar head group type. In sum-

mary, for amastigotes, (i) head groups containing *N*-methyl-piperidine rings displayed high activity (C₁₆ and C₁₈), (ii) trimethylammonium groups gave high activity (C₁₆) and moderate activity (C₁₈), (iii) modified trimethylammonium groups showed moderate activity (C₁₆) and low activity (C₁₆ and C₁₈), and (iv) *N*-methyl-morpholine rings led to high activity (C₁₆)

TABLE 2. In vivo activities of selected phospholipid derivatives D-20317, D-22883, and D-22830 and miltefosine against *L. donovani*^a

Compound	Structure	No. of expts	Administration route ^b	Dose response	ED ₅₀ (mg/kg) ^c
Miltefosine		1	p.o.	Obtained	8.58
		2	p.o.	Obtained	10.31
D-20317		1	p.o.	Obtained	28.54
		2	p.o.	Obtained	85.03†
D-22883		1	p.o.	Not obtained	NO
		2	p.o.	Obtained	35.32†
Miltefosine		3	p.o.	Obtained	8.09
D-22830		3	p.o.**	Not obtained	NO
Miltefosine		3	i.p.	Obtained	7.72
D-22830		3	i.p.*	Obtained	32.97

^a Antiamastigote TIs for D-20317 were 95.71 against THP-1 cells and 23.44 against KB cells, TIs for D-22830 were >219.51 against THP-1 cells and 179.01 against KB cells, and TIs for D-22883 were 11.67 against THP-1 cells and 6.89 against KB cells. TIs for miltefosine, based on respective experiments, were 5.48 against THP-1 cells and 0.83 against KB cells.

^b *, administered as suspension; **, administered as solution.^c †, value was obtained by line extrapolation. NO, not obtained.

and low activity (C_{18}). A similar trend was found for *N,N*-dimethyl-substituted piperidine rings. Data were similar for promastigotes. When the volume of amino groups was calculated, the optimum for antiamastigote activity was found to be between 400 and 540 Å³ and that for antipromastigote activity between 393 and 512 Å³.

Clustering of head groups bearing ring systems (e.g., *N*-alkylated piperidine and *N*-alkylated morpholine rings) was also observed when data were analyzed on the basis of activity ratios (EC_{50} phospholipid/ EC_{50} miltefosine in respective experiments), choosing a cutoff of ≤ 0.5 (Table 1). In other studies, Avlonitis et al. (2) and Kapou et al. (11) showed that the *N,N,N*-trimethylammonium moiety gave greater antileishmanial activity than piperidine- and morpholine-substituted head groups. However, in these studies the phospholipid tail groups contained bulky substituents, which was not the case with the compounds in this paper. This could be a reason for some of the different conclusions. In the present study, selective compounds were identified in the amastigote screen. This was not reflected in the promastigote screen, and potencies differed between the two life cycle stages (for promastigote data, see the supplemental material).

Out of five compounds tested in vivo (D-22830, D-23156, D-22883, D-21298, and D-20317, with miltefosine as a comparator), only D-20317 gave a dose-response effect in repeated experiments, with various degrees of activity (50% effective doses [ED_{50} s] of 28.54 mg/kg and 85.03 mg/kg). For D-22883, a dose response and moderate activity (ED_{50} of 35.32 mg/kg) were obtained in a single experiment. Dose responses were obtained only for soluble phospholipid derivatives. A third experiment was carried out comparing p.o. and intraperitoneal (i.p.) administrations of D-22830 and miltefosine. For i.p. administration, compounds were dissolved in 10% dimethyl sulfoxide in phosphate-buffered saline, pH 7.4. Moderate activity was obtained after i.p. administration (ED_{50} of 32.97 mg/kg) but none after p.o. administration (Table 2). The poor in vivo activity is most probably due to pharmacokinetic/solubility issues and possibly poor absorption after p.o. administration, based on a demonstration of direct antiparasitic activity in vitro and a difference in activity for D-22830 after p.o. and i.p. administration. No compounds were as good as miltefosine.

It is noteworthy that perifosine (D-21266), an oral novel anticancer alkylphospholipid (10), displayed improved intrinsic antileishmanial activity in vitro compared to miltefosine (ratio of 0.63, EC_{50} of phospholipid/ EC_{50} of miltefosine). Perifosine also exhibited moderate activity in vivo after p.o. administration when tested previously (ED_{50} of 25.24 mg/kg) (S. L. Croft, supplemental material).

In conclusion, (i) head groups containing ring systems like *N*-piperidino or *N*-morpholino can increase antileishmanial activity in vitro, and (ii) the optimal lengths of the lipid chain

in APCs tested were 16 and 18 carbon atoms. Other trends in agreement with earlier studies were the blockage of the C-2 position of alkylglycerophosphocholines, leading to higher activity than that of a free hydroxyl group (1), and the fact that elongation of the distance between N and O decreased antileishmanial activity (15).

Further structure-activity relationship studies of these synthetic phospholipid derivatives should include (i) systematic comparison between choline head groups and ring system head groups of APCs, (ii) determination of solubility/lipophilicity (log P), and (iii) determination of bioavailability.

This work was supported by EC grant QLRT-2000-01404.

We are grateful to Zentaris GmbH for providing miltefosine and the phospholipid derivatives tested in this study.

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