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Hexadecylphosphocholine: Oral Treatment of Visceral Leishmaniasis in Mice

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Hexadecylphosphocholine (He-PC), a novel phospholipid derivative, was tested against *Leishmania donovani* and *Leishmania infantum*, the causative agents of visceral leishmaniasis. In vitro, promastigotes were highly susceptible to He-PC; the 50% inhibitory concentrations were between 0.89 and 2.25 μg/ml for the different leishmanial strains. In vivo, a marked antileishmanial activity in infected BALB/c mice could be demonstrated after oral administration of He-PC. Whereas parasite suppression and killing in the liver were comparable after 5 days of treatment with He-PC (10 or 20 mg/kg of body weight per day administered orally) and sodium stibogluconate (120 mg of pentavalent antimonal agent per kg/day administered subcutaneously), a superior reduction in the parasite load in the spleen and bone marrow was observed after oral treatment with He-PC. After a 4-week treatment period, parasite suppression in the spleen was better than that observed with standard sodium stibogluconate therapy by a factor of more than 600.

Leishmaniasis is a protozoan parasitic disease of the tropics and subtropics which causes considerable morbidity and mortality. Besides a visceral form, cutaneous and mucocutaneous types of the disease are recognized. The estimated number of cases is 12 million. More than 400,000 new cases per year have been reported in the past decade. Approximately 350 million people live in endemic areas (34). The *Leishmania donovani* complex causes the visceral type of the disease (kala azar), which is usually fatal if it is not treated. Pentavalent antimonal agents (Sb^V) such as sodium stibogluconate (pentostam) and *N*-methylglucamine antimonate (glucantime) are first-line drugs for treating leishmanial infections. Second-line drugs, such as pentamidine and amphotericin B, are less satisfactory because of their unacceptable toxicities at effective therapeutic doses. Other experimental agents such as bis(benzyl)polyamine analogs (1), allopurinol and related compounds, azole derivatives (ketoconazole, itraconazole, fluconazole), and 8-aminoquinolines are in various stages of evaluation (for reviews, see references 3 and 19).

Depending on the drug and treatment duration, first- and second-line drugs can cause a variety of untoward reactions which, in some cases, are severe and life-threatening. Unfortunately, all drugs must be administered via the parenteral route and are impracticable for wide-scale use. Recently, increased resistance to antimonal drugs has been reported; of 200,000 patients suffering from kala azar in the state of Bihar, India, more than 10,000 cases were unresponsive to antimonal drugs (33). In addition, visceral leishmaniasis in patients with AIDS is generally refractory to treatment with classical antileishmanial drugs (20, 35). Therefore, a search for new chemotherapeutic agents against visceral leishmaniasis is extremely important.

We studied alkylphosphocholines as a new group of membrane-active agents. Hexadecylphosphocholine (He-PC) displays a variety of biological properties (28). The chemical structure of the molecule is shown in Fig. 1. Besides its pronounced antiproliferative activity in vitro and in vivo (10, 29), the compound induces differentiation of leukemic cell lines into mature myeloid cells (15), and some data suggest that induction of differentiation may contribute to the in vivo antitumor activity of He-PC (13). From experiments on normal peripheral mononuclear cells, it was concluded that He-PC may possess immunomodulatory activity, acting as a costimulator for the interleukin-2-mediated T-cell activation process (32). The antileishmanial activity of alkylphosphocholines and their derivatives has been reported in a recent study (8). Although some compounds showed distinct in vitro activity, subcutaneous injection in mice produced unacceptable side effects, like skin edema and necrosis (8). We demonstrate in this report that orally applied He-PC exhibits outstanding antileishmanial activity in mice and that the activity is superior to the effect of sodium stibogluconate. In addition, no remarkable side effects were observed at therapeutically active dosages of He-PC.

MATERIALS AND METHODS

Chemicals. He-PC was synthesized as described previously (9). Sodium stibogluconate (pentostam; 100 mg of Sb^V/ml) was purchased from Deutsche Wellcome GmbH, Burgwedel, Germany. All cell culture reagents for preparation of HOSMEM medium were from Sigma Chemie GmbH, Deisenhofen, Germany.

Parasites. The following three leishmanial strains were used in this study: *L. donovani* MHOM/IN/54/LRC-L.51 (LRC-L.51) and MHOM/IN/80/DD8 (DD8) and *L. infantum* MHOM/ES/86/STI-172 (STI-172). All strains were isolated from patients with visceral leishmaniasis. Stabilates of these strains were kept in liquid nitrogen. Before they were used for experiments, the strains were passaged through BALB/c mice by intravenous injection.

Promastigotes. Promastigotes were cultivated in vitro in a medium consisting of one part HOSMEM (2) and one part RPMI 1640 (041-02400; GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (011-06290; GIBCO) at

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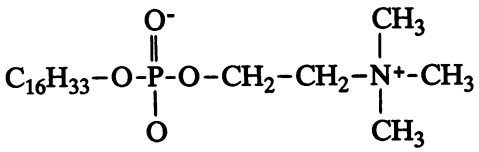

FIG. 1. Chemical structure of He-PC.

26°C. Cultures were maintained by seeding 5×10^6 promastigotes from 4-day-old cultures into 10 ml of fresh medium in 25-cm^2 tissue culture flasks (690160; Greiner).

In vitro studies. In vitro experiments were done as described by Berman and Wyler (4), with minor modifications. Briefly, promastigotes were cultured in 24-well multiplates (3047; Becton Dickinson). After 96 h of incubation in control or drug-containing media, the number of promastigotes was determined in a Neubauer counting chamber. The 50% inhibitory concentration (IC_{50}) was calculated graphically. Experiments were repeated three times, and each set of three experiments was done in duplicate.

In vivo studies. Female BALB/c mice (Charles River Wiga, Sulzfeld, Germany) were infected on day 0 by intravenous injection of a mixture of 10^7 promastigotes from the stationary growth phase and 10^7 amastigotes which were isolated from the livers or spleens of leishmania-infected BALB/c mice, washed, and resuspended in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (042-04200; GIBCO). Treatment was initiated on day 7, at which time the mean parasite burden was 100×10^6 to 200×10^6 amastigotes per liver, 1×10^6 to 2×10^6 per spleen, and 3 to 6 amastigotes per microscopic field of 100 hematopoetic cells in the bone marrow. Pentostam was diluted in 0.9% saline for subcutaneous injection. He-PC was dissolved in water, and the solution was administered orally through a stomach tube. Drugs were given at doses of 10 or 20 mg of He-PC per kg of body weight per day, whereas pentostam was administered subcutaneously at a dose of 120 mg of Sb^V per kg of body weight as described by Peters et al. (21). The respective doses were administered for 5 consecutive days a week; doses were not administered on weekend days.

Assessment of drug efficacy. The parasite burdens in spleens and livers were determined in Giemsa-stained impression smears from cut sections by counting the number of amastigotes per 1,000 spleen or liver cell nuclei. The total number of amastigotes per organ was calculated as described by Stauber et al. (25). Briefly, organ weight (in milligrams) × number of amastigotes per nucleus × 200,000 corresponded to the total parasite burden per organ. Parasite suppression (parasite burden in drug-treated mice to parasite burden in untreated mice) and parasite killing (parasite burden at the end of treatment to parasite burden at the start of treatment) were calculated as described by Baumann et al. (1) for all strains on day 14 postinfection and for strain LRC-L.51 on days 21, 28, and 35 postinfection as well. The parasite burden (strain LRC-L.51) in the bone marrow was examined on days 7 and 35 postinfection. The femurs of treated and untreated mice were removed and denuded. The femurs were gently broken with a forceps and the bone marrow was carefully removed with a scalpel. Smear preparations were prepared, stained with Giemsa solution, and examined microscopically for Leishman-Donovan's bodies.

To determine whether the microscopically detected parasites were viable or whether parasites were present even though they could not be detected in the stained impression smears, homogenates of spleen, liver, and bone marrow were cultivated for a maximum of 3 weeks in HOSMEM-RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 26°C. Cultures were examined daily for the presence of promastigotes.

TABLE 1. Development of parasite burden in leishmania-infected BALB/c mice[a]

Day after infection	Mean ± SD no. of leishmania (10^6) per:		Splenic wt (mg) (mean ± SD)
	Liver	Spleen	
0	0 ± 0	0.0 ± 0	90 ± 10
7	140 ± 20	1.8 ± 0.2	96 ± 10
14	579 ± 47	11.8 ± 2.8	130 ± 30
21	643 ± 32	17.4 ± 3.5	280 ± 10
28	538 ± 19	80.7 ± 12.4	600 ± 50
35	929 ± 90	120.6 ± 33.2	820 ± 30

[a] Female BALB/c mice ($n = 3$) were infected intravenously on day 0 with 10^7 *L. donovani* LRC-L.51 amastigotes and promastigotes. Liver and spleen impression smears were prepared on the indicated days. The number of amastigotes per 1,000 organ nuclei was counted under oil immersion, and the parasite burden was calculated as described by Stauber et al. (25).

Statistics. The two-sided *t* test for small numbers was used to calculate the significance of the differences between the means (14).

RESULTS

In vitro IC_{50}s and IC_{90}s for leishmania promastigotes were determined after 4 days of exposure to different concentrations of He-PC. The data represent the means ± standard deviations (SDs) of three independent experiments done in duplicate. All three strains showed a marked susceptibility to He-PC, with IC_{50}s of between 0.89 and 2.25 μg/ml and IC_{90}s of between 2.60 and 4.90 μg/ml (the mean ± SD IC_{50}s and IC_{90}s for *L. donovani* DD8 were 0.89 ± 0.08 and 2.60 ± 0.07 μg/ml, respectively; for *L. donovani* LRC-L.51 they were 2.07 ± 0.23 and 3.13 ± 0.29, respectively; and for *L. infantum* STI-172 they were 2.25 ± 0.21 and 4.90 ± 0.40, respectively).

On day 0, BALB/c mice were infected by intravenous injection of 10^7 amastigotes and 10^7 promastigotes. As demonstrated in Table 1, this procedure resulted in a reproducible infection rate. Treatment was initiated on day 7 for 5 consecutive days. The mice were killed on day 14, and the parasite burdens in the spleens and livers were determined. As shown in Table 2, pentostam (120 mg of Sb^V per kg/day administered subcutaneously) or He-PC (10 or 20 mg/kg/day administered orally) produced a similar suppression and reduction of the parasite load in the liver. In contrast, a significant difference ($P < 0.01$) in parasite suppression and killing could be observed in the spleens between pentostam- and He-PC-treated mice. Both 10 and 20 mg of He-PC per kg administered orally exerted superior effects on parasite suppression in the spleen when the doses were compared with the effects of pentostam.

Results of a 4-week treatment of leishmania-infected mice are shown in Fig. 2. There was a continuous decrease in the parasite load in the livers of pentostam-treated mice, which was similar to the effect of He-PC at a dose of 10 mg/kg. However, 20 mg of He-PC per kg was significantly more effective, resulting in a 5- to 10-fold-higher suppression of parasites ($P < 0.02$). However, the most striking effect of He-PC compared with that of pentostam was observed in the spleen. In this organ, pentostam exerted only a moderate

TABLE 2. Parasite suppression and parasite killing in leishmania-infected mice treated with He-PC orally for 5 days

Parasite status, organ, and strain[a]	% Suppression or killing after the following treatment		
	120 mg of Sb^V/kg[b]	10 mg of He-PC/kg	20 mg of He-PC/kg
Parasite suppression[c]			
Liver			
LRC-L.51	97.2	91.7	97.8
DD8	96.9	93.6	99.9
STI-172	90.1	87.3	94.9
Spleen			
LRC-L.51	83.0	94.9	98.3
DD8	66.8	94.1	99.8
STI-172	18.3	70.7	75.3
Parasite killing[d]			
Liver			
LRC-L.51	88.3	65.2	90.9
DD8	90.4	80.4	99.5
STI-172	56.4	44.3	77.8
Spleen			
LRC-L.51	−11.1	66.7	88.9
DD8	−31.1	77.2	98.7
STI-172	−20.8	56.7	63.5

[a] Leishmania strains are described in detail in the text.

[b] Mice (n = 3) were given an injection of 0.2 ml of a pentostam solution (120 mg of Sb^V per kg) subcutaneously or 0.2 ml of an aqueous solution of He-PC (10 or 20 mg/kg) orally by gastric gavage once daily for 5 consecutive days. Organ impression smears were made 3 days after the end of treatment. The approximate amastigote count per liver and spleen was determined as described in footnote *a* of Table 1.

[c] Parasite suppression is the ratio of parasite load in drug-treated groups to the parasite load in untreated control groups. Organ impression smears were made 3 days after 5 days of treatment, and amastigote counts per organ were determined as described in footnote *a* of Table 1.

[d] Parasite killing is the ratio of parasite load in drug-treated groups after the end of treatment in comparison with the parasite load before the start of treatment.

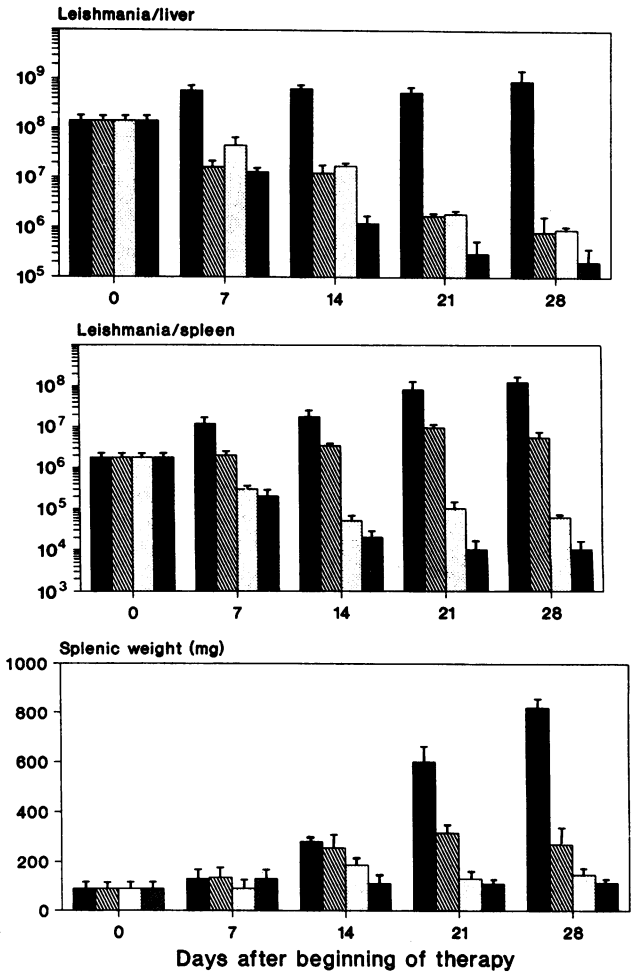

FIG. 2. Female BALB/c mice (n = 3) were infected intravenously with 10^7 amastigotes and promastigotes of the leishmania strain LRC-L.51 on day −7. On day 0, treatment was begun with He-PC (10 or 20 mg/kg) orally or 120 mg of pentavalent antimony (pentostam) per kg subcutaneously. Drugs were given five times a week for up to 4 weeks. The parasite burdens in livers and spleens were determined weekly as described in footnote *a* of Table 1. Bars represent standard errors of the means. ■, control; ▨, pentostam; ▦, He-PC (10 mg/kg); ▨, He-PC (20 mg/kg).

suppression of parasite growth in comparison with parasite growth in untreated control mice. Altogether, the parasite load increased slightly during therapy, which corresponds to the development of splenomegaly over time. In contrast, oral treatment with 20 mg of He-PC per kg resulted in more than a 3-log rank reduction of parasites and was 630-fold more effective than pentostam. Accordingly, spleen weights remained normal, as they did in uninfected control mice.

Bone marrow specimens from femurs were evaluated for parasites at the start (day 7) and at the end (day 35) of therapy. On day 7, 3 to 6 leishmania parasites per 100 bone marrow cells could be detected. Under treatment with pentostam, however, the parasite load on day 35 increased to 20 to 30 leishmania parasites per 100 bone marrow cells. In contrast, He-PC led to a remarkable parasite suppression and killing. At a dose of 10 mg of He-PC per kg, less than 1 parasite per 100 bone marrow cells could be registered on day 35, whereas at a dose of 20 mg of He-PC per kg, the number of parasites was below the detection limit of the light microscope.

Homogenates from the livers, spleens, and bone marrow of mice treated for 4 weeks with He-PC or pentostam were cultivated and investigated for parasite growth. All cultures were promastigote positive after a few days of cultivation. However, whereas in the case of pentostam-treated mice cultures were positive within 3 days, cultures of bone marrow and spleens from He-PC (20 mg/kg)-treated mice were not positive before 8 days of incubation.

DISCUSSION

In a recent report, Croft et al. (8) reported on the antileishmanial activities of some alkylphosphocholines and their derivatives. Although marked in vitro effects were found, the subcutaneous injection of these compounds into mice produced unacceptable toxicity, particularly skin edema and necrosis. Therefore, an in vivo application of these compounds does not seem possible because of their strong adverse effects.

In previous studies, we demonstrated that alkylphosphocholines can be applied orally without overt side effects (5, 13). After a daily oral application of the alkylphosphocholine He-PC (10 mg/kg) to rats, a steady-state level of about 100 μM was obtained in serum (30), indicating that He-PC is well

absorbed from the gut. Furthermore, biodistribution studies of He-PC in mice demonstrated an accumulation of the compound in spleen and liver (5), the main target organs of visceral leishmaniasis. Therefore, an evaluation of the efficacy of oral treatment with He-PC in humans with visceral leishmaniasis should be initiated.

In vitro, a strong effect of He-PC on the three strains of leishmania was observed, with IC_{50}s of about 1 to 2 μg/ml. Oral administration of He-PC to mice suppressed parasites by as much as 99.9%. Whereas He-PC was at least as effective as pentostam in reducing the parasite load in the mouse livers, a substantially greater effect on suppression and killing of parasites in the spleens and bone marrow could be achieved. This may be of importance, since parasites in the bone marrow and spleens of mice are scarcely affected by antimonal drugs (6). However, the relevance of this observation in the mouse model to human infections must be proved in further experiments.

An outstanding advantage of He-PC is its significant activity after oral administration, since few other antileishmanial drugs that are effective by oral administration are known. Ketoconazole, allopurinol, and allopurinol riboside are effective in vitro; however, clinical trials showed that cures could be achieved in only a few patients (23, 24). Administration of allopurinol in the treatment of visceral leishmaniasis in the state of Bihar in India was correlated with more than 50% failures or relapses (17), and administration of ketoconazole did not result in any clinical improvement or in any improvement in the parasitological load (26). On the other hand, pentostam in combination with allopurinol was shown to be effective in the treatment of pentostam-resistant kala azar (7). A bis(benzyl)polyamine analog (MDL 27,695) (1) and a primaquine analog (WR 6026) (22) are also effective in leishmania-infected mice, but to our knowledge, they have not been tested in humans.

The remarkable activity of He-PC in the spleens and bone marrow of mice might be explained, at least in part, by a favorable distribution of the compound in the reticuloendothelial system (5, 18). Specifically, destruction of intracellular amastigotes in the reticuloendothelial system is achieved by activated macrophages; and different cytokines, such as gamma interferon, interleukin-4, or tumor necrosis factor alpha, contribute to the induction of antileishmanial macrophage activation (12, 27). It is noteworthy in this respect that He-PC enhanced the production of gamma interferon in human T cells in a dose-dependent manner (16, 32). Furthermore, He-PC acts synergistically with different growth hormones such as granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, and interleukin-3 (31). It is thus possible that the compound interacts with the signal transduction pathways of hormones or regulatory peptides, which ultimately leads to macrophage activation for antiparasitic activity. On the other hand, since promastigotes are markedly susceptible to He-PC, direct cytotoxic effects on the parasitic membranes may be involved. He-PC could interfere with phospholipid biosynthesis in the parasites, since the compound has strong suppressive effects on the biosynthesis of phosphatidylcholine in mammalian cells (11).

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