Antiprotozoal Lysophospholipid Analogues: A Comparison of their Activity Against Trypanosomatid Parasites and Tumor Cells

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Abstract: Lysophospholipid analogues (LPAs), originally developed as anti-cancer agents, have shown significant activity against *Leishmania* spp. and *Trypanosoma cruzi*, both *in vitro* and *in vivo*. Miltefosine, used as a topical formulation (Miltex™) for metastases, was registered in 2002 for the oral treatment of visceral leishmaniasis. LPAs interfere with lipid synthesis in *T. cruzi* and cancer cells, but the activity is about >20-fold higher against the parasite.

1. INTRODUCTION

Leishmaniasis, South American trypanosomiasis (Chagas disease) and human African trypanosomiasis (sleeping sickness) are parasitic diseases with a wide distribution throughout the tropical and subtropical regions with an estimated prevalence of 2 million, 18 million and 0.5 million cases respectively [1]. These diseases are caused by closely related trypanosomatid protozoa, a group characterised by unique organelles and biochemistry, for example the kinetoplast, the glycosome and, thiol metabolism [2]. Another common feature of these diseases is that drugs used in their treatment are inadequate [3].

The recommended drugs for leishmaniasis remain the pentavalent antimonials, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) despite long courses of parenteral administration, toxicity and the threat of resistance in India [4]. The polyene antibiotic amphotericin B offers alternative treatment and lipid formulations, with reduced toxicity, are highly effective (and highly expensive) for the treatment of visceral leishmaniasis. Other alternatives include paromomycin and pentamidine and two oral drugs, sitamaquine and miltefosine (HePC), that are currently on clinical trial. The fifteen *Leishmania* species that cause this disease show variation in drug sensitivity [5]. Available therapies for Chagas disease are the nitroheterocylic drugs nifurtimox and benznidazole introduced in the 1970s. Their use is limited to the early acute phase of the disease and is restricted by toxicity and by the variable efficacy of strains of the causative agent, *Trypanosoma cruzi* [6,7]. A series of antifungal imidazoles and triazoles that inhibit sterol biosynthesis in *T. cruzi* have high activity against acute and chronic disease in experimental models and show potential for the treatment of human disease [8]. The treatment of human African trypanosomiasis is even more desperate being reliant upon pentamidine and suramin for the treatment of the early haemolymphatic stage and the arsenical melarsoprol for the late CNS stage of the disease. Melarsoprol has significant side effects and there are increasing indications of acquired resistance in Central Africa [9]. Eflornithine and nifurtimox are alternatives to melarsoprol but also have significant side effects. Eflornithine is active against *Trypanosoma brucei gambiense*, the cause of sleeping sickness in West and Central Africa, but not *T. b. rhodesiense*, the causative agent in East Africa. Structures for all of these compounds except for miltefosine are given in Fig. (**1**).

It is within this context there has been a search for new drugs for leishmaniasis and the trypanosomiases, exploiting rational approaches for drug design, for example glycolytic enzyme inhibitors [10], novel chemistry, for example bisphosphonates and farnesyl transferase inhibitors [11,12], drug delivery systems [13-15] and immunomodulation, especially in leishmaniasis [16]. This review focuses on lysophopsholipid analogues (LPAs), both alkylphospho-cholines (APCs) and alkylglycerophosphocholines (AGPCs), compounds that have shown significant activity against *Leishmania* spp. and *T. cruzi*, but not *T. brucei*, in experimental models. These compounds were originally developed as anti-cancer agents but gastrointestinal toxicity and lack of efficacy has limited their use [17,18]. One LPA, the APC miltefosine is used topically for metastases and another APC perifosine remains on clinical trial for cancer. The fact that some of these compounds had already passed through the expensive preclinical and early clinical stages as anticancer drugs has greatly helped the switching of one LPA, miltefosine, to clinical trial for the treatment of visceral and cutaneous leishmaniasis.

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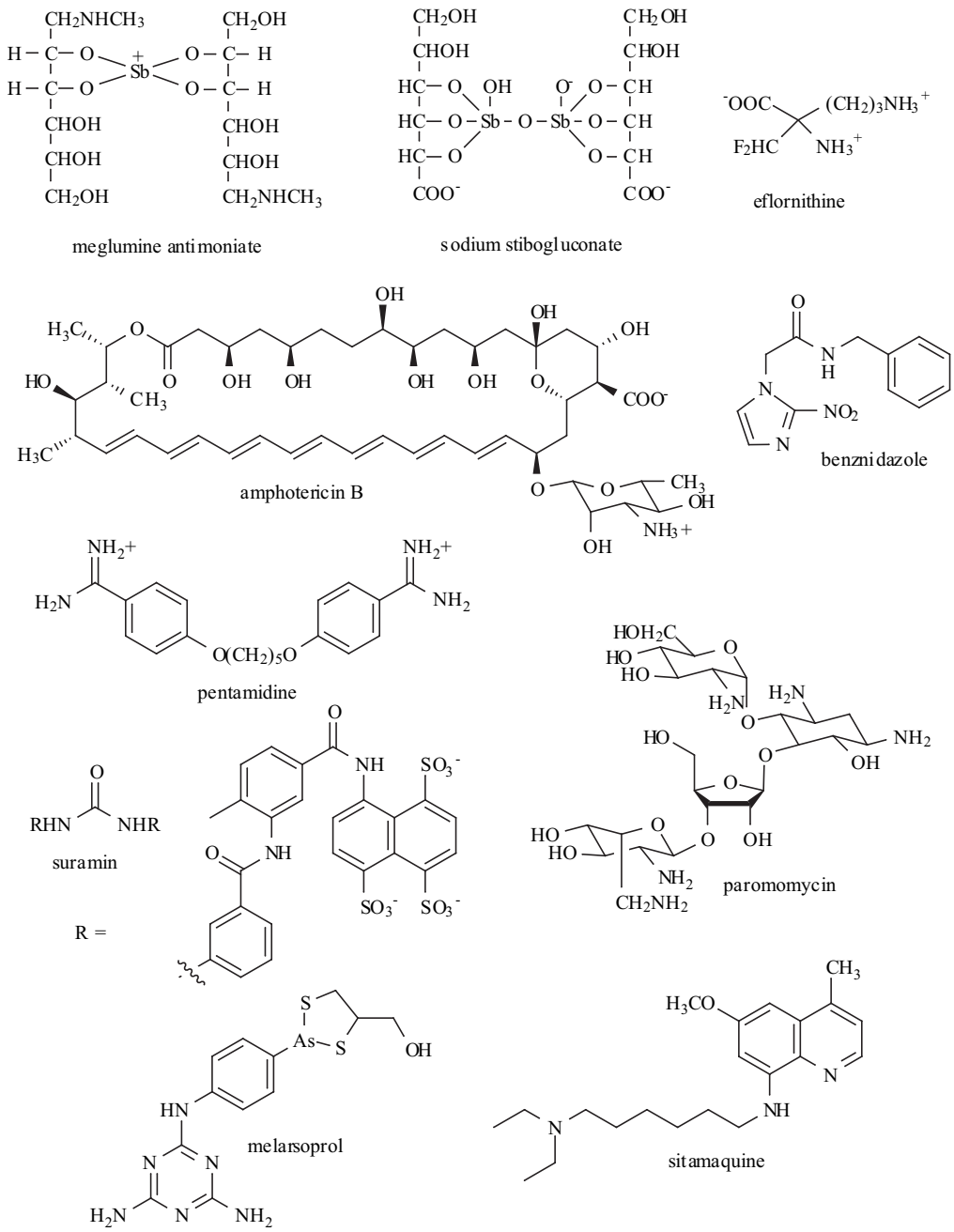

Fig. (1). Chemical structures of antileishmanial and antitrypanosomal drugs.

2. ANTICANCER ACTIVITIES OF LYSOPHOSPHOLIPID ANALOGUES

Background and Clinical Studies

Initially studies on the immunomodulatory effects of 2-lysophosphatidylcholine [19] led to the search for phospholipid analogues that could avoid rapid metabolism by phospholipases or acyltransferases. A series of ether lipids was synthesized, for example compounds with a glycerol backbone and a lateral aliphatic chain linked to C1 and a moiety resistant to hydrolysis at C2 like the AGPC ET-18-OCH_3 (Fig. (**2a**)) [20-22]. These compounds also showed an inhibitory effect on the growth of tumor cells *in vitro*. Later, a thioether substituted AGPC, ilmofosine (BM 41,440) (Fig. (**2b**)), was developed and also proved to be active both *in vitro* against neoplastic lineages [23-25] and *in vivo* in different models of leukemia, melanoma, lung and breast carcinona [26,27]. The anti-tumor activities of a series alkylphosphocholines (APCs) showed that the glycerol moiety was not essential for these effects and that their metabolism was even lower than that of AGPCs [28,29]. One APC, hexadecylphosphocholine (HePC, miltefosine) (Fig. (**2c**)) showed a pronounced antitumor effect both *in vitro* and *in vivo* [30-32]. More recently, another more lipophilic APC, erucylphosphocholine (ErPC) (Fig. (**2d**)) was investigated, which formed lamellar structures and had reduced hemolytic effects when administered intravenously [33,34]. ErPC was active *in vitro* and also *in vivo* in different tumor models [35-37] and due to its accumulation in the CNS it was considered a candidate drug for brain tumors [38]. Another promising anticancer agent compound is the alkylphosphocholine perifosine (D-21266) [39,40].

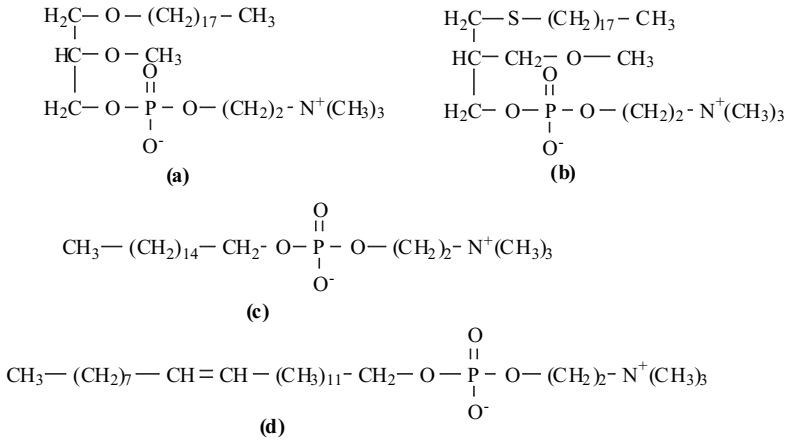

Fig. (2). Chemical structures of LPAs: (**a**) ET-18-OCH_3 (edelfosine); (**b**) ilmofosine (BM 41,440); (**c**) hexadecylphosphocholine (HePC, mitelfosine); (**d**)erucylphosphocholine (ErPC).

In the 1980s clinical trials in cancer patients were initiated [41]. Due to the selective activity of ET-18-OCH_3 it was tried as a purging agent in remission cases to rid bone marrows of residual leukemic cells in the treatment of acute cases of leukemia with promising results [42,43]. BM 41,440 was evaluated in a multicenter phase I/II study in patients with different neoplastic disorders [44]. The systemic use of miltefosine has been limited by its side effects, especially a high gastrointestinal toxicity after oral administration [45] and the results of systemic administration in cases of carcinoma were disappointing [45,46]. However, miltefosine is effective in a topical formulation, Miltex™, for use against cutaneous lymphomas [47] and has been licensed for use in Europe for treatment of skin metastases due to breast cancer [48-50]. Further details on the origins and chemistry of anticancer LPAs are covered in several reviews [18,41,51-55].

Mechanisms of Action of LPAs as Anticancer Compounds

Although the action of LPAs on tumor cells has been known for over 20 years, it is still unclear why they have a selective effect on malignant cells in comparison to normal counterparts [18,55]. Many mechanisms of action at the cellular and molecular levels have been described but no adequate explanation has emerged.

(I) Immunomodulation

The activity of LPAs against tumor cells was originally considered to be associated with an increase of the non-specific activation of macrophages [56,57]. Further studies have described the involvement of cytokines and other mediators in the effect of LPAs on macrophage function [58-61]. However, it is unlikely that immunomodulatory effects are important in the anticancer profile of LPAs as these drugs work in immunodeficient mouse cells models [62,63].

(II) Cell Sensitivity

Differences in sensitivity were also observed between different tumor lineages, and these differences have been exploited to better understand the mode of action of LPAs. The most studied cell lines have been the promyelocytic leukemia cell line HL-60 (susceptible) and the erythroleukaemic cell line K562 (resistant). Several hypotheses have been presented to explain differences in sensitivity, including (**a**) higher/faster uptake of the LPA [64-67], including endocytosis with internalization faster into the susceptible lineage [68]; (**b**) lower activity of enzymes that cleave the O-alkyl linkage [69-71]; (**c**) higher affinity for acyltransferases [72,73]; (**d**) membrane composition including higher levels of ether lipids [74] and lower levels of cholesterol [75]; (**e**) higher levels of free radical generation and lipid peroxidation [76]; (**f**) pattern of membrane accumulation, as [^{3}H]-ET-18-OCH_3 showed labeling mainly located at the plasma membrane of HL-60 cells while in K-562 cells it was uniformly distributed [77]. Many of these proposals do not appear to be supported for miltefosine where in a recent study on 13 tumor cell lines sensitivity was shown not to be related to endocytosis, drug uptake, membrane composition or cell cycle alterations [78].

(III) Membrane Interaction

A fundamental component on the antitumor activity of LPAs is their direct effect at the plasma membrane [79]. Due to their highly lipophilic nature, LPAs affect the physical properties of neoplastic cells by permeabilizing and increasing plasma membrane fluidity with consequent morphological alterations [80-82]. A detergent property of LPAs has also been reported causing cell lysis but at high concentrations [83,84], above those required for anti-tumor activity.

The activity of LPAs was not associated with interaction with membrane PAF receptor, since several comparative studies showed that PAF is not able to interfere with production of cytokines [85,86] and does not lead to ultrastructural damage [23] as occurs with LPAs. However, other studies suggest that they act through the same binding site [87-89].

(IV) Phospholipid Metabolism

Due to structural similarities of LPAs with acyl-lysophospholipds it is not unexpected that these compounds affect phospholipid metabolism, in particular PC biosynthesis [90-92]. Several studies suggest that the enzyme CTP: phosphocholine cytidylyl transferase (CT) is the target in tumor cells [93-97].

(V) Signal Transduction

Enzymes involved in cell signal transduction, for example phospholipase C [98-101], phospholipase D [102-104] and phosphatidylinositol-3-kinase (PI-3K) [105,106] have all been suggested as targets of LPAs. However, most studies have focused on protein kinase C (PKC), a key regulatory enzyme that is abundant in tumor cells. LPAs inhibited PKC activity in experiments with intact cells, homogenates and purified enzyme [99,107,108]. ET-18-OCH_3 inhibited the phosphotransferase activity of PKC acting as a competitor of phosphatidylserine (PS) and phorbol ester-induced phosphorylation of endogenous proteins [109-111]. However, it was noted that the mode of addition of this LPA in PKC assays influenced its effects: when added from an ethanol solution, the compound inhibited PKC activity [112,113], whereas it stimulated the enzyme when added together with PS and 1,2-diacylglycerol as liposomes [114].

Whether the reported effect of LPAs on components of signal transduction system is associated with the toxicity to tumor cells is still a controversial issue [54,55].

(VI) Generation of Free Radicals

Although the chemical structures of LPAs do not suggest the generation of free radicals, they could arise as secondary event to the damage of plasma membrane. The presence of an alkyl radical and lipid-derived radicals were detected after the treatment of leukaemic cells with ET-18-OCH_3 or ilmofosine and was associated with the cytotoxicity effect with LPAs [115,116]. This group suggested that the higher susceptibility of HL-60 was due to higher levels of poly-unsaturated fatty acids in comparison with K-562 cells [76].

(VII) Induction of Apoptosis

Diomede and coworkers [117] detected in several leukaemic lineages treated with ET-18-OCH_3, morphological alterations and DNA fragmentation compatible with apoptosis. This study was then extended to other LPAs and other tumor lineages [118-123]. Through a different approach, it was suggested that physico-chemical stress leading to reactive oxygen species was involved in the apoptotic process triggered by LPAs in tumor cells [124]. It was also reported that LPAs enhanced radiation-induced apoptosis in tumor cell lines [125]. During the last few years, increasing evidence has shown that ceramides, formed by sphingomyelin hydrolysis, induce apoptosis [126]. Synthetic ceramide analogues were shown to induce apoptosis and cleavage of the caspase 3 substrate poly-(ADP-ribose) polymerase and these effects are antagonized by Bcl-2 [127]. The low percentage of apoptosis induced in MCF7 cells lacking caspase 3 indicated that this enzyme seems to play an essential role in miltefosine-induced apoptosis [78].

3. EXPERIMENTAL STUDIES ON TRYPANOSOMATIDS

In Vitro and *In Vivo* Activities

The research on the activity of LPAs against the pathogenic trypanosomatids, that cause the leishmaniasis and trypanosomiasis, followed earlier studies by Tsushima *et al.* [128] who reported that LPAs were active against a variety of fungi, including human pathogens, and the protozoan *Tetrahymena pyriformis,* and the work of Herrmann & Gercken [129], which suggested the possibility of inhibitors of lipid biosynthesis in *Leishmania*.

The first reports in 1987 described the activities of both AGPCs and APCs on *Leishmania donovani* promastigotes. The susceptibility of this parasite to ET-18-OCH_3 and other AGPCs was shown at concentrations comparable to those active against tumor cells [130] and that radiolabelled compound was rapidly taken and incorporated into lipids [131]. At the same time a series of APCs, including hexadecylphosphoholine (HePC), were shown to possess selective activity against *L. donovani* amastigotes in murine macrophages in the range of 0.2 to 3.9 μM. HePC was also active against *L. donovani* in the mouse model with an ED_{50} of 12.8 mg/kg following parenteral administration [132]. These studies on HePC in mouse models were extended to demonstrate oral activity against *L. donovani* [133] and *L. infantum* [133, 134]. In a comparative study on the activities of four LPAs that reached clinical trial as anticancer drugs, HePC was the most active compound against *L. donovani*, when compared with the AGPCs ET-18-OCH_3, ilmofosine and SRI 62-834 *in vitro* and *in vivo* [135,136]. In mouse studies HePC gave higher activity against liver and spleen infections than the control antimonial drugs with the advantage of oral administration. Unilamellar HePC liposomes were also effective in clearing visceral *L. donovani* infections in mice [137], but required intravenous administration; this offers no advantage for a drug with oral activity.

There have been limited SAR studies on the activity of LPAs against *L. donovani* and none on other trypanosomatids. The early study of [132] showed that both straight chain and branched APCs and one alkylphosphoethanolamine had activity against amastigotes in macrophages in the 1 to 10 μM range; bromo-derivatives were inactive. Three of the APCs were also active against *L. donovani in vivo* whereas the ethanolamine derivative was inactive. More recently the activity of a series of 12 APCs against *L. donovani* was reported. The octadecyl- was more active than hexadecyl-, tetradecyl-, erucyl- or docodecyl- phosphocholines; the configuration of double bonds (*cis*- or *trans*-) did not affect activity, nor did replacement of the quaternary ammonium group by pyrrolidine or piperidine groups [138]. However, *in vivo* the choline-containing compounds were most active. In studies on *L. donovani* promastigotes both alkylglycerophosphocholines and ethanolamines were active with ED_{50}s in the 5-10 μM range, whereas the ether linked (including ET-18-OCH_3) were more active than ester linked derivatives with ED_{50}s in the 2-3 μM range [130]. An analogue of ET-18-OCH_3, *rac*-1-do-decyl-2-octanamide-2-deoxy-glycerophosphocholine, showed a similar level of activity against *L. donovani* and *Leishmania major* promastigotes and amastigotes in macrophages [139]. ET-18-OCH_3 is an analogue of PAF. Although PAF and lyso-PAF are only active against *L. donovani* above 25 μM (*Matu, S. & Croft, S. L. unpublished results*), slight effects on *T. cruzi* differentiation were observed at 1 μM [140].

In a comparative *in vitro* study including six *Leishmania* species, those that cause cutaneous diseases were less sensitive to both HePC and ET-18-OCH3 than *L. donovani*, with the sensitivity of *L. major* significantly lower than

other species [141]. In a BALB/c-*L. major* mouse model neither topical nor oral administration of HePC or ET-18-OCH_3 caused regression of cutaneous lesions (*Yardley, V. & Croft S.L., unpublished results*). This is in contrast to a topical study where Miltex®, 6% HePc in a propylene glycol formulation, reduced the parasite burden and healed established lesions of mice infected with *Leishmania mexicana* or *L. major* [142].

In a comparative study of the activity of LPAs against *Trypanosoma* spp. and *Leishmania*, ET-18-OCH_3, HePC, ilmofosine and SRI 62-834 has higher activity against *T. cruzi* amastigotes in macrophages than against *L. donovani* [136]. Further studies extended this data, showing: (**a**) that all three forms of *T. cruzi* (amastigotes, epimastigotes and trypomastigotes) were sensitive to HePC, ET-18-OCH3 and ilmofosine; (**b**) extensive blebbing of the flagellar membrane of epimastigotes after treatment with these LPAs; (**c**) a dose-dependent inhibition of the intracellular proliferation of amastigotes in heart muscle cells by ET-18-OCH_3 and (**d**) inhibition of the differentiation of epimastigotes to trypomastigotes by ET-18-OCH_3 [143]. In another study it was found that LPAs are potent inhibitors of PC synthesis in *T.cruzi* epimastigotes, which takes place in these cells through the Greenberg (transmethylation) pathway, in contrast with the situation in vertebrate cells, where CDP-choline pathway is predominant [144]. However, *in vivo* experiments showed that LPAs had only a suppressive effect on the parasitaemia of *T. cruzi*-infected mice [136].

In initial studies *Trypanosoma brucei* spp. proved to be the least susceptible trypanosomatid to LPAs; the most active compound in tests on bloodstream form trypomastigotes, ilmofosine, was about 10-fold less active against *T. b. brucei* than against *L. donovani* amastigotes [136]. In another report, several LPAs also showed low activity *in vitro* against *T. brucei* [142]. *In vivo* there was no activity against this trypanosomatid [136,145]. Interestingly, *T. brucei* procyclic trypomastigotes (the form found in tsetse flies) were about 100 fold more sensitive to these drugs than the bloodstream form trypomastigotes (*Croft, S.L. unpublished results*). However, these are not the clinically relevant forms.

Mechanisms of Action

As HePC and ET-18-OCH_3 have been shown to stimulate T cells and macrophages to respond to and to secrete cytokines and the production of microbicidal reactive nitrogen and oxygen intermediates, it was necessary to determine whether the antileishmanial activities of LPAs *in vivo* was direct or indirect via macrophages. In studies on *L. donovani*-infected mice lacking or deficient in T cells, IFN-γ and a range of macrophage deficiencies generation of such intermediates, the efficacy of HePC was similar to that observed in normal mice [62]. These observations were extended in T and B cell deficient *scid* mice where HePC showed a similar dose-response effect in the normal BALB/c and immunodeficient mice [63]. The activity of HePC therefore is direct and does not require host T cell or macrophage-dependent activation (Table 1).

The selective activity of ET-18-OCH_3 against epimastigotes of *T. cruzi* was explained by the observation that at the ED_{50} value the blockade of PC synthesis in vertebrate cells, probably at the level of CT [89], is more than one order of magnitude higher than those in parasite

Table 1. Interference of LPA on Mammalian Tumor Cells and Pathogenic Trypanosomatids

Targets	Tumor cells	*Leishmania* spp.	*T. cruzi*	*T. brucei*
Inhibition of differentiation	+		+ [143]	
Inhibition of proliferation	+	+ [130, 133,136,139,142]	+ [132,136,143,144]	
Induction of lysis			+ [141,143,144]	+/- [135,145]
Macrophages (non-specific activation)	+			
Macrophages (production of mediators)	+			
Plasma membrane (ultrastructural damage)	+		+ [143]	
Plasma membrane (generation of free radicals)	+			
Plasma membrane (inhibition of plasmatic enzymes)	+			
Transduction signaling (inhibition of PKC)	+		+ [147]	
PC synthesis (inhibition of CT)	+			
PC synthesis (inhibition of Greenberg route)			+ [144]	
Sterol synthesis (inhibition of sterol C-22 desaturase)			+ [144]	
Ether lipids remodelling (inhibition of alkyl-acyl-CoA acyltransferase)		+ [148]		
Metabolism of phosphoinositides	+			
Calcium levels	+			
Induction of apoptosis	+			

cells. It was observed that in LPAs-treated *T. cruzi*, ergosterol and its 24-ethyl analogue were replaced by its Δ^{22}-saturated analogues, indicating that inhibition of sterol C-22 desaturase is also involved in the mode of action of LPAs on *T. cruzi*. Another interesting point is a synergistic effect observed between ketoconazole, a known sterol biosynthesis inhibitor of this parasite [146] and ET-18-OCH_3 [144]. It was also suggested that ET-18-OCH_3 caused inhibition of phospholipase C in epimastigotes, preventing the hydrolysis of inositol biphosphate after stimulus by fetal calf serum [147].

In studies with *L. mexicana*, HePC and ET-18-OCH_3 inhibited the glycosomal alkyl-acyl-CoA-acyltransferase, suggesting a perturbation of ether-lipid remodelling [148]. However, the correlation between ether-lipid remodeling and cytotoxicity was not directly established as the IC_{50} for the former effect was about 4-fold higher than the concentration required to inhibit cell growth.

As yet there have been no published studies on the accumulation of LPAs by trypanosomatids or the nature of the initial interaction with membranes. However, there is evidence that LPAs can be removed from parasites by ABC transporters. Promastigotes of a multidrug-resistant strain of *L. tropica* with over-expression of a P-glycoprotein-like transporter were found to 9.2 and 7.1 fold less sensitive to HePC and ET-18-OCH3, respectively, than wild-type cells [149].

Experimental Studies on Other Protozoa

The useful activities of LPAs on protozoa appear to be mostly limited to trypanosomatids. No significant activity was found for HePC, ilmofosine or ET-18-OCH_3 against *Plasmodium falciparum in vitro* or *P. berghei in vivo* in a mouse model (*Croft, S.L. unpublished results*). However, a series of alkylphosphocholines was tested against *Entamoeba histolytica*; HePC was less active than C_{18}- and C_{19}-analogues that had ED_{50} values in the order of 15-20 μM against one of the strains used [150]. Among a series of APCs, HePC showed the highest activity against different strains of *Acanthamoeba* spp, being suggested that this compound is a potential candidate for the treatment of keratitis caused by amoebae of this genus [151]. Earlier work on protozoa was limited to tests of ET-18-OCH_3 and other lysophospholipids against *Tetrahymena pyriformis* with MIC values of < 1 μg/ml reported, more active than against pathogenic fungi and inactive against bacteria [128].

Clinical Studies on Leishmaniasis with HePC

The reported studies in rodent models, together with available pharmacological and toxicological data from cancer clinical trials, led both Zentaris (originally Asta Medica) and WHO/Tropical Diseases Programme in 1996 to consider HePC for clinical trials against visceral leishmaniasis. In a first phase I/II study in Bihar, India, 30 visceral leishmaniasis cases received daily or every other day oral doses of 50 to 250 mg administered for 28 days. Eight months after treatment 7 out 10 (70%) of the patients treated with 50mg or 100 mg every other day relapsed, while 18 out 19 (94.5%) treated with 100, 200 or 250 mg/day were cured, according to the criteria of parasite-free bone-marrow aspirate and no clinical evidence of relapse [152]. In a subsequent phase II study 45 patients were treated orally with HePC at 100-200 mg/day for 28 days including 17 patients for whom previous therapy with antimonial drug had failed. In this study the cure rate was 98% including the antimony-unresponsive cases [153]. A phase II trial including 120 patients using oral administration of HePC at 50 to 150 mg/day for 28-42 days, with clinical response assessed 6 months after treatment, gave a 95% cure rate at 100 mg /day

Table 2a. Direct *In Vitro* Effect of LPAs on Extracellular Trypanosomatids

Trypanosomatid	Parasite strain	Parasite form	ET-18-OCH_3		Ilmofosine		HePC		ref.
			ED_{50} (μM)	% serum	ED_{50} (μM)	% serum	ED_{50} (μM)	% serum	
L. donovani	L51	Promastigote	0.2 (4d)[1]	0[2]					[130]
	LRCL.51, DD8, STI172						0.89-2.25 (4d)	10	[133]
	L82						12 (2d)	20	[132]
T. b. brucei	S427	Bloodstream trypomastigote	44.0 (2d)	20	7.0 (2d)	20	35.5 (2d)	20	[136]
	STIB920						MIC[3] 76 (3d)	15	[145]
T. b. rhodesiense	STIB900	Trypomastigote	40.7 (2d)	20	18.1 μM (2d)	20	47.0 (2d)	20	[136]
							MIC 88 (3d)	15	[145]
T. b. gambiense	STIB930	Trypomastigote					MIC 273 (3d)	15	[145]
T. cruzi	Y	Trypomastigote	MIC 1000 (1d)	20	MIC 1000 (1d)	20	MIC 1000 (1d)	20	[136]
			29. 0 (1d)	10	29.5 (1d)	10	55.4 (1d)	10	[143]
		Epimastigote	11.7 (1d) 3 (5 d)	10 10	26.6 (1d) 3 (5 d)	10 10	17.4 (1d) 1(5 d)	10 10	[143] [144]
		Axenic amastigote	13.4 (1d)	10	11.6 (1d)	10	18.6 (1d)	10	[143]

[1]days of treatment; [2]percent of serum in the assay; [3]MIC: lowest concentration causing abnormal morphology or motility

Table 2b. ***In vitro*** **Effect of LPAs on Intracellular Trypanosomatids**

Trypanosomatid	parasite strain	Host cell	ET-18-OCH_3 ED_{50} (μM)	Ilmofosine ED_{50} (μM)	HePC ED_{50} (μM)	ref.
L. donovani	L82	Macrophages		3.73 (7d)[1]	12.3 (7d)	[132,135]
	NandiII L82		0.7 (5d) 5.0 (5d)	0.6 (5d) 2.6 (5d)	0.2 (5d) 3.9 (5d)	[136]
	L82			2.96 (5d)		[139]
L. infantum	-[2]	Macrophages		3.46 (7d)		[135]
T. cruzi	Y	Macrophages	1.4 (3d)	0.2 (3d)	0.5 (3d)	[136]
		heart muscle cells	6.8 (3d)			[143]
		Vero cells	MEC[3] 0.1 μM (4d)	MEC 1 μM (4d)	MEC 0.1 μM (4d)	[144]

[1]days of treatment; [2]antimony resistant line; [3]MEC: lowest concentration causing complete erradication of intracellular parasites

Table 2c. ***In vivo*** **Effect of LPAs in Experimentally Infected Mice**

Trypanosomatid	parasite strain	parasite form-mouse lineage	ET-18-OCH_3 (mg/kg)	Ilmofosine (mg/kg)	HePC (mg/kg)	HePC route, treatment time	ref.
L. donovani	LV9 PatnaI	Amastigote-BALB/c	ED_{50} >30[1] ED_{50} >30	ED_{50} = 14.5 ED_{50} = 12.3	ED_{50} = 9.16 ED_{50} = 2.9	or, 7-11 dpi	[136]
	L82				ED_{50} = 10.6	or, 7-11 dpi	[135]
	L82	Amastigote-BALB/C Amastigote-scid mice			ED_{50} = 3.98 ED_{50} = 4.53	or, 15-21 dpi	[63]
	DD8 LRC-L.51	Amastigote-BALB/C			20 (99.5%) (90.9%)[2]	or, 15- 20 dpi	[133]
L. infantum	STI-172	Amastigote-BALB/C			20[2]	or, 1-4 wpi	[133]
	LPN101	Promastigote-Balb/C			30 (94.0%)[3]		[134]
L. mexicana	M379	Amastigote-Balb/C			1.5 mg/day[4]	tp, 22-25 wpi	[142]
		Amastigote-CBAJ					
		Amastigote-C57/BL6					
L. major	IR76	Promastigote-C57/BL6			1.5 mg/day[5]	tp, 4-5 wpi	[142]
T. b. brucei	S427	Trypomastigote-Balb/C	30 inactive[6]	30 inactive[6]	30 inactive[6]	tp, 22-25 wpi	[136]
	STIB920	Trypomastigote-NMRI			30 (35%)[7]	or, 1–11 dpi	[145]
T. cruzi	Y	Trypomastigote-Balb/C	15 inactive[6]	15 inactive[6]	15 inactive[6]	tp, 22-25 wpi	[136]

or: oral route; tp: topical application 5 days/week; dpi: days post-infection; wpi: weeks post-infection

[1]value for ED_{50} for the inhibition of number of amastigotes/liver; [2]at 20 mg/kg the decrease in the parasite load in liver was 600X higher than that of sodium stibogluconate; [3]percent of inhibition of number of amastigotes/liver; [4]parasite burden assessed in draining lymph nodes and spleen, lesions healed; [5]parasite burden assessed in draining lymph nodes and spleen, lesions healed; [6]no increase in survival in relation to untreated mice; [7] percent of survival

again including patients resistant to pentavalent antimony [154]. Mild to moderate gastrointestinal side effects were reported in 62 % patients but no patient discontinued the treatment. An editorial comment on this trial considered HePC as the long-awaited oral drug for visceral leishmaniasis (VL) [155]. Clinical studies continue: 54 patients treated orally with 50 mg given twice daily for 14, 21 and 28 days achieved cure rates of, respectively 89%, 100% and 100%, while adverse reactions were limited and mild [156]. Phase III trials have been completed but have not yet been reported and miltefosine registered in India in March 2002 for use against VL. The potential of HePC in the treatment of immunosuppressed VL patients has also been considered. One HIV positive/VL case that failed to respond to Pentostam showed clinical and parasitological cure after 28 days of treatment with HePC at 100 mg/day [157].

A special formulation of 6% HePC, Miltex®, that can penetrate the skin has been developed for the topical treatment of breast cancer skin lesions and other cutaneous malignancies and approved for use in several European countries [158]. Following studies in mice [142] there have been clinical trials of Miltex® for the treatment of cutaneous leishmaniasis; the results have not been published but were

disappointing (*P. Bachmann, pers. comm.*). However, results from a phase I/II trial in Colombia involving 32 CL patients showed oral HePC to be be effective at 100 - 150 mg/day for up to 28 days giving a cure rate of 94% [159].

4. CONCLUDING REMARKS: COMPARISON OF THE EFFECT OF LPAS ON CANCER CELLS AND PATHOGENIC TRYPANOSOMATIDS

The antiprotozoal and anticancer activities of LPAs were discovered independently in the 1980s. Experimental and clinical studies on leishmaniasis in the 1990s saw the two lines of research coming together. This review compares the novel activity of LPAs on pathogenic trypanosomatids with the better-characterized activity on tumor cells (**Tables 2** & **3**). When making comparisons several points need to be considered:

(I) A factor of critical importance when determining the *in vitro* activity of LPAs against different types of cells is the presence of blood components in the assay, which can lead to a marked reduction of the amount of free drug. This has been observed in studies both in the areas of cancer and protozoology. For example a 60-fold reduction of on the activity of LPAs against fibroblast was achieved by addition of 10% fetal bovine serum in relation to serum-free medium [160]. Decrease of LPA activity by increasing levels of serum was also reported by other groups [161-164]. This effect was also observed with *T. cruzi* when comparing the effect of LPAs in

Table 3. Effect of LPAs on Mammalian Tumor Cells

cells	Type	ET-18-OCH_3		ilmofosine	HePC	ref.
		ED_{50} (μM)	% serum	ED_{50} (μM)	ED_{50} (μM)	
HL-60	promyelocytic leukemia (Hu)[1]	1.5-13.2 (1d)[2] 1.2-6.4 (2d)	10[3]	2 (1d)	3.6-7.7 (2d)	[65,68, 74-76,97, 117, 118,120,121]
		30.5 (1d)	20			[64]
		1.5 (1d)	0			[77]
K-562	erythroblastic leukemia (Hu)	21.0 to > 60 (1d) 9.6 -18.4 (2d)	10	60 (1d)	90.0 (2d)	[36,74-76,91, 117, 120,121,161]
		>> 20 (1d)	20			[64]
		28 (1d)	0			[77]
KG-1	myeloblastic leukemia (Hu)	107.9 (1d)	10			[162]
ALL	acute myeloid leukemia (Hu)	3.7 (1d)	10			[23]
AMML	acute myelomonocytic leukemia (Hu)	1.6 (1d) 25.4 (1d)	10 20	1.5 (1d)		[64]
CML	chronic myelocytic leukemia (Hu)	50 (1d)	10			[23]
CLL	chronic lymphocytic leukemia (Hu)			4.6-7.6 (2d)		[23]
WEH1-3B	myelomonocytic leukemia (Mu)[4]	5.4 (2d)	10			[65]
Daudi	B-cell leukemia (Hu)	78.0 (1d)	10			[162]
Jurkat	T cell (Hu)				10. 1 (2d)	[121]
Molt-4	leukemia (Hu)	16.4 (1d)	10		22.9 (2d)	[120,121]
U-937	leukemia (Hu)	21.1 (1d)	10		21.0 (2d)	[120,121]
ABLS-8.1	Abelson "pre-B"-lymphoma	3.2 (1d)	10			[91]
MethA	sarcoma (Mu)	26.6 (1d); 4,7 (2d)	10			[90,91]
MO_4	sarcoma (Mu)	51.4 (2d)	10			[80]
YAC	lymphoma (Mu)	5.6 (1d)	10			[91]
Raji	Burkitt lymphoma (Hu)				23.3 (2d)	[121]
MCF-7	mammary gland-derived (Hu)	≅ 11 (12h)	10		54.0 (3d)	[71]
5637	bladder carcinoma (Hu)				1.4 (3d)	[36]
EJ	bladder carcinoma (Hu)				85.0 (3d)	[36]

[1]Hu: human; [2]time of treatment; [3]percent of serum in the assay; [4]Mu: murine

culture medium and in phosphate buffered saline. For example, for trypomastigotes the ED_{50}/24h was 29.0 ± 2.8 μM in the appropriated medium, while in the buffer total lysis of the parasites occurred at the same concentration after only 30 min of treatment [143].

(II) The scarcity of the knowledge about cell signalling in trypanosomatids [165-167] in comparison to that in cancer cells hampers "bona fide" comparisons between the mechanism of action of LPAs operating in these two models. Although evidences for apoptosis in protozoa are still controversial, recent papers point in this direction [168-170], but only further studies will determine if such mechanism could be involved in the activity of LPAs against *T. cruzi* and *Leishmania*. In this context, interference in phospholipid and sterol biosynthetic pathways has been associated with the effect of LPAs against cancer cells and trypanosomatids, but PC biosynthesis inhibition in vertebrate cells requires >20-fold higher concentrations than that effective in *T. cruzi*

(III) No information is available about the intracellular levels of LPAs in trypanosomatids and the drug concentrations to which amastigote forms are exposed. It is possible that differences in sensitivity to LPAs when comparing *T. cruzi* and *L. donovani* could be associated with their intracellular localization cytoplasmic versus phagosomal compartments.

(IV) Although development of LPAs as anticancer agents dates from 1980´s, the only approved application in this field has been the topical use of mitelfosine (Miltex™) for the treatment of metastatic breast carcinoma. On the other hand, a remarkable efficacy of this drug has been demonstrated in the oral treatment of visceral lesihmaniasis and its use was approved in March 2002, for the oral treatment of visceral leishmaniasis in India. The different efficacies of LPAs in these two applications could be due to distinct intrinsic susceptibilities of the biological targets and/or pharmacokinectic characteristics relevant to these applications.

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ABBREVIATIONS

LPC = 2-Lysophosphatidylcholine

PC = Phosphatidylcholine

APCs = Alkylphosphocholines

AGPCs = Alkylglycerophosphocholines

HePC = Hexadecylphosphocholine

LPAs = Lysophospholipid analogues

ErPC = Erucylphosphocholine

PAF = Platelet activating factor

CTP = Phosphocholine

CT = Cytidylyl transferase

PI-3K = Phosphatidylinositol-3-kinase

PKC = Protein kinase C

PS = Phosphatidylserine

IFN-γ = Interferon-γ

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