Miltefosine — discovery of the antileishmanial activity of phospholipid derivatives

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KEYWORDS
Leishmaniasis;
Antileishmanial;
Miltefosine;
Alkyl-
phosphocholines;
Anticancer drug

Summary   Miltefosine (hexadecylphosphocholine, Impavido[TM]), a novel antiprotozoal drug used for the treatment of visceral and cutaneous leishmaniasis, was identified and evaluated independently in the early 1980s as a potential anticancer drug in Germany and as an antileishmanial drug in the UK. Although miltefosine is not the most active compound of its class against *Leishmania* parasites in vitro, the early demonstration of activity after oral administration in experimental models of visceral leishmaniasis helped to bring this compound to the attention of WHO TDR for further development in a unique collaboration model with the pharmaceutical industry (Zentaris GmbH). Miltefosine is active against most *Leishmania* species, including those that cause cutaneous disease.
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1. Introduction

Miltefosine (hexadecylphosphocholine) is the first oral drug to be registered for the treatment of visceral leishmaniasis (VL) (Ganguly, 2002) and more recently for the cutaneous form of the disease. Although several oral drugs have been undergoing clinical trials for the treatment of VL, for example, allopurinol and sitamaquine (Croft and Yardley, 2002), no other has been successfully registered for this indication. The development of phospholipid derivatives as drugs, essentially based upon their antitumour activities, involves two closely related groups of compounds: the alkylglycerophosphocholines (AGPs), also termed ether-lipids, and the alkylphosphocholines (APCs) (Brachwitz and Vollgraf, 1995). The identification of miltefosine and other phospholipid derivatives as potential antileishmanials came in the late 1980s as a result of three independent lines of research.

The first pathway to miltefosine followed observations on the anticancer activity of the AGP rac-1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine, also known as ET-18-OCH$_3$ and later called edelfosine, and a subsequent programme of medicinal chemistry. The second followed studies on the phospholipid metabolism of *Leishmania* promastigotes, which led to the identification of several AGPs, including ET-18-OCH$_3$, that were cytotoxic to promastigotes. The third pathway, which actually identified hexadecylphosphocholine (miltefosine) as a lead antileishmanial compound, came through a pharmaceutical company screening programme. These routes are described in the next sections of this review. A final section covers comparative studies on miltefosine against species of *Leishmania* and a consideration of other phospholipid drugs.

2. Discovery of miltefosine — the anticancer drug

The group of Westphal and Munder (Westphal, 1987) first described the in vitro and in vivo anticancer activity of

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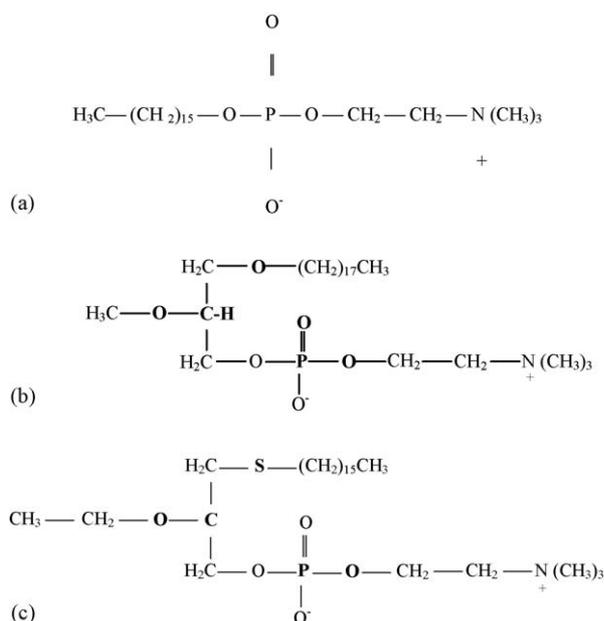 *E-mail address:* scroft@dndi.org (S.L. Croft).

Figure 1   Phospholipid drugs with antileishmanial activity: (a) miltefosine, (b) edelfosine, (c) ilmofosine.

Figure 2   Putative binding of PAF-acether, edelfosine, ilmofosine and miltefosine into the PDF-receptor model proposed by Godfroid and co-workers (see references in text). Miltefosine is coloured green to distinguish it from the other molecules which are coloured by atom type. The electrostatic isopotential surfaces for PAF (yellow) edelfosine (red) ilmofosine (blue) and miltefosine (green) are shown at -30 kcal/mol. The red cylinders indicate areas of the receptor with which negative electrostatic potential surfaces of the ligands may interact; the green cylinder indicates a putative hydrophobic pocket.

the new substance class, alkyl lysophospholipids (ALPs), which are structurally related to platelet aggregation factor (PAF) (Demopoulos et al., 1979). Edelfosine (Figure 1) (Munder et al., 1977, 1981) — the most prominent compound out of this class — was tested in clinical studies in cancer patients but did not reach the market for various reasons including but not limited to reduced oral bioavailability, lack of demonstration of clear anticancer activity and its weak PAF agonistic potency. Ilmofosine (Figure 1), another candidate from this class of alkyl lysophospholipids, was also tested clinically in cancer patients (Giantonio et al., 2004).

It was Hansjoerg Eibl from the Max-Planck-Institute for Biophysical Chemistry in Goettingen who suggested replacing the glycerol backbone with a simple alkyl chain; this retained the antitumour activity and led to the discovery of miltefosine (hexadecylphosphocholine, D-18506) (Figure 1) as a new type of anticancer agent (Eibl and Unger, 1990). In molecular modelling studies and calculations of isopotential surfaces, we were able to demonstrate that alkylphosphocholines no longer retained PAF agonistic activities. Figure 2 shows the putative binding of PAF-acether, edelfosine, ilmofosine and miltefosine to the PAF-receptor model proposed by Godfroid and co-workers (Braquet and Godfroid, 1986; Dive et al., 1989; Godfroid and Braquet, 1986; Godfroid et al., 1991; Lamotte-Brasseur et al., 1991). According to the model there are two attractive potential areas of the receptor (red cylinders) with which the negative electrostatic potential surfaces of the ligands may interact. In contrast to the other molecules, miltefosine does not have a side chain at position 2 of the glyceryl backbone, and since it has only one negative electrostatic potential surface around the phosphate group, it can interact with only one of the attractive potential areas of the receptor. Furthermore, miltefosine does not interact with the putative hydrophobic pocket (green cylinder) inside the receptor.
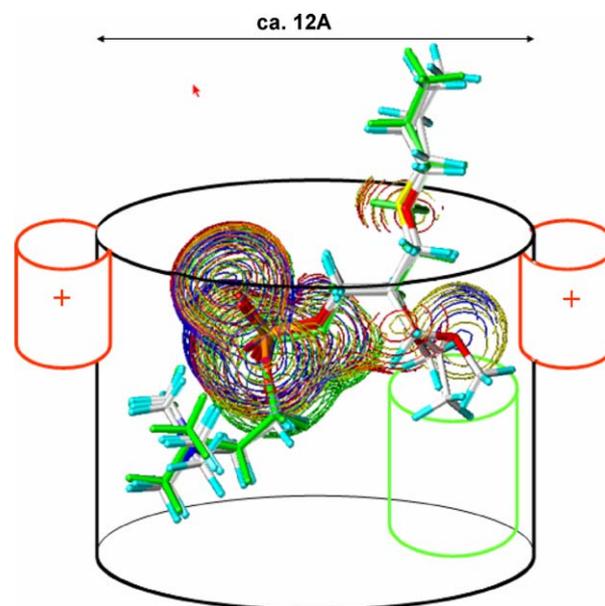
The antitumour activity of miltefosine was proven in a variety of in vitro and in vivo models including authochtonous tumour models, such as the nitrosomethylurea and dimethylbenzanthracene-induced mammary carcinoma of the rat, by various groups (Hilgard et al., 1988; Unger et al., 1989). Miltefosine was selected for clinical development for oral use against solid tumours and for the topical treatment of cutaneous metastases in breast cancer patients. After successful clinical development the topical treatment was approved as Miltex® in several countries in Europe (Burk et al., 1994).

In phase II studies of oral miltefosine dose-limiting gastrointestinal side effects were described. Because of rather low plasma levels — not in the expected range of in vitro antitumour activity — development for this indication was abandoned. Through structure—activity relationship analysis, perifosine was identified with an enhanced gastrointestinal tolerability and increased anticancer activity (Hilgard et al., 1997). This new alkylphosphocholine is now in several phase II studies in Europe and the USA as the first oral protein kinase B inhibitor in clinical development.

3. Basis for activity of alkyl-glycerophosphocholines

In the early 1980s, a group in the Department of Biochemistry, University of Hamburg, Germany, began to study ether lipid biosynthesis in *L. donovani* promastigotes. Initial studies (Herrmann and Gercken, 1982) showed that

1-O-alkylglycerols at $25\,\mu M$ killed the parasites within 5 h of exposure. These studies on cytotoxic ether lipid derivatives were extended by Achterberg and Gercken (1987a, 1987b) who showed that 1-O-alkylglycerols, ester and ether lysophospholipids were active against *L. donovani* promastigotes at low μM levels. Of these, the 1-O-alkylglycerophosphocholines, 1-O-alkylglycerophosphoethanolamines and 1-O-hexadecyl-*sn*-glycerol were the most active compounds with $<3\,\mu M$ ED_{50} values. By contrast, 1-stearoylglycerol was inactive suggesting that the ether linkage at the *sn*-1 position in the glycerol was important for activity. The highest activity identified by Achterberg and Gercken (1987a) was by the antitumour compound rac-1-O-octadecyl-2-O-methylglycero-3-phosphocholine (edelfosine) which has a methoxy residue at the *sn*-2 position. The authors acknowledged that the potential for these compounds in the chemotherapy of leishmaniasis was not clear as their studies were performed on promastigotes. Further aspects of the basis of the mechanisms of action of this class of compounds are described by Urbina (2006).

4. Alkylphosphocholines as antileishmanial compounds

Miltefosine (hexadecylphosphocholine) was also synthesized independently in 1982 by Bill Pendergast and Joseph Chan at Burroughs Wellcome, RTP, NC, USA, as part of an anti-inflammatory programme. Miltefosine was one of a series of simple analogues of PAF that had recently been described (Benveniste et al., 1979). Compounds were selected for screening against *Leishmania* and trypanosomes at the Wellcome Research Laboratories, Beckenham, UK (WRL) in 1984. At that time knowledge of the antimicrobial activities of phospholipid drugs was limited to a Japanese report on the antifungal activity of AGPs that also mentioned their in vitro activity against the protozoan *Tetrahymena pyriformis* (Tsushima et al., 1982). This was also a period when there was an interest in compounds like muramyl dipeptide that could activate macrophages to kill intracellular *Leishmania*. Reviews also described macrophage activation by compounds such as lysophosphatidylcholine and its derivative $ET-18-O-CH_3$ (edelfosine) (Munder et al., 1977, 1981). The approach was later exemplified in studies with edelfosine and another ether lipid derivative, the lipoidal amine CP-46,665-1, which had immunopotentiating activity in an *L. donovani* mouse model of infection (Adinolfi and Bonventre, 1985).

Meanwhile, Pendergast and Chan had synthesized several APCs, with different alkyl chain lengths and distances between P and N of the phosphocholine moiety. Seven APCs, including hexadecylphosphocholine (miltefosine), and one alkyl phosphoethanolamine were reported active at $<10\,\mu g/ml$ against *L. donovani* amastigotes and promastigotes (Croft et al., 1987). Miltefosine had an activity of $5.0\,\mu g/ml$ in the mouse macrophage model, not the highest activity but the best tolerated by host cells. Four compounds were selected for in vivo study, of which the three APCs were active, but the phosphoethanolamine was inactive. In a subsequent experiment, the ED_{50} of miltefosine was determined as $12.8\,mg/kg$ body weight $\times 5$ administrations (subcutaneous route). This information was published in 1987 with the closure of the antileishmanial and antitrypanosomal programme at the WRL, Beckenham.

Subsequently, during a night shift at University Hospital Goettingen, C. Unger and A. Kuhlencord, knowing the results from Croft et al. (1987) and the oral bioavailability of miltefosine from the phase II studies in tumour patients, planned oral testing of miltefosine against *Leishmania* and other protozoa using existing in vivo models in the group of W. Bommer (Institute of Hygiene, University of Goettingen, Germany). They were able to confirm the excellent antileishmanial activity after oral treatment in BALB/c mice (Kuhlencord et al., 1992). Miltefosine produced >95% suppression of both *L. donovani* and *L. infantum* amastigotes in the liver, spleen and bone marrow at $20\,mg/kg \times 5$ administrations (oral). The activity of miltefosine was markedly superior to that of the standard drug sodium stibogluconate and this study, demonstrating the advantage of oral administration, helped to establish miltefosine as a lead compound for the treatment of VL. These data were the basis for one of us (J.E.) to decide to initiate a development programme within ASTA Medica (later Zentaris) for miltefosine in VL. This decision consequently led to the first phase II study in India initiated by H. Murray, S. Sundar and ASTA Medica in 1997 with favourable results after only 28 days of treatment (Sundar et al., 1998). This proof-of-concept study paved the way for the further clinical development of miltefosine in an unique collaboration between the pharmaceutical industry, WHO/TDR and the Indian Government (Engel, 2002; Ganguly, 2002 and other articles in this supplement). This clinical programme led to the approval of miltefosine as Impavido™, as the first oral treatment for leishmaniasis in several countries.

5. Further experimental studies on the antileishmanial activity of miltefosine

The oral activity of miltefosine against *L. infantum* in mice was confirmed by Le Fichoux et al. (1998). Studies were also made in immune-deficient mice as these could be relevant to problems associated with treatment of patients with *L. infantum*—HIV co-infections. Miltefosine was shown to be highly active in T-cell deficient nude mice at $25\,mg/kg \times 5$ administrations (oral) (Murray, 2000), as well as in knock-out mice lacking specific macrophage killing mechanisms (Murray and Delph-Etienne, 2000). These observations were extended by Escobar et al. (2001) who showed that miltefosine had similar ED_{50} and ED_{90} values against *L. donovani* in both BALB/c and CB-17 *scid* mice. In comparison the standard antileishmanial sodium stibogluconate was inactive in the immunodeficient mouse models.

Miltefosine was also active in experimental models of cutaneous leishmaniasis. A 6% miltefosine ointment (Miltex™), developed for treatment of breast cancer skin metastases, was applied topically to established lesions of *L. mexicana* or *L. major* on either BALB/c, CBA/J or C57Bl/6 mice (Schmidt-Ott et al., 1999). Lesions healed following application over 2—5 weeks, accompanied by a corresponding reduction in the number of parasites in the spleen and the draining lymph nodes. However, there was no promotion of a Th1-type response in the miltefosine-treated mice.

6. Comparative studies on miltefosine and other phospholipids drugs

During the same period that miltefosine was in development as an anticancer drug, three AGPs also reached clinical trials (Brachwitz and Vollgraf, 1995): rac-1-O-octadecyl-2-O-methylglycero-3-phosphocholine (edelfosine) which has a methoxy residue at the *sn*-2 position; 1-hexadecyl-mercapto-2-methoxymethyl-2-deoxy-rac-glycero-3-phosphocholine (ilmofosine) and rac-2-(hydroxy-[tetrahydro-2-(octadecyloxy)methylfuran-2-yl]methoxyl)-phosphinoxy-N,N,N-trimethylethaniminium hydroxide (SRI 62,834). All these compounds showed comparable activity to miltefosine at $<5\,\mu M$ against *L. donovani* amastigotes in macrophage models (Croft et al., 1993, 1996). However, in the BALB/c mouse model of infection, miltefosine was the most active oral compound followed by the AGP ilmofosine, with ED_{50} values in the range of 4 to $20\,mg/kg \times 5$ administrations (Croft et al., 1996).

A dialkyl AGP, rac-1-dodecyl-2-octanamido-2-deoxy-glycerophosphocholine, was also active against *L. donovani* promastigotes and amastigotes in similar models in the same range as edelfosine (Bourass et al., 1996). In contrast, C_{18} and C_{16} lyso and acyl analogues of PAF were poorly active ($ED_{50}s > 30\,\mu M$) against *L. donovani* promastigotes and had no selective activity in the amastigote—macrophage model (Seifert et al., 2003).

In a further extension of studies on antileishmanial activity of miltefosine, Escobar et al. (2002) showed that both promastigote and amastigote stages of *L. donovani*, *L. major*, *L. tropica*, *L. aethiopica*, *L. mexicana* and *L. panamensis* vary in their in vitro sensitivity. In all assays, *L. donovani* was the most sensitive species, with ED_{50} values in the range $0.12–1.32\,\mu M$ for promastigotes and $1.2–4.6\,\mu M$ for amastigotes, whilst *L. major* was the least sensitive species in the majority of assays with ED_{50} values for miltefosine in the range of $4.8–13.1\,\mu M$ for promastigotes and in the range of $7.5–37.1\,\mu M$ for amastigotes. In separate study *L. tarentolae* promastigotes were reported to be 10-fold less sensitive to miltefosine than these *Leishmania* species, the difference being related to the functioning of a phospholipid translocase (Perez-Victoria et al., 2003). More recently, studies on clinical isolates have confirmed the high sensitivity of *L. donovani* amastigotes, but have shown a reduced sensitivity of *L. braziliensis* and *L. guyanensis* clinical isolates in contrast to high sensitivity of *L. lainsoni* isolates (Yardley et al., 2005).

7. Conclusion — the future

The discovery and development of miltefosine for the treatment of leishmaniasis has heralded the identification of a novel group of antiprotozoal drugs. Miltefosine is not the most active APC or AGP but displays excellent bioavailability. Unger et al. (1998) and Croft et al. (1987) reported other highly active antileishmanial compounds from the same series as miltefosine. Other substituted phospholipids with piperidine and morpholine groups have been synthesised and the possibility of designing more effective antileishmanials explored (Avlonitis et al., 2003). There is a possibility that more efficacious antileishmanial phospholipids derived

drugs could be identified. It is not known why gastrointestinal side effects were not a problem in treatment for leishmaniasis whereas they were when miltefosine was evaluated as an anticancer agent. Further research is needed on this aspect.

Conflicts of interest statement
Some of the funding received by S.L.C. (see Acknowledgements) was specifically allocated for miltefosine. S.L.C. has no conflicts of interest concerning the work reported in this paper. J.E. is an employee of Zentaris.

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