Chapter 19

Alkylphospholipids and Leishmaniasis

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Additional information is available at the end of the chapter

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1. Introduction

Leishmaniasias is a complex of diseases caused by intracellular protozoan parasites that belong to the genus *Leishmania* (class: Kinetoplastida; order: Trypanosomatidae), for which there are more than 20 different species, and is transmitted by the bite of female phlebotomine sand flies (order: Diptera; family: Psychodidae; subfamily: Phlebotominae). Various species of phlebotomine sand flies of the genus *Phlebotomus* are responsible for transmission of leishmaniasis in the Old World, and of the genus *Lutzomyia* in the New World [1]. *Leishmania* parasite has a digenetic life-cycle alternating between a mammalian host and insect vectors, phlebotomine sand flies, which are small (1.5-2 mm body length) insects mainly found in tropical and subtropical regions. *Leishmania* lives extracellularly as flagellated promastigotes in the gut and salivary glands of the sand fly vector, and intracellularly as amastigotes in the vertebrate host macrophages. *Leishmania* promastigotes, transmitted to mammalian skin by the bite of a female phlebotomine sand fly, invade human macrophages as the main host for the parasites, where *Leishmania* transforms into amastigotes and replicate intracellularly. Leishmaniasis represents a major international health problem, has a high morbidity and mortality rate, and is classified as an emerging and uncontrolled disease by the World Health Organization (WHO). The disease burden of leishmaniasis is high, with about 350 million people in 98 countries considered at risk. Among parasitic diseases, leishmaniasis accounts for the second highest burden of disease after malaria, with a loss of about 2.4 million disability-adjusted life years (DALYs) [2, 3]. There are an estimated 1.5-2 million new cases per year, with 1.5 million cases of self-healing, but disfiguring, (muco-)cutaneous leishmaniasis, and 500,000 cases of life-threatening visceral leishmaniasis [2, 3]. However, more than 90% of the world's cases of visceral leishmaniasis are in India, Bangladesh, Nepal, Sudan, and Brazil. Some species tend to cause cutaneous leishmaniasis (e.g., *L. major* and *L.tropica*), whereas others lead to cause visceral leishmaniasis (e.g., *L. infantum* and *L. donovani*). Leishmaniases are usually classified based on the clinical manifestations, leading to three major clinical forms, namely:

- Cutaneous leishmaniasis. It is the most common form of the disease, leading to a skin sore at the bite site, which erupts weeks to months after the person affected is bitten by sand flies, and then heals in a few months to a year, leaving an unpleasant-looking scar.
- Mucocutaneous leishmaniasis. It occurs predominantly in the New World, starting with skin ulcers which spread, causing tissue damage and destruction, and certain *Leishmania* species migrate to the upper respiratory tract where destruction of the oropharynx and nose ensues, resulting in extensive midfacial destruction and, occasionally, in death.
- Visceral leishmaniasis. It is classically known as kala-azar, and also referred to as black fever, and Dumdum fever. It is the most serious and devastating form of the disease, where the parasites localize to the reticuloendothelial system, rather than to the skin, and migrate to and affect internal organs (usually spleen, liver, and bone marrow), producing a potentially lethal widespread systemic disease that is fatal if untreated. Visceral leishmaniasis is characterized by irregular bouts of fever, weight loss, substantial swelling of the spleen and liver, fatigue and anemia. The systemic infection of the liver, spleen and bone marrow leads to hepatomegaly, splenomegaly, lymph node enlargement, thrombocytopenia, and anemia.

Cutaneous leishmaniasis can be further divided into: a) localized (crusted papules or ulcers occur several weeks to months after sand fly bite inoculation on exposed skin, and lesions usually heal spontaneously); b) diffuse cutaneous (producing widespread skin lesions which resemble leprosy, being particularly difficult to treat; and patients cannot mount a cell-mediated immune response to the *Leishmania* parasite, developing multiple, widespread cutaneous papules and nodules); c) recidivans (appearing as a recurrence of lesions at the site of apparently healed disease years after the original infection; and occurring typically on the face as an enlarging papule, plaque, or coalescence of papules that heals with central scarring, leading to facial destruction in some cases); d) post-kala-azar dermal leishmaniasis (a complication of visceral leishmaniasis in areas where *L. donovani* is endemic, and characterized by a hypopigmented macular, maculopapular, and nodular rash that usually appears 6 months to 1 or more years after apparent cure of visceral leishmaniasis).

Another way to categorize leishmaniasis is based on geographic occurrence. Thus, Old World leishmaniasis, caused by *Leishmania* species found in Africa, Asia, the Middle East, and the Mediterranean, mainly leads to cutaneous or visceral forms of disease; and New World leishmaniasis, caused by *Leishmania* species found in endemic regions extending from southern USA to northern Argentina, mainly in Central and South America, generates cutaneous, mucocutaneous, and visceral forms of disease. It is interesting to note that distinct forms of leishmaniasis follow different clinical courses of the disease depending on the geographical location. Thus, post-kala-azar dermal leishmaniasis heals spontaneously in the majority of cases in Africa, but rarely in patients in India. This form of leishmaniasis, endemic to India and Sudan, is considered to have an important role in maintaining and contributing to transmission of the disease particularly in interepidemic periods of visceral leishmaniasis, acting as a reservoir for parasites. Post-kala-azar dermal leishmaniasis reflects the immune response of the individual to the *Leishmania* organism, and lesions may be numerous and persist for decades. Most forms of the disease are transmissible from non-human animals to people

(zoonotic transmission), but some can be spread between humans (anthroponotic transmission).

The chemotherapy currently available for the treatment of leishmaniasis is far from satisfactory and shows a series of problems, including toxicity, adverse side-effects, high costs and development of drug resistance [2, 4]. Thus, search for new antilesihmanial drugs is urgently needed.

2. Miltefosine as a new antileishmanial drug

The control of leishmaniasis in the absence of vaccine depends solely on the choice of chemotherapy. Additional complications in the treatment of leishmaniasis include intrinsic species-specific differences in drug susceptibility [5, 6] as well as differences in drug efficacy between geographical areas [7], which can reflect the genetic differences between *Leishmania* parasites at species and strain levels [8, 9]. The generation of drug resistance is a major concern in the treatment of leishmaniasis that can be worsened by the rather low number of drugs currently available in therapy, thus leading to the lack of a putative alternative for a drug to which resistance has arisen. In this regard, it is well known the increasing resistance against the widely used pentavalent antimonial compounds in India, where resistance rates have been shown to be higher than 60% in parts of the state of Bihar, in north-east India [10, 11]. Thus, search for novel anti-*Leishmania* drugs is desperately needed. In the last years a new drug has been included in the clinical arsenal of antileishmanial drugs named miltefosine (hexadecylphoshocholine) (Figure 1), which is orally administered and is effective against pentavalent antimonial compound-resistant *Leishmania* parasites. Following a number of successful clinical trials ranging from phase I/II to phase IV in the period 1996-2004, miltefosine was registered as a new antileishmanial drug in India in 2002 [12]. Miltefosine, registered under the trade name of Impavido®, is the first oral drug in leishmaniasis therapy, having being developed by Zentaris (Frankfurt, Germany) in close cooperation with WHO/Special Programme for Research & Training in Tropical Diseases (TDR), and currently being manufactured by Paladin (Quebec, Canada). The standard miltefosine treatment includes oral administration of 100-150 mg/day, depending on the body weight, for 28 days and is well tolerated, except for mild gastrointestinal side effects.

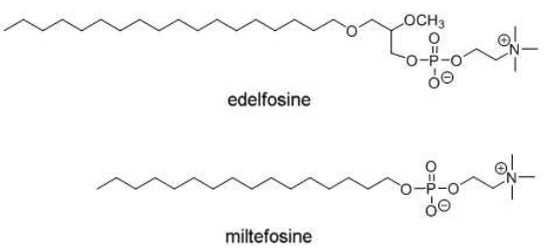

Figure 1. Chemical structures of edelfosine and miltefosine.

Miltefosine treatment leads to high cure rates in visceral leishmaniasis in India (*L. donovani*; 94% cure) [13]. However, different trials regarding the efficacy of miltefosine against cutaneous leishmaniasis in Colombia led to distinct outcomes ranging from a 90% cure [14] to an unsatisfactory cure rate of 69.8% [15]. Sensitivity to miltefosine is known to vary between *Leishmania* species [16]. In this regard, *L. braziliensis* seems to be somewhat refractory to miltefosine as shown in a number of clinical studies [14, 17-19]. Miltefosine efficacy against leishmaniasis lesions caused by *L. braziliensis*, which comprise more than 60% of cutaneous leishmaniasis in Colombia, fell to 49% [15], and was only 33% in Guatemala [14]. Additional clinical trials in Brazil showed a miltefosine cure rate of 75% and 71% for the treatment of cutaneous leishmaniasis caused by *L. braziliensis* [20] and *L. guyanensis* [21], respectively. Thus, these trials have challenged the therapeutical potential of miltefosine for the treatment of American cutaneous leishmaniasis. Miltefosine treatment has also led to approximately 70% cure rate for mucosal leishmaniasis due to *L. braziliensis* in Bolivia [18, 22], 53% for cutaneous leishmaniasis (33% for *L. braziliensis* infection, and 60% for *L. mexicana* infection) in Guatemala [14, 17, 23], and 63% for *L. tropica* infection in Afghanistan [23]. The above cure rates contrast with those reported for the treatment of visceral leishmaniasis (kala-azar) in India [12, 24] and Bangladesh [25] that were higher than 82%. These data highlight the great variability in the clinical outcome depending on the geographical area for reasons that are not well understood.

The main toxicity for miltefosine involves gastrointestinal organs in both animal and human studies. Thus, miltefosine frequently induces gastrointestinal side effects, such as anorexia, nausea, vomiting and diarrhea, that sometimes lead to drop out from treatment [2, 3, 24]. The testis and retina have been identified as target organs in rats, but the expected and corresponding effects and symptoms based on these observations have not been detected in clinical studies in humans [26]. Miltefosine distributes widely in body organs and is not metabolized by cytochrome P450 enzymes *in vitro*. Miltefosine has been found to be embryotoxic and fetotoxic in rats and rabbits, and teratogenic in rats, but not in rabbits [26]. Thus, miltefosine is potentially teratogenic, being contraindicated for use during pregnancy, and adequate contraception is required during treatment and for up to 3 months afterwards in women of child-bearing age [2, 3, 26]. An additional concern is the rapid *in vitro* generation of resistance to miltefosine [27-30] that could limit its clinical use.

Miltefosine is a member of a family of structurally-related compounds collectively known as synthetic alkylphospholipids (APLs), that target cell membranes and show pleiotropic actions with multiple biomedical applications in addition to their antitumor effect, which have been widely studied [31-35]. The advent of miltefosine as a new antileishmanial drug introduces APLs as putative novel drugs for the treatment of leishmaniasis. In addition, because of the numerous studies reported on the antitumor action of these compounds, it could be envisaged that the insight acquired for their antitumor action might be of use in the treatment of leishmaniasis.

3. Alkylphospholipids and leishmaniasis

As stated above miltefosine is a member of a series of synthetic lipids structurally related and known collectively as APLs, which in turn can be classified in two major categories [31]: a) the alkyl ether phospholipids (AEPs), widely referred to collectively as alkyl-lysophospholipid analogs (ALPs), containing ether bonds in the glycerol backbone of the phospholipid, as exemplified by 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3; edelfosine) (Figure 1); b) and the alkylphosphocholines (APCs), lacking the glycerol backbone and formed by a simple long-chain alcohol esterified to a phosphobase, as exemplified by hexadecylphosphocholine (HPC; miltefosine) (Figure 1). All of these APLs show low rates of metabolism both *in vitro* and *in vivo*. APLs were initially shown to elicit actions on the immune system and to kill cancer cells [31, 33, 35]. The first studies of the antiprotozoal activity of APLs in the 1980s against *Tetrahymena pyriformis* [36] and *L. donovani* [37, 38] were reported soon after their development as anticancer drugs [39-41]. APLs have been reported to be effective against different *Leishmania* species, including *L. donovani, L. infantum* (Old World)/*chagasi* (New World), *L. major, L. braziliensis, L. amazonensis, L. mexicana, L. panamensis, L. guyanensis, L. tropica, L. aethiopica* and *L. lainsoni* [16, 42-44].

In 1987, Croft *et al*. [38] reported the antiparasitic action of miltefosine against *L. donovani* amastigotes. Subsequently, *in vivo* studies showed the antileishmanial activity of miltefosine (10-20 mg/kg of body weight/day, orally administered) in mice infected with *L. donovani* and *L. infantum* [45]. Later on, following eight successful clinical trials [12], miltefosine (Impavido®) became the first oral treatment of visceral leishmaniasis and is the most recent drug to come to the market for the treatment of this disease.

Within APLs, edelfosine has been considered as the long-standing prototype of these compounds, and recent evidences have unveiled part of its mechanism of action as a promising antitumor drug. Another APL named perifosine, a miltefosine analog, is currently in clinical trials for different types of cancer. Thus, the insights gained in the last years on both the mechanism of action of APLs as antitumor agents, especially edelfosine, and the clinical experience acquired with some of these compounds, particularly miltefosine and perifosine, constitute an invaluable source of information for the putative use of APLs as antiparasitic drugs, as well as for the development of novel APL-related compounds as antileishmanial drugs. Very recently, edelfosine has been shown to be effective against different types of *Leishmania* parasites in *in vitro* and *in vivo* studies, displaying a better profile than miltefosine on the generation of drug resistance [30]. In addition, edelfosine exerts a potent anti-inflammatory effect [46], which is of importance as several of the clinical signs of leishmaniasis are due to an inflammatory response. In this regard, it is also worth mentioning that current evidence suggests that *Leishmania* parasites are initially engulfed by neutrophils, a major inflammatory cell type, and delivered to cytoplasmic neutrophil granules [47], where they survive and retain infectivity, thus using neutrophils as transport vehicles before they enter safely and silently their ultimate host cell, the macrophage [48, 49]. Increasing evidence suggests that APLs are new promising oral leishmanicidal drugs that could act either as single drugs or as combination therapy.

The antileishmanial efficacy of miltefosine in T cell-deficient athymic mice infected with *L. donovani* (25 mg/kg of body weight dissolved in a volume of 0.3 ml tap water, orally administered) was similar to that found in normal mice [50, 51]. These observations were extended in T and B-cell deficient severe combined immunodeficiency (SCID) mice, where miltefosine showed a similar effect to that detected in normal BALB/c mice [52]. These results raise the possibility that miltefosine may be of interest as an initial oral treatment approach to the growing problem of AIDS-associated visceral leishmaniasis in CD4 cell-depleted patients [53].

Currently, miltefosine is the only APL in the market for the treatment of leishmaniasis, but edelfosine and perifosine show promising and potent anti-*Leishmania* activity both *in vitro* and *in vivo* [30, 54, 55], warranting further studies for their putative clinical use in the future. The mechanism of action of miltefosine is not properly understood, but a clear correlation between the accumulation of the drug within the parasite and its toxic effects has already been described [27], this notion being identical to that previously advanced for edelfosine in its antitumor activity [32, 56-58], and therefore the ability of APLs to kill different *Leishmania* species and cancer cells is critically dependent on the drug uptake.

4. Mechanism of action of miltefosine as a leishmanicidal drug

The studies directed to unravel the underlying mechanism of miltefosine as an antiparasitic drug have followed to great extent the same trends previously reported and advanced in the analysis of the molecular and cellular pathways involved in the antitumor action of APLs. Thus, the insights generated from the antitumor action of APLs on cancer cells are being extrapolated to the APL killing action on *Leishmania* parasites. In this regard, a stark example lies in the direct induction of cell death by APLs in both tumor cells and *Leishmania* parasites. Following the studies reported simultaneously in 1993 by Diomede's group in Milan [59] and Mollinedo's group in Madrid [60], showing that the APL edelfosine induced apoptosis in a wide variety of tumor cells, subsequent studies extended this proapoptotic activity against cancer cells to other APLs, including miltefosine and perifosine [61, 62]. Later on in the new millennium, a number of studies have started to unveil the underlying mechanisms, signaling pathways and subcellular structures involved in the antitumor activity of APLs, including reorganization of lipid raft membrane domains, death receptors and mitochondria [32, 34, 63, 64]. These studies have provided a conceptual framework for a better understanding of the processes involved in the anti-*Leishmania* activity of APLs. Thus, two reports came out in 2004 showing that miltefosine induced a cell death process showing several features of metazoan apoptosis in *L. donovani* promastigotes and amastigotes [65, 66], including cell shrinkage, DNA fragmentation into oligonucleosome-sized fragments, and phosphatidylserine exposure. Then, subsequent studies have reported the miltefosine-mediated induction of an apoptosis-like cell death in promastigotes from different species of *Leishmania* promastigotes, including *L. amazonensis* [67], *L. infantum* [68], *L. tropica* [69], *L. major* [69], *L. panamensis* and many others [30]. The fact that an apoptosis-related process seems to be involved in the death of *Leishmania* parasites upon APL addition is further supported by the recent finding that tolerance to undergo apoptosis-like cell death in *Leishmania* is linked to multidrug resistance within the

parasite *in vitro* [70]. This raises the concern that cross-tolerance to drug-induced apoptosis-like cell death, not only against a particular selective drug promoting resistance, but also against additional drugs sharing a similar mode of killing, might lead to a facilitated emergence of cross-resistance against other drugs that have different cellular targets but analogous ways of killing [70]. However the mechanism by which APLs induce an apoptosis-like cell death in *Leishmania* parasites remains to be fully elucidated.

One critical organelle that seems to be involved in the killing process in *Leishmania* is the mitochondrion. Miltefosine treatment has been found to lead to loss of mitochondrial membrane potential and the release of cytochrome *c* with consequent activation of cellular proteases in *L. donovani* promastigotes, even in arsenite-resistant L. *donovani* promastigotes displaying a multidrug resistance phenotype and overexpressing Pgp-like protein [71]. The finding that edelfosine-induced cell death in *L. infantum* promastigotes can be regulated by the ectopic expression of the antiapoptotic and proapoptotic members of the Bcl-2 family of proteins Bcl-X_L and Hrk, which affect mitochondria-related processes, suggests that this process shows certain similarities to apoptosis in eukaryotic cells and that mitochondria are involved in the killing process [72]. Furthermore, miltefosine inhibits mitochondrial cytochrome *c* oxidase in *L. donovani* promastigotes, and this enzyme was suggested to act as a target for this APL [73]. In this regard, cytochrome *c* oxidase has also been identified as a potential target of miltefosine from a genomic library screen of the model yeast *Saccharomyces cerevisiae* [74]. Miltefosine inhibited cytochrome *c* oxidase activity in a dose-dependent manner, and this inhibition most likely contributed to the miltefosine-induced apoptosis-like cell death in *S. cerevisiae* [74]. Figure 2 depicts a schematic view for the antileishmanial activity of miltefosine based on current data.

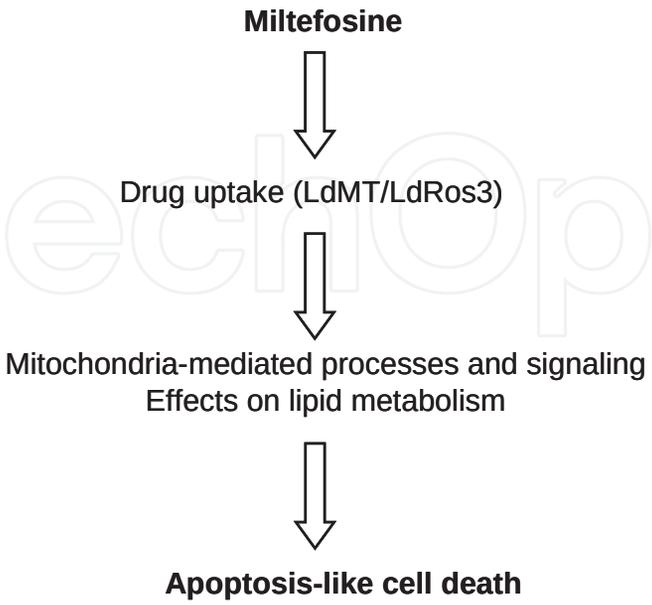

Figure 2. Schematic view of the antileishmanial mechanism of action of miltefosine. See text for further details.

5. Miltefosine effects on lipid metabolism

APLs, including miltefosine, have been found to interact with membrane lipids and affect lipid metabolism [75-77], these actions having been suggested being involved in their mechanism of action. In lipid monolayer studies, miltefosine molecules were inserted into the monolayer of lipids as monomers until the critical micellar concentration, and a high condensation was found between miltefosine and sterols showing a high affinity between miltefosine and sterols [78]. However, miltefosine did not act as detergent disturbing membrane integrity [78]. *Leishmania* parasites have high levels of ether-phospholipids [79-83], and these are mainly found in the glycosylphosphatidylinositol-anchored glycolipids and glycoproteins present on the surface of the parasites [84-86]. Because edelfosine and other APLs are ether lipids, it could be suggested that the biosynthesis of ether lipids occurring in the glycosomes of *Leishmania* might be affected. Miltefosine and edelfosine did not affect enzymes involved in early steps in ether lipid biosynthesis in *L. mexicana* promastigotes, including dihydroxyacetonephosphate acyltransferase, *sn*-l-acyl-2-*lyso*-glycero-3-phosphocholine and *sn*-l-alkyl-2-*lyso*-glycero-3-phosphocholine acyltransferases activities [87]. However, both miltefosine and edelfosine affected the later metabolism of alkyl-phosphatidylcholine intermediates by inhibiting the glycosomal located alkyl-specific-acyl-CoA acyltransferase in a dose-dependent manner with an inhibitory concentration of 50 μM, thus suggesting these drugs can perturb ether-lipid remodelling [87]. However, the fact that inhibition of alkyl-specific-acyl-CoA acyltransferase required drug concentrations higher than those showing cytotoxicity to *L. mexicana* (IC_{50}, 14 μM and 18 μM for miltefosine and edelfosine, respectively) [87] challenges the putative involvement of this pathway as the primary target of these drugs. In addition, the role of glycosylphosphatidylinositols and ether phospholipids in the survival of *Leishmania* amastigotes is questioned by the viability of *L. major* null mutants for alkyldihydroxyacetonephosphate synthase (ADS), the first committed step of ether lipid synthesis. These mutants lacked all ether phospholipids, including plasmalogens, lipophosphoglycan (LPG), and smaller glycosylphosphatidylinositols (GIPLs) [88].

Treatment of *L. donovani* promastigotes with 10 μM miltefosine significantly reduced the phosphatidylcholine content and enhanced the phosphatidylethanolamine content in parasite membranes, suggesting a partial inactivation of phosphatidylethanolamine-N-methyltransferase [89]. Phospholipase D activity was not affected by miltefosine, whereas the enhancement of the lysophosphatidylcholine content could be ascribed to phospholipase A2 activation. No effect was observed in the fatty acid alkyl chain length or the fatty acid unsaturation rate upon miltefosine treatment, whereas a two-fold increase was detected in the amount of cholesterol within the membranes [89]. Because cholesterol is not biosynthesized by the *Leishmania* parasite, but is taken from the external medium, it might be envisaged that miltefosine promotes cholesterol uptake in promastigotes perhaps by the condensation effect between miltefosine and cholesterol [78]. In contrast, a strong reduction of about two times in the C24 alkylated sterol content was detected in miltefosine-treated membranes, even though the level of the final C24 sterol alkylating product, ergosterol, the predominant plasma membrane sterol in fungi and *Leishmania*, was not changed [89].

Interestingly, the content of unsaturated phospholipid alkyl chains was lower in miltefosine-resistant parasite plasma membranes than in those of the wild type, suggesting a lower fluidity of miltefosine-resistant parasite membranes, and rendering the miltefosine interaction with the external monolayer of miltefosine-resistant parasites more difficult. Miltefosine-resistant parasite membranes displayed a higher content of short alkyl chain fatty acids, suggesting a partial inactivation of the fatty acid elongation enzyme system in miltefosine-resistant parasites, and the C24-alkylated sterol content was halved in miltefosine-resistant parasites, but this modification was not related to miltefosine sensitivity [90]. Thus, miltefosine resistance affects three lipid biochemical pathways: fatty acid elongation, the desaturase system responsible for fatty acid alkyl chain unsaturation, and the C-24-alkylation of sterols. [90]. Because of the differences detected in the lipid composition of miltefosine-treated *Leishmania* and miltefosine-resistant parasites, it could be hypothesized that continuous *in vitro* drug pressure affects the regulation of *Leishmania* lipid metabolism [89], but the real implication of these actions on parasite killing remains a topic of much debate.

6. Drug resistance

A major hurdle in successful leishmanial chemotherapy is emergence of drug resistance. Miltefosine, the first orally administrable anti-leishmanial drug, has shown the potential against drug-resistant strains of *Leishmania*. However, at the same time the readiness in generating miltefosine-resistant parasites *in vitro* in the laboratory [27-29] raises some concerns about the life span for an efficient use of miltefosine. The major underlying mechanisms involved in the generation of miltefosine-resistant parasites seem to reside in defective drug uptake into the parasite and increased drug efflux. In addition, its long half-life (approximately 150 hours) [91] might facilitate the emergence of resistance.

L. donovani promastigotes resistant to up to 40 μM miltefosine have been generated *in vitro* in the laboratory by continuous step-wise drug pressure, resulting in being 15-fold more resistant to miltefosine than wild-type promastigotes [28]. A drastic reduction (>95%) in the ability of resistant *L. donovani* promastigotes to internalize [^{14}C]miltefosine was detected, whereas binding of miltefosine to the plasma membrane and drug efflux from preloaded cells were similar in both drug-sensitive and -resistant cells, and no metabolism of [^{14}C]miltefosine was evident in either sensitive and resistant cells [92]. This miltefosine uptake was temperature and energy dependent and sensitive to the thiol-reactive agent N-ethylmaleimide [92]. Resistant parasites were also unable to take up other short-chain phospholipid analogs, independently of their polar head group, even though endocytosis remained unaltered [92]. The underlying basis for the generation of drug-resistant *L. donovani* promastigotes turned out to be a decrease in the uptake of miltefosine mediated by the plasma membrane P-type transporter *L. donovani* miltefosine transporter (LdMT) from the aminophospholipid translocase subfamily and by LdRos3 [93, 94]. LdMT is an inward-directed lipid translocase belonging to the P4 subfamily of P-type ATPases, which comprises lipid flippases that catalyze the translocation of phospholipids from the exoplasmic to the cytosolic leaflet of cell membranes; and LdRos3 is a non-catalytic subunit of LdMT that belongs to the CDC50/Lem3 family, which

includes proteins proposed as likely beta subunits for P4-ATPases [93, 94]. LdMT and LdRos3 proteins are primarily localized to the *Leishmania* plasma membrane and required for the rapid intracellular uptake of miltefosine and additional related choline-bearing lipids. Likewise, in the budding yeast *S. cerevisiae*, members of the two protein families have been found to form stable transporter complexes that function in the translocation of phospholipids from the exoplasmic to the cytoplasmic leaflet of cellular membranes [95, 96]. Despite both LdMT and LdRos3 normally localize to the plasma membrane, they are retained at the endoplasmic reticulum in the absence of the other protein or in the presence of inactivating point mutations in LdMT [94]. Both LdMT and the Cdc50-like protein LdRos3 form a stable complex that plays an essential role in maintaining phospholipid asymmetry in the parasite plasma membrane [97], and constitute part of the same translocation machinery that determines flippase activity, responsible for miltefosine uptake, as well as miltefosine sensitivity in *Leishmania* [94]. Loss of either LdMT or LdRos3 blocks ATP-dependent transport of NBD-labeled phosphatidylethanolamine and phosphatidylcholine from the outer to the inner plasma membrane leaflet in *L. donovani* promastigotes and results in an increased cell surface exposure of endogenous phosphatidylethanolamine, whereas infectivity was not compromised [97]. A promastigote line, M-mutR, that shows mutations in LdMT, thus leading to defective miltefosine internalization, is infective to macrophages *in vitro* and in BALB/c mice *in vivo*, and displays a good correlation of *in vitro* resistance between promastigotes and intracellular amastigotes [29]. The fact that M-mutR parasites retain the resistant phenotype *in vivo* indicate that miltefosine-resistant *L. donovani* promastigotes transform to miltefosine-resistant amastigotes [29]. It was also observed no cross-resistance to other antileishmanial drugs in M-mutR amastigotes [29]. Some clinical studies have suggested, as indicated above, that miltefosine shows significantly less efficiency against the cutaneous and mucocutaneous leishmaniasis caused by *L. braziliensis* parasites, mainly due to their inability to internalize the drug because of the low expression levels of the beta subunit LbRos3 [98]. Overexpression of LbRos3 induced increased miltefosine sensitivity in both *L. braziliensis* promastigotes and intracellular amastigotes, further supporting the notion that miltefosine uptake is a major event in determining miltefosine antileishmanial potency [98]. Miltefosine-resistant promastigotes, displaying cross-resistance to the ether lipid edelfosine, but not to the standard anti-leishmanial drugs, shows no amplification of specific genes, including the multidrug resistance P-glycoprotein gene, and resistance has been found to be stable up to 12 weeks in drug-free culture medium [28].

The evidence gathered so far has shown that reduced miltefosine incorporation has always led to a resistant phenotype. This lower accumulation of miltefosine can be achieved by two processes: a) a decrease in drug uptake, rendered by inactivation of any one of the two proteins responsible for the miltefosine uptake, namely LdMT and its beta subunit LdRos3; b) an increase in drug efflux, mediated by the overexpression of the ABC transporter P-glycoprotein [27]. Thus, in addition to a flaw in the uptake of miltefosine as stated above, an increased efflux of miltefosine has also been implicated in miltefosine resistance. A multidrug resistance (MDR) *L. tropica* line overexpressing a P-glycoprotein-like transporter was found to display significant cross-resistance to the ALP miltefosine and edelfosine, with resistant indices of 9.2- and 7.1-fold, respectively [99]. This resistance was mediated through overexpression of an ABC transporter, namely the *Leishmania* P-glycoprotein-like transporter (*Leishmania* ABCB1 or

LtrMDR1) [99, 100]. ATP-binding cassette (ABC) transporters constitute one of the largest and most conserved protein families and have been considered major players in drug resistance during the treatment of cancer and infectious diseases. Interestingly, sesquiterpene C-3 completely sensitizes MDR parasites to APLs, acting as an inhibitor of LtrMDR1 [99]. In addition, overexpression of two *Leishmania*-specific ABC subfamily G (ABCG)-like transporters localized at the plasma membrane of *Leishmania* protozoan parasites, LiABCG6 and LiABCG4 half-transporters, conferred resistance to APLs in *Leishmania* parasites and *S. cerevisiae* [101, 102]. Overexpression of LiABCG6 not only leads to miltefosine resistance *in vitro*, but also to the antileishmanial oral drug 8-aminoquinoline analog sitamaquine [102].

Additional genes associated with miltefosine resistance have been identified by generating *L. major* promastigote mutants highly resistant to miltefosine (80-100 μM) in a step-by-step manner and subsequent analysis of the short-read whole genome sequencing [103]. In addition to the previously described P-type ATPase involved in phospholipid translocation, another new gene coding for pyridoxal kinase, involved in the formation of pyridoxal-5′-phophate (active vitamin B6), has been implicated in miltefosine susceptibility [103]. Following this genetic approach, it was clear the polyclonal nature of a resistant population with varying susceptibilities and genotypes, indicating that miltefosine resistance can be genetically and phenotypically highly heterogeneous [103].

7. Effects of APLs on the immune system

Even though miltefosine retains its antiparasitic activity against *Leishmania* infection in immunodeficient SCID mice, leading to similar levels of activity in both SCID and BALB/c mouse-*L. donovani* models [52], the immunomodulatory properties of miltefosine have been proposed as an additional factor to its antileishmanial action [104]. Thus, miltefosine's antileishmanial function has been reported to be significantly compromised in interferon-gamma (IFNγ)-deficient macrophages, suggesting the importance of endogenous IFNγ in the miltefosine-induced antileishmanial functions of macrophages. IFNγ responsiveness is reduced in *L. donovani*-infected macrophages but is significantly restored by miltefosine, as it induces IFNγ, enhances IFNγ receptors, and IFNγ induces STAT-1 phosphorylation but reduces activation of SHP-1, the phosphatase implicated in the downregulation of STAT-1 phosphorylation [104]. *L. donovani*-infected macrophages induced Th2 response, but miltefosine treatment reversed the response to Th1-type [104].

Miltefosine is able to form stable multilamellar vesicles (MLVs liposomes) to deliver the APL to monocytes/macrophages. Both micellar and liposomal miltefosine have been found to interact with human monocytes and upregulate specific adhesion molecules, including intracellular adhesion molecule-1 and class 1 major histocompatibility complex (MHC-1) antigen in a dose-dependent manner in U937 cells [105], used as a cell line model system to study human monocytes. These actions could be involved in the initial steps of miltefosine-mediated recruitment of macrophages [105]. Miltefosine, better in liposomal than in free (micellar) form, has also been reported to induce an increase in tumor necrosis factor (TNF)

release and nitric oxide (NO) generation after *in vitro* co-culture of mouse peritoneal macrophages or U937 cells with lipopolysaccharide (LPS) [106-108]. Miltefosine has also been reported to enhance the immune response of IL-2-stimulated mononuclear cells resulting in granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFNγ gene expression and IFNγ secretion [109].

However, recent studies on the effects of miltefosine on dendritic cells (DC) in *L. major* infection have challenged the putative role of the immunomodulatory action of miltefosine on its antiparasitic action, and suggest that miltefosine functions independently of the immune system, mostly through direct toxicity against the *Leishmania* parasite [110]. DC are critical for initiation of protective immunity against *Leishmania* through induction of Th1 immunity via interleukin 12 (IL-12), and when co-cultured with miltefosine for 4 days, most of the *in vitro*-infected DC were free of parasites. However, miltefosine treatment did not influence DC maturation (upregulation of major histocompatibility complex II [MHC II] or co-stimulatory molecules, e.g., CD40, CD54, and CD86), did not significantly alter cytokine release (IL-12, tumor necrosis factor alpha [TNF-α], or IL-10), antigen presentation, or NO production [110].

8. Canine leishmaniasis

Miltefosine is marketed as Milteforan® (Virbac, Carros, France) for the treatment of canine visceral leishmaniasis that is the result of infection with *L. infantum* in the Old World and *L. chagasi* in the New World. These two *Leishmania* species are considered sibling and indistinguishable species, and several genetic studies have shown evidence for the synonym of *L. infantum* and *L. chagasi*, and suggest the introduction of *L. infantum* from Southwest Europe into the New World in recent history [111-114]. Thus, *L. infantum* (Old World) and *L. chagasi* (New World) belong to the same species, and therefore *L. chagasi* has been synonymized with *L. infantum*. Dogs are considered the primary reservoir hosts *of L. infantum/chagasi*, and infection of dogs with *L. infantum/chagasi* involves cells of the lymphatic series resulting in visceralization of infection. The domestic dog seems to be a main reservoir for human visceral leishmaniasis, rendering canine disease control a critical issue. Unfortunately efforts to control leishmaniasis in dogs have been largely unsuccessful so far. Oral administration of miltefosine at a dose of 2 mg/kg body weight once a day for 28 days leads to significant reduction of parasite loads and clinical symptoms, whereas adverse reactions were not serious and observed in less that 12% of the dogs, the most frequent one being vomiting, which was transient, self-limiting, and reversible [115-117]. *Leishmania* DNA quantification by real-time PCR has shown that miltefosine treatment of dogs leads to a drastic and progressive reduction of parasite load in lymph node aspirates, but does not suppress the parasite in lymph nodes [118]. Miró et al. [119] has shown that the treatment of miltefosine-allopurinol combination therapy (2 mg/kg miltefosine orally once daily for 28 days and 10 mg/kg of allopurinol orally twice daily for 7 months) behaved similarly to the current reference combination therapy, namely meglumine antimoniate-allopurinol (50 mg/kg of meglumine antimoniate sub-cutaneously twice daily for 28 days and 10 mg/kg of allopurinol orally twice daily for 7 months), in promoting a significant reduction in total clinical score and parasite load over the 7-month study period. These

observations together with the lack of effect on renal and hepatic parameters and adverse reactions suggests that miltefosine, in combination with allopurinol, might offer a safe and effective alternative treatment option for canine leishmaniasis compared to the reference therapy [119]. A recent study [117], where dogs naturally infected with *L. infantum/chagasi* were treated with miltefosine using different therapeutic regimens, has shown that after treatment and during the following 24 months, there was progressive clinical improvement and complete recovery in 50% (7/14) of the treated animals. There was a decrease in the smear positivity of the bone marrow after treatment, and there was also a gradual and constant decrease in positive cultures at the end of the follow-up period. However, the PCR detection of parasite DNA remained positive, and the animals presented a significant increase in parasite load 6 months after treatment. Thus, the fact that the improvement in the clinical symptoms is not followed by total parasitological clearance, raises some doubts about the use of this drug in endemic areas where the dogs are involved in the maintenance of the parasite cycle.

9. Conclusions

At present the control of protozoan parasite *Leishmania* infections relies primarily on chemotherapy, but the armoury of drugs available for treating *Leishmania* infections is rather limited and includes a few drugs with unknown cellular targets and unclear mode of action. These drugs include pentavalent antimonials, pentamidine, amphotericin B, miltefosine, paromomycin, fluconazole, allopurinol, and few other drugs at various stages of their development process. The recent inclusion of miltefosine as a new antileishmanial drug has been a breakthrough in the treatment of leishmaniasis, as it constituted the first effective oral drug, thus facilitating medical access and making treatment more accessible to rural and remote areas. Interestingly, miltefosine belongs to a family of lipid compounds collectively known as APLs, some of them showing also interesting and promising antileishmanial activities in addition to their well known antitumor action. Thus, additional APLs or APL-related compounds might be of interest to identify novel drugs to combat *Leishmania* infections both in humans and animals, especially in dogs as major reservoirs for human visceral leishmaniasis (*L. infantum/chagasi*). Identification of the death signaling pathways activated in miltefosine-sensitive parasites will be essential for a better understanding of the molecular mechanisms of action and resistance in these parasites. In this regard, the knowledge acquired for the antitumor action of APLs is being and will be of further aid to unveil the mode of action of miltefosine and putative additional APLs with potent antileishmanial activities. Furthemore, elucidation of the molecular mechanisms underlying miltefosine- and APL-mediated cell death will facilitate the design of new therapeutic strategies against *Leishmania* parasites. The proneness to generate *in vitro* resistance to miltefosine raises some concerns about its putative life span in clinical use, thus favoring research on additional APLs that could improve the current antileishmanial features of miltefosine as well as on combination therapy regimens. APLs have shown themselves as potent antileishmanial drugs, and current evidence warrants further research on these promising lipid drugs that could make a difference in the clinical setting and medical care of leishmaniasis.

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References

[1] Bates PA. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol 2007;37:1097-106.

[2] Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. Lancet 2005;366:1561-77.

[3] WHO. Control of the leishmaniases. World Health Organization technical report series 2010;949:1-186.

[4] van Griensven J, Balasegaram M, Meheus F, Alvar J, Lynen L, Boelaert M. Combination therapy for visceral leishmaniasis. Lancet Infect Dis 2010;10:184-94.

[5] Croft SL. Monitoring drug resistance in leishmaniasis. Trop Med Int Health 2001;6:899-905.

[6] Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. Clin Microbiol Rev 2006;19:111-26.

[7] Hailu A, Musa A, Wasunna M, Balasegaram M, Yifru S, Mengistu G, et al. Geographical variation in the response of visceral leishmaniasis to paromomycin in East Africa: a multicentre, open-label, randomized trial. PLoS Negl Trop Dis 2010;4:e709.

[8] Schonian G, Kuhls K, Mauricio IL. Molecular approaches for a better understanding of the epidemiology and population genetics of *Leishmania*. Parasitology 2011;138:405-25.

[9] Subba Raju BV, Gurumurthy S, Kuhls K, Bhandari V, Schnonian G, Salotra P. Genetic typing reveals monomorphism between antimony sensitive and resistant *Leishmania donovani* isolates from visceral leishmaniasis or post kala-azar dermal leishmaniasis cases in India. Parasitol Res 2012;111:1559-68.

[10] Sundar S. Drug resistance in Indian visceral leishmaniasis. Trop Med Int Health 2001;6:849-54.

[11] Sundar S, Chatterjee M. Visceral leishmaniasis - current therapeutic modalities. Indian J Med Res 2006;123:345-52.

[12] Sundar S, Jha TK, Thakur CP, Bhattacharya SK, Rai M. Oral miltefosine for the treatment of Indian visceral leishmaniasis. Trans R Soc Trop Med Hyg 2006;100 Suppl 1:S26-33.

[13] Sundar S, Jha TK, Thakur CP, Engel J, Sindermann H, Fischer C, et al. Oral miltefosine for Indian visceral leishmaniasis. N Engl J Med 2002;347:1739-46.

[14] Soto J, Arana BA, Toledo J, Rizzo N, Vega JC, Diaz A, et al. Miltefosine for new world cutaneous leishmaniasis. Clin Infect Dis 2004;38:1266-72.

[15] Velez I, Lopez L, Sanchez X, Mestra L, Rojas C, Rodriguez E. Efficacy of miltefosine for the treatment of American cutaneous leishmaniasis. Am J Trop Med Hyg 2010;83:351-6.

[16] Escobar P, Matu S, Marques C, Croft SL. Sensitivities of *Leishmania* species to hexadecylphosphocholine (miltefosine), ET-18-OCH_3 (edelfosine) and amphotericin B. Acta Trop 2002;81:151-7.

[17] Soto J, Berman J. Treatment of New World cutaneous leishmaniasis with miltefosine. Trans R Soc Trop Med Hyg 2006;100 Suppl 1:S34-40.

[18] Soto J, Toledo J, Valda L, Balderrama M, Rea I, Parra R, et al. Treatment of Bolivian mucosal leishmaniasis with miltefosine. Clin Infect Dis 2007;44:350-6.

[19] Minodier P, Parola P. Cutaneous leishmaniasis treatment. Travel medicine and infectious disease 2007;5:150-8.

[20] Machado PR, Ampuero J, Guimaraes LH, Villasboas L, Rocha AT, Schriefer A, et al. Miltefosine in the treatment of cutaneous leishmaniasis caused by *Leishmania braziliensis* in Brazil: a randomized and controlled trial. PLoS Negl Trop Dis 2010;4:e912.

[21] Chrusciak-Talhari A, Dietze R, Chrusciak Talhari C, da Silva RM, Gadelha Yamashita EP, de Oliveira Penna G, et al. Randomized controlled clinical trial to access efficacy and safety of miltefosine in the treatment of cutaneous leishmaniasis caused by *Leishmania* (*Viannia*) *guyanensis* in Manaus, Brazil. Am J Trop Med Hyg 2011;84:255-60.

[22] Soto J, Rea J, Valderrama M, Toledo J, Valda L, Ardiles J, et al. Efficacy of extended (six weeks) treatment with miltefosine for mucosal leishmaniasis in Bolivia. Am J Trop Med Hyg 2009;81:387-9.

[23] Soto J, Soto P. Oral miltefosine to treat leishmaniasis. Biomedica 2006;26 Suppl 1:207-17.

[24] Bhattacharya SK, Sinha PK, Sundar S, Thakur CP, Jha TK, Pandey K, et al. Phase 4 trial of miltefosine for the treatment of Indian visceral leishmaniasis. J Infect Dis 2007;196:591-8.

[25] Rahman M, Ahmed BN, Faiz MA, Chowdhury MZ, Islam QT, Sayeedur R, et al. Phase IV trial of miltefosine in adults and children for treatment of visceral leishmaniasis (kala-azar) in Bangladesh. Am J Trop Med Hyg 2011;85:66-9.

[26] Sindermann H, Engel J. Development of miltefosine as an oral treatment for leishmaniasis. Trans R Soc Trop Med Hyg 2006;100 Suppl 1:S17-20.

[27] Perez-Victoria FJ, Sanchez-Canete MP, Seifert K, Croft SL, Sundar S, Castanys S, et al. Mechanisms of experimental resistance of *Leishmania* to miltefosine: Implications for clinical use. Drug Resist Updat 2006;9:26-39.

[28] Seifert K, Matu S, Javier Perez-Victoria F, Castanys S, Gamarro F, Croft SL. Characterisation of *Leishmania donovani* promastigotes resistant to hexadecylphosphocholine (miltefosine). International journal of antimicrobial agents 2003;22:380-7.

[29] Seifert K, Perez-Victoria FJ, Stettler M, Sanchez-Canete MP, Castanys S, Gamarro F, et al. Inactivation of the miltefosine transporter, LdMT, causes miltefosine resistance that is conferred to the amastigote stage of *Leishmania donovani* and persists *in vivo*. International journal of antimicrobial agents 2007;30:229-35.

[30] Varela-M RE, Villa-Pulgarin JA, Yepes E, Muller I, Modolell M, Munoz DL, et al. *In vitro* and *in vivo* efficacy of ether lipid edelfosine against *Leishmania* spp. and SbV-resistant parasites. PLoS Negl Trop Dis 2012;6:e1612.

[31] Gajate C, Mollinedo F. Biological Activities, Mechanisms of Action and biomedical prospect of the antitumor ether phospholipid ET-18-OCH_3 (edelfosine), a proapoptotic agent in tumor cells. Curr Drug Metab 2002;3:491-525.

[32] Gajate C, Del Canto-Janez E, Acuna AU, Amat-Guerri F, Geijo E, Santos-Beneit AM, et al. Intracellular triggering of Fas aggregation and recruitment of apoptotic molecules into Fas-enriched rafts in selective tumor cell apoptosis. J Exp Med 2004;200:353-65.

[33] Mollinedo F, Gajate C, Martin-Santamaria S, Gago F. ET-18-OCH_3 (edelfosine): a selective antitumour lipid targeting apoptosis through intracellular activation of Fas/CD95 death receptor. Curr Med Chem 2004;11:3163-84.

[34] Gajate C, Mollinedo F. Edelfosine and perifosine induce selective apoptosis in multiple myeloma by recruitment of death receptors and downstream signaling molecules into lipid rafts. Blood 2007;109:711-9.

[35] Mollinedo F. Antitumor ether lipids: proapoptotic agents with multiple therapeutic indications. Expert Opin Ther Patents 2007;17:385-405.

[36] Tsushima S, Yoshioka Y, Tanida S, Nomura H, Nojima S, Hozumi M. Syntheses and antimicrobial activities of alkyl lysophospholipids. Chem Pharm Bull (Tokyo) 1982;30:3260-70.

[37] Achterberg V, Gercken G. Cytotoxicity of ester and ether lysophospholipids on *Leishmania donovani* promastigotes. Mol Biochem Parasitol 1987;23:117-22.

[38] Croft SL, Neal RA, Pendergast W, Chan JH. The activity of alkyl phosphorylcholines and related derivatives against *Leishmania donovani*. Biochem Pharmacol 1987;36:2633-6.

[39] Berdel WE. Membrane-interactive lipids as experimental anticancer drugs. Br J Cancer 1991;64:208-11.

[40] Berdel WE, Bausert WR, Fink U, Rastetter J, Munder PG. Anti-tumor action of alkyl-lysophospholipids. Anticancer Res 1981;1:345-52.

[41] Berdel WE, Fink U, Rastetter J. Clinical phase I pilot study of the alkyl lysophospholipid derivative ET-18-OCH_3. Lipids 1987;22:967-9.

[42] Croft SL, Neal RA, Thornton EA, Herrmann DB. Antileishmanial activity of the ether phospholipid ilmofosine. Trans R Soc Trop Med Hyg 1993;87:217-9.

[43] Croft SL, Seifert K, Duchene M. Antiprotozoal activities of phospholipid analogues. Mol Biochem Parasitol 2003;126:165-72.

[44] Yardley V, Croft SL, De Doncker S, Dujardin JC, Koirala S, Rijal S, et al. The sensitivity of clinical isolates of *Leishmania* from Peru and Nepal to miltefosine. Am J Trop Med Hyg 2005;73:272-5.

[45] Kuhlencord A, Maniera T, Eibl H, Unger C. Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. Antimicrob Agents Chemother 1992;36:1630-4.

[46] Mollinedo F, Gajate C, Morales AI, del Canto-Janez E, Justies N, Collia F, et al. Novel anti-inflammatory action of edelfosine lacking toxicity with protective effect in experimental colitis. J Pharmacol Exp Ther 2009;329:439-49.

[47] Mollinedo F, Janssen H, de la Iglesia-Vicente J, Villa-Pulgarin JA, Calafat J. Selective fusion of azurophilic granules with *Leishmania*-containing phagosomes in human neutrophils. J Biol Chem 2010;285:34528-36.

[48] Laskay T, van Zandbergen G, Solbach W. Neutrophil granulocytes--Trojan horses for Leishmania major and other intracellular microbes? Trends in microbiology 2003;11:210-4.

[49] van Zandbergen G, Klinger M, Mueller A, Dannenberg S, Gebert A, Solbach W, et al. Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. J Immunol 2004;173:6521-5.

[50] Murray HW. Suppression of posttreatment recurrence of experimental visceral leishmaniasis in T-cell-deficient mice by oral miltefosine. Antimicrob Agents Chemother 2000;44:3235-6.

[51] Murray HW, Delph-Etienne S. Visceral leishmanicidal activity of hexadecylphosphocholine (miltefosine) in mice deficient in T cells and activated macrophage microbicidal mechanisms. J Infect Dis 2000;181:795-9.

[52] Escobar P, Yardley V, Croft SL. Activities of hexadecylphosphocholine (miltefosine), AmBisome, and sodium stibogluconate (Pentostam) against *Leishmania donovani* in immunodeficient scid mice. Antimicrob Agents Chemother 2001;45:1872-5.

[53] Lawn SD, Wilkinson RJ. Immune reconstitution disease associated with parasitic infections following antiretroviral treatment. Parasite Immunol 2006;28:625-33.

[54] Cabrera-Serra MG, Lorenzo-Morales J, Romero M, Valladares B, Pinero JE. *In vitro* activity of perifosine: a novel alkylphospholipid against the promastigote stage of *Leishmania* species. Parasitol Res 2007;100:1155-7.

[55] Cabrera-Serra MG, Valladares B, Pinero JE. *In vivo* activity of perifosine against *Leishmania amazonensis*. Acta Trop 2008;108:20-5.

[56] Mollinedo F, Fernandez-Luna JL, Gajate C, Martin-Martin B, Benito A, Martinez-Dalmau R, et al. Selective induction of apoptosis in cancer cells by the ether lipid ET-18-OCH_3 (Edelfosine): molecular structure requirements, cellular uptake, and protection by Bcl-2 and Bcl-X_L. Cancer Res 1997;57:1320-8.

[57] Gajate C, Fonteriz RI, Cabaner C, Alvarez-Noves G, Alvarez-Rodriguez Y, Modolell M, et al. Intracellular triggering of Fas, independently of FasL, as a new mechanism of antitumor ether lipid-induced apoptosis. Int J Cancer 2000;85:674-82.

[58] Mollinedo F, Gajate C. Fas/CD95 death receptor and lipid rafts: New targets for apoptosis-directed cancer therapy. Drug Resist Updat 2006;9:51-73.

[59] Diomede L, Colotta F, Piovani B, Re F, Modest EJ, Salmona M. Induction of apoptosis in human leukemic cells by the ether lipid 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine. A possible basis for its selective action. Int J Cancer 1993;53:124-30.

[60] Mollinedo F, Martinez-Dalmau R, Modolell M. Early and selective induction of apoptosis in human leukemic cells by the alkyl-lysophospholipid ET-18-OCH_3. Biochem Biophys Res Commun 1993;192:603-9.

[61] Konstantinov SM, Topashka-Ancheva M, Benner A, Berger MR. Alkylphosphocholines: Effects on human leukemic cell lines and normal bone marrow cells. Int J Cancer 1998;77:778-86.

[62] Konstantinov SM, Eibl H, Berger MR. BCR-ABL influences the antileukaemic efficacy of alkylphosphocholines. Br J Haematol 1999;107:365-80.

[63] Gajate C, Gonzalez-Camacho F, Mollinedo F. Lipid raft connection between extrinsic and intrinsic apoptotic pathways. Biochem Biophys Res Commun 2009;380:780-4.

[64] Gajate C, Mollinedo F. The antitumor ether lipid ET-18-OCH_3 induces apoptosis through translocation and capping of Fas/CD95 into membrane rafts in human leukemic cells. Blood 2001;98:3860-3.

[65] Paris C, Loiseau PM, Bories C, Breard J. Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. Antimicrob Agents Chemother 2004;48:852-9.

[66] Verma NK, Dey CS. Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. Antimicrob Agents Chemother 2004;48:3010-5.

[67] Marinho Fde A, Goncalves KC, Oliveira SS, Oliveira AC, Bellio M, d'Avila-Levy CM, et al. Miltefosine induces programmed cell death in *Leishmania amazonensis* promastigotes. Memorias do Instituto Oswaldo Cruz 2011;106:507-9.

[68] Khademvatan S, Gharavi MJ, Saki J. Miltefosine induces metacaspase and PARP genes expression in *Leishmania infantum*. Braz J Infect Dis 2011;15:442-8.

[69] Khademvatan S, Gharavi MJ, Rahim F, Saki J. Miltefosine-induced apoptotic cell death on *Leishmania major* and *L. tropica* strains. The Korean journal of parasitology 2011;49:17-23.

[70] Moreira W, Leprohon P, Ouellette M. Tolerance to drug-induced cell death favours the acquisition of multidrug resistance in *Leishmania*. Cell death & disease 2011;2:e201.

[71] Verma NK, Singh G, Dey CS. Miltefosine induces apoptosis in arsenite-resistant *Leishmania donovani* promastigotes through mitochondrial dysfunction. Exp Parasitol 2007;116:1-13.

[72] Alzate JF, Arias A, Mollinedo F, Rico E, de la Iglesia-Vicente J, Jimenez-Ruiz A. Edelfosine induces an apoptotic process in *Leishmania infantum* that is regulated by the ectopic expression of Bcl-X_L and Hrk. Antimicrob Agents Chemother 2008;52:3779-82.

[73] Luque-Ortega JR, Rivas L. Miltefosine (hexadecylphosphocholine) inhibits cytochrome *c* oxidase in *Leishmania donovani* promastigotes. Antimicrob Agents Chemother 2007;51:1327-32.

[74] Zuo X, Djordjevic JT, Bijosono Oei J, Desmarini D, Schibeci SD, Jolliffe KA, et al. Miltefosine induces apoptosis-like cell death in yeast via Cox9p in cytochrome *c* oxidase. Mol Pharmacol 2011;80:476-85.

[75] Boggs K, Rock CO, Jackowski S. The antiproliferative effect of hexadecylphosphocholine toward HL60 cells is prevented by exogenous lysophosphatidylcholine. Biochim Biophys Acta 1998;1389:1-12.

[76] Boggs KP, Rock CO, Jackowski S. Lysophosphatidylcholine attenuates the cytotoxic effects of the antineoplastic phospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine. J Biol Chem 1995;270:11612-8.

[77] Boggs KP, Rock CO, Jackowski S. Lysophosphatidylcholine and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine inhibit the CDP-choline pathway of phosphatidylcholine synthesis at the CTP:phosphocholine cytidylyltransferase step. J Biol Chem 1995;270:7757-64.

[78] Rakotomanga M, Loiseau PM, Saint-Pierre-Chazalet M. Hexadecylphosphocholine interaction with lipid monolayers. Biochim Biophys Acta 2004;1661:212-8.

[79] Beach DH, Holz GG, Jr., Anekwe GE. Lipids of *Leishmania* promastigotes. J Parasitol 1979;65:201-16.

[80] Herrmann H, Gercken G. Synthesis of phospholipids in *Leishmania donovani*. Hoppe-Seyler's Zeitschrift fur physiologische Chemie 1980;361:1735-42.

[81] Wassef MK, Fioretti TB, Dwyer DM. Lipid analyses of isolated surface membranes of *Leishmania donovani* promastigotes. Lipids 1985;20:108-15.

[82] Heise N, Opperdoes FR. The dihydroxyacetonephosphate pathway for biosynthesis of ether lipids in *Leishmania mexicana* promastigotes. Mol Biochem Parasitol 1997;89:61-72.

[83] Zufferey R, Ben Mamoun C. *Leishmania major* expresses a single dihydroxyacetone phosphate acyltransferase localized in the glycosome, important for rapid growth and survival at high cell density and essential for virulence. J Biol Chem 2006;281:7952-9.

[84] McConville MJ, Collidge TA, Ferguson MA, Schneider P. The glycoinositol phospholipids of *Leishmania mexicana* promastigotes. Evidence for the presence of three distinct pathways of glycolipid biosynthesis. J Biol Chem 1993;268:15595-604.

[85] McConville MJ, Ferguson MA. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem J 1993;294 (Pt 2):305-24.

[86] Schneider P, Rosat JP, Ransijn A, Ferguson MA, McConville MJ. Characterization of glycoinositol phospholipids in the amastigote stage of the protozoan parasite *Leishmania major*. Biochem J 1993;295 (Pt 2):555-64.

[87] Lux H, Heise N, Klenner T, Hart D, Opperdoes FR. Ether-lipid (alkyl-phospholipid) metabolism and the mechanism of action of ether-lipid analogues in *Leishmania*. Mol Biochem Parasitol 2000;111:1-14.

[88] Zufferey R, Allen S, Barron T, Sullivan DR, Denny PW, Almeida IC, et al. Ether phospholipids and glycosylinositolphospholipids are not required for amastigote virulence or for inhibition of macrophage activation by *Leishmania major*. J Biol Chem 2003;278:44708-18.

[89] Rakotomanga M, Blanc S, Gaudin K, Chaminade P, Loiseau PM. Miltefosine affects lipid metabolism in *Leishmania donovani* promastigotes. Antimicrob Agents Chemother 2007;51:1425-30.

[90] Rakotomanga M, Saint-Pierre-Chazalet M, Loiseau PM. Alteration of fatty acid and sterol metabolism in miltefosine-resistant *Leishmania donovani* promastigotes and consequences for drug-membrane interactions. Antimicrob Agents Chemother 2005;49:2677-86.

[91] Maltezou HC. Drug resistance in visceral leishmaniasis. Journal of biomedicine & biotechnology 2010;2010:617521.

[92] Perez-Victoria FJ, Castanys S, Gamarro F. *Leishmania donovani* resistance to miltefosine involves a defective inward translocation of the drug. Antimicrob Agents Chemother 2003;47:2397-403.

[93] Perez-Victoria FJ, Gamarro F, Ouellette M, Castanys S. Functional cloning of the miltefosine transporter. A novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. J Biol Chem 2003;278:49965-71.

[94] Perez-Victoria FJ, Sanchez-Canete MP, Castanys S, Gamarro F. Phospholipid translocation and miltefosine potency require both *L. donovani* miltefosine transporter and the new protein LdRos3 in *Leishmania* parasites. J Biol Chem 2006;281:23766-75.

[95] Saito K, Fujimura-Kamada K, Furuta N, Kato U, Umeda M, Tanaka K. Cdc50p, a protein required for polarized growth, associates with the Drs2p P-type ATPase implicated in phospholipid translocation in *Saccharomyces cerevisiae*. Mol Biol Cell 2004;15:3418-32.

[96] Furuta N, Fujimura-Kamada K, Saito K, Yamamoto T, Tanaka K. Endocytic recycling in yeast is regulated by putative phospholipid translocases and the Ypt31p/32p-Rcy1p pathway. Mol Biol Cell 2007;18:295-312.

[97] Weingartner A, Drobot B, Herrmann A, Sanchez-Canete MP, Gamarro F, Castanys S, et al. Disruption of the lipid-transporting LdMT-LdRos3 complex in *Leishmania donovani* affects membrane lipid asymmetry but not host cell invasion. PLoS One 2010;5:e12443.

[98] Sanchez-Canete MP, Carvalho L, Perez-Victoria FJ, Gamarro F, Castanys S. Low plasma membrane expression of the miltefosine transport complex renders *Leishmania braziliensis* refractory to the drug. Antimicrob Agents Chemother 2009;53:1305-13.

[99] Perez-Victoria JM, Perez-Victoria FJ, Parodi-Talice A, Jimenez IA, Ravelo AG, Castanys S, et al. Alkyl-lysophospholipid resistance in multidrug-resistant *Leishmania tropica* and chemosensitization by a novel P-glycoprotein-like transporter modulator. Antimicrob Agents Chemother 2001;45:2468-74.

[100] Perez-Victoria JM, Cortes-Selva F, Parodi-Talice A, Bavchvarov BI, Perez-Victoria FJ, Munoz-Martinez F, et al. Combination of suboptimal doses of inhibitors targeting different domains of LtrMDR1 efficiently overcomes resistance of *Leishmania* spp. to Miltefosine by inhibiting drug efflux. Antimicrob Agents Chemother 2006;50:3102-10.

[101] Castanys-Munoz E, Alder-Baerens N, Pomorski T, Gamarro F, Castanys S. A novel ATP-binding cassette transporter from *Leishmania* is involved in transport of phosphatidylcholine analogues and resistance to alkyl-phospholipids. Mol Microbiol 2007;64:1141-53.

[102] Castanys-Munoz E, Perez-Victoria JM, Gamarro F, Castanys S. Characterization of an ABCG-like transporter from the protozoan parasite *Leishmania* with a role in drug re-

sistance and transbilayer lipid movement. Antimicrob Agents Chemother 2008;52:3573-9.

[103] Coelho AC, Boisvert S, Mukherjee A, Leprohon P, Corbeil J, Ouellette M. Multiple mutations in heterogeneous miltefosine-resistant *Leishmania major* population as determined by whole genome sequencing. PLoS Negl Trop Dis 2012;6:e1512.

[104] Wadhone P, Maiti M, Agarwal R, Kamat V, Martin S, Saha B. Miltefosine promotes IFN-gamma-dominated anti-leishmanial immune response. J Immunol 2009;182:7146-54.

[105] Eue I. Hexadecylphosphocholine selectively upregulates expression of intracellular adhesion molecule-1 and class I major histocompatibility complex antigen in human monocytes. J Exp Ther Oncol 2002;2:333-6.

[106] Zeisig R, Rudolf M, Eue I, Arndt D. Influence of hexadecylphosphocholine on the release of tumor necrosis factor and nitroxide from peritoneal macrophages in vitro. J Cancer Res Clin Oncol 1995;121:69-75.

[107] Zeisig R, Eue I, Kosch M, Fichtner I, Arndt D. Preparation and properties of sterically stabilized hexadecylphosphocholine (miltefosine)-liposomes and influence of this modification on macrophage activation. Biochim Biophys Acta 1996;1283:177-84.

[108] Eue I, Zeisig R, Arndt D. Alkylphosphocholine-induced production of nitric oxide and tumor necrosis factor alpha by U937 cells. J Cancer Res Clin Oncol 1995;121:350-6.

[109] Hochhuth CH, Vehmeyer K, Eibl H, Unger C. Hexadecylphosphocholine induces interferon-gamma secretion and expression of GM-CSF mRNA in human mononuclear cells. Cell Immunol 1992;141:161-8.

[110] Griewank K, Gazeau C, Eichhorn A, von Stebut E. Miltefosine efficiently eliminates *Leishmania major* amastigotes from infected murine dendritic cells without altering their immune functions. Antimicrob Agents Chemother 2010;54:652-9.

[111] Mauricio IL, Howard MK, Stothard JR, Miles MA. Genomic diversity in the *Leishmania donovani* complex. Parasitology 1999;119 (Pt 3):237-46.

[112] Mauricio IL, Stothard JR, Miles MA. The strange case of *Leishmania chagasi*. Parasitology today (Personal ed 2000;16:188-9.

[113] Mauricio IL, Gaunt MW, Stothard JR, Miles MA. Genetic typing and phylogeny of the *Leishmania donovani* complex by restriction analysis of PCR amplified gp63 intergenic regions. Parasitology 2001;122:393-403.

[114] Kuhls K, Alam MZ, Cupolillo E, Ferreira GE, Mauricio IL, Oddone R, et al. Comparative microsatellite typing of new world *Leishmania infantum* reveals low heterogeneity among populations and its recent old world origin. PLoS Negl Trop Dis 2011;5:e1155.

[115] Woerly V, Maynard L, Sanquer A, Eun HM. Clinical efficacy and tolerance of miltefosine in the treatment of canine leishmaniosis. Parasitol Res 2009;105:463-9.

[116] Mateo M, Maynard L, Vischer C, Bianciardi P, Miro G. Comparative study on the short term efficacy and adverse effects of miltefosine and meglumine antimoniate in dogs with natural leishmaniosis. Parasitol Res 2009;105:155-62.

[117] Andrade HM, Toledo VP, Pinheiro MB, Guimaraes TM, Oliveira NC, Castro JA, et al. Evaluation of miltefosine for the treatment of dogs naturally infected with *L. infantum* (=*L. chagasi*) in Brazil. Veterinary parasitology 2011;181:83-90.

[118] Manna L, Gravino AE, Picillo E, Decaro N, Buonavoglia C. *Leishmania* DNA quantification by real-time PCR in naturally infected dogs treated with miltefosine. Ann N Y Acad Sci 2008;1149:358-60.

[119] Miro G, Oliva G, Cruz I, Canavate C, Mortarino M, Vischer C, et al. Multicentric, controlled clinical study to evaluate effectiveness and safety of miltefosine and allopurinol for canine leishmaniosis. Veterinary dermatology 2009;20:397-404.

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