Alkylphospholipid – A Promising Class of Chemotherapeutic Agents with a Broad Pharmacological Spectrum

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ABSTRACT - PURPOSE: Since when alkylphospholipids (ALPs) were discovered and, even further after miltefosine’s approval for the treatment of cutaneous metastasis of breast cancer and leishmaniasis, their activity against many other diseases have been extensively studied. This review aims to provide a summary of the alkylphospholipid’s applications investigated so far. RESULTS: The mechanism of action of ALPs is not fully understood, however it is believed that they interfere with lipid homeostasis leading to cell apoptosis. Due to ALPs cytotoxic activity, this class of molecules has shown to be effective against many diseases and conditions. Besides the approval of miltefosine for application in cutaneous metastasis of breast cancer and visceral and cutaneous leishmaniasis, several other analogs have proved efficacy and are promising as less toxic alternatives. ALPs have also shown in vitro and in vivo activity against Trypanosoma spp., amoebae, Trichomonas vaginalis, Schistosoma mansoni, HIV, and some fungi and bacteria species. The use of ALPs for intraocular lens coating is also under investigation. In addition, a clinical trial comparing miltefosine with usual treatments to spontaneous urticaria is also being conducted. CONCLUSIONS: Alkylphospholipid present such a broad pharmacological spectrum that justifies the need for further investigations of the drug class mechanisms of action, as well as the search for new analogs with improved activity and toxicological profiles.

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INTRODUCTION

The use of synthetic metabolically stable analogs of lysophosphatidylcholines (LysoPC) as biological response modifiers is quite old (1). The idea of using lysophosphatidylcholine analogs for this purpose comes from the fact that LysoPC, an endogenous cell membrane component produced from the hydrolytic breakdown of phosphatidylcholines by phospholipases, acts as a modulator of several signaling pathways and some biochemical routes. In the late 60s, through the change of a glycerol C1 ester bond in Lyso PC to an ether bond, and the addition of another ether-linked methyl group at the C2 position, Hansjörg Eibl synthesized edelfosine (an ether lipid, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine, Figure 1), the first molecule of this drug class known as ALPs (synthetic alkylphospholipids) (2). More recently, ALPs were divided into two groups: alkyl-lysophospholipids and alkylphospholipids (1).

Edelfosine was initially considered a very promising molecule owing to its immunomodulatory properties and inhibitory activity over tumor cell proliferation (3, 4). However, cytotoxicity assays carried out on a large variety of tumors (solid and hemathological, such as leukemia) and normal cell lines, showed that edelfosine is not highly selective for tumor cells (5, 6, 7, 8). Nonetheless, the molecule was also evaluated on its antiproliferative activity in vitro and in vivo (in murine models) and was proven to be effective (6, 9, 10). However, edelfosine clinical use is limited mainly due to the molecule metabolic instability, high hemolytic potential and gastrointestinal toxicity. Edelfosine is used only for bone marrow purging in patients with acute leukemia (11, 12). New molecules have then been proposed and ilmofosine (1-hexadecylthio-2-methoxymethyl-rac-glycero-3-phosphocholine) was synthesized as a thio-ether analog.

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This chemical modification, however, did not alter significantly its metabolic stability or cytotoxic effect, preventing the clinical use of ilmofosine. Nonetheless, preliminary studies showed that this molecule is as an effective inhibitor of tumor cell proliferation (3, 4), encouraging the search for more stable analogs.

It was only in the late 80s, with the discovery of a new analog lacking the glycerol motif, that ALPs reached a clinical important level. Miltefosine (hexadecylphosphocholine, HePC, Figure 1) was simultaneously synthesized by two different groups, one screening platelet-aggregation analogs for anti-inflammatory purposes in the UK, and another searching for molecules with anti-tumor activity in Germany (13). Miltefosine became the prototype of a new class, called alkylphosphocholines (APCs), and led the way in the search for new molecules. Similarly to the earlier ALPs, miltefosine presented appreciable in vitro antiproliferative activity (14, 15). However, miltefosine was found to be highly hemolytic when administered parenterally and its oral application was associated with cumulative gastrointestinal toxicity (16). These severe side effects limited its maximum daily dose (MTD = 200 mg/day), preventing in vivo observation of antiproliferative effects (17).

Another structural ALP modification aiming to improve the therapeutic potency refers to the replacement of the choline moiety of miltefosine by a heterocyclic piperidine group, resulting in perifosine (octadecyl-1-(1,1-dimethyl-piperidinio-4-yl)-phosphate) (18). The insertion of the piperidine group increased stability and half-life by approximately 140 hours, preventing perifosine rapid metabolic degradation (19). This is actually an advantage over miltefosine, which is metabolized by phospholipases, yielding choline, phosphocholine and 1,2-diacylphosphocholine (14). Despite the favorable pharmacokinetic profile and the in vitro cytotoxic effect of perifosine against a wide range of tumor cell lines (19), phase I clinical trials revealed a toxicological profile similar to miltefosine’s, with a maximum tolerated dose of 200 mg/day (17).

More recently, two other molecules, erucylphosphocholine (ErPC) and its homocholine analog erufosine (ErPC3) were developed, having in common a longer 22 carbon chain with a ω-9-cis-double bond. This modification resulted in reduced hemolytic activity, thereby enabling intravenous administration, not feasible with previous ALPs (20, 21). The reduction of the hemolytic potential can be explained by the fact that ErPC and ErPC3 form preferentially lamellar structures (less hemolytic) in aqueous solutions instead of micelles (1). Another very interesting feature of these new analogs refers to their ability to cross the blood-brain barrier. It has been demonstrated that ErPC is able to cross the blood-brain barrier and thus accumulates in the brain, reaching sufficient concentrations to kill human glioblastoma cell lines in vitro without observed severe toxic side effects (22). Moreover, studies with ErPC3 point to lower bone marrow toxicity when compared to other compounds of this class, allowing intravenous infusion at higher doses and, therefore, holding out possibilities for monotherapy or combination regimens (23). This fourth generation (ErPC and ErPC3) comprises the most promising ALPs up to date, and has been suggested as an interesting therapeutic option not only for cancer, but also for the treatment of several other diseases (1).

Mechanisms of action
Unlike other conventional anticancer agents, whose mechanisms of action are mainly based on interaction with the genetic machinery of the cell, it is proposed that the primary target of ALPs is the cellular membrane due to their similarity with endogenous phospholipids (24, 25). Structurally, they correspond to classical surfactants and may cause cell lysis at high concentrations. However, at clinical concentrations, ALPs insert into the lipid bilayer producing a biophysical disturbance of the cell membranes. The presence of micelles is important to the cell lysis mechanism, which involves micellar dissolution and partition into the membrane, phase transition between lamellae and micelles and decrease in the size of micelles (26), as shown in Figure 2. Micellar aggregation depends on the space occupied by the hydrophilic and hydrophobic groups of the surfactants (27). In general, it is accepted that the amount of surfactant required to solubilize a membrane increases with the ease of forming micelles, i.e., with the critical micellar concentration (CMC) (28).

There are few hypotheses to explain the activity of ALPs as shown in Figure 3. When metabolically stable ALPs insert into the membrane, they generate a biophysical perturbation, interfering with phospholipid metabolism, proliferation and cell survival signaling pathways, while simultaneously
**Figure 1.** Timeline of alkylphospholipids development (Based on Blitterswijk and Verheij, 2012).

**Figure 2.** Schematic illustration of the micelle fusion and bilayer disruption process. The black and white circles represent the surfactant and phospholipids heads, respectively (hydrophilic moieties). The black curved lines represent the surfactant tails (hydrophobic moieties).
activating several stress pathways that promote apoptosis (29). ALPs are easily inserted into the outer leaflet of the plasma membrane and pass through it with the help of a “flippase” ATP-dependent complex. Another possible mechanism refers to an ALP endocytosis-based internalization by means of lipid rafts (6, 8, 30, 31).

The interference of ALPs with the metabolism of cell membranes leads to a cellular stress that, in turn, triggers apoptosis. The mechanism by which the inhibition of phosphocholine (PC) biosynthesis initiates apoptosis is not clear yet. It has been shown that the lack of PC in the endoplasmic reticulum somehow induces a stress of the pro-apoptotic transcription factor CHOP/GADD 153 (32, 33). It is well known that apoptosis can be triggered by a shift in the overall balance of apoptotic and survival signals. ALPs can also affect this balance via crosstalk between these pathways (8, 30, 34). Not only do ALPs inhibit the PC biosynthesis, but can also, under certain signaling conditions, prevent its breakdown to phosphatidic acid and further degradation to diacylglycerol. The phosphatidic acid and diacylglycerol are second messengers involved in the mitogen activated protein kinase (MAPK) and Ras/Raf/MEK/ERK signaling pathways, which are involved in cell proliferation (8, 30, 34, 35).

Besides the self-accumulation in membrane microdomains (lipid rafts), ALPs can also affect cholesterol homeostasis and promote lipid rafts perturbations, what would increase its uptake and lead to apoptotic signaling (36, 31, 37). ALPs inhibit the cholesterol transport from the cellular membrane to endoplasmic reticulum and, thereby, prevent its esterification. This leads to an accumulation of free cholesterol in cell membrane lipid rafts, which may affect signaling processes that are vital to cells survival and growth (38, 39, 1).

The best ALPs characterized target is the AKT enzyme, a serine/threonine kinase which is a key regulator of various cell survival pathways (40, 41, 42, 43). AKT is activated in most human cancers and contributes to cell growth, proliferation and cellular survival pathways, being an attractive target for anticancer therapy (44). ALPs prevents AKT activation either by disrupting membrane microdomains that are crucial to growth factor signaling, or by displacing the natural AKT ligands, PIP2 (phosphatidylinositol-4,5-biphosphate) and PIP3 (phosphatidylinositol-3,4,5-triphosphate) (42, 45, 46).

As a result, AKT is no longer capable of adopting the favorable conformation for its phosphorylation and activation (47, 48). Perifosine, in particular, is known to target the lipid-binding pleckstrin homology (PH) domain of AKT and to inhibit its translocation to cell membrane, an essential step for the enzyme activation (49). Studies about induced stress and pro-apoptotic pathway interference mechanisms have indicated that edelfosine can also promote the endoplasmic reticulum stress and generate ROS (reactive oxygen species), leading to ASK1 (apoptosis signal-regulating kinase 1) activation and apoptosis induction (32, 50, 51, 52).

Figure 3. Schematic representation of the hypotheses for ALPs mechanism of action.
ASK1 plays a crucial role on an apoptotic signaling pathway activated in response to some types of stress, such as ROS, tumoral necrosis factor (TNF-α), and endoplasmic reticulum stress (53, 54).

Another possible mechanism is the stimulation of Fas/CD95 that induces apoptosis by promoting the formation of a complex named DISC (death inducing signaling complex) (55, 56). The DISC formation and resulting apoptosis are based on Fas redistribution in lipid rafts (56, 57). Mollinedo et al. has shown that ALPs can induce Fas redistribution in lipid rafts at certain lymphoid tumoral cells, independently of endogenous ligands. This theory is strongly supported by the fact that treatment with methyl-β-ciclodextrine (a cholesterol remover from cell membranes) caused lipid raft rupture and prevented Fas redistribution induced by ALPs (58, 59, 60).

A mechanism related to PKC (protein kinase C) inhibition is also described, although this may not be the main mechanism leading to apoptosis (61). Owing to the similarity between ALPs and the endogenous substrates of PKC, namely PS (phosphatidyl serine) and PIP2 (phosphatidylinositol-4,5-biphosphate), it is proposed that ALPs can inhibit PKC by interacting with the regulatory domain C2, whereas the regulatory domain C1 recognizes the endogenous compound diacylglicerol (DAG) and phorbol esters. The inhibition, or regulation, of any of these domains modulates the phosphorylation of endogenous proteins, and can prevent cell growth by interfering with signaling pathways (62, 63, 64, 65).

Resistance mechanisms
Resistance to ALPs usually involves one of the two mechanisms of internalization described for this class. One refers to the trans-bilayer movement from the outer to the inner leaflet of the cell membrane that usually involves an ATP-dependent lipid translocator/flippase. The other mechanism corresponds to the internalization by endocytosis. The resistance against ALPs in squamous cell carcinoma and in RAW cells 264.7 (mouse leukemic monocyte macrophage cell line) is related to a dysfunctional flippase and a higher expression of Bcl-2 (B-cell lymphoma 2) anti-apoptotic protein. Without a functional flippase, ALPs are not significantly internalized into the cell and, consequently, cannot act on molecular pathways leading to apoptosis (66, 67). According to recent studies, resistant lymphoma rat cells S49 and S49AR that does not show ALPs internalization, have lower expression of Fas/CD95 and greater ERK/AKT signaling (31, 68, 69). S49AR cells also lack sphingomyelin, one of the main constituents of lipid bilayers, as cholesterol, due to a lower expression of sphingomyelin synthase (SM1). Sphingomyelin is essential for lipid synthesis and for the cell membrane vesicular traffic. Without sphingomyelin, ALPs cannot not form lipid rafts and, therefore, be internalized by endocytosis (69).

CLINICAL APPLICATIONS
Cancer
The first synthetic alkylphopholipid, edelfosine, had its use restricted to bone marrow purging in acute leukemia patients due to its high toxicity and lack of selectivity (11, 12). Nonetheless, studies with this molecule were resumed in 2010 when Molinedo et al. showed that, in an in vitro trial, in which edelfosine was able to induce apoptosis in mantle lymphoma and chronic lymphocytic leukemia cells via lipid raft with greater efficiency than other ALPs (7). The thioether analog of edelfosine, ilmofosine, has also showed anti-tumoral in vivo activity in pre-clinical trials, reaching phases I and II. Despite the primary promising results, phase I clinical trials of ilmofosine limited its maximum dose for both oral and intravenous administration mainly due to gastrointestinal toxicity (70, 71), and phase II trial ended up finding no objective results (72, 73).

Clinical studies were also carried out to evaluate the activity of miltefosine against soft tissue sarcoma (16), metastatic colorectal cancer (74), and head and neck squamous cell carcinoma (16). However, these studies were ceased in phase II trial since the oral doses required for the systemic effect were toxic to the gastrointestinal tract. For this reason, since its approval as Miltex® in 1992 in Germany, the clinical use of miltefosine is limited to the topical treatment of breast cancer cutaneous metastasis. The oral application of miltefosine is approved in some countries for treatment of visceral and cutaneous leishmaniasis (75) and the drug is currently under investigation for other diseases, such as some parasitosis.

In addition, phase II clinical trials with perifosine (table1) as a single agent for the treatment of advanced or metastatic breast cancer, head and neck cancer, soft tissue sarcomas, metastatic melanoma, prostate cancer, and
adenocarcinoma were disappointing (1, 14, 15, 71, 73, 76, 77). In spite of that, several studies with this drug as an anticancer agent in association with other drugs showed that such therapeutic approach can result in numerous clinical benefits. Perifosine can increase the antineoplastic effect of other drugs on tumor cells, producing better results. The combined therapy of perifosine with different agents, such as lenalidomide (thalidomide analog) and dexamethasone, is currently in phase I study for multiple myeloma and good results have been observed so far (78). In a more advanced phase II trial, perifosine was used in combination with bortezomib, a reversible proteasome inhibitor, in association or not with dexamethasone, to treat multiple myeloma; results are also promising. Perifosine has been given Fast Track status by the FDA for this indication and is currently in phase III clinical trials (23). The best results have been observed when perifosine is combined with temsirolimus, an mTOR inhibitor, to treat patients with neuroblastoma. Based on preclinical data and the premise that direct AKT inhibition may overcome AKT activation secondary to mTOR inhibition, perifosine-temsirolimus combined therapy shows a synergistic effect and have been considered very promising (49).

As well as perifosine, the fourth generation of ALPs, currently represented by erucylphosphocholine and erufosine, has been highlighted on in vitro and pre-clinical assays (Table 1), which can indicate a new perspective in cancer treatment. Rübel et al studied the in vitro putative beneficial effects of ErPC and ErPC3 in combination with radiation on human astrocytoma and glioblastoma cell lines (T98G, A172 and U87MG). The combined treatment enhanced the induced damage to mitochondria and caspase-activation, besides increasing the radiation-induced eradication of clonogenic T98G cells (79). Other studies have tested ErPC and ErPC3 against choriocarcinoma, acute myeloid leukemia, chronic lymphocytic leukemia and oral squamous cell carcinoma (Table 1).

| Table 1. Studies conducted with more recent ALPs (perifosine, erucylphosphocholine and erufosine). |
|---------------------------------------------------------------|
| **Type of Cancer**                                             | **Study Phase** | **References** |
| Relapsed and refractory multiple myeloma                      | Phase III       | Aeterna Zentaris, 2013 (80) |
| Taxane and platinum-resistant or refractory epithelial ovarian cancer | Phase I         | Fu S et al, 2012 (81) |
| Advanced renal cell carcinoma                                 | Phase II        | Cho D C et al, 2012 (82) |
| Metastatic colorectal cancer                                   | Phase II        | Bendell J C et al, 2011 (83) |
| Relapsed and refractory Waldenstrom’s macroglobulinemia       | Phase II        | Ghobrial I M et al, 2010 (84) |
| Solid tumors                                                  | Phase I         | Unger C et al, 2010 (85) |
| Recurrent prostate cancer                                     | Phase II        | Chee K G et al, 2007 (86) |
| Metastatic pancreatic adenocarcinoma                          | Phase II        | Marsh R W et al, 2007 (87) |
| Head and neck cancer                                          | Phase II        | Argiris A et al, 2006 (88) |
| Advanced soft tissue sarcoma                                  | Phase II        | Bailey H H et al, 2006 (89) |
| Metastatic melanoma                                           | Phase II        | Ernst D S et al, 2005 (90) |
| Androgen independent prostate cancer                          | Phase II        | Posadas E M et al, 2005 (91) |

**Perifosine**

Choriocarcinoma                                               in vitro                    Takai N et al, 2011 (92)
Breast carcinoma, pancreatic carcinoma and multiple myeloma    in vitro                    Bagley R G et al, 2011 (23)
Prostate cancer                                               in vitro                    Rudner J et al, 2010 (93)
Endometrial and ovarian cancer                                 in vitro                    Takai N et al, 2008 (94)
Astrocytoma and glioblastoma                                  in vitro                    Rübel A et al., 2006 (79)

**Erucylphosphocholine**

Breast cancer                                                 Pre-clinical                Dineva I K et al, 2012 (35)
Oral squamous cell carcinoma                                   in vitro                    Kapoor V., 2012 (95)
Multiple myeloma                                               in vitro                    Yosifiv D Y et al, 2011 (96)
Multiple myeloma                                               in vitro                    Königs S K et al, 2011 (97)
Acute myeloid leukemia                                         in vitro                    Fiegl M et al., 2007 (24)
Astrocytoma and glioblastoma                                   in vitro                    Rübel A et al., 2006 (79)
Breast carcinoma and leukemia                                   in vitro                    Berger M R et al, 2003 (98)

**Erufosine**

Breast cancer                                                 Pre-clinical                Dineva I K et al, 2012 (35)
Oral squamous cell carcinoma                                   in vitro                    Kapoor V., 2012 (95)
Multiple myeloma                                               in vitro                    Yosifiv D Y et al, 2011 (96)
Multiple myeloma                                               in vitro                    Königs S K et al, 2011 (97)
Acute myeloid leukemia                                         in vitro                    Fiegl M et al., 2007 (24)
Astrocytoma and glioblastoma                                   in vitro                    Rübel A et al., 2006 (79)
Breast carcinoma and leukemia                                   in vitro                    Berger M R et al, 2003 (98)
Leishmaniasis
The activity against Leishmania protozoan was first described by Croft et al., who found out that miltefosine was able to inhibit 100% of *Leishmania donovani* infection in mice, on a dose of 100 mg/kg/day (99). An oral presentation of miltefosine, Impavido®, is registered since early 2000 for the treatment of visceral leishmaniasis in Nepal and also for the cutaneous form of the disease in other countries such as Argentina, Bangladesh, Bolivia, Colombia, Ecuador, Germany, Guatemala, Honduras, India, Mexico, Pakistan, Paraguay and Peru (75). In India, there is also a generic miltefosine product available for the treatment of the visceral form of the disease (13). Miltefosine is the only oral drug available for the treatment of leishmaniasis so far. The World Health Organization (WHO) does not recommend miltefosine as first choice for the treatment of any type of leishmaniasis in any region, giving preference to pentavalent antimony and liposomal amphotericin B, alone and in association (100). Nonetheless, miltefosine monotherapy has been suggested as first-line choice for the treatment of visceral leishmaniasis at the Bihar district, in India (101).

Miltefosine is efficient against antimony resistant Leishmania, yet, its effectiveness is different for each protozoan species and each geographic location. Escobar et al. showed that miltefosine sensitivity was greater for intracelluar amastigotes of *L. donovani* (ED₅₀ of 3.32- 4.56 μM), followed by *L. aethiopica*, *L. tropica*, *L. mexicana*, and *L. panamensis*. The *L. major* amastigote was significantly less sensible (ED₅₀ of 31.56 – 37.17 μM) (102). Another *in vitro* study also showed a different profile of sensitivity of amastigotes among the species of *L. donovani* (ED₅₀ = 8.7 – 0.04 μg/mL), *L. braziliensis* (ED₅₀ = >30 – 8.4 μg/mL), *L. guyanensis* (ED₅₀ = >30 – 1.9 μg/mL), *L. mexicana* (ED₅₀ = 30 μg/mL) and *L. lainsoni* (ED₅₀ = 3.4 – 1.9 μg/mL) (103). Data from clinical trials of different countries also suggested that different isolates from the same species have variable responses to miltefosine. While a clinical trial carried out in Bolivia presented 88% cure rate of cutaneous leishmaniasis caused by *L. braziliensis* (104), another performed in Guatemala, where *L. braziliensis* is also the most common form, resulted in 53% cure rate (105).

The best perspective for miltefosine as an effective treatment for both visceral and cutaneous leishmaniasis consists on its association with other drugs (13). Some clinical trials have been made in the last decade with different associations in a search for more efficient, tolerable, shorter and cheaper treatments for visceral leishmaniasis. A phase III clinical trial performed in India concluded that all combination therapies tested (single dose liposomal amphotericin B followed by miltefosine or paromomycin, and 10 days miltefosine followed by 10 days paromomycin) had similar or higher cure rates and were more tolerable than the standard first choice treatment with amphotericin B alone (106).

Although clinical trials for leishmaniasis with other ALPs have not been conducted so far, there are evidences of their antiparasitic activity. Escobar et al. have shown that intracellular amastigotes were more sensitive to edelfosine compared to miltefosine (102). A more recent study compared the activity of four ALPs and concluded that edelfosine was the most active against different leishmania species, followed by perifosine, miltefosine and erucylphosphocholine. Edelfosine also proved to be an efficient alternative against antimonial resistant strains (107). The activity of perifosine has also been demonstrated *in vitro* against *L. braziliensis*, *L. infantum*, *L. major* and *L. amazonensis* strains (108). In a subsequent study on mice with cutaneous leishmaniasis caused by *L. amazonensis*, perifosine presented a higher degree of lesions reduction in comparison to miltefosine (109).

Miltefosine has also been successfully used for the treatment of visceral leishmaniasis on individuals co-infected with human immunodeficiency virus type-1 (HIV-1) (110, 111). Interestingly, several studies have shown that miltefosine exerts significant immunomodulatory properties in different cell types, being also a promising drug against HIV (112, 113). A recent study performed by Garg and Tremblay showed that miltefosine was able to reduce HIV-1 replication inside CD4(+)T cells partially due to secretion of type I Interferon by dendritic cells (114). Other study proved that miltefosine combined with sodium nitroprusside (SNP), which generates cytotoxic nitric oxide, reduces HIV-1 replication in macrophages, via downstream activation of AKT kinase. The same effect was observed with perifosine (115). These studies show that miltefosine, in addition to its anti-leishmanial property, can also prevent the spread of HIV-1. Hence, the *in vitro* inhibitory activity of miltefosine against HIV-1 and its efficacy on other cellular targets (eg,
macrophages) are important to validate its use on a larger scale on individuals co-infected with HIV-1 and Leishmania spp. The clinical relevance of these studies, however, remains to be determined (114).

**Chagas disease and sleeping sickness**

It is reasonable that drugs that are effective against leishmaniasis may also be useful against Chagas disease and sleeping sickness, given the fact that Leishmania spp. and Trypanosoma spp. are parasites of the Kinetoplastid order (116, 117). The ALP’s action against Trypanosoma spp. has been investigated since 1996, when Croft et al. described that Trypanosoma cruzi was more sensitive to miltefosine, ilmofosine and edelfosine, with ED₅₀ of 0.5 μM, 0.2 μM and 1.4 μM respectively, than to nifurtimox, with ED₅₀ of 2.7 μM (118).

Miltefosine is the most studied ALP against both T. cruzi and T. brucei species, probably because it is already approved for leishmaniasis. Various in vitro and in vivo studies have demonstrated that this drug is more active against T. cruzi than the standard drugs, nifurtimox and benznidazole (119, 120). Saraiva et al. showed that miltefosine produced higher reduction on the parasitemia level than benznidazol in mice with acute Chagas disease (119). Although alkylphosphocholines were less active against T. brucei than T. cruzi (118), miltefosine on a dose of 30 mg/kg/day was able to increase the lifetime of mice infected by T. brucei by 35%. In addition, the effect of the drug seems to vary according to the protozoan subspecies, being greater against T. b. brucei, followed by T. b. rhodesiense and T. b. gambiense (121). Although other ALPs such as edelfosine and ilmofosine were less studied for antitrypanosomal activity, they also present activity against this protozoan, but not as powerful as miltefosine (122).

Despite the great action of miltefosine against T. cruzi and T. brucei, its use is still restricted due to gastrointestinal toxicity and hemolytic effect observed with the required dose for therapeutic effect. Therefore, no clinical trials for ALPs involving Chagas disease or sleeping sickness have been conducted so far. Some studies considered drug association therapies, allowing the administration of lower doses of miltefosine and, as a result, lower incidence of side effects (121, 123). Santa-Rita et al. observed better results in terms of T. cruzi lysis with an association of ketoconazole and one ALP (miltefosine, edelfosine or ilmofosine), in comparison with the isolated drugs. This result can be explained as a synergistic effect of the drugs inhibiting the protozoan’s phospholipid metabolism (123).

**Other parasitosis**

The great effect of alkylphosphocholines against Leishmania and Trypanosoma gave rise to new research on these molecules into others parasitosis. In 2001, the activity of this class against amoebae was first described by Seifert et al. The authors showed that miltefosine and other ALPs analogs were able to kill Entamoeba histolytica, with the best results corresponding to an oleyl-PC derivative presenting an EC₅₀ for SFL-3 strain of 15 μM after two days of treatment. Miltefosine was the least effective among all analogs tested, with EC₅₀ between 57 and 225 μM (124). However, on another study performed with different clinical isolates of Acanthamoeba spp., miltefosine proved to be the most effective ALP, corresponding to a maximum log reduction of 100% at 40 μM. These results have enabled future investigations of ALPs for the treatment of Acanthamoeba keratitis and granulomatous amoebic encephalitis (125).

Another potentiality of ALPs already demonstrated refers to the treatment of Trichomonas vaginalis, an anaerobic flagellate protozoan. This application was first described in 2002 by Blaha et al., who investigated miltefosine activity against four strains of T. vaginalis, two metronidazole-resistant and two metronidazole-susceptible. The results showed that T. vaginalis susceptibility to miltefosine is not strain-dependent and does not correlate well with metronidazole susceptibility, which can be understood based on their different mechanisms of action. While metronidazole is activated in the hydrogenosomes of the organisms, resulting in a nitro radical damage to the genomic DNA and inhibiting cell division, miltefosine interacts with the cell membrane resulting in cell lysis. More specifically, miltefosine resulted in rounding up, immobility, blebbing and total lysis of the organisms. Blebbing of the cell membrane has also been observed in Entamoeba and Acanthamoeba treated with miltefosine and may reinforce an apoptotic mechanism. An important observation of this study is that no effect was recorded after incubation times <30 min, indicating that the cytotoxic effect of miltefosine on T. vaginalis is not immediate. This is reinforced by the lower values of EC₅₀ and EC₉₀ after longer incubation times. A time dependent
cytotoxic effect of miltefosine had already been found in *E. histolytica* and is also consistent with the proposed mode of action of this drug (126).

Several studies also support the use of miltefosine for the treatment of Schistosomiasis. The hypothesis that this drug can act against *Schistosoma mansoni* is mainly related to the fact that this parasite presents an uncommon lipid bilayer, which is important for the host-parasite relationship (127). Miltefosine presents a lipophilic alkyl chain that enables the molecule to penetrate into the parasite lipid bilayer and a polar quaternary nitrogen which facilitates the transport through an aquaporin (SmAQP) (128). In addition, the choline portion of miltefosine structurally resembles metrifonate, an irreversible organophosphate acetylcholinesterase inhibitor that used to be employed against *Schistosoma mansoni* (129). Miltefosine on an oral daily dose of 20 mg/kg on a five-day treatment of *Schistosoma mansoni* infected mouse showed to be more efficient than praziquantel, resulting in significant parasite load reduction and hepatic pathology improvement. Electronic microscopy analysis also revealed severe damage on adult *Schistosoma* tegument after miltefosine treatment (130).

**Fungi infection**

In 2006, Widmer *et al.* tested miltefosine against some fungi species and significant activity was observed against *Scedosporium prolificans*, an infectious agent with few effective therapeutic alternatives (131). In the same work, it was suggested that the treatment of *Cryptococcus* spp. with miltefosine might cause inhibition of LPTA (lyso phospholipase-transacylase), an enzyme involved in membrane synthesis, in a concentration dependent manner. It has also been reported an inhibitory activity of PLB1, phospholipase B, which is responsible for hematogenic spread and for the fungus adhesion to mammalian cells (132, 133). The antifungal activity of a series of ALPs analogs against *Cryptococcus neoformans* and *Candida albicans* has also been demonstrated and it was shown to correlate with the alkyl chain size, with the 18 carbons analog being more active than miltefosine (134).

Regarding the activity against *Candida albicans*, a work by Lukáč *et al.* compared miltefosine to Isophol-PC analogs (ALPs with branched alkyl chains), and a MIC of 5 to 20μM was observed for miltefosine, while the compounds Isophol<sub>16</sub>-PC and Isophol<sub>20</sub>-PC showed MIC values between 40 and 150μM. In another work, the same authors compared APCs and quaternary ammonium analogs and demonstrated that miltefosine was among the most active compounds, although all analogs studied presented relevant activity (135). In a recent work, the ability of miltefosine to inhibit *Candida albicans* biofilm formation was demonstrated (136).

The *in vitro* activity of miltefosine was also tested against nine species of dermatophytic fungi (*T. rubrum, T. mentagrophytes, T. tonsurans, T. soudanense, T. violaceum, E. floccosum, M. canis, M. gypseum and M. cookei*) and it was shown to be up to four times more active than itraconazole, a drug used for the treatment of chronic and severe infections caused by *Trichophyton* spp. and *Microsporum* spp. (137).

**Bacterial infection**

The activity of alkylphosphocholines and alkylglycerophosphocholines against fungi and protozoa had encouraged the research for their antibacterial activity (134).

Miltefosine was proved to be active against *Streptococcus* spp., but the activity significantly varied among species. For *Streptococcus pneumoniae*, MIC values ranging from 5 to 6.25 mM were observed, while for other streptococci (*S. mitis, S. oralis, S. pyogenes, S. agalactiae, S. mutans*) MIC values ranged from 10 to 20μM. There is evidence that miltefosine’s action against these bacteria is primarily related to its surfactant properties, which enable the solubilization of lipoteichoic acid present in the pneumococcal membrane and a natural inhibitor of autolysin LytA, an enzyme responsible for the degradation of the cell wall peptidoglycan (138, 139, 140).

In a study performed by Huelves *et al.* (2008), miltefosine activity against pneumococcal strains was tested both *in vitro* and *in vivo*. Miltefosine showed *in vitro* effect, however, according to the authors, MICs varied in a disparate manner under the influence of different culture media. One can infer that, giving the amphiphilic nature of miltefosine, it may have interacted with several constituents of culture media, interfering with the results. In the *in vivo* test, the drug was not effective (141).

In 2012, Lukáč *et al.* investigated the activity of miltefosine and other alkylphosphocholines with branched chains (Isophol-PC) against not only fungi, as mentioned before, but also *Staphylococcus aureus* and *Escherichia coli*. Among the compounds tested, Isophol<sub>16</sub>-PC and
Isophol20-PC showed relevant activity against *S. aureus*, corresponding to MIC of 0.15 mM and 0.31 mM, respectively. Miltefosine, on the contrary, did not present significant activity against *S. aureus* (135). Despite the results obtained by Lukáč *et al.*, previous studies by Obando *et al.* revealed a MIC of miltefosine against *S. aureus* isolate of 44 µM. The same study also showed ALPs activity against two clinically significant gram-positive bacteria: methicillin-resistant *S. aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE). The authors present a MIC of 44 µM for miltefosine against VRE and 22 µM against MRSA (134). It is clear, therefore, that new studies are needed in order to prove the effectiveness of ALPs against these strains.

It is also worth to mention that ALPs did not affect the growth of the gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, indicating that the outer membrane barrier prevents the drug from reaching the cytoplasmic membrane of these organisms (134, 140).

### Other applications

#### Intraocular lens coating

ALPs have also been investigated for other non-chemotherapeutically related applications, such as coating agent for intraocular lens. Previous studies considered the possibility of using coated intraocular lens as a polymeric drug-delivery device. A good coating agent should be able to inhibit epithelial cell growth over the lens, since posterior capsule opacification (PCO) is caused by cell proliferation, migration and attachment in the capsular bag (142). In this sense, ALPs would be appropriate coating agents of foldable hydrophilic acrylic intraocular lens to be used in cataract surgery (143). It was shown that ALPs in concentrations in the range of 1 mM are not only well tolerated by human corneal endothelial cells but also capable of reducing human ocular cell proliferation. Clearly, more investigation is still necessary to ensure biocompatibility and physiochemical properties of ALPs as coating agents, but there is a good potential for this novel application (143).

#### Chronic spontaneous urticaria

The first evidence that miltefosine could be used for chronic spontaneous urticaria was reported by Grosman, who described inhibition of histamine release from rat mast cells (144). Weller *et al.*, in 2009, demonstrated that processes mediated by signal transduction receptors, such as those involved in immunoglobulin E (IgE) - receptor (FceRI) mast cell-dependent activation with subsequent degranulation, are regulated by lipid rafts in the cell membrane (145). The same study also showed that miltefosine can inhibit the activation of degranulation and inflammatory mediator release by human mast cells in vitro, being able to avoid inflammatory reactions mediated by these cells. In addition, a previous study has shown that miltefosine causes inhibition of signal transduction pathways at cell membrane level (146). These observations support the theory that miltefosine acts as a lipid raft modulator and, thereby, interferes with the response of mast cell activation and degranulation (147). All these findings justify further investigations of miltefosine and other ALPs to treat other inflammatory disease than cancer, such as allergies.

Magerl *et al.* performed a randomized, double-blind, placebo-controlled multicentre study and found that miltefosine was as effective as antihistamines in preventing urticaria symptoms when compared to placebo, in a 4-week treatment period. Miltefosine is usually well tolerated and safe for systemic use in the required dosage for the treatment of urticaria, despite some adverse events as abdominal discomfort, nausea and vomiting. Therefore, miltefosine seems to be an interesting treatment option for chronic spontaneous urticaria patients who do not respond to standard-dosed antihistamines (147).

#### Ongoing and anticipated research

Considering the potential of alkylphospholipids as chemotherapeutic agents, as well as the new applications under investigation (Figure 4) such as intraocular lens coating, our group has been carrying out research on this topic, with special attention to the synthesis of molecules with lower hemolytic potential (148, 149). Moreover, we investigated the physical-chemical and structural characteristics of ALPs that most influence the hemolysis (150). Regarding amphiphilic compounds, it is noteworthy to mention that hemolysis occurs through mechanisms involving surfactant-membrane interaction and membrane disruption/solubilization. The presence of micelles is important in this mechanism, which involves micellar dissolution and partition into membrane, phase transition between lamellae and micelles and decrease in the size of micelles (26). It is generally accepted that the amount of surfactant required to solubilize a membrane increases with
the ease of forming micelles, expressed by the critical micellar concentration (CMC) (28). Therefore, our group focuses on developing molecules that will preserve the minimum requirements for chemotherapeutic activity but present higher CMC values, i.e., that will be more difficult to aggregate into micelles. Additionally, we are also carrying out coarse-grained molecular dynamics simulations to investigate the interaction and partition of monomers in different lipid bilayers, as well as the changes in the membrane caused by monomers and micelles insertion. Developing a molecular understanding of the mechanisms mentioned above is extremely relevant for the future design and development of ALPs-based drugs.

The unique mechanism of action of ALPs on cellular membranes and their interference with the lipid metabolism and rafts, apart from their action on enzymes responsible for the cellular signaling, give this class an advantage over the currently used chemotherapeutic agents. Apart from it, ALPs can be synergistically combined with other chemotherapeutic drugs so as to overcome pathogens mechanisms of resistance. The many therapeutic applications show the potentials of this class and thus the development of a molecular understanding of the mechanisms mentioned above is extremely relevant for the future design and development of ALPs-based drugs.

DECLARATIONS

Conflict of interest
The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Figure 4. Alkylphospholipid’s applications.
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