The development of novel anti-inflammatory drugs for IBD

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Repurposing miltefosine for the treatment of immune mediated disease?

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Journal of Pharmacology and Experimental Therapeutics
(2014) 350:189-195.

Abstract

Miltefosine is an ether lipid initially developed for cancer treatment in the early eighties. Miltefosine largely failed development for oncology although it has been approved for the topical treatment of breast cancer metastasis. It was subsequently discovered that miltefosine is a highly effective treatment for visceral Leishmaniasis, a parasitic disease which affects millions worldwide and causes an estimated 30.000 fatalities each year. Oral treatment with miltefosine is in general well tolerated and has relatively few adverse effects. The exact mechanism of action of miltefosine treatment is still a matter of investigation. Its close resemblance to phospholipids allows it to be quickly taken up by cell membranes and affect related processes such as lipid metabolism and signaling through lipid rafts. These processes play an important role in the immune response and it comes as no surprise that miltefosine has been successfully tested for the treatment of a number of immune mediated diseases in preclinical models of disease. Drug repurposing of miltefosine for immune mediated diseases may provide an opportunity to expand the limited number of drugs that is currently available for therapeutic use.

Miltefosine or hexadecylphosphocholine (HePC) was developed in the early eighties as a potential cytostatic drug. The drug largely failed development for oncology and is now best known as an oral treatment for Visceral Leishmaniasis, a lethal disease that is the result from infection with the parasite Leishmania. Discovery of its potential as an anti-parasitic drug has in recent years renewed scientific interest in the drug and it's mechanism of action. Development of new drugs is a costly and time-consuming process with a high risk of failure at multiple steps along the road of preclinical and clinical development. This makes repurposing of existing drugs with established side effects for novel indications an attractive alternative to classical drug development. Miltefosine has been extensively investigated in large clinical trials and appears to be relatively safe with limited and reversible side effects. In this review we will focus on the potential to repurpose miltefosine as an anti-inflammatory drug for immune mediated diseases. Of note, we will not cover perifosine, an alkyl-phospholipid developed to improve oral tolerability. Assuming that the mechanism of action of perifosine is similar to that of miltefosine, perifosine could be an alternative candidate for repurposing. However, the number of studies that address perifosine for other applications than the treatment of cancer is very limited.

Miltefosine (hexadecylphosphocholine) is an alkyl-phospholipid or ether-lipid with structural resemblance to lysophosphatidylcholine (LPC), a type of phospholipid that is present in the cell membrane and makes up around 3% of the cellular membrane. LPCs have a very short half-life as they are quickly metabolized by phospholipases and acyltransferases. In the LPC analogues miltefosine, perfosine and edelfosine an acyl group is replaced

by an alkyl group (Fig. 1) which makes the compounds metabolically stable (Eibl and Unger 1990).

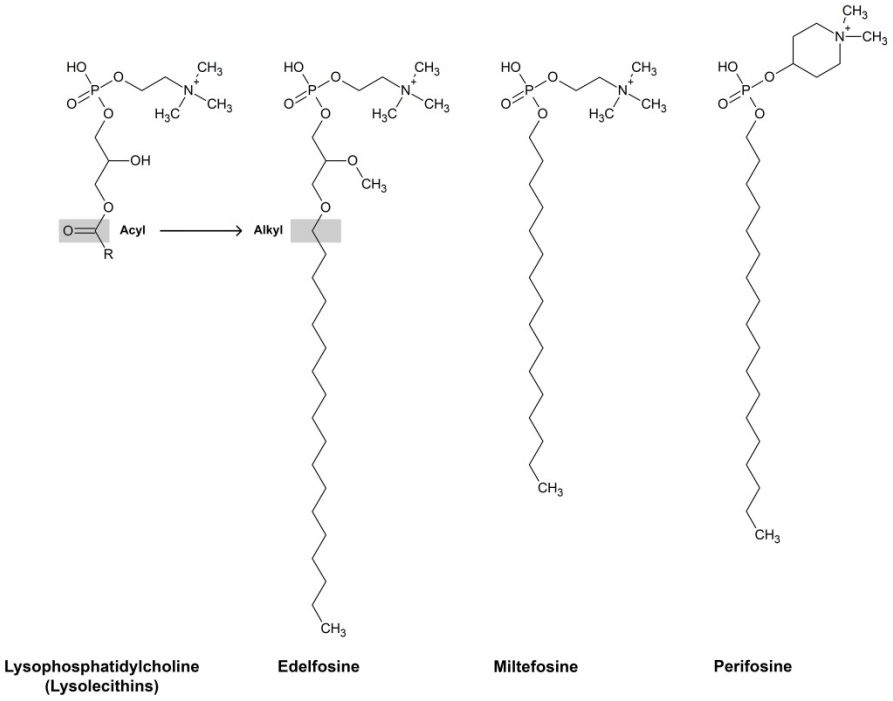

Fig. 1. Chemical structure of lysophosphatidylcholine and synthetic analogues. Lysophosphatidylcholine (also known as lysolecithin) is a natural occurring phospholipid present in cellular membranes. The lipid is easily metabolized, a problem that was solved in synthetic analogues by replacing the acyl group with a more resistant alkyl group. This change causes the ester, linking oxygen to the acyl group, to be changed into an ether.

The first stable LPC analogue was synthesized with an interest in enhancing macrophage mediated anti-tumor immunity (Munder PG, Fischer H, Weltzien HV, Oettgen HF, Westphal O (1976) Lysolecithin analogs: a new class of immunopotentiators with antitumor activity. Proc Am Assoc Cancer

Res 17:174) as it was found that LPCs increased the phagocytic capacity of peritoneal macrophages (Burdzy, Munder et al. 1964). It was subsequently discovered that some of these alkyl-phospholipids possessed direct cytostatic properties, shifting the interest away from the immunomodulatory properties. As the ability to inhibit cell growth appeared to be related to structural properties it was attempted to create the most minimal structure required. This resulted in a new group of molecules, the alkylphosphocholines and the discovery of miltefosine and edelfosine (Munder, Modolell et al. 1979).

Screening of miltefosine in cancer cell lines suggested that miltefosine possessed potent anti-neoplastic properties. Miltefosine inhibited cell growth in various leukemic cell lines (Unger, Damenz et al. 1989) and reduced tumor weight and size in dimethylbenzanthracene induced mammary carcinomas and transplanted mammary tumors in rats *in vivo* (Scherf, Schuler et al. 1987). However, miltefosine appeared only effective against some cell types, as no effect was observed in a variety of other cancer cell lines such as rat DS-carcinosarcoma, AH 13s sarcoma and L5222 leukemia, and mouse L1210 and P388 lymphocytic leukemia cells, Lewis lung carcinoma cells and B16 melanoma cells. The reason for this differential sensitivity of cancer cell lines to miltefosine has not been established,

Development of miltefosine for oncology

Intravenous administration of miltefosine is hemolytic and thus clinical applications have been limited to topical and oral treatments. A phase II dose-finding study in cancer patients confirmed previous phase I data that

the major dose limiting toxicity were nausea and vomiting and suggested a maximum dose of 150 mg daily split into three doses (Verweij, Planting et al. 1992). Oral treatment was subsequently tested in a number of oncological trials, including a phase II study for treatment of soft tissue sarcomas (Verweij, Krzemieniecki et al. 1993), a phase II study in advanced non-small cell lung cancer (Berdel, Becher et al. 1992), a phase II study in advanced colorectal cancer (Planting, Stoter et al. 1993), a phase II study in advanced breast cancer (Unger, Becher et al. 1993) and a phase II study for squamous cell head and neck cancer (Verweij, Gandia et al. 1993). None of these phase II clinical trials showed a sign that miltefosine treatment might affect disease progression. The only trials that showed an effect of miltefosine are studies in which miltefosine was applied topically. A 6% topical miltefosine solution was successfully applied in a phase II study for cutaneous T cell lymphomas (Dumontet, Thomas et al. 2006) and a multicenter randomized placebo-controlled phase III study for the treatment of skin metastasis of mammary carcinoma (Leonard, Hardy et al. 2001). Reasonable efficacy was observed in the latter trial with 2 out of 24 patients achieving a complete response of the skin lesions (0/27 for placebo) and 6 out of 24 showing partial response (1/27 for placebo).

Leishmaniasis

Leishmaniasis is a disease caused by infection of macrophages by a protozoan parasite belonging to the genus Leishmania. The parasites are transferred by a type of sandflies that only lives in (sub)tropical regions, restricting the disease to those areas. Although the infection can lead to cutaneous, mucocutaneous or visceral disease (also called kala-azar).

Visceral leishmaniasis is characterized by bouts of fever, weight loss, hepatosplenomegaly and anemia. It is a lethal disease if left untreated (Chappuis, Sundar et al. 2007). Unfortunately the yearly incidence of visceral leishmaniasis is estimated at 200.000 to 400.000 per year worldwide, with around 20.000 to 30.000 fatalities a year (Alvar, Velez et al. 2012). In 95% of the cases the disease can be quite effectively treated with intravenous injections of amphotericin B or pentavalent antimonial drugs (sodium stibogluconate and meglumine antimoniate). These drugs are inexpensive but treatment requires a trained physician to perform the injection and patients often succumb before reaching a doctor. More importantly, the number of patients showing resistance to treatment is increasing and patients with VL in combination with AIDS are generally refractory to treatment with pentavalent antimonial drugs.

The development of an in vitro model for infecting primary mouse peritoneal macrophages with *Leishmania donovani* in the 1980-ies allowed screening of large libraries of compounds for possible new treatments (Croft 1986). These screenings resulted in the discovery of alkyl phospholipids as possible candidates (Croft, Neal et al. 1987). In 1992 it was first shown that Leishmaniasis can indeed be treated with miltefosine in vivo. Mice infected with *Leishmania donovani* and *Leishmania infantum* were treated orally with miltefosine and compared to standard treatment using sodium stibogluconate. Parasitic levels in spleen and liver after four weeks of treatment showed that miltefosine is more potent in both suppressing and killing Leishmania parasites than sodium stibogluconate in mice (Kuhlencord, Maniera et al. 1992).

The use of miltefosine as an oral treatment for visceral leishmaniasis has been tested in numerous trials (Sundar, Rosenkaimer et al. 1998; Jha, Sundar et al. 1999; Sundar, Jha et al. 2002; Sundar, Sinha et al. 2011). Different treatment strategies have been used but in general patients with visceral leishmaniasis receive 50 or 100 mg of miltefosine daily for a duration of 28 days. On average treatment was well tolerated and adverse effects were limited to gastro-intestinal side effects such as vomiting and diarrhea. Treatment with miltefosine resulted in a 90 to 100 percent cure rate.

Immunomodulatory effects of miltefosine

The development of alkyl phospholipids initially started with the aim to create an immunomodulator that would increase anti-tumor phagocytic capacity of tumor associated macrophages. Similar to the original compounds that development started out with, miltefosine exhibits immunomodulatory properties.

Stimulatory effects on the innate immune response

There are a number of reports that suggest that miltefosine may stimulate multiple aspects of monocyte and macrophage mediated immunity. Such effects may explain the potent clearance of Leishmania parasites that have their niche inside macrophages.. For example, Balb/c mouse monocytes and macrophages treated with miltefosine showed a more potent response to LPS, with enhanced secretion of TNFα and release of NO (Eue, Zeisig et al. 1995; Zeisig, Rudolf et al. 1995; Ghosh, Roy et al. 2013). In a similar experiment with macrophages isolated from C57BL/6 mice there was no

increase in NO release but miltefosine did increase their phagocytic capacity. Not only did sensitized macrophages phagocytize more *S. cerevisiae* after miltefosine treatment but miltefosine also increased the number of macrophages that participated in the process (Ponte, Alves et al. 2012).

One report investigated the effect of miltefosine on circulating monocytes and cytokine levels in patients suffering from cutaneous Leishmaniasis (Mukhopadhyay, Das et al. 2011). In 12 patients that completed 4 months of miltefosine treatment (100 mg/day) the percentage of CD14+ monocytes decreased while CD16+ monocytes increased. This shift indicated that miltefosine treatment may have triggered maturation of the monocyte population towards a more pro-inflammatory phenotype. Levels of monocyte associated pro-inflammatory cytokines such as TNF-a, IL-6, IL-1b and IL-8 were significantly increased compared to these levels at the moment of presentation of the disease. Of course it cannot be excluded in this study that some of these effect were indirect and a systemic response to the eradication of the Leishmania parasites.

In a case study of one patient suffering from cutaneous Leishmaniasis treated with miltefosine, tissue biopsies were taken from the lesional area. Quantitative analysis of mRNA expression showed a significant decrease of TNFα, IL-10 and TGFβ levels while IFNγ and CD40 were significantly increased when comparing before to after treatment (Ansari, Ramesh et al. 2008). CD40 is a costimulatory molecule found on antigen presenting cells that helps to stimulate T helper cells which in turn produce IFNγ stimulating macrophages. Again, this was a single patient and it is difficult to judge if

the changes in expression are the direct result of the miltefosine treatment or alternatively result from the eradication of parasite-laden macrophages.

The initial development of alkyl phospholipids as compounds that stimulate macrophage phagocytosis, the potent parasite clearance by Leismania infected macrophages together with some of the experiments above suggest that treatment with miltefosine potentiates monocyte and macrophage mediated innate immunity.

Inhibitory effects on the adaptive immune response

The reason for a potential role for miltefosine and other alkyl phospholipids in immune mediated diseases is that miltefosine potently inhibits T cell activation (Baumer, Wlaz et al. 2010; Verhaar, Wildenberg et al. 2013). This effect has been verified in animal models where inflammation was successfully reduced using miltefosine. We investigated the effect of miltefosine in a CD4CD45RB^{high} transfer mouse model of inflammatory bowel disease (IBD). In this model, SCID mice, which lack T and B cells, are transplanted with CD45RB^{high} T cells resulting in chronic inflammation of the gut that develops over the course of several weeks. Miltefosine, given twice weekly, greatly reduced colonic inflammation in this model. Miltefosine improved clinical parameters such as weight loss, significantly improved the pathology score and reduced the expression of pro-inflammatory cytokines il-6, tnf-α and ifnγ to levels no longer significantly different from control mice without colitis (Verhaar, Wildenberg et al. 2013). These findings may make miltefosine an attractive candidate for the treatment of IBD. Although the cause of IBD remains unclear there are many indications that suggest that a reduced function of

the innate immune system predisposes to an excessive response of the adaptive immune response to intestinal microbiota (Marks, Harbord et al. 2006; Hayee, Rahman et al. 2010; Uhlig 2013). Given the results discussed above miltefosine may have a dual therapeutic effect in IBD by both stimulating macrophage mediated innate immunity and reducing excessive activation of T cell mediated adaptive immunity.

Baumer et al. investigated the effect of miltefosine treatment in a number of animal models addressing its effect on Th1 or Th2 related inflammatory responses and feasibility for use in atopic dermatitis. The models use ear thickness as a measure of inflammation. In an ovalbumin induced delayed type hypersensitivity model (DTH) miltefosine was able to significantly reduce ear swelling. Likewise in the more Th2 orientated mouse model of toluene diisocyanate induced hypersensitivity treatment with miltefosine significantly reduced the ear swelling and pro-inflammatory cytokine expression, both when administered systemically as well as topically (Baumer, Wlaz et al. 2010).

Topical application of a 6% miltefosine solution was compared with 1% hydrocortisone in a small clinical trial of 16 patients (Dolle, Hoser et al. 2010). Patients with at least two different target lesions were treated topically with one drug on each lesion for three weeks. Both treatments effectively reduced the severity of the disease and reduced the number of infiltrating lymphocytes in the lesions. Follow-up of the patients revealed a more sustained effect of miltefosine treatment. Interestingly, closer examination revealed a local increase in the number of FoxP3+ regulatory T cells in the miltefosine treated lesions while the number of FoxP3+ cells

was reduced by hydrocortisone. An important potential advantage of miltefosine over hydrocortisone was that in contrast with hydrocortisone, miltefosine did not affect proliferation of cells in the basal layer of the epidermis. Indeed, again in contrast with hydrocortisone, no reduction in epidermal thickness was observed with miltefosine.

Miltefosine and allergic disease

A potential role for miltefosine in allergic disease was suggested when it was shown that miltefosine can reduce histamine release from rat primary mast cells (Grosman 1990). Mast cells contain large granules with inflammatory mediators such as cytokines, proteases, histamine, serotonin and eicosanoids. Mast cells release their granules in response to activation by pattern recognition receptors or cross-linking of the IgE receptor. Degranulation causes redness of the skin, swelling and itching.

Miltefosine has also been shown to inhibit histamine release in human mast cells (Weller, Artuc et al. 2009; Batista, Friedrichson et al. 2010; Batista, Schlechtingen et al. 2011). Ten minutes of pretreatment with 25 µM miltefosine reduced anti-IgE induced histamine release by more than 50%. These findings were translated into a small study in which the effect of miltefosine was tested on the allergic response to a standard skin prick test. Five allergic patients were treated on both arms with either placebo or a 6% miltefosine solution. The inflammatory response was measured by the diameter of the resulting wheal and erythema. Miltefosine was able to significantly reduce the inflammatory response that resulted from injection with an allergen. A control condition in which the patients were injected with histamine showed no effect (Weller, Artuc et al. 2009).

Miltefosine has also been tested in a number of other mast cell related conditions. One such condition, mastocytosis, involves the accumulation of mast cells in various organs, most commonly in the skin. This rare condition affects mostly children and skin manifestations causes symptoms related to mast cell mediator release such as pruritus and flushing. 39 Adult patients were tested in a double-blind, placebo-controlled trial with a topical 6% miltefosine solution and the glucocorticoid clobetasol (0.5 mg/ml) for 2 weeks. Although it seems that miltefosine may have reduced weal formation in this study none of the differences reached statistical significance and it seems the study was under powered to detect a meaningful difference (Hartmann, Siebenhaar et al. 2010).

Chronic urticaria, is a kind of idiopathic rash that is relatively common affecting up to 1% of the population in the US. In most cases the underlying cause is unclear but the rash is the result of activated mast cells. Urticaria can be treated with anti-histamines but with chronic urticaria most patients respond poorly and are often resistant. In a multicenter randomized, double-blind, placebo-controlled study that included 54 anti-histamine resistant chronic urticaria patients, miltefosine was tested as an oral drug (Magerl, Rother et al. 2012). Patients took increasing dosages miltefosine (up to 150 mg/daily unless intolerable adverse effects) daily for 4 weeks. At the end of the treatment period, miltefosine treated patients showed substantial improvement as was clear from a specific urticaria activity score and a decrease in the number of wheals. Miltefosine treatment did not reduce the intensity of pruritus.

Collectively these data suggest that miltefosine could be a potential drug for mast cell related conditions.

Side effects

Miltefosine has few side effects but those found may hamper long term use. From the phase II trials for the oral use of miltefosine with cancer patients it has become clear that daily dosages of 150 mg and higher cause potentially severe gastrointestinal side effects resulting in loss of appetite, nausea and vomiting (Verweij, Planting et al. 1992). This can be solved by combining with anti-emetics but in some cases the side effects are dose limiting. Development of a better tolerated formulation for oncological indications was suspended due to the discovery of perifosine, a better tolerated oral anticancer drug (Crul, Rosing et al. 2002).

Miltefosine treatment may have teratogenic effects. Studies on embryonic development and organogenesis of rats suggested an embryotoxic, fetotoxic and teratogenic risk (Sindermann and Engel 2006). The same was reported from studies in rabbits, although with the exception of the teratogenic effect. Due to these findings and because of a lack of human study results, use of miltefosine during pregnancy is contraindicated. This is especially a problem due to its long half-life. Six months after treatment miltefosine can still be measured in serum and it is therefore advisable to use contraception for half a year after stopping treatment.

In some patients the renal function is affected and a rise in creatinine levels is observed. In most cases this effect is reversible. Studies in dogs showed no lasting effect of miltefosine on the kidney (Bianciardi, Brovida et al.

2009). Miltefosine might have a direct effect on renal function but change in fluid balance due to gastrointestinal effects might contribute. Supporting this hypothesis, Leishmaniasis patients treated with miltefosine sometimes show elevated creatinine serum levels during episodes of vomiting and dehydration (Sindermann and Engel 2006).

To summarize, a treatment schedule of 50-100 mg miltefosine daily is in general well tolerated and rarely leads to serious or treatment limiting adverse effects. Studies on the miltefosine treatment of VL clearly indicate that such a treatment schedule can be maintained for at least 4 weeks. These data indicate that the potential of miltefosine as a maintenance treatment for IBD could be investigated.

Mechanism of action

The precise mechanism of action of miltefosine is lacking and may be different for different cell types. Because miltefosine was originally selected as a cytostatic drug, most of the research is focused on explaining how miltefosine induces apoptosis in cancer cells. And even though the effect of miltefosine on macrophages, lymphocytes and mast cells appears to be different, may involve similar pathways.

Miltefosine shows resemblance to the membrane phospholipid lysophosphatidylcholine. It's structural properties allow miltefosine to integrate into the cellular membrane (Rakotomanga, Loiseau et al. 2004). From there it redistributes to endoplasmic reticulum, Golgi-, nuclear- and mitochondrial membranes. How this happens is unclear but in Caco-2 colorectal cancer cells the process appears to be partly ATP dependent

suggesting active transport. The normal membrane recycling process may be responsible for the remainder of the intracellular transport of the drug (Menez, Buyse et al. 2007). Because alkyl-phopholipids, such as miltefosine, are more resistant to metabolizing enzymes, they accumulate in the membranes possibly affecting the continuous turnover of endogenous phospholipids.

One of the most important effects of miltefosine incorporation into the cell membrane is believed to be inhibition of phosphatidylcholine (PC) synthesis. PC is the most abundant phospholipid in cellular membranes of eukaryotic cells and interference with its production may predispose the cell to undergo apoptosis (Wieder, Orfanos et al. 1998; van der Luit, Budde et al. 2002). Miltefosine affects PC synthesis by inhibiting CTP:phosphocholine cytidylyltransferase, the rate limiting enzyme in the PC biosynthesis pathway at the endoplasmatic reticulum. This is supported by the fact that apoptosis can be prevented by supplementing miltefosine treated cells with exogenous lysoPC, an alternative precursor for the synthesis of PC. As apoptosis induced by other triggers could not be rescued by adding lysoPC, this is a strong suggestion that indeed inhibition of PC synthesis is the mechanism of miltefosine induced apoptosis (Boggs, Rock et al. 1995; Baburina and Jackowski 1998; Van Der Luit, Budde et al. 2003).

In addition to inhibition of PC synthesis, miltefosine also impairs signaling molecules through inhibition of phospholipases. Phospholipases hydrolyze phospholipids and inhibition results in a decrease in breakdown products, including the important second messengers diacylglycerol (DAG) and

phosphatidic acid (PA). Further downstream, alkyl-phospholipids may impact proliferation and cell survival through effects on the kinase Akt (Song, Ouyang et al. 2005). Under normal conditions, once recruited and positioned at the plasma membrane, Akt can be activated. Although this has not been described for miltefosine, studies on perifosine suggest that treatment affects the recruitment of Akt to the plasma membrane and displacements of its ligands PIP3 (phosphatidylinositol (3,4,5)-trisphosphate, PtdIns(3,4,5)P3) or PIP2 (phosphatidylinositol (3,4)-bisphosphate, PtdIns(3,4)P2).

All of these effects could be cause by disturbance of membrane integrity and the functionality of lipid rafts (Kondapaka, Singh et al. 2003). Miltefosine increases the membrane fluidity in macrophages (Ghosh, Roy et al. 2013) an effect that has been reported to decreases antigen presentation and stimulation of T cells (Chakraborty, Banerjee et al. 2005). On the other hand miltefosine has an affinity for sterols and is known to stabilize sterol rich areas such as lipid rafts which increase antigen presentation (Jimenez-Lopez, Rios-Marco et al. 2010). And indeed macrophages treated with miltefosine show an enhance ability to stimulate T cells resulting in a higher production of IL-2, IL-12, TNFα and NO production (Ghosh, Roy et al. 2013). The effect of miltefosine on membrane integrity could also very well explain why mast cells are inhibited in their IgE dependent histamine release. Upon activation, IgE receptors cluster in organized microdomains (Weller, Artuc et al. 2009; Silveira, Mazucato et al. 2011). Likewise T cell activation is dependent on the formation and organization of T cell receptor microclusters and formation of the immunological synapse (Kabouridis and Jury 2008).

Signaling molecules that have been implicated as targets for miltefosine treatment in cancer, such as Akt, Ras/Raf-1, PLC and second messengers like PA and DAG, are also important mediators in cells of the immune system. Inhibition of even one of these molecules could explain for example the inhibition of lymphocyte proliferation. Of course future investigations will have to shed more light on this.

Conclusion

Miltefosine is an alkyl-phospholipid that was initially tried unsuccessfully in several phase II trials in oncology and hematology. Originally this class of compounds was identified for its stimulatory effect on macrophage phagocytosis and several groups have observed that miltefosine may stimulate myeloid cell mediated immunity. At the same time miltefosine shows profound inhibition of T cell activation and miltefosine was successfully applied in preclinical models of atopic dermatitis and inflammatory bowel disease. Given the well-established and relatively limited side effects and oral route of administration miltefosine may be a candidate for drug repurposing for immune mediated diseases that are in dire need for novel therapeutic options such as inflammatory bowel disease.

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