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***Leishmania* parasitophorous vacuole membranes display phosphoinositides that create conditions for continuous Akt activation and a target for miltefosine in *Leishmania* infections**

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Abstract

Miltefosine is an important drug for the treatment of leishmaniasis; however, its mechanism of action is still poorly understood. In these studies, we tested the hypothesis that like in cancer cells, miltefosine's efficacy in leishmaniasis is due to its inhibition of Akt activation in host cells. We show using pharmacologic agents that block Akt activation by different mechanisms and also using an inducible knockdown approach, that miltefosine loses its efficacy when its access to Akt1 is limited. Interestingly, limitation of Akt activation results in clearance of established *Leishmania* infections. We then show, using fluorophore-tagged probes that bind to phosphoinositides, that *Leishmania* parasitophorous vacuole membranes (LPVMs) display the relevant phosphoinositides to which Akt can be recruited and activated continuously. Taken together, we propose that the acquisition of PI(4)P and the display of PI(3,4)P₂ on LPVMs initiates the machinery that supports continuous Akt activation and sensitivity to miltefosine.

Introduction

It is estimated that there are 1.2 million and 0.4 million new cases of cutaneous and visceral leishmaniasis, respectively spread amongst 98 countries each year (Alvar et al., 2012). In the mammalian host, *Leishmania* are phagocytosed by macrophages and dendritic cells into intracellular compartments called *Leishmania* parasitophorous vacuoles (LPVs). The biogenesis and composition of LPVs is not fully understood. Internalization of *Leishmania* parasites engages phosphatidyl inositol 3-kinase (PI3K) signaling, which plays an important role in the infection (Kima, 2016). Activation of PI3K signaling in cells is initiated by PI3K enzymes at the site of hormone or phagocytic receptor engagement in the plasma membrane. There, PI3Ks catalyze the phosphorylation of phosphatidyl-inositol lipids at the 3-position of the inositol ring resulting in the display of phosphatidyl-inositol 3,4 phosphate [PI(3,4)P₂] or phosphatidyl-inositol 3,4,5 phosphate [PI(3,4,5)P₃]. It is to these lipids that the serine-threonine kinase, Akt (also called protein kinase B), binds (Manning and Cantley, 2007). While anchored to the membrane lipids, Akt becomes activated by phosphorylation at threonine 308 (T308) and at serine 473 (S473); thereafter, it detaches from the cell

membrane and traffics to the various locations in the cell where pAkt engages its substrates. Through those substrates, PI3K/Akt signaling regulates several critical cellular functions, including prolongation of cell survival, resistance to apoptotic signals and actin reorganization (Manning and Cantley, 2007). Under physiologic conditions, PI3K/Akt signaling ceases when the stimulus at the plasma membrane dissipates or upon the induction of phosphatases such as phosphatase and tensin homolog deleted on chromosome 10 (PTEN) that dephosphorylates the 3-position of phosphatidyl-inositols and by so doing prevents Akt activation (Liu et al., 2009).

Miltefosine is an orally administered drug that was originally identified as an anti-breast cancer agent. It and other related alkylphospholipids including perifosine were shown to act by disrupting PI3K/Akt signaling in cancer cells (Ruiter et al., 2003, Chakrabandhu et al., 2008, Jangir et al., 2014). More recently, miltefosine was re-purposed for the treatment of leishmaniasis (Croft and Engel, 2006). Miltefosine is now the indicated drug for the treatment of visceral leishmaniasis (McGwire and Satoskar, 2014). It is also indicated for the treatment of cutaneous leishmaniasis; however, its efficacy for treating cutaneous *Leishmania* infections is variable (Castro et al., 2017, Fernandez et al., 2014). The basis for the variable sensitivity of some *Leishmanias* to miltefosine is not understood. Moreover, there is concern for the potential appearance and spread of parasites that are resistant to miltefosine (Bhandari et al., 2012, Ostyn et al., 2014). Since the adoption of miltefosine for treatment of leishmaniasis, several studies have investigated miltefosine's mechanism of action in *Leishmania* infections. Most of those studies have focused on the direct effects of miltefosine on the parasite; these studies have shown that miltefosine affects lipid metabolism in the parasite, induces mitochondrial dysfunction and induces apoptosis of amastigotes (Santa-Rita et al., 2004, Paris et al., 2004, Luque-Ortega et al., 2007, Turner et al., 2015). Although some of these observations are controversial, together, they suggest that miltefosine may have several targets in the parasite that could explain why parasite species and strains differ in their susceptibility to the drug. It has also been proposed that miltefosine's mechanism of action in *Leishmania* infections may be through its targeting of host processes including Akt mediated signaling (Seifert et al., 2010, Dorlo et al., 2012); however, no published studies have evaluated host Akt signaling as the target of miltefosine in *Leishmania* infections. Knowledge of miltefosine's mechanism of action is useful if adequate strategies to overcome the development of resistant organisms are to be developed.

If host Akt is the target of miltefosine in *Leishmania* infected cells, there are several outstanding questions that need to be addressed: 1) Does miltefosine treatment block Akt activation in the host cell at concentrations that are distinct from those that are required to target parasite processes? 2) How does targeting host cell Akt activation lead to the control of *Leishmania* infections? 3) Under physiologic conditions, Akt activation occurs at the plasma membrane at sites where the appropriate phosphoinositides are displayed; what is the situation in *Leishmania*-infected cells where Akt activation would have to be sustained for continued sensitivity to miltefosine? 4) In light of the fact the availability of PI(3,4)P₂ or PI(3,4,5)P₃ is the rate limiting step of Akt activation, what is the distribution of these phosphoinositides in *Leishmania*-infected cells?

In this study, we initially show that miltefosine is effective at lower concentrations against intracellular parasites as compared to extracellular parasites, which suggests that the targeting of a host cell component mediates the action of miltefosine against *Leishmania* parasites at low miltefosine concentrations. Those studies reveal that limitation of Akt activation reduces *Leishmania* survival in macrophages in the absence of additional stimuli, which implies that modulation of Akt activation is a viable strategy for controlling *Leishmania* infections. We then show that blocking Akt availability or its activation results in reduced efficacy of miltefosine. We tracked the distribution of the relevant phosphoinositides that are required for Akt activation, in order to gain insight on the intracellular sites in infected cells that can support continuous Akt activation. Specifically, using fluorophore-tagged lipid-binding probes that bind to PI(3,4)P2 or PI(3,4,5)P3 and their precursors, we show that *Leishmania* PV membranes (LPVM) display PI(3,4)P2 to which Akt can bind and be activated. LPVMs also display PI(4)P, which is an important precursor of PI(3,4)P2.

Results

Miltefosine has direct effects on *Leishmania* parasites

Leishmania spp. exhibit differences in their sensitivity to miltefosine (Castro et al., 2017, Paris et al., 2004). To commence our studies, we tested the efficacy of miltefosine on two parasite lines that are in wide use. The susceptibility of promastigote forms of *L. donovani* (MHOM/S.D./62/1S-CL2_D) and *L. amazonensis* (RAT/BA/74/LV78) to miltefosine was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. After incubation of parasites for 24 hr with increasing concentrations of miltefosine, survival curves were plotted (Figure 1A) establishing the EC₅₀ values for *L. donovani* promastigotes of 13.7±1.6 KM and for *L. amazonensis* of 37.2±4.8 KM. This range of miltefosine efficacy on *Leishmania* parasites is consistent with previous reports (Castro et al., 2017).

Several studies have reported that *Leishmania* amastigote forms within macrophages are more sensitive to miltefosine as compared to promastigotes in axenic culture (Escobar et al., 2002, Seifert et al., 2010, Dorlo et al., 2012). We proceeded therefore to evaluate the sensitivity of the *L. donovani* and *L. amazonensis* parasites lines to miltefosine after infections of RAW264.7 mouse macrophages. It had previously been reported that at concentrations above 20KM, miltefosine is increasingly toxic to macrophages; specifically, on J774, a mouse macrophage-like cell line, miltefosine toxicity was estimated at IC₅₀'s of 24 to 26KM (Azzouz et al., 2005). To ensure that we evaluate the effects of miltefosine on *Leishmania* infections at concentrations that are not toxic to macrophages, we limited evaluations of miltefosine to maximum concentrations of 10–15KM. At those concentrations, which result in clearance of approximately 90% of parasites from within macrophages, miltefosine is not toxic to RAW264.7 cells (Supplemental Figure 1a). The efficacy of miltefosine on intracellular parasites after 24 hr infections was estimated by microscopy after Giemsa staining. Survival curves were generated (Figure 1B). The estimated EC₅₀ for miltefosine on *L. donovani* infections was 6KM ±0.56KM and for *L. amazonensis* the EC₅₀ of miltefosine was estimated at 6±1.03KM. Infections were also

performed on peritoneal exudate cells (PECs) that were obtained 4 days after intraperitoneal injection of thioglycolate into BALB/c mice. The EC₅₀ of miltefosine on *L. amazonensis*-infected PECs was 6.16+/-1.86KM. These observations show that in the laboratory maintained parasites lines as well, intracellular amastigotes are more sensitive to miltefosine as compared to the extracellular promastigote forms. In addition, these results show that sensitivity of parasites to miltefosine in the RAW264.7 macrophage cell line was comparable to sensitivity in primary macrophages. The observations above are in agreement with several published studies that have reported that *Leishmania* species (and isolates) exhibit differences in sensitivity to miltefosine and that intracellular parasites are more sensitive than extracellular parasites to miltefosine (Seifert et al., 2010, Coelho et al., 2014). Based on those observations, we hypothesized that the increased efficacy of miltefosine on intracellular parasites is mediated by its effects on a host target of the drug. In light of studies on the mechanism of action of miltefosine on cancer cells that implicated Akt as its primary target, the subsequent studies presented below were designed to evaluate Akt as the target of miltefosine in *Leishmania*-infected host cells.

Akt activation is sustained in *Leishmania*-infected cells

To commence the evaluation of Akt activation in infected cells, we evaluated the time course of Akt activation in macrophages after infection with *Leishmania* parasites. Macrophages incubated with *L. donovani* or *L. amazonensis* parasites showed an increase in both the T308 and S473 pAkt forms within 15 minutes that peaked at 30 minutes (Figure 2A). A second activation phase was sustained through 72 hr post infection. Glycogen synthase kinase 3(GSK3 β) is a downstream substrate of Akt, whose phosphorylation can be assessed as a functional readout of Akt activation (Manning and Toker, 2014). *Leishmania* infection resulted in sustained phosphorylation of GSK3 β (Figure 2A). Akt activation in *Leishmania*-infected cells is apparently biphasic, which is highlighted by the line plot through the compiled densitometry data from Western blot experiments (Figure 2A). These results are in agreement with observations by Ruhland et al., (2007), but those studies evaluated other parasite species and a shorter time course. In typical infection experiments of RAW264.7 macrophages over a 72 hr time course, there is an increase in the number of parasites per macrophage (Figure 2B). It is known that the uptake of *Zymosan* particles by macrophages is mediated by phagocytic receptors, the ligation of which activates PI3K signaling that leads to the phosphorylation of Akt (Tamura et al., 2009). We observed that peak activation of Akt in macrophages in response to *Zymosan* was after 15 min incubation (Figure 2). pAkt levels returned to resting levels once the particles were internalized and the events at the plasma membrane dissipated. Similarly, after a quick increase, pGSK3 β levels also returned to resting stage levels in macrophages harboring *Zymosan* particles (Figure 2). Taken together, these results show that in contrast to *Leishmania* infection, the uptake of *Zymosan* particles does not result in the biphasic increase in pAkt levels. The second wave of Akt activation in *Leishmania*-infected cells is evidently distinct from classical phagocytosis, suggesting the possible development of a new intracellular site from which Akt activation is sustained.

Miltefosine blocks akt activation

In tumor cells, miltefosine was shown to block Akt activation (Chakrabandhu et al., 2008, Jangir et al., 2014, Gradziel et al., 2014). In light of those studies, we sought to confirm that miltefosine blocks Akt activation in macrophages in response to hormonal activation and also in response to *Leishmania* infection. For assessment of hormonal activation, macrophages were pre-incubated with miltefosine or medium alone (control) prior to their incubation with insulin, which is a potent activator of Akt (Liang et al., 2007). As shown in Figure 3a, within 15 minutes, insulin induces an appreciable increase in Akt phosphorylation at both T308 and S473. Incubation with 10KM miltefosine blocks insulin-induced Akt phosphorylation as only basal levels of Akt were detected. Un-phosphorylated Akt levels remained unchanged. Comparable experiments were performed on *Leishmania*-infected cells. For evaluation of *Leishmania*-infected macrophages, infections were allowed to establish for 12 hr, at which time 10KM miltefosine or medium (control) was added to the cultures. After an additional 24 hr incubation, lysates were prepared and evaluated in Western blots for pAkt. Both the T308 and S473 pAkt forms were reduced after miltefosine treatment (Figure 3b). The plot of band densities shown in the figure show a consistently greater reduction in the S473 phospho-form in infected cells as compared to hormone-treated cells. There is a consensus that phosphorylation at T308 is accomplished by Phosphoinositide-Dependent Kinase-1 (PDK1); in contrast, several kinases including PDK1 have been implicated in the phosphorylation of the S473 site. In infected cells, the second phase of Akt activation is most probably occurring at an alternative membrane site within the infected cell. In light of studies that had shown that the action of alkylphospholipids is influenced by the composition of lipid rafts (Reis-Sobreiro et al., 2013), to which PDK1 and other relevant kinases are partitioned, the differential effects of miltefosine on pAkt sites would suggest that the lipid environment at that alternate site differs from the environment in the plasma membrane.

Miltefosine is less effective against *Leishmania* after Akt knock down

There is increasing evidence that the 3 isoforms of Akt have distinguishable functions (Toker and Marmiroli, 2014). We had found previously that the knock down of Akt1 was sufficient to render *Leishmania*-infected cells susceptible to apoptosis inducers (Ruhland et al., 2007). Burleigh and colleagues (Caradonna et al., 2013) also found that knock down of Akt1 had an appreciable effect on *T. cruzi* survival in mammalian cells. Together, those studies suggested that in infections with protozoan parasites, Akt1 plays an important role. In the current study, to further establish that the host cell Akt is the primary target of miltefosine in *Leishmania*-infected macrophages, we developed cell lines in which Akt1 could be knocked down on demand. For the studies here, Smartvector lentiviral inducible shRNAs that target mouse Akt1 were custom created (Dharmacon). These vectors contain the Tet-On 3G tetracycline-inducible expression system. Three stable cell lines that each expressed a different Akt1-specific shRNA sequence were developed. A control macrophage line stably transfected with a lentiviral vector with an mCMV promoter controlling non-target shRNAs was also created. Induction of shRNA synthesis and eventual knockdown was achieved by growing cells in doxycycline. All three Akt1 shRNA cell lines exhibited some level of Akt knockdown as shown in Supplemental Figures 2a,b. We elected to perform subsequent analyses with the Akt shRNA-2 line because it exhibited more consistent growth

characteristics. Figure 4A shows a representative Western blot of lysates from the Akt1 shRNA-2 line and also the control mCMV cell line when cells were grown in doxycycline. In the Akt1 shRNA-2 line, induction of knockdown reduced Akt1 levels by up to 60% by 48hr, which is about when most infection experiments were evaluated. In contrast there was no change in Akt1 levels in the mCMV line upon incubation with doxycycline.

We proceeded to infect the Akt1 shRNA-2 line with *L. donovani* parasites. After 12 hr infection, doxycycline was added to the infection cultures to induce shRNA synthesis. After 24 hr, miltefosine or vehicle was added to the cultures. The cultures were incubated for an additional 24 hr after which they were fixed and evaluated after Geimsa staining. Figure 4b shows that there was no effect on parasite viability when doxycycline was added to wild type macrophages infected with *Leishmania*. In contrast, induction of Akt1 shRNA with doxycycline resulted in a 40% reduction in the number of infected cells (Figure 4C). This result was in agreement with the results of Calejari-Silva et al., (2015) who showed that in THP-1 human cells transfected with Akt1 shRNA, the survival of *Leishmania* parasites was significantly reduced. We then proceeded to assess the efficacy of miltefosine in these cells with doxycycline co-incubation. There was no change in miltefosine efficacy in control cell lines after doxycycline induction or in the Akt1 shRNA-2 lines without doxycycline treatment (Figure 4D). For evaluation of the efficacy of miltefosine in Akt1 shRNA cells co-incubated with doxycycline, parasite survival values were normalized to accommodate for parasite loss that resulted from diminished Akt1 levels. The survival curves after miltefosine treatment showed that the efficacy of miltefosine is significantly reduced in the Akt1 shRNA-2 cell lines co-incubated with doxycycline. The EC_{50} for miltefosine in wild type cells was 6.2 ± 0.47 KM. In the Akt1 shRNA-2 cells without doxycycline induction, the EC_{50} was 5.56 ± 0.32 KM. In contrast, the EC_{50} for miltefosine was estimated at 26.45 ± 9 KM after doxycycline co-incubation in the Akt1 shRNA-2 cells lines. In mCMV cells, the EC_{50} s of miltefosine were 6.14 ± 0.61 KM and 6.56 ± 0.49 KM in doxycycline or no doxycycline induction, respectively. Taken together, the observations using cell lines whose Akt1 levels were knocked down on demand showed that when the cellular levels of Akt1 are reduced, not only is parasite survival in macrophages limited but also that miltefosine loses its efficacy to control the remaining *Leishmania* infections. Infections with the Akt1 shRNA-2 line were also evaluated after *L. amazonensis* infections. The results of the *L. amazonensis* experiments are in Supplemental figure 2C. The EC_{50} of miltefosine on *L. amazonensis* with doxycycline co-incubation was estimated at 37.86 ± 0.94 KM as compared to 6.3 ± 0.56 KM in cells without doxycycline. Finally, we ruled out the possibility that knock down of Akt1 predisposed cells to apoptosis upon treatment with miltefosine (Supplemental figure 2D).

Evaluations of Akt as the target of miltefosine in macrophage infections

For another approach to evaluate the hypothesis that Akt is the primary target of miltefosine in *Leishmania* infections, the effect of pharmacological inhibitors was evaluated. We showed earlier that incubation of cells with miltefosine results in the inhibition of Akt phosphorylation (Figure 3). However, the mechanism by which miltefosine disrupts Akt activation is still not well understood. A report by Gradziel et al., (2014) showed that miltefosine most likely blocks Akt activation by interfering with the binding of Akt's

pleckstrin homology domain to PI(3,4)P2 or PI(3,4,5)P3. PDK1, which phosphorylates Akt at T308, also binds to these phosphoinositides. We reasoned that if miltefosine's action was directed at Akt, then incubation of miltefosine with co-inhibition of PKD1 or other inhibitors of Akt would alter the efficacy of miltefosine.

PDK1 inhibitor II inhibits the phosphorylation of Akt at T308 by PDK1 (Islam et al., 2007). At the concentrations and incubation conditions evaluated in these experiments, the PDK1 inhibitor II is not toxic to macrophages (Supplement figure 1B). First, Western blots were performed to evaluate the effect of PDK1 inhibitor II on Akt phosphorylation (Figure 5a). On average, 40% and 60% inhibition of Akt phosphorylation was achieved at 24 and 48 hr, respectively. Then, prior to adding miltefosine, incubation of infected cells with the PDK1 II inhibitor resulted in a 30% reduction in *L. donovani* infected cells (Figure 5B) and a 24% reduction in the number of *L. amazonensis* infected cells (Supplemental figure 3a). The reduction in the number of infected cells was statistically significant. This result is consistent with previous observations that showed that reduced Akt activation limits *Leishmania* parasite survival in macrophages. We proceeded to assess the killing efficacy of miltefosine on parasites in infected macrophages while actively inhibiting Akt activation with the PDK1 II inhibitor. In light of the fact that PDK1 inhibitor II treatment alone results in reduced infection levels, the infections with co-incubation of PDK1 inhibitor II and miltefosine were normalized so that any additional effects resulting from miltefosine treatment would be apparent. In infection cultures to which DMSO but no PDK1 inhibitor II was added, the EC₅₀ of miltefosine on *L. donovani* infections was 5.4+/-0.3KM while in the presence of the PDK1 inhibitor II, the efficacy of miltefosine on *L. donovani* infections rose to an estimated EC₅₀ of 40.19+/- 5KM. Identical experiments were performed on *L. amazonensis* infections. The results are shown in Supplemental figure 3b. When infections were incubated with the diluent (DMSO), the EC₅₀ of miltefosine was 8.07 +/-0.4KM. In contrast, in the presence of the PDK1 inhibitor II, the efficacy of miltefosine was estimated at >50KM. These results show that miltefosine loses its efficacy to kill intracellular *Leishmania* when the activation of Akt is impaired.

Inhibition of Akt activation with other pharmacological inhibitors reduces the efficacy of miltefosine killing of *L. donovani* parasites

We next evaluated additional pharmacological inhibitors that block the activation of Akt by other mechanisms as compared to the PDK1 inhibitor II. Akt inhibitor IV and Akt inhibitor VIII (Calbiochem) that exert direct effects on all 3 Akt isoforms or 2 Akt isoforms, respectively, were tested. In addition, inhibitors of the mammalian target of rapamycin (mTOR) were also tested. The two mTOR complexes, mTORC1 and mTORC2 are downstream kinases in the PI3K/Akt signaling pathway (Saxton et al., 2017). mTORC2 has been identified as one amongst other kinases that can phosphorylate Akt at S473. Most commercially available pharmacological inhibitors of mTOR inhibit both mTORC1 and mTORC2. For the studies here, we evaluated the effects of the mTOR inhibitor XI Torin 1 (Torin 1). The toxicity of each of these inhibitors to RAW264.7 macrophages was evaluated at the concentration and incubation times in these experiments (Supplemental figure 1a). Only AktIV inhibitor was found to be toxic to macrophages Consequently, it wasn't used in subsequent experiments. Co-incubation with each of these Akt inhibitors reduced the

efficacy of miltefosine on *Leishmania* infections The results of those studies are shown in Supplemental figure 3B.

Other signaling molecules such as the MAP kinases and molecules in the toll-like receptor pathway have been implicated in the survival of *Leishmania* within macrophages (Martinez and Petersen, 2014, Terrazas et al., 2015). To determine whether inhibition of MAP kinase or toll-like receptor pathways has a comparable effect on the efficacy of miltefosine, we assessed the effect of inhibition of those signaling pathways on the efficacy of miltefosine. Inhibition of MAP kinase p38 was accomplished with SB202190 (evaluated previously in *Leishmania* infections, Ruhland et al., 2007). Toll like receptor 4 (TLR4) dependent signaling was inhibited with CLI-095, which also inhibits ERK and JNK Map kinase (Lee et al., 2016). As shown in Figure 5C, neither CLI-095 nor SP202190 resulted in reduced levels of Akt phosphorylation. Unlike the inhibitors of Akt, incubation with CLI-095 and SP202190 did not change the rate of established infections. Moreover, co-incubation with these inhibitors did not alter the efficacy of miltefosine. The EC_{50} 's of miltefosine after co-incubation with SB202190 or CLI-095 were 7.38 ± 2.67 and 6.91 ± 3.31 KM, respectively (Figure 5D). Together, the results of these experiments show that inhibition of Akt activation alters the efficacy of miltefosine, while inhibition of other signaling pathways that have also been shown to be activated during *Leishmania* infection, does not alter the efficacy of miltefosine.

Killing of intracellular *Leishmania* by miltefosine is mediated by reactive oxygen species (ROS)

Several studies have described a cross talk between the induction of reactive oxygen species (ROS) and Akt activation (reviewed by Zhang et al., 2016). Studies on the alkylphospholipids have also shown a link between their actions and activation of ROS (Canuto et al., 2014, Shen et al., 2016). In light of the observations reported above that showed that miltefosine efficacy was dependent on Akt availability, we assessed the impact of blocking ROS on miltefosine's efficacy. Infected macrophages were incubated with N-acetyl-L-cysteine (NALC), an inhibitor of ROS, with or without co-incubation with miltefosine. When *L. donovani* infections were incubated with NALC alone it had no effect on the course of *Leishmania* infection (Figure 6A). However, when miltefosine was co-incubated with NALC, the efficacy of miltefosine was reduced. The estimated EC_{50} of miltefosine without NALC co-incubation was 5.01 ± 0.21 KM as compared to 17.07 ± 3.63 KM with NALC co-incubation. Comparable observations were made with *L. amazonensis* infections. Incubation of infected cells with NALC did not affect the infection. The EC_{50} of miltefosine in those parasites without NALC was $8 \text{ KM} \pm 0.4 \text{ KM}$. However, when co-incubated with NALC the EC_{50} of miltefosine could not be estimated because miltefosine was completely ineffective. This result is in agreement with observations by others (Canuto et al., 2014). Together, these experiments suggest that the leishmanicidal effects of miltefosine are mediated by Akt activation and ROS.

LPVMs display phosphoinositides that required for Akt activation in *Leishmania* infected cells

We showed earlier that unlike *Zymosan* particles that activate Akt transiently upon internalization, *Leishmania* infection results in sustained Akt activation through several days with a biphasic course (Figure 2). The early activation phase is presumed to be due to phagocytic receptor engagement during *Leishmania* internalization. The second phase of activation is apparently essential for the survival of parasites within infected cells. The question that we began to consider is “how is Akt activation sustained in *Leishmania*-infected cells?”. A critical and rate-limiting step in the activation scheme of Akt is the presence on the plasma membrane of PI(3,4)P2 or PI(3,4,5)P3, to which Akt is recruited (Manning and Cantley, 2007). In light of the critical role of these phosphoinositides, we proceeded to determine whether the LPVM is an internal site within infected cells where these lipids are displayed and to which Akt could be recruited and activated. The pleckstrin homology domain probe (PH-Akt(GFP)) is a well characterized probe for evaluating the distribution of PI(3,4)P2 and PI(3,4,5)P3 (Balla and Varnai, 2002, Balla and Varnai, 2009). To determine the distribution of these phosphoinositides in infected macrophages, PH-Akt(GFP) was transiently expressed in RAW264.7 macrophages, which were then infected with either *L. donovani* or *L. amazonensis* parasites. Infections were evaluated at 2 hr and 24 hr post infection. At 2 hr, it is expected that most events associated with parasite entry would have dissipated. At 24 hr post infection, *Leishmania* promastigote forms that initiated the infection in macrophages should have fully transformed into amastigote forms and parasite replication should have commenced (Doyle et al., 1989). Figure 7a shows representative cells expressing PH-Akt(GFP) and infected with *L. donovani* or *L. amazonensis* parasites for the indicated times. There is some labelling on the cell plasma membrane, which is expected, and also some diffuse cytosolic fluorescence that is most likely due to non-physiological levels of the PH-Akt(GFP) probe. There is intense binding of the PH-Akt(GFP) probe to the LPVM of individual vacuoles that harbor *L. donovani* parasites, and also to the communal vacuoles that harbor *L. amazonensis* parasites. Preferential labeling of the LPVM by the PH-Akt(GFP) probe is highlighted by the line trace of fluorescence intensity through a representative LPV and shown in the histogram plot (Figure 7a). The contours of the LPVM are also delineated by anti-LAMP-1 reactivity. In previous studies, it was shown that >95% of LPVs are LAMP-1 positive after 1 hr infection (Ndjamen et al., 2010), although the intensity of LAMP-1 staining on early *L. donovani* LPVs can be very low, in agreement with the observations of Desjardins and Descoteaux, 1997. The number of infected cells that contained at least one LPV that labeled positively for PH-Akt(GFP) was estimated at 62% for *L. amazonensis*-infected cells and 59% for *L. donovani* infected cells. In cells transfected with PH-Akt(R25C)(GFP), which is mutated in the PH domain and hence has lost lipid binding (Balla and Varnai, 2002), we estimated that approximately 20% of cells have LPVs that are reactive (albeit faint) with this probe (not shown). This established that 20% reactivity is the approximate false positive estimation rate with the PH-Akt(GFP) probe. It is noteworthy that only 65% of LPVs labeled positively with the PH-Akt(GFP) probe. In contrast, greater than 95% of LPVs are LAMP-1 positive (Ndjamen et al., 2010). We interpret this observation to imply that LAMP-1 is delivered to LPVs via a mechanism that is distinct from the mechanism by which the lipid precursors of PI(3,4)P2 and PI(3,4,5)P3 are delivered to LPVs.

To determine whether the display of PI(3,4)P₂ and PI(3,4,5)P₃ on a phagosome-like compartment is restricted to LPVs, cells expressing PH-Akt(GFP) were allowed to internalize *Zymosan* particles and the distribution of the lipid reporter on *Zymosan*-containing phagosomes (ZCP) was monitored as well. Although *Zymosan* particles were found within LAMP-1 positive phagosomes, these ZCPs were for the most part not labeled with the PH-Akt(GFP) at the indicated times (Figure 7B). Less than 20% of ZCPs were reactive with the PH-Akt(GFP) probe (Figure 8C), which is at the false positive threshold estimated with the PH-Akt(R25C)(GFP) mutant probe. Nigorikawa et al., (2015) using *Zymosan* particles and Bohdanowicz et al., (2010) using opsonized red blood cells, had shown that after internalization of those particles by RAW 264.7 macrophages, PI(3,4,5)P₃ and also PI(3,4)P₂ are lost from nascent phagosomes soon after internalization. Our observations that these phosphoinositides are displayed on 2 hr and 24 hr old LPVs underscores the differences between LPVs and classical phagosomes.

Phosphoinositide precursor of PI(3,4)P₂ but not the precursor of PI(3,4,5) is displayed on LPVMs

The AktPH(GFP) probe is reactive to both PI(3,4)P₂ and PI(3,4,5)P₃ lipids (Manning and Cantley, 2007). These lipids have distinct biosynthetic and metabolic schemes (De Craene et al., 2017). To gain more insight into which of these phosphoinositides is displayed on LPVMs, we sought to determine whether the precursor lipids for PI(3,4)P₂ and PI(3,4,5)P₃ are also displayed on LPVs. Several studies have shown that PI(4,5)P, which is abundant on the plasma membrane (PM) is the most important precursor of PI(3,4,5)P₃ (Manning and Cantley, 2007, De Craene et al., 2017). To determine whether PI(4,5)P₂ accumulates on LPVs, macrophages were transfected with the (PLC δ)-PH-GFP probe that is a widely used reporter for PI(4,5)P₂ (Balla and Varnai, 2002). The cultures were then infected with *L. donovani* or *L. amazonensis* parasites and the distribution of PI(4,5)P₂ was evaluated after 2 and 24 hr. Representative images of cells expressing (PLC δ)-PH-GFP and infected with either *L. donovani* or *L. amazonensis* are shown (Figure 8a). As expected with this probe, the plasma membrane of transfected cells was prominently labeled. However, there was limited to no evidence of (PLC δ)-PH-GFP labeling of LPVMs. The proportion of cells with LPVs that labeled positively for (PLC δ)-PH-GFP was below 20% as shown (Figure 8B). A comparable proportion of cells contained LPVs that were deemed to be labeled with the (PLC δ)-PH-GFP as did cells that labeled with PH-PLCD1(R40L)-GFP mutant (Balla and Varnai, 2002) (not shown). Based on these results, our interpretation is that PI(4,5)P is not displayed on the LPVM. PI(4,5)P is therefore unlikely to be the precursor of the phosphoinositide species on LPVMs to which the PH-Akt(GFP) probe is reactive. We did not rule out the possibility that the absence or limited presence of PI(4,5)P could be explained by the unsuitability of this probe to detect this lipid in non-PM membranes; however, Botelho et al., (2000) showed that this lipid is barely detected on nascent phagosomes after sealing of the phagocytic cup.

PI(4)P can be converted to PI(3,4)P₂ by Class II PI3kinases (Hawkins and Stephens, 2016). PI(4)P is an abundant lipid in secretory pathway compartments including the ER and the Golgi (Graham and Bird, 2011, Mesmin et al., 2013). To determine whether PI(4)P is displayed on LPVMs, we evaluated the distribution of the *Legionella pneumophila*

PtdIns4P-interacting effector SidM *Legionella* (sidM-GFP) (Hammond et al., 2014) in *Leishmania* infected cells. Balla and colleagues showed that the sidM-GFP is a reliable probe for PI(4)P (Hammond et al., 2014). Our initial studies with sidM-GFP yielded poorly expressing RAW264.7 macrophages. Better expressing cells and unambiguous results were obtained with the GFP-P4M-SidMx2 which consists of two P4M binding domains fused in tandem and tagged with GFP (Hammond et al., 2014). Although in the initial description of this probe, GFP-P4M-SidMx2 was believed to cause distressed Golgi, a subsequent study by Levin et al., (2017), found this probe to function adequately in RAW264.7 cells that expressed low to moderate levels of this probe. We first assessed the distribution of the GFP-P4M-SidMx2 in uninfected cells counterstained with either the Golgi reassembly stacking protein of 65 kDa (GRASP65) or LAMP-1. We observed that GFP-P4M-SidMx2 co-localizes exquisitely with Grasp65. Hammond and colleagues showed that GFP-P4M-SidMx2 labeled a subset of late endocytic compartments as well (Hammond et al., 2014). In our studies, we also found that some GFP-P4M-SidMx2-positive vesicles were also LAMP-1-positive. Upon infection with *Leishmania* parasites, the GFP-P4M-SidMx2 was abundantly recruited to LPVs. Figure 8b shows representative images of *L. amazonensis* and *L. donovani* LPVs at 2 hr and at 24 hr post infection. The LPVM is conspicuously labeled by the GFP-P4M-SidMx2 probe. The white arrow in the GFP-P4M-SidMx2 expressing cells points to positively labeled LPVs. The GFP-P4M-SidMx2 probe also labels the Golgi, which are counter stained for Grasp65. At 2hr post-infection greater than 80% of infected cells have at least 1 LPV that is reactive with the GFP-P4M-SidMx2 probe (Figure 8D). There was a statistically insignificant decrease in the number of infected cells with at least 1 GFP-P4M-SidMx2 positive LPV at 24 hr post infection. Comparable observations were made with *L. donovani*-infected cells. To assess the specificity of this recruitment, GFP-P4M-SidMx2 transfected cells that internalized *Zymosan* particles were evaluated. Representative images are shown in Figure 8C. Some ZCPs were conspicuously labeled with GFP-P4M-SidMx2 probe. The white arrow points to a labeled ZCP. We enumerated the number of cells with at least 1 positively labeled ZCP. The result in Figure 8D shows that at 24 hr, approximately 60% of cells that took up *Zymosan* particles had at least 1 ZCP that was reactive with the GFP-P4M-SidMx2 probe. Although there is a significant pool of PI(4)P in the Golgi, this probe also detects PI(4)P pools in endocytic pathway compartments, which are the most likely source of PI(4)P on ZCPs. Taken together, the results above show that PI(4,5)P₂, which is the immediate precursor for PI(3,4,5)P₃ is absent from LPVs. In contrast, PI(4)P, which is the immediate precursor for PI(3,4)P₂, is displayed on LPVs in most infected cells.

LPVs display PI(3,4)P₂

As discussed above, the PH-Akt(GFP) probe binds equally well to PI(3,4)P₂ and PI(3,4,5)P₃ (Hawkins and Stephens, 2016). In light of the observation that PI(4,5)P, the immediate precursor of PI(3,4,5)P₃, is not displayed on LPVs, it opened the possibility that it is PI(3,4)P₂ and not PI(3,4,5)P₃ that accounts for the reactivity of the PH-Akt(GFP) probe on LPVs. To investigate this issue further, the labeling of LPVs with Bruton's tyrosine kinase (Btk-PH-GFP), a more specific probe for PI(3,4,5)P₃ (Balla and Varnai, 2002) was evaluated. Transfected cells expressing Btk-PH-GFP were infected with *L. donovani* or *L. amazonensis* parasites and the recruitment of Btk-PH-GFP to LPVMs was determined.

Representative images showing the distribution of the Btk-PH-GFP probe in infected cells at the indicated times are shown (Figure 9). LPVs exhibited limited labeling with Btk-PH-GFP. Enumeration of the proportion of cells with at least 1 Btk-PH-GFP reactive LPV revealed that approximately 30% of *L. donovani* infected cells contain LPVs that are reactive with Btk-PH-GFP. The reactivity of the PH-Btk(R28C)-GFP mutant was only slightly lower than the wild type probe. These studies support the conclusion that PI(3,4,5)P3 is not a major lipid on LPVs.

PH-Akt(GFP) transfected cells are less sensitive to miltefosine

Upon binding to either PI(3,4)P2 or PI(3,4,5)P3, the PH-Akt(GFP) probe or antibodies specific for the PH domain of Akt, competitively inhibit the binding of endogenous Akt (Varnai et al, 2005, Barnett et al, 2005). This then results in inhibition of Akt activation. We reasoned that in cells transfected with PH-Akt(GFP) and infected with *Leishmania*, activation of endogenous Akt will be inhibited, which will reduce the sensitivity of those infections to miltefosine. Experiments to test this hypothesis included cells transfected with the PH-Akt mutant, PH-Akt(R25C)(GFP). Figure 10 shows that in contrast to control infected cells, where miltefosine treatment results in a substantial and significant reduction in the number of infected cells, there were fewer infected cells in PH-Akt(GFP) transfected cells. Moreover, there was no further loss of infected cells in the PH-Akt transfected cells upon treatment with miltefosine. The infection profile of PH-Akt(R25C)(GFP) transfected cells mirrors the control infected cells. Infection in cells expressing the PH-Akt mutant remained sensitive to miltefosine treatment. Together these results affirm that the binding of Akt to phosphoinositides through their PH-domain is essential for sensitivity of the infection to miltefosine treatment.

Discussion

The mechanism of action of miltefosine in leishmaniasis is still unknown. In this manuscript we present findings that support the hypothesis that in *Leishmania* infections, miltefosine inhibits Akt activation, which results in the control of *Leishmania* infections. By displaying PI(3,4)P2, LPVs can sustain Akt activation in infected cells. It is acknowledged that miltefosine can exert direct effects on *Leishmania* parasites. Those direct effects have been shown to include alteration of lipid metabolism in the parasite, induction of mitochondrial dysfunction and induction of apoptosis in amastigotes that leads to parasite death (Santa-Rita et al., 2004, Paris et al., 2004, Luque-Ortega et al., 2007, Turner et al., 2015). Most of the direct effects on parasites occur at miltefosine concentrations greater than 25KM, which are concentrations at which miltefosine toxicity to macrophages has been reported (Azzouz et al., 2014). However, the efficacy of miltefosine on *Leishmania*-infected cells has been shown to be as low as 2KM, which is consistent with observations reported in this manuscript. There are at least 3 major explanations for why distinct concentrations of miltefosine are effective on extracellular organisms as compared to intracellular parasites in infected cells: 1) the intracellular amastigotes express novel activities that are more sensitive to miltefosine, 2) miltefosine preferentially accumulates in LPVs, which results in higher intra-vacuolar concentrations that are comparable to levels to which extracellular parasites are sensitive, and 3) miltefosine primarily targets host cell molecules or processes in infected

cells that are critical for the intracellular survival of the parasite. In light of previous studies on cancer cells that showed that inhibition of Akt activation is the primary target of miltefosine and other alkylphospholipids, we evaluated the possibility that in *Leishmania* infections as well, miltefosine acts primarily by targeting host Akt activation at concentrations that are much lower than is required to kill axenic parasites.

Several studies had observed that the PI3K/Akt signaling pathway is activated in *Leishmania* infections and that inhibition of this pathway has a negative impact on the course of *Leishmania* infections (reviewed by Kima (2016)). In the current studies, we evaluated tetracycline inducible Akt1 shRNA cell lines that could be induced to knock down Akt1 levels. We showed that in established macrophage infections with either *L. donovani* or *L. amazonensis*, knock down of Akt1 levels by greater than 50% upon addition of doxycycline resulted in greater than 50% reduction in the number of infected cells. Under these conditions that limited the availability of Akt1, miltefosine lost its efficacy against *Leishmania* infections. Experiments with pharmacological inhibitors that target Akt activation as compared to inhibitors that target other signaling pathways showed that perturbation of Akt activation also diminished the efficacy of miltefosine. It is possible that metabolic processes for survival of *Leishmania* parasites are also dependent on Akt1 activation that are disrupted by miltefosine. Along these lines, in this study we considered the possibility that miltefosine's leishmanicidal effects could be through the induction of reactive oxygen species (ROS). Incubation of infected cells with the ROS scavenger NALC, did not result in any changes to established infections. However, upon co-incubation of NALC with miltefosine, the efficacy of miltefosine on both *L. donovani* and *L. amazonensis* infections was significantly reduced. These observations are in agreement with a previous study, where the effects of miltefosine on heme oxygenase were explored. Those studies found that addition of miltefosine to cultures induced ROS (Das et al., 2013). Together, these studies imply that although infection doesn't induce significant ROS, miltefosine treatment induces elevated ROS production, which contributes in the killing of *Leishmania* parasites.

Although the likelihood that Akt is the primary target of miltefosine in *Leishmania* infections has been proposed previously (Dorlo et al., 2013), we are not aware of any study that has investigated this possibility experimentally. An early event that is the rate limiting step in Akt activation is the availability on the plasma membrane of PI(3,4)P2 or PI(3,4,5)P3. Both of these phosphoinositides are displayed transiently on the plasma membrane during the phagocytic uptake of particles (Nigorikawa et al., 2015; Bohdanowicz et al., (2010)). Using phosphoinositide probes that bind to PI(3,4)P2 and/or PI(3,4,5)P3, we showed that these lipids are displayed on the LPVM. In light of the strong reactivity of the PH-Akt(GFP) probe that binds to both PI(3,4)P2 and PI(3,4,5)P3 we concluded that PI(3,4)P2 is most likely the predominant phosphoinositide that is displayed on LPVMs. This conclusion is supported by the observation that the precursor of PI(3,4)P, PI(4)P, is detected on LPVs observed by the reactivity of GFP-P4M-SidMx2 probe. In contrast the precursor of PI(3,4,5)P3, PI(4,5)P2 is not detected on LPVs. Several schemes for the biogenesis of PI(3,4)P2 have been proposed. First, dephosphorylation of PI(3,4,5)P3 by SH2-containing inositol polyphosphate 5-phosphatase can lead to the production of PI(3,4)P2 (Li et al., 2015). In light of the fact that PI(3,4,5)P3 does not appear to be abundant on the LPVM, it is unlikely that PI(3,4)P2 is generated by this scheme. Second, PI(3)P can be phosphorylated

by PIPKII to produce PI(3,4)P2 (Li et al., 2015). PI(3)P is an abundant lipid in endocytic compartments. Using the EEA1-GFP probe that binds to PI(3)P we observed no significant labeling at the LPVs at 2 and 24hr infection times (not shown). Moreover, in light of the fact that some LAMP-1 containing compartments also display PI3P, the absence of PI3P on LPVMs, which are LAMP1 positive, suggests that PI(3)P is unlikely to be the precursor of PI(3,4)P2 on LPVMs. Lastly, PI(4)P can be converted to PI(3,4)P2 by Class II PI3kinases (Hawkins and Stephens, 2016). Based on the reactivity of the GFP-P4M-SidMx2 probe with the LPVM, we confirmed that PI(4)P is displayed on LPVs, which suggests that conversion of PI(4)P to PI(3,4)P2 is the most likely scheme for its biogenesis on LPVMs.

Hammond et al., (2014) and Levin et al (2017) have recently shown that in addition to the Golgi complex, there are other PI(4)P pools in a cell. In our studies the precise organellar origin of PI(4)P was not determined. The surprising finding was made that ZCPs also display PI(4)P. But in contrast to LPVs, ZCPs exhibited limited reactivity to the PH-Akt(GFP) probe, which suggested that neither PI(3,4)P2 nor PI(3,4,5)P3 is displayed on ZCPs. This was interpreted to imply that PI(4)P on the limiting membranes of ZCPs is not converted to PI(3,4)P2. These observations bring to the forefront several questions that could be addressed in future studies. For example, is the class II PI3kinase that converts PI(4)P to PI(3,4)P2 a parasite derived enzyme? Also, what is the organellar origin of the PI(4)P on LPVs? Could it be derived from the Golgi pool? Which accessory molecules are co-trafficked with PI(4)P that promote its conversion PI(3,4)P2?

In summary, we evaluated the hypothesis that miltefosine controls *Leishmania* infections by disrupting host Akt activation. These studies affirmed the critical role that Akt plays in the survival of *Leishmania* parasites in infected cells. Through several approaches we showed that miltefosine's efficacy was dependent on the availability of Akt for activation. Relative sensitivity to miltefosine may be determined by the capacity of parasites to modulate host Akt activation. The differential capacity of parasites to activate Akt may underpin differences in parasite pathogenicity.

Material and methods

Parasites

L. amazonensis strain RAT/BA/74/LV78 (LV78) was cultivated in Schneider's Drosophila Medium (BioWhittaker) with 10% heat inactivated fetal bovine serum (FBS) and 0.2% gentamycin (Complete Schneider Medium) at 23 C. Parasite infectivity was assured by passing through mice. *L. donovani* (MHOM/S.D./62/1S-CL2D) was obtained from Nakhasi's lab and cultivated at 26 C in M199 (Sigma) containing 15% FBS, 0.1mM Adenosine, 0.1mg/ml Folic Acid, 2mM Glutamine, 25mM HEPES, 1% Penicillin/streptomycin, 1x BME Vitamins, and 1mg/ml Sodium bicarbonate in PH 6.8.

Macrophage culture

RAW264.7 macrophages were obtained from ATCC and maintain in DMEM medium with 10% FBS and 1% Penicillin/streptomycin (complete DMEM) at 37° C under 5% CO₂ atmosphere. *L. amazonensis* infected macrophages were incubated in complete DMEM at

34° C under 5% CO₂ atmosphere. *L. donovani* infected macrophages were incubated in complete DMEM at 37 C under 5% CO₂ atmosphere.

Macrophage infections

These experiments were performed as described previously (Ndjamen et al., 2010). Briefly, macrophages were plated in 100 mm Petri dishes containing sterile glass coverslips and allowed to adhere overnight in complete DMEM media at 37°C with 5% CO₂. For infections with *L. donovani*, the peanut agglutination (PNA) protocol (Sacks and Melby, 2001) was implemented to enrich for metacyclic parasites (PNA-) from stationary stage promastigote cultures. PNA- parasites were then incubated with macrophages. For *L. amazonensis* infections were initiated with mid-stationary phase promastigotes at 1:5 or 1:20 ratio for 12 hours. Coverslips were then washed and treated with the varying concentrations of miltefosine or with co-incubation with the indicated compounds as described in the experiments. Some coverslips were incubated in DMSO as vehicle control. After incubation with drugs for an additional 24 hours at 34°C, coverslips were fixed in methanol for 5 minutes prior to Giemsa staining. After methanol fixation, infected cells were stained with Wright-Giemsa stain at a 1:20 dilution for 15 minutes made fresh with NanoPure diH₂O. Cells were then washed twice with NanoPure diH₂O and allowed to dry for 1–2 hours. Coverslips were dehydrated with xylenes for 1 minute and mounted in Permount on glass slides for viewing under a bright field light microscope. The percentage of infected macrophages and the average number of parasites was determined by counting at least 200 macrophages per coverslip. Counts were done in duplicate over at least three experiments and EC₅₀ values were determined in GraphPad Prism using a three parameter dose-response best-fit curve line.

Sustained Akt activation

Macrophages in DMEM were plated in 6-well plates at 1×10⁶cells/ml overnight in 37 °C incubator with 5% CO₂. Then, macrophages were incubated with *L. amazonensis*, *L. donovani* (after PNA selection), or *Zymosan* at 1:20 ratio. Parasites or *Zymosan* were washed away 30 minutes after infection. Cell lysates were collected at 0hr, 0.25hr, 0.5hr, 1hr, 4hr, 12hr, 24hr, 48hr, 72hr, and 96hr after infections. Equivalent protein amount of each sample was analyzed in Western blot experiments. The relative density of each band was obtained relative to the density of the control band, all calculations were performed in GraphPad Prism 6. Statistical significance of difference between time points of each concentration was measured using a student's t test.

Western blot

Cells were lysed in RIPA buffer and protein concentration was measure by BCA assay (Thermo Scientific). Aliquots with equal protein amounts were suspended in SDS PAGE loading buffer and run in 140V for 1hr. Protein was transferred onto a nitrocellulose membrane (Bio-Rad) with 100V for 1hr in 4°C. Membranes were blocked with 5% milk in 1hr then incubated with primary antibody overnight in 4°C. Primary antibodies included Anti-Akt1, Anti-pAkt(S473), Anti-pAkt(T308), Anti-pGSK3b, Anti-pPTEN, GAPDH, Anti-pPDK1, from Cell Signaling, and Anti-Akt1 from Thermo Scientific. Actin was obtained from Secondary antibodies: Anti-Rabbit IgG from Novus Biologicals (Littleton, CO).

Membranes were then washed and incubated with secondary antibody for 1hr at room temperature. Membranes were washed and incubated in Western blot substrate (Thermo Scientific) for 2 min. Membranes were exposed on X-ray film.

shAkt macrophage lines

Macrophage lines expressing shAkt or controls were generated by transduction of SMARTvector Inducible Lentiviral shRNA system (Dharmacon, Lafayette CO). Custom made lentiviruses that included one of three sequences: shAkt1: ATCGGAAGTCCATCGTCTC, shAkt2: GGGACTCTCGCTGATCCAC shAkt3: CGTTTGTGCAGCCAGCCCT were developed. A control Lentiviral recombinant containing the sequence TGGTTTACATGTTGTGTGA was also used to generate a control cell line. Lentiviral particles prepared by Dharmacon were used to transduce RAW264.7 macrophages. After 24 hr, transduced macrophages were selected by growth in 6ug/ml puromycin in complete DMEM at 37° C under 5% CO₂ following protocols from Dharmacon. After at least 2 passages under antibiotic selection, each cell line was then assessed for Akt shRNA expression. The expression of Akt shRNA was induced by growth in 1uM doxycycline.

Transfections with phosphoinositide probes

The following probes GFP-P4M-SidM (Addgene plasmid # 51469); GFP-P4M-SidMx2(Addgene plasmid #51472); PH-Akt-GFP (Addgene plasmid # 51465); PH-Akt(R25C)-GFP (Addgene plasmid # 51466) PH-PLCD1-GFP (Addgene plasmid # 51407); PH-PLCD1(R40L)-GFP (Addgene plasmid # 51408); PH-Btk-GFP (Addgene plasmid # 51463); PH-Btk(R28C)-GFP (Addgene plasmid # 51464) were gifts from Tamas Balla; GFP-EEA1 wt (Addgene plasmid # 42307) was a gift from Silvia Corvera; pBGPα-CMV-GFP-OSBP PH domain (Addgene plasmid # 58840) was a gift from Tim Levine & Sean Munro. mKate2-P2A-APEX2-TAPP1-PH (Addgene Plasmids #67662) was a gift was a gift from Rob Parton. Plasmids were isolated and test digested for confirmation. They were then introduced into RAW264.7 by nucleofection using reagents from Mirus Bio (Madison, Wisconsin). After seeding transfected cells and overnight culture, they were infected. Infection of transfectants were evaluated after 2 or 24hr.

Determination of Drug EC₅₀s and promastigotes survival

To determine the EC₅₀ of miltefosine on axenic parasites, an MTT Cell Viability Assay Kit was used. Early stationary phase promastigotes were seeded at 1×10⁵ parasites/well in a 96-well tissue culture plate and allowed to grow for 24 hours at room temperature in the presence of miltefosine at concentrations ranging from 0–100μM. Parasite susceptibility to the DMSO vehicle alone was assessed by treating parasites at an equal concentration of DMSO to the highest concentration of drug used in each experiment. Promastigotes were incubated with MTT for 2 hours and formazan product was read at 570 nm wavelength and a 630 nm background wavelength as described by the Biotium protocol. Viable parasites were estimated from an MTT standard curve that was made from serial dilutions of parasites and correlation of those values to the relative amount of formazan product. Plots of % Cell Viability as compared to controls vs Log Molar Concentration were generated in GraphPad Prism 6. EC₅₀'s were calculated by non-linear regression analysis of the sigmoidal curves

that were generated. Significance of the differences in parasite growth was determined using the multiple t-tests function. Statistical significance between time points of each concentration was measured using a student's t test

Immunofluorescence Assays were as described in (Ndjamen et al., 2010). Briefly, labeled coverslips were examined and images captured using a QImaging Retiga 1300C cooled CCD camera mounted on an Olympus BX50 microscope equipped with automated filters with 100x NA 1.30 oil-immersion objective. Images were also examined on a Zeiss Confocal laser scanning microscope (Axiovert 200M inverted microscope) with LCM 5 Pascal Vario One system. Image series over a defined Z-focus range acquired on the Olympus BX50 were processed with three-dimensional deconvolution software supplied by AxioVision. An extended focus function in ImageJ was used to merge optical sections to generate the images presented in the figures. Z- series optical sections from the confocal microscope were merged in ImageJ.

Inhibitors and drugs treatment

Infected cells were treated with 1 or 2KM PDK1 inhibitor II (Calbiochem), 250nM Torin 1 (Calbiochem), 1uM AKT inhibitor IV(Calbiochem), 1–5 uM AKT inhibitor VIII(Calbiochem), 3uM CLI-095 (InvivoGen), 5uM SB202190 (Sigma) for 24hr. For Rapamycin, cells were treated with 1uM Rapamycin (Calbiochem) for 1 hour. Following inhibitor treatment, cells were treated with miltefosine [HePC] (Cayman Chemicals) for another 24 hr before fixing cells. For miltefosine inhibition of Akt activation by insulin, cells were incubated with 10KM miltefosine or water (control) prior to incubation in 1ug insulin (R&D Systems Cat.7544-MR) for 15min. Cell lysates were collected and western blots of p-Akt S473, p-Akt T308, and Akt were performed on samples. Band densities from scanned blots were measured with the ImageJ program. The relative density of each band was obtained relative to the density of the control band, all calculations were performed in GraphPad Prism 6. Statistical significance of difference between time points of each concentration was measured using a student's t test.

For miltefosine inhibition of Akt activation in infected cells, macrophages were incubated with stationary stage *L. amazonensis* promastigotes 1:10 ratio macrophages:parasites. Cultures were washed after 4 hr to remove free parasites. 10KM of miltefosine or water were added to the culture after 24 hr infection. Cell lysates were collected at after an additional of miltefosine treatment. Western blots were performed for S473 and T308 pAkt forms. The relative density of each band was obtained relative to the density of the control band, all calculations were performed in GraphPad Prism 6. Statistical significance of differences between time points of each concentration was measured using a student's t test.

Statistical analysis of results

Data analysis and the generation of graphs were performed using Microsoft Excel and GraphPad Prism 7 (La Jolla, CA). Data are presented as the mean $\pm$ standard error. Statistical significance between time points of each concentration and also between treated infected and uninfected cells was measured using the student t-test or a one-way or two-way

ANOVA (dependent on the experimental details) in GraphPad Prism 7. This was followed by either the Holm-Sidak or the Dunnett post hoc tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Miltefosine is effective on extracellular *Leishmania* and also on intracellular *Leishmania* in infected macrophages

(A). The efficacy of miltefosine on promastigote forms of *Leishmania* was measured in MTT assays. Parasites were incubated with increasing concentrations of miltefosine. Proportion of surviving parasites was analyzed by GraphPad prism and the EC₅₀ was calculated from those plots. B) RAW264.7 macrophages were infected with either *L. amazonensis* or *L. donovani* promastigotes. After 24 hr, infections were treated with indicated concentrations of miltefosine and infected cells fixed after an additional 24 hr. After Geimsa staining, infected cells were enumerated. The data is presented as the relative infection rate. C) The data from the miltefosine treatment experiments were also scored as the number of parasites per 100 cells. The data presented in this figure was from at least 4 experiments that were compiled and plotted in GraphPad.

Figure 2. Akt is activated continuously in *Leishmania* infections

After incubation of macrophages with *Leishmania* parasites or serum-opsonized Zymosan particles, lysates were prepared at indicated times and equivalent amounts of protein from each lysate was processed for Western blot analysis (A). Blots were initially probed with antibodies to T308 or with antibodies to S473. Most blots were stripped and probed for total Akt. Western blots with equal amounts of those lysates were also probed for GSK-3 β . Density of bands from at least 4 experiments were compiled. The density of bands for each experiment was normalized to the band density in resting cells. This data was compiled in GraphPad, which is where plots were generated in GraphPad. The activation of Akt in *Leishmania* infections is biphasic. A line plot generated from the data was overlaid to highlight the activation pattern of Akt. B) The progress of infections in infected cells are routinely measured. Coverslips recovered after each infection are stained with Giemsa. Then the number of parasites per infected macrophage are enumerated. The plot is of the mean number of parasites per macrophage from at least 4 experiments.

Figure 3.

Miltefosine inhibits Akt activation after hormonal treatment and also after *Leishmania* infection

A. Macrophages were pre-incubated with miltefosine or medium (con) before co-incubation with Insulin. After 15 minutes cell lysates were prepared and analyzed in Western blots. Blots were probed for T308 and S473 pAkt forms. The pAkt band densities were determined and normalized to band densities in control treated cells. The data was compiled in GraphPad. Determination of statistical significant difference between with or without miltefosine treatment was by student's t-test. B. For *Leishmania* infections, macrophages were infected with *L. amazonensis* for 24 hr. Then miltefosine or vehicle was added and incubated for an additional 24 hr. Lysates were prepared and analyzed in Western blots for T308 and S473 pAkt forms. The pAkt band densities were determined and normalized to band densities in control treated cells. The data was compiled in GraphPad. Determination of statistical significant difference between with or without miltefosine treatment was by student's t-test.

Figure 4. Inducible knock down of Akt1 reduces *Leishmania* viability in infected cells and also reduces miltefosine efficacy

The RAW264.7 macrophage line was transduced with recombinant lentiviruses expressing one of 3 shAkt1 sequences (materials and methods) and selected by growth in puromycin. The line with the shAkt1-2 sequence was incubated with doxycycline after which lysates were prepared at indicated times. A). Western blots were probed with anti-Akt1 or actin. A control mCMV cell line was analyzed in parallel experiments. Densitometric scans of bands from several experiments were compiled and plotted in GraphPad. B) Wild type cells and the shAkt1-2 line were infected with *L. donovani* and infections were scored after 48 hr. C) Infections were scored after incubation with or without doxycycline to induce Akt1 knockdown. D) Infections were incubated with doxycycline then co-incubated with increasing concentrations of miltefosine. Infections with co-incubation in miltefosine were normalized with infections with doxycycline. Survival curves from at least 3 experiments were compiled and curves plotted Graph pad 7. Statistical significance between treated infected cells and the control was measured using a one-way ANOVA with the Dunnett test for multiple comparisons.

Figure 5. Inhibition of PDK1 reduces miltefosine efficacy

PDK1 inhibitor II was added to 12 hr old infected cells. In parallel cultures, miltefosine at 5KM was co-incubated with the PDK1 inhibitor II on infected cells. Lysates were prepared after an additional 24 or 48 hr. A) Western blot of the samples were probed with antibodies to T308 followed after stripping with a pan Akt antibody. Densitometric scans of bands from at least 4 experiments were compiled. B) Proportion of *L. donovani* infected cells was estimated in cultures without or with addition of PDK1 inhibitor II addition; C) *L. donovani*-infected cells were estimated in cultures with DMSO or with PDK1 inhibitor II and co-incubation with increasing concentrations of miltefosine. Infections were normalized to the maximum infection rates that were obtained after PDK1 inhibitor II treatment. Data from at least 4 repetitions was compiled analyzed in GraphPad Prism 7, where graphs were generated. The EC₅₀ of miltefosine under these conditions was estimated from the data used to generate these curves. Significance of difference was determined by multiple t-tests. (Key: NI is non-infected resting cells; I is infected cells at 24 hr; PII (24) or (48) is infected cells incubated with PDK1 inhibitor II after indicated times; PII(24)M or PII(48)M is infected cells co-incubated with PDKI inhibitor II and miltefosine after indicated times). C) Infected cells were incubated with DMSO vehicle or pharmacological inhibitors of MAPkinase (SB202190 at 5KM) and TLR4 (CLI-095 at 3KM). After 24 hr lysates were prepared and analyzed by Western blots for reactivity to T(308) or S(473) antibodies. (D) *L. donovani*-infected cells were co-incubated with increasing concentrations of miltefosine and the above inhibitors and the proportions of infected cells were determined. Infection rates were used to plot survival curves in GraphPad and EC₅₀ values for miltefosine were calculated. Data was compiled from at least 3 experiments.

Figure 6. Inhibition of ROS reduces efficacy of miltefosine

L. donovani-infected cells were incubated with 2nM N-acteyl-L-cysteine (NALC) or with DMSO (diluent). The number of infected cells after 24 hr was enumerated (A). The killing efficacy of increasing concentrations of miltefosine co-incubated with NALC was determined (B). Data was compiled and plotted in GraphPad. The EC₅₀ of miltefosine was determined from these curves. The results were compiled from 3 experiments.

Figure 7. Reactivity of PH-Akt-GFP probe reveals that PI(3,4)P2 or PI3,4,5)P are displayed on LPVs

Representative images show the distribution of the PH-Akt-GFP probe and of LAMP1 in infected cells. Images are Zprojections at average intensities of deconvolved Z series sections. Red arrows point to LPVs that are reactive with LAMP1 but they are not reactive with the PH-Akt-GFP probe. White arrows show representative LPVs that are reactive with both probes. A line was drawn across representative LPVs and the fluorescence intensity along that line is shown in a histogram to highlight the recruitment of the probe to LPVMs. B) PH-Akt-GFP transfected cells were allowed to phagocytose *Zymosan* particles. Stars are placed next to representative parasites within phagosomes. C) The proportion of transfected and infected cells with at least one PH-Akt-GFP reactive LPV or ZCP was determined. The plot is from the means $\pm$ from at least 3 experiments.

Figure 8. PI(4,5)P₂ probed with (PLCδ)-PH-GFP and PI(4)P probed with sidM

A). Representative images show the distribution of the (PLCδ)-PH-GFP probe and of LAMP1 in RAW264.7 cells were transfected with the (PLCδ)-PH-GFP probe and infected with either *L. donovani* or *L. amazonensis* parasites. Images are Zprojections at average intensities of deconvolved Z series sections. White arrows point to representative LPVs. Red arrows point to representative LPVs that are not labeled with the (PLCδ)-PH-GFP probe. Stars are placed next to representative parasites within phagosomes. The proportion of transfected and infected cells with at least one (PLCδ)-PH-GFP reactive LPV was determined. The plot is from the means+/- from at least 3 experiments. B) Representative images show the distribution of the GFP-P4M-SidMx2 probe and of Grasp65 in RAW264.7 cells that were transfected with the GFP-P4M-SidMx2 probe and infected with either *L. donovani* or *L. amazonensis* parasites. Images are Zprojections at maximal intensities of Z series sections from a confocal microscope. In C), transfected cells were incubated with *Zymosan* particles. White arrows point to the representative LPVs or ZCPs positively labeled with the GFP-P4M-SidMx2 probe. D) The proportion of transfected and infected cells with at least one SidMx2 reactive LPV or ZCP was determined. The plot is from the means+/- from at least 3 experiments.

Figure 9. Btk-PH-GFP exhibits limited reactivity with LPVs

Representative images show the distribution of the Btk-PH-GFP probe and of LAMP1 in RAW264.7 cells that were transfected with the Btk-PH-GFP probe and infected with either *L. donovani* or *L. amazonensis* parasites. Images are Zprojections at average intensities of deconvolved Z series sections. Reactivity with DAPI was also included. White arrows point to representative LPVs and the parasites within them. We enumerated the number of Btk-PH-GFP transfected cells and infected with *L. donovani* with at least 1 positive LPV. Cells transfected with the PH-Btk(R28C)-GFP a non PI(3,4,5)P3 binding mutant of PH-Btk were also infected and cells with at least 1 positively labeled LPV were enumerated. A plot generated in GraphPad is shown.

Figure 10. PH-Akt(GFP) transfected cells are less sensitive to miltefosine

RAW264.7 macrophages were transfected with PH-Akt(GFP) or with PH-Akt(R25C)(GFP) plasmids. They were infected on coverslips with *L. amazonensis* promastigotes for 24 hrs before incubation with 10 KM miltefosine for an additional 24 hrs. The coverslips were processed for fluorescence microscope evaluation by mounting on slides and DAPI stained. Infected cells that express PH-AKT(GFP) or the mutant were enumerated. Non-transfected cells were processed similarly and served as the infection control. The data from three experiments was analyzed in GraphPad. Determination of statistical significant difference between with or without miltefosine treatment was by student's t-test.