Miltefosine increases macrophage cholesterol release and inhibits NLRP3-inflammasome assembly and IL-1β release

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Miltefosine is an FDA approved oral drug for treating cutaneous and visceral leishmaniasis. Leishmania is a flagellated protozoa, which infects and differentiates in macrophages. Here, we studied the effects of Miltefosine on macrophage’s lipid homeostasis, autophagy, and NLRP3 inflammasome assembly/activity. Miltefosine treatment conferred multiple effects on macrophage lipid homeostasis leading to increased cholesterol release from cells, increased lipid-raft disruption, decreased phosphatidylserine (PS) flip from the cell-surface, and redistribution of phosphatidylinositol 4,5-bisphosphate (PIP2) from the plasma membrane to actin rich regions in the cells. Enhanced basal autophagy, lipophagy and mitophagy was observed in cells treated with Miltefosine vs. control. Miltefosine treated cells showed marked increased in phosphorylation of kinases involved in autophagy induction such as; Adenosine monophosphate-activated protein kinase (AMPK) and Unc-51 like autophagy activating kinase (ULK1). The Toll like receptor (TLR) signaling pathway was blunted by Miltefosine treatment, resulting in decreased TLR4 recruitment to cell-surface and ~75% reduction in LPS induced pro-IL-1β mRNA levels. Miltefosine reduced endotoxin-mediated mitochondrial reactive oxygen species and protected the mitochondrial membrane potential. Miltefosine treatment induced mitophagy and dampened NLRP3 inflammasome assembly. Collectively, our data shows that Miltefosine induced ABCA1 mediated cholesterol release, induced AMPK phosphorylation and mitophagy, while damping NLRP3 inflammasome assembly and IL-1β release.

Atherosclerosis, a sterile inflammatory disease, is the major cause of coronary artery disease (CAD). LDL cholesterol drives atherosclerosis by depositing LDL into the arterial intima, where it can be modified to induce endothelial cell activation and the recruitment of leukocytes; and, the uptake of modified LDL into macrophages leads to foam cell formation and a further amplification of inflammation. Accumulation of oxidized lipids and cholesterol crystals in plaques activate toll-like receptor (TLR) pathways and the assembly of the NLRP3 inflammasome. The NLRP3 inflammasome plays a key role in processing procaspase 1 resulting in subsequent caspase 1 mediated processing of pro IL-1β to generate active interleukin-1β (IL-1β). The role of IL-1β in promoting human CAD was highlighted by the recently concluded CANTOS trial, showing that anti-IL-1β therapy met the primary endpoint, a reduction in a composite of heart attack, stroke and cardiovascular death.

In contrast to atherogenic pathways, the atheroprotective pathways such as autophagy and cholesterol efflux become increasingly dysfunctional in aging, advanced atherosclerotic plaques, and in animal models of atherosclerosis and diabetes. Thus, the simultaneous induction of atheroprotective pathways, along with dampening of atherogenic pathways may serve as a potent therapeutic treatment for CAD patients. Here, we report that Miltefosine, an FDA approved drug for treating visceral and cutaneous leishmaniasis, promoted cholesterol release, disrupted lipid-rafts and TLR4 signaling, increased cell-surface phosphatidylserine (PS) exposure, induced phosphatidylinositol 4,5-bisphosphate (PIP2) trafficking from plasma membrane (PM) to the cell interior. Miltefosine treated cells exhibited increased phosphorylation of autophagy inducing kinases AMPK1 and ULK1, leading to increased basal autophagy, lipophagy and mitophagy. The lipopolysaccharide (LPS) induced

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mitochondrial reactive oxygen species (ROS) generation and NLRP3 inflammasome assembly was blunted in cells pretreated with Miltefosine, leading to decreased caspase 1 cleavage and mature IL-1β release. The structure of Miltefosine is similar to lyso-PC and it is known to integrate in the cell membrane and redistribute in intracellular membranes of ER, Golgi and mitochondria. It is widely believed that most of the downstream effects of Miltefosine are dependent on cell-type, with a range of activities such as anticancer, antimicrobial, effects on cholesterol homeostasis, and inhibition of Mast cell activation. The detailed investigation of mechanisms involved in Miltefosine’s action may lead to novel therapeutic targets for preventing and treating cardiovascular and inflammatory metabolic diseases.

Results
Miltefosine induced ABCA1 mediated cholesterol release from cells. Previous study have shown that Miltefosine and other alkylphospholipids induced cholesterol efflux from the HepG2 cells. We tested effect of Miltefosine treatment on ABCA1 mediated cholesterol release in RAW264.7 macrophages. Control and ABCA1 expressing cells were incubated with different doses of Miltefosine for 4 h and cholesterol release to serum-free media in the absence of acceptor containing either vehicle or 7.5 μM Miltefosine. Values are % cholesterol release mean ± SD, N = 5, different letters above the bars show p < 0.01 by ANOVA Bonferroni posttest, with separate analyses for ± ABCA1 induction. (B) Western blot analysis of 8Br-cAMP induced RAW264.7 cell total and cell-surface ABCA1 ± 7.5 μM Miltefosine treatment for 4 h. (C) Cholesterol release (4 h at 37 °C) from ABCA1 stably transfected and control HEK293 to serum-free DMEM without addition of acceptor containing either vehicle or 7.5 μM Miltefosine. Values are % cholesterol release mean ± SD, N = 3–5, different letters above the bars show p < 0.01 by ANOVA Bonferroni posttest, with separate analyses for ± ABCA1 induction. (D) Cholesterol release (4 h at 37 °C) from ABCA1 and ABCA1 (W590S, C1477R) double mutant (DM) stably transfected HEK293 to serum-free DMEM ± 7.5 μM Miltefosine. Values are % cholesterol release mean ± SD, N = 3, different letters above the bars show p < 0.01 by ANOVA Bonferroni posttest.
levels (Fig. 1B), instead a trend toward decreased ABCA1 levels was observed in Miltefosine treated cells. This data indicated that the Miltefosine mediated increase in cholesterol release was not due to increased ABCA1 levels. Further studies used 7.5 μM or less of Miltefosine, as these doses did not lead to cell death in RAW264.7 macrophages or in mouse bone–marrow derived macrophages (BMDMs) (Fig. S1A,B). We observed the Miltefosine induction of cholesterol release in three additional cell lines, HEK293, BHK cells and THP-1 macrophages. In all cases, addition of Miltefosine induced basal cholesterol release, and markedly induced ABCA1-mediated cholesterol release to the acceptor-free media (Figs 1C, S1C,D). Addition of cholesterol acceptor apoA1 to Miltefosine treated cells did not lead to further significant increase in cholesterol release (Fig. S1D–F). To test if the lipid flip-pase activity (outward translocation across the plasma membrane) of ABCA1 is required for increased cholesterol release in Miltefosine treated cells, we used HEK293 cells stably expressing WT-ABCA1 isoform or a Tangier disease double mutant W590S-C1477R-ABCA1 isoform. The WT ABCA1 can flop phosphatidylserine (PS) and phosphatidylcholine 4.5-bisphosphate (PIP2) across plasma membrane15. The W590S mutation in ABCA1 decreases PS flop16 while C1477R decreases PIP2 flop16. The W590S-C1477R double mutant (DM) isoform of ABCA1, with protein expression levels slightly higher than WT ABCA1 (Fig. S1G), showed significantly lower cholesterol release to acceptor-free media compared to WT-ABCA1 (Fig. 1D), indicating that lipid flip-pase activity of ABCA1 promotes the Miltefosine induced cholesterol release to media. It has previously been shown that ABCA1 expression promotes membrane blebbing and the release of microparticles17,18. Microparticles are generated by ABCA1 activity, but unlike nascent HDL particles, the microparticles are formed even in the absence of apoA117. We determined the effect of Miltefosine on generation of microparticles by cells. The media from RAW264.7 macrophages incubated ± Miltefosine for 4 h was collected and analyzed by a small particle tracking analyzer. The media from the cells treated with Miltefosine had significant 2-fold more microparticles compared to control cells (P < 0.001, Fig. S2A). These data indicated that Miltefosine mediated remodeling of the plasma membrane promotes generation of microparticles leading to cellular cholesterol release to the media, which was even greater in the presence of ABCA1 expression.

Miltefosine disrupts lipid rafts and increased PS exposure by inhibiting PS flip. Lipid rafts are comprised mainly of PC, cholesterol and sphingomyelin20, and play a role in AKT and TLR signaling pathways. Disruption of lipid-rafts by ABCA1 is proposed to provide a free cholesterol pool for efflux and hamper TLR signaling via reduced recruitment of TLR2/4 to lipid-rafts21–23. Previous studies have also shown that Miltefosine uses lipid-rafts as entry portals to cells24 and can act as a lipid-raft disrupting agent to inhibit human mast cell activation13. We determined the status of AKT phosphorylation in RAW264.7 macrophages and found that Miltefosine potently inhibited basal AKT phosphorylation (Fig. S2B,C). The lipid-rafts in Miltefosine treated RAW264.7 macrophages were probed by staining for the ganglioside GM1 using Alexa647-labeled cholera toxin B, followed by either fluorescent microscopy or flow cytometry. As shown in Fig. 2A cells treated with 7.5 μM Miltefosine for 16 h displayed less GM1 vs. control cells. Flow cytometry quantification showed ~26% GM1 decrease in Miltefosine treated vs. control cells (P < 0.001, Fig. 2B). These data indicated that the Miltefosine mediated increase in cholesterol release was not due to increased ABCA1 levels (Fig. 1B), instead a trend toward decreased ABCA1 levels was observed in Miltefosine treated cells. This data indicated that the Miltefosine mediated increase in cholesterol release was not due to increased ABCA1 levels. Further studies used 7.5 μM or less of Miltefosine, as these doses did not lead to cell death in RAW264.7 macrophages or in mouse bone–marrow derived macrophages (BMDMs) (Fig. S1A,B). We observed the Miltefosine induction of cholesterol release in three additional cell lines, HEK293, BHK cells and THP-1 macrophages. In all cases, addition of Miltefosine induced basal cholesterol release, and markedly induced ABCA1-mediated cholesterol release to the acceptor-free media (Figs 1C, S1C,D). Addition of cholesterol acceptor apoA1 to Miltefosine treated cells did not lead to further significant increase in cholesterol release (Fig. S1D–F). To test if the lipid flip-pase activity (outward translocation across the plasma membrane) of ABCA1 is required for increased cholesterol release in Miltefosine treated cells, we used HEK293 cells stably expressing WT-ABCA1 isoform or a Tangier disease double mutant W590S-C1477R-ABCA1 isoform. The WT ABCA1 can flop phosphatidylserine (PS) and phosphatidylcholine 4.5-bisphosphate (PIP2) across plasma membrane15. The W590S mutation in ABCA1 decreases PS flop16 while C1477R decreases PIP2 flop16. The W590S-C1477R double mutant (DM) isoform of ABCA1, with protein expression levels slightly higher than WT ABCA1 (Fig. S1G), showed significantly lower cholesterol release to acceptor-free media compared to WT-ABCA1 (Fig. 1D), indicating that lipid flip-pase activity of ABCA1 promotes the Miltefosine induced cholesterol release to media. It has previously been shown that ABCA1 expression promotes membrane blebbing and the release of microparticles17,18. Microparticles are generated by ABCA1 activity, but unlike nascent HDL particles, the microparticles are formed even in the absence of apoA117. We determined the effect of Miltefosine on generation of microparticles by cells. The media from RAW264.7 macrophages incubated ± Miltefosine for 4 h was collected and analyzed by a small particle tracking analyzer. The media from the cells treated with Miltefosine had significant 2-fold more microparticles compared to control cells (P < 0.001, Fig. S2A). These data indicated that Miltefosine mediated remodeling of the plasma membrane promotes generation of microparticles leading to cellular cholesterol release to the media, which was even greater in the presence of ABCA1 expression.

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PIP2 is redistributed from plasma membrane upon Miltefosine treatment. Similar to PS, phosphatidylcholine 4, 5-bisphosphate (PIP2) is also sequestered in the inner leaflet of the plasma membrane. To determine the effect of Miltefosine on PIP2 trafficking and localization, RAW264.7 cells stably transfected with a PIP2 reporter plasmid containing 2 copies of the pleckstrin homology domain from phospholipase C fused to GFP (2X-PH-PLC-cGFP) were treated with ± 7.5 μM Miltefosine for 16 h and PIP2 reporter localization was determined by fluorescent microscopy. As shown in Fig. 3A, control cells showed uniform localization of the PIP2 reporter at the plasma membrane while the cells treated with Miltefosine showed an additional localization of the reporter in a specific actin rich region of the cytoplasm in both fixed and live cells (Figs 3B,C, S3).

Miltefosine induced basal and lipid-droplet autophagy. Autophagy plays a protective role against atherosclerosis26 and becomes increasingly impaired in advanced human plaques7. Autophagy is required for hydrolyzing macrophage cholesterol ester stored in lipid droplets to generate free cholesterol that can be effluxed from cells27. As Miltefosine increases cholesterol release, we tested if Miltefosine induced autophagy. We observed that the RAW264.7 macrophages treated with 7.5 μM Miltefosine for 16 h showed multiple p62 puncta...
in cytoplasm, while control cells had evenly distributed weak cytoplasmic signal with almost no p62 puncta signal (Fig. 4A, upper panel). Miltefosine treatment also increased LC3 puncta formation in macrophages transfected with LC3-GFP plasmid (Fig. 4A lower panel). Next, we evaluated whether the LC3 puncta formation was due to increased autophagic initiation or due to decreased autophagic flux. Decreased or blocked autophagy flux can also lead to accumulation of autophagic markers. If western blot analysis shows increased LC3-II after chloroquine (an autophagy flux inhibitor) treatment, then autophagic flux is occurring. We found that the LC3-II protein levels were increased by 3.5-fold in Miltefosine treatment alone vs. control cells (Figs 4B,C, S4), while there was an additional 1.8 fold increase in LC3-II protein levels with Miltefosine + chloroquine treatment compared to cells treated with Miltefosine alone (Fig. 4C). These data indicated that Miltefosine increased autophagic flux. Next, we tested the effect of Miltefosine on generation of free cholesterol from cholesterol ester rich lipid droplets. RAW264.7 macrophages were loaded with 100 μg/ml acetylated LDL (AcLDL) for 24 h followed by a 4 h chase with an acyl-co A cholesterol O-acyltransferase (ACAT) inhibitor (ACATi) to prevent cholesterol re-esterification and free cholesterol levels were determined by enzymatic assay described previously. As expected, the AcLDL loaded cells had a higher ratio of cholesterol ester to free cholesterol. Miltefosine treatment led to decreased CE:FC ratio, indicating that Miltefosine treated cell had higher free cholesterol and less cholesterol esters as compared to control cell (P < 0.05 vs. control) (Fig. 4D). The cells chased with ACATi had reduced CE:FC ratio (P < 0.001 vs. control), while the Miltefosine + ACATi treated cells showed further lowering of CE:FC ratio (p < 0.001 vs. control) (Fig. 4D). We also tested the effect of Miltefosine on lipid-droplets by

Figure 2. Miltefosine disrupts lipid-rafts and inhibits PS flip across plasma membrane. (A) GM1 levels assessed by binding of cholera toxin B (CTB) in live RAW macrophages ±7.5 μM Miltefosine for 16 h at 37°C. (B) Flow cytometry quantification of CTB binding of RAW cells treated ±7.5 μM Miltefosine for 16 h at 37°C. Values are the mean ± SD of the median fluorescence from 3 independent wells (**p < 0.01 by two-tailed t-test). (C) RAW macrophages were incubated with or without 8Br-cAMP to induce ABCA1 and ±7.5 μM Miltefosine for 16 hrs. PS exposure was determined Annexin V binding via flow cytometry (different letters above the bars show p < 0.01 by ANOVA Bonferroni posttest). (D) Cells were pretreated ±7.5 μM Miltefosine and incubated with 25 μM NBD-PS at RT for 15 min to assess cellular association of PS. (E) Quantification of NBD-PS translocated inside the cells. RAW macrophages were pretreated ±7.5 μM Miltefosine and incubated with 25 μM NBD-PS at 37°C for 15 min in phen’red free DMEM. The cells were subjected to flow cytometry analysis ± sodium dithionite to quench extracellular NBD fluorescence, yielding only intracellular NBD fluorescence (mean ± SD % NBD-PS translocated into the cells; N = 3; ***p < 0.001 by two-tailed t-test).
loading RAW264.7 cells with 100 μg/ml AcLDL for 24 h, followed by a 4 h chase with ± Miltefosine and staining with Nile-red dye. Cells treated with Miltefosine showed reduced Nile-red staining as compared to control cells (Fig. S4D). To quantify the lipid-droplets, AcLDL loaded cells were chased for 4 h with ACATi ± Miltefosine and subjected to flow-cytometry analysis. A shown in Fig. 4E, the cells chased with ACATi showed decreased lipid droplets that were further decreased by Miltefosine (n = 4, p < 0.0001). These data indicate that Miltefosine induced lipid-droplet autophagy as ACATi prevents new CE formation.

Autophagy induction is promoted by energy sensing Adenosine monophosphate-activated protein kinase (AMPK), while mammalian target of rapamycin (mTOR) acts to inhibit autophagy. The AMPK substrate ‘Unc-51 like autophagy activating kinase (ULK1)’ is also phosphorylated by AMPK during autophagy and both AMPK1 and ULK1 have been shown to be essential for mitophagy and cell survival during starvation27–29. In order to determine the mechanism of autophagy induction by Miltefosine, we tested the phosphorylation status of AMPK and ULK1 and found that the phosphorylation levels of both AMPK and ULK1 were significantly higher in Miltefosine treated cells vs. control cells (Figs 4F, G, S4E, F). These data indicated that Miltefosine induced autophagy and did not block cellular autophagy flux.

**Miltefosine decreased LPS mediated induction in IL-1β levels.** We demonstrated in Fig. 2A that Miltefosine reduces lipid-rafts. The lipid rafts have been shown to play an essential role in TLR2/4 signaling pathway and atherosclerosis progression31,22,30–32. We tested if LPS mediated recruitment of TLR4 to the cell surface is impaired in Miltefosine treated cells. The BMDMs ± pretreatment with Miltefosine for 16 h were analyzed for TLR4 cell-surface recruitment by flow cytometry. As expected, the LPS treated control cells showed increased binding of TLR4 antibody, indicating robust TLR4 recruitment to the cell-surface. In contrast, the cells pretreated with Miltefosine showed significantly reduced TLR4 recruitment to cell-surface (Figs 5A, S5A, B). Next, we tested if LPS mediated induction of pro IL-1β mRNA was affected by Miltefosine. Bone-marrow derived macrophages
Miltefosine inhibited NLRP3 inflammasome assembly and IL-1β release. Cholesterol crystals can activate NLRP3 inflammasome and promote atherosclerosis. Cholesterol crystals and other treatments including extracellular ATP can promote NLRP3 inflammasome assembly, which processes pro-IL-1β to its mature form for secretion from cells. We tested if Miltefosine inhibited NLRP3 inflammasome assembly. The BMDMs pretreated with ±5 μM Miltefosine for 16 h, were primed with LPS for 4 h and incubated with ATP for 20 min, followed by probing with anti-ASC antibody for visualization of NLRP3 inflammasome assembly. BMDMs treated with LPS and ATP formed ASC specks in 41% cells (n = 308 cells analyzed) while in BMDMs pretreated with Miltefosine only 12% of cells showed ASC specks (523 cells analyzed, p < 0.001 by two-tailed Fisher’s exact test) (Figs 6A, S6, S7). Miltefosine treatment led to markedly reduced release of cleaved caspase-1 in media and ~75% decrease in release of mature IL-1β to media as compared to control macrophages (n = 3, p < 0.0039) (Figs 6B, S8A). To decipher the mechanism by which Miltefosine inhibited NLRP3 inflammasome assembly, we determined the mRNA and protein levels of the constituents of the NLRP3 inflammasome and one substrate Gasdermin D (Gsdmd) in BMDMs with or without LPS priming. Miltefosine with LPS actually slightly increased the mRNA levels, beyond the effects of LPS alone, for NLRP3, procaspase 1 and Gasdermin D (Fig. 6C). In agreement with earlier published studies, western blot analysis showed robust induction in protein levels of NLRP3 upon LPS treatment, which was not reduced by Miltefosine treatment (Fig. S8B). Miltefosine did not appreciably alter the protein levels of ASC or procaspase 1 (Fig. S8C, D). To test if Miltefosine specifically inhibited NLRP3 inflammasome assembly and IL-1β release, we determined the effects of Miltefosine on the AIM2 inflammasome. The control
and Miltefosine treated macrophages were primed with LPS (1 mg/ml) for 4hrs followed by lipofectamine mediated transfection of poly (dA:dT) for 3 h. As shown in Fig. 6D, there was no difference in levels of AIM2 induced IL-1β released from cells ± Miltefosine treatment (n = 3, n.s.); thus, Miltefosine did not alter AIM2 inflammasome activity. The release of mature IL-1β from cells is regulated by Gasdermin D mediated pore formation on the plasma membrane35,36 and previous studies have shown that the deficiency of ABCA1/ABCG1 increases inflammation in macrophages while ABCA1 mediated cholesterol efflux in dendritic cells dampens inflammasome assembly37,38. Though, we used unloaded BMDMs for these studies, there is a possibility that Miltefosine inhibits NLRP3 inflammasome assembly via cholesterol depletion. To directly test the role of cholesterol depletion on NLRP3 inflammasome assembly, we treated BMDMs with 5 μM Miltefosine for 16 h or with 1 mM cyclodextrin for 45 minutes at 37 °C, followed by LPS/ATP incubation. Although cholesterol levels in BMDMs treated with cyclodextrin were significantly lower compared to Miltefosine treated cells (n = 3, p < 0.01, Fig. 6E), Miltefosine was much more effective than cyclodextrin in inhibiting inflammasome activity as assayed by IL-1β release (Fig. 6F) and ASC speck formation (Figs S6, S9). Thus, NLRP3 inflammasome inhibition by Miltefosine cannot be attributed solely to cholesterol depletion.

Miltefosine reduced endotoxin mediated mitochondrial ROS production and loss of mitochondrial membrane potential. Mitochondrial ROS drives NLRP3 inflammasome assembly39 and mitochondrial cardiolipin provides a docking site for NLRP3 protein binding and inflammasome assembly40. As shown in Fig. 7A, LPS treatment induced ROS production in control BMDMs as evident by increased staining with MitoSox Red while the Miltefosine pretreated cells showed decreased MitoSox staining upon LPS treatment. To quantify the MitoSox signal, we performed flow-cytometry and found that Miltefosine treated cells showed a significant 43% reduction in LPS-induced MitoSox staining (Fig. 7B). Next, we tested the effect of Miltefosine on the mitochondrial membrane potential. Control and Miltefosine pretreated BMDMs were incubated with ± LPS for 1 h and stained with tetramethylrhodamine (TMRM) for 30 min at 37 °C, followed by fluorescent microscopy of live cells. The cells treated with LPS showed marked reduction in TMRM staining while cells pretreated with Miltefosine partially rescued the mitochondrial membrane potential (Fig. S10). To quantify the TMRM signal, we performed flow-cytometry and found that LPS treated cells showed a significant 41% reduction in TMRM staining signal while cells pretreated with Miltefosine showed only a 24% reduction (Fig. 7C). Given that Miltefosine induced autophagy in macrophages, we tested role of Miltefosine in removing damaged mitochondria from via

![Figure 5](https://example.com/figure5.png)
mitophagy. Live RAW264.7 macrophages were loaded with 500 nM mitophagy indicator dye for 30 min at 37 °C, followed by treatment with ±Miltefosine for 4 h. The control cells showed weak fluorescence while cells treated with Miltefosine showed bright fluorescence (Fig. S11). To determine if the mitochondria were indeed fused with lysosomes, a lysosomal stain was used. As compared to control cells, the cells treated with Miltefosine showed markedly increased co-localization of the mitophagy and lysosome dyes (Fig. 7D). Thus, Miltefosine may inhibit inflammasome assembly by decreasing mitochondria ROS, preventing LPS mediated damage to mitochondria, and by removing damaged mitochondria through mitophagy.

Discussion

We found that Miltefosine has multiple activities in macrophages. Cholesterol release in the absence of an exogenous acceptor has been shown to be due to microparticle generation that is increased by ABCA1 expression17, and Miltefosine effects on the plasma membrane may alter membrane fluidity to increase microparticle generation. The Miltefosine increase in ABCA1-mediated cholesterol release requires functional lipid floppase activity as the ABCA1 mutant isoform defective in PS and PIP2 floppase activity showed lower cholesterol release to media in
presence of Miltefosine. Addition of apoA1 to ABCA1 expressing cells in presence of Miltefosine did not showing additive effect, indicating that apoA1 may compete with pathways that are effluxing cholesterol in apoA1 independent manner. In addition Miltefosine delocalize PIP2 and we have shown before that apoA1 needs PIP2 for binding to cell-surface, thus apoA1 may have restricted access to ABCA1 in Miltefosine treated cells15. Thus, the Miltefosine mediated increase in cell surface PS and the disruption of lipid-rafts may lead to a redistribution of lipids to promote microparticle release.

PIP2 plays an important role in membrane ruffling, microvilli formation, endocytosis, phagocytosis and the attachment of membrane to cytoskeleton41. Miltefosine was initially identified as anti-cancer compound and is known to inhibit AKT signaling pathway10. Alkylphosphocholines such as Miltefosine are proposed to prevent plasma membrane recruitment of the PH domain of AKT by disrupting plasma membrane microdomains42. We speculate that Miltefosine effects on PIP2 localization could also play a role in AKT inhibition as reduced localization of PIP2 at plasma membrane by Miltefosine can deplete PIP3 pool at plasma membrane and reduce AKT signaling.

Autophagy plays a protective role in atherosclerosis and it is impaired in advanced human plaques7,8,43. Autophagy is controlled by multiple pathways responding to stimuli such as the status of cellular energy (AMP-dependent protein kinase, AMPK) or amino acid availability (target of rapamycin, TOR). Miltefosine induced basal autophagy in macrophages as evident by increased cytoplasmic p62 and LC3-GFP puncta. We showed that Miltefosine treatment increased the turnover of cholesterol esters in cholesterol loaded cells, supporting the notion that Miltefosine can deplete PIP3 pool at plasma membrane and reduce AKT signaling.

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regulated differently at posttranscriptional level. The NLRP3 protein is stabilized via deubiquitination, similarly there is possibility of NLRP3 mRNA is being stabilized while IL-1β mRNA is not. In addition, the synthesis of NLRP3 mRNA and IL-1β mRNAs, while both under control of NF-kB, may require different nuclear factors for transcription and Miltefosine treatment may affect these factors differentially. Thus, the detailed mechanism for the TLR-mediated induction of these two genes may differ.

The levels of NLRP3 inflammasome components were unaltered in control vs. Miltefosine treated cells. The intriguing question raised by these data is that if all inflammasome components are present and more or less equally expressed in control vs. Miltefosine treated macrophages, why is the NLRP3 inflammasome not assembled in Miltefosine treated cells? Recently it was shown that NLRP3 is localized to the trans golgi network via binding to PLP2 prior to inflammasome assembly. Miltefosine may work at this or other steps to block inflammasome assembly. Alternatively, Miltefosine may diminish K+ efflux which is required for activation of inflammasome or alter NLRP3 binding proteins such as NIMA-related kinase 7 (NEK7) that acts downstream of potassium efflux to regulate NLRP3 oligomerization and activation. Another important factor regulating inflammasome function is mitochondria. Mitochondrial dysfunction or oxidized mitochondrial DNA fragments can also serve as signal for NLRP3 inflammasome activation. Similar to our work, a recent paper from Karin lab showed inhibiting choline uptake leads to activation of AMPK and mitophagy and dampening of NLRP3 inflammasome activity and IL-1β release. Miltefosine, a phosphocholine analogue can inhibit phosphatidylcholine (PC) biosynthesis, thus Miltefosine may be activating AMPK via PC depletion, leading to induced mitophagy and NLRP3 inflammasome inhibition.

Material and Methods
A detailed description of the materials and methods used in this study is provided in SI Materials and Methods. Basic methods are summarized below.

Cholesterol release assay. Cholesterol release assays were performed in HEK293-ABCA1-GFP cells, BHK cells, or RAW264.7 murine macrophages as described earlier. Other details are described in SI Materials and Methods.

Lipid-Raft quantification. The lipid-raft were visualized using Alexa647-labeled cholera toxin B subunit and fluorescent microscopy, with quantification by flow-cytometry assay as described before. Other details are described in SI Materials and Methods.

Total/FC measurements. RAW264.7 cells were loaded with 100 µg/ml AcLDL for 16 h at 37 °C with or without 2 µg/ml ACA/TSi (Sandoz 53–035, Sigma) treatment for 2 h. The total cholesterol and free cholesterol levels were determined by using enzymatic assay as described earlier.

Cell-surface PS and NBD-PS translocation. Cell-surface PS and translocation of NBD-PS were described earlier. Other details are described in SI Materials and Methods.

Western blotting. Details are described in SI Materials and Methods. Total and cell-surface ABCA1 levels were determined as described earlier.

PIP2 cellular reporter assay. RAW264.7 macrophage cell lines stably transfected with 2PH-PLCδ-GFP plasmid was described earlier.

Significance statement. Atherosclerosis is driven by cholesterol accumulation and inflammation, and the arterial macrophage is a key cell type in both of these processes. The macrophage characteristics that protect against atherosclerosis include increased cholesterol efflux/reverse cholesterol transport, increased autophagy, and decreased inflammatory cytokine production and signaling. Here, we show that one single orally available compound, Miltefosine, can target multiple macrophage pathways involved in lipid homeostasis and inflammation. Miltefosine activated cholesterol release and autophagy while inhibiting pro IL-1β gene expression and NLRP3 inflammasome assembly. Miltefosine activated AMPK signaling pathway and mitophagy, leading to reduced NLRP3 inflammasome assembly and IL-1β release.

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Author Contributions
K.G. conceived, designed, performed and directed research. A.J.I., H.L. and J.H. performed research. A.J.I, H.L., J.H., H.A., J.D.S. and K.G. analyzed data. A.J.I., J.D.S. and K.G. drafted the manuscript. All authors critically reviewed the manuscript.

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Competing Interests: A patent application related to this work has been filed by the Cleveland Clinic that lists K.G. and J.D.S. as inventors. Authors declare no non-financial competing interests.

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