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Platelet-Activating Factor Receptor Contributes to Antileishmanial Function of Miltefosine

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Miltefosine [hexadecylphosphocholine (HPC)] is the only orally bioavailable drug for the disease visceral leishmaniasis, which is caused by the protozoan parasite Leishmania donovani. Although miltefosine has direct leishmanicidal effects, evidence is mounting for its immune system-dependent effects. The mechanism of such indirect antileishmanial effects of miltefosine remains to be discovered. As platelet-activating factor and HPC share structural semblances and both induce killing of intracellular Leishmania, we surmised that platelet-activating factor (PAF) receptor had a significant role in the antileishmanial function of miltefosine. The proposition was supported by molecular dynamic simulation of HPC docking into PAF receptor and by comparison of its leishmanicidal function on PAF receptor–deficient macrophages and mice under HPC treatment. We observed that compared with wild-type macrophages, the PAF receptor–deficient macrophages showed 1) reduced binding of a fluorescent analog of HPC, 2) decreased TNF-α production, and 3) lower miltefosine-induced killing of L. donovani. Miltefosine exhibited significantly compromised leishmanicidal function in PAF receptor–deficient mice. An anti-PAF receptor Ab led to a significant decrease in miltefosine-induced intracellular Leishmania killing and IFN-γ production in a macrophage–T cell coculture system. These results indicate significant roles for PAF receptor in the leishmanicidal activity of HPC. The findings open new avenues for a more rational understanding of the mechanism of action of this drug as well as for improved therapeutic strategies. The Journal of Immunology, 2015, 194: 000–000.

Visceral leishmaniasis (VL), a fatal disease with an annual worldwide incidence of 0.5 million new cases (1), is caused by infection with the protozoan species Leishmania infantum, L. chagassi, and L. donovani, the last-named with the highest relevance in terms of mortality and number of patients. Treatment with antimonial drugs has declined rapidly with increased resistance of the parasite. One alternative is liposomal amphotericin B, which requires parenteral administration and is prohibitively expensive (2). So, a new orally bioavailable antileishmanial drug was a pressing need, when miltefosine [hexadecylphosphocholine (HPC)], an alkyl-phosphocholine originally developed as an antitumor drug (3), was proved to be an effective antileishmanial agent in clinics (4). Miltefosine is reported to trigger apoptosis-like effects (5), lipid-dependent cell signaling in cancer cells (6), and impaired phospholipid synthesis in the parasite (7), as well as acting as an immunomodulator, promoting macrophage activation (8–11). Together with our previous report on the host cell signaling–dependent antileishmanial activity of miltefosine (12), these observations imply that miltefosine may work through a cell surface receptor.

HPC shared important structural features with platelet-activating factor (PAF), such as a single long fatty acyl chain and a phosphocholine polar head. Although PAF enhanced killing of L. amazonensis, some of its antagonists inhibited the parasite clearance in mouse macrophages (13–15). Accordingly, PAF receptor (PAF receptor) is perhaps the most feasible candidate to serve as an HPC receptor. Indeed, PAF receptor–deficient mice were more susceptible to L. amazonensis infection and showed a delayed IFN-γ production and higher arginase activity, suggesting an essential role for PAF receptor signaling in the control of L. amazonensis infection in mice (16). Thus, consistent with its structural similarities to PAF, our hypothesis was that HPC had at least partial requirement for PAF receptor to exert its leishmanicidal function through immunomodulation of macrophages, the obligate host cell for Leishmania. Therefore, we examined whether PAF receptor blockade or PAF receptor deficiency reduced the antileishmanial functions of miltefosine.

Our observations showed that an anti-PAF receptor Ab significantly reduced the miltefosine-triggered Th1 response, TNF-α production, and parasite killing in macrophages. In corroborration of these observations, miltefosine-induced L. donovani clearance was significantly impaired in PAF receptor–deficient macrophages and mice. Thus, PAF receptor contributes to miltefosine-induced antileishmanial functions. The PAF receptor–dependent component of HPC perhaps accounts for the heightened inflammation observed in miltefosine-treated mice and patients (17, 18). Altogether, involvement of PAF receptor in the miltefosine-induced

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Abbreviations used in this article: CSA, crude soluble Ag; HPC, hexadecylphosphocholine; IM, infected macrophage; KO, knockout; MD, molecular dynamic; PAF, platelet-activating factor; UIM, uninfected macrophage; VL, visceral leishmaniasis; WT, wild-type.

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elimination of intracellular parasites is a novel finding that stressesthe dual role of miltefosine as a direct antiparasitic effector and as an immunomodulator that may expand its range of activities on intracellular pathogens.

Materials and Methods

Animals, parasite infection, and reagents
BALB/c mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred in the Institute’s (National Centre for Cell Science) experimental animal facility in individually ventilated cages (Thoren Caging Systems, Hazleton, PA). PAF receptor–deficient mice (19) on the BALB/c background were a kind gift from Prof. Mauro Martins Teixeira (Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil) and were reared under SPF conditions at Instituto de Ciências Biológicas. L. donovani (MHOM/IN/80/DD8) was maintained in vitro in RPMI 1640 medium, supplemented with 10% FCS (Life Technologies, Grand Island, NY), and virulence was maintained by passage through BALB/c mice, as described earlier (12). Stationary phase promastigotes (2 × 10^7 per mouse, i.v.) were used for infection of mice. Mice were treated with miltefosine (20 mg/kg body weight) for 7 d consecutively by gavage 2 wk after L. donovani infection. Parabiosis in mice was assessed by limiting dilution and expressed as the negative log of the titer (16). Experimental protocols were approved by the ethical committee CTEEA/UFMG protocol 150/09.

Miltefosine was purchased from Sigma-Aldrich (St. Louis, MO) and AG Scientific (San Diego, CA). Anti–TNF-α Ab was from BD Biosciences, and anti-PAF receptor Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

PAF receptor–HPC and PAF receptor–PAF interaction by molecular dynamic simulation. The molecular dynamic (MD) simulations were performed using the GROMACS package version 4.5.3 with the GROMOS96 force field (20). The molecular topology file was generated with PRODRG (21). The partial atomic charges were determined using the CHelpG force field (20). The molecular topology file was generated with PRODRG (21). The partial atomic charges were determined using the Gaussian09 program with the DFT/B3LYP/6-311G** basis set. Electrostatic interactions between charged groups within 9 Å were calculated explicitly, and long-range electrostatic interactions were calculated using the particle mesh Ewald method (23) with a grid width of 1.2 Å and a fourth-order spline interpolation. A cutoff distance of 14 Å was applied for the Lennard–Jones interactions. Numerical integration of the equations of motion used a time step of 2 fs with atomic coordinates saved every 1 ps for further analysis. The molecular docking program AutoDock 4.0 was employed to probe the possible binding sites of the energy-minimum conformations of the PAF receptor derived from the MD simulations.

Macrophage culture and L. donovani infection in vitro

Thioglycollate-elicted macrophages (99% pure, as assessed by FACS) from BALB/c or PAF receptor–deficient mice were cultured in 16-well tissue culture slides with promastigotes at a 1:30 macrophage–promastigote ratio for 12 h. The cultures were incubated for 60 h without treatment. In some cases, 48-h infected cultures were infected with the indicated concentrations of miltefosine for 24 h. The cultures were terminated, fixed with methanol, and stained with Giemsa. The parasites were counted under a light microscope (E600, Nikon, Tokyo, Japan) and expressed as the number of amastigotes per 100 macrophages (12). For comparing miltefosine’s leishmanicidal effects on PAF receptor–deficient and BALB/c macrophages, the cells were infected with promastigotes at a ratio of 1:10 for 4 h. The extracellular promastigotes were washed out, and the cultures were treated with the indicated concentrations of miltefosine for different periods (4 h, 24 h, 72 h) and processed as stated above.

Macrophage–T cell coculture. Thioglycollate-elicted BALB/c-derived macrophages were infected with L. donovani promastigotes for 48 h. The infected macrophages (IM) were treated with HPC in the presence or absence of anti-PAF receptor Ab for 24 h. The macrophages were washed and pulsed with leishmanial crude soluble Ag (CSA), 10 μg/ml, for 2 h followed by coculture with CD4+ T cells from 10-d L. donovani–infected BALB/c mice. The cells were cultured for 24 h.

Confocal microscopy for binding of miltefosine–BODIPY to PAF receptor–deficient and wild-type macrophages. Miltefosine–BODIPY (11’4’)-difluoro-1,3,5,7-tetramethyl-4’-bora-3’4’a-diaza-7-indacen-2’-yl) undecylyl-phosphocholine was synthesized, as previously described (24). This fluorescent analog of miltefosine showed antiparasitic activity similar to that of miltefosine. This analog was used in confocal time-lapse imaging to address the binding of miltefosine to PAF receptor–deficient or wild-type (WT) macrophages. Thioglycollate-elicted macrophages from naive WT or PAF receptor–deficient mice were cultured on cover slips in RPMI 1640–10% FCS in six-well tissue culture plates for 24 h. The cover slips with the adherent cells were placed in a perfusion chamber. The perfusion was made using perfusion solution (1% fatty acid–free BSA in PBS) or miltefosine–BODIPY (0.8 μM) in the same perfusion solution. After 1 min, miltefosine–BODIPY was added directly to the imaging chamber via a capillary tube. After 7 min, perfusion was reinitiated to stabilize the fluorescence. The nonbound miltefosine–BODIPY was removed by washing the cells with perfusion solution. All confocal microscopy images were collected with a Zeiss LSM 510 confocal microscope using a ×63, 1.4 numerical aperture objective lens with excitation at 488 nm and emission λ-max at 526 nm.

Statistical analyses
Each experiment was performed at least three times. The in vitro cultures were set in triplicates, and the in vivo experiments were performed with five mice per group. The Student t test was performed to ascertain the significance of the differences between the means of the control and the experimental groups. Alternatively, statistical analysis was performed by ANOVA (two-way ANOVA) followed by the Tukey test.

Results

Miltefosine fulfills the conformational requirements to act as a ligand for the PAF receptor

As miltefosine and PAF shared a significant structural similarity—both containing a hexadecyl chain and the phosphocholine group (Fig. 1A)—we first modeled miltefosine to examine if it was conformationally similar to PAF. As they exhibited a significant conformational similarity (Fig. 1B), both molecules were docked into the murine PAF receptor (Fig. 1C). During the first 9 ns of the dynamics, the conformation of PAF in the PAF receptor binding cavity becomes quite different from that for miltefosine. Relevant
The role of H-3.92 in stabilizing the bent miltefosine conformation in the PAF receptor binding cavity was more important, as the N-4.93 at the same position in the PAF-PAF receptor was not involved in any interaction with PAF. In agreement with the conformational similarities and capacity to be recognized by PAF receptor, PAF and miltefosine additionally shared biological activities as well, including their strong antileishmanial activity (12, 14, 15) or p38 MAPK and ERK-1/2 phosphorylation in macrophages (P.R. Gangalum and B. Saha, unpublished observations).

An anti-PAF receptor Ab inhibits miltefosine-induced leishmanicidal activity in macrophages

To verify whether PAF receptor is required for the leishmanicidal activity of miltefosine, the effect of an anti-PAF receptor Ab on parasite elimination and TNF-α production by the macrophage was tested. Both activities (Fig. 2A, 2B) were inhibited by the anti-PAF receptor Ab in a dose-dependent manner. In response to miltefosine, PAF receptor–deficient macrophages failed to enhance the production of TNF-α (Fig. 2C), a cytokine required for miltefosine-induced antileishmanial activity (Fig. 2D), and the anti-PAF receptor Ab reduced the TNF-α–dependent antileishmanial activity of miltefosine (Fig. 2D) in BALB/c macrophages. Whereas the TNF-α–inducing function of HPC was completely inhibited by the anti-PAF receptor Ab (Fig. 2B) and the absence of PAF receptor on macrophages (Fig. 2C), both anti-TNF-α Ab and anti-PAF receptor Ab inhibited the leishmanicidal function of HPC completely (Fig. 2D). Taken together, these observations suggest that PAF receptor–mediated induction of TNF-α is a key factor in the HPC-induced leishmanicidal function. TNF-α production in response to miltefosine was completely impaired in PAF receptor–deficient macrophages. Although TNF-α was previously shown to be a strong antileishmanial cytokine (25, 26), the involvement of TNF-α in miltefosine-induced PAF receptor–mediated antileishmanial function was never implicated.

In this article, our observations report for the first time, to our knowledge, that PAF receptor is involved in miltefosine-induced TNF-α production and subsequent antileishmanial functions. These data suggested that PAF receptor contributed to the antileishmanial activity of miltefosine.

The anti-PAF receptor Ab reduces IFN-γ production by CD4+ T cells elicited with leishmanial Ags

Miltefosine exhibits proinflammatory effects and host-protective antileishmanial functions (12). As the latter was inhibited by PAF receptor blockade, we examined whether this was a consequence of impairment of the miltefosine-induced Th1 functions (12) under these conditions. Therefore, BALB/c-derived macrophages were pulsed with Leishmania CSA and cocultured with CD4+ T cells, isolated from the spleen of BALB/c mice 10 d after L. donovani infection, in the presence of anti-PAF receptor Ab or its control isotype Ab. The anti-PAF receptor Ab significantly reduced miltefosine-induced IFN-γ, but increased IL-4, production by leishmanial Ag–specific T cells (Fig. 3A). The parallel culture examining leishmanicidal activity showed that the PAF receptor blockade significantly reduced the leishmanicidal efficacy of HPC (Fig. 3B). These data suggested a significant role for PAF receptor in miltefosine-enhanced T cell IFN-γ production, perhaps through the induction of IL-12 (12), which is required for L. donovani elimination.

PAF receptor–deficient macrophages showed a significantly impaired miltefosine binding

The above-stated results suggested binding of miltefosine to the PAF receptor–deficient macrophages. Therefore, we examined miltefosine–BODIPY binding to WT and PAF receptor–deficient macrophages. The fluorescence associated with PAF receptor–deficient macrophages was significantly lower (<50%) than for the control.
BALB/c macrophages (Fig. 4A–C), stressing the importance of PAF receptor in the binding of miltefosine onto murine macrophages. The residual binding could be due to nonspecific adsorption of miltefosine–BODIPY through its fatty acyl chains to the membrane.

PAF receptor deficiency significantly reduced the leishmanicidal function of miltefosine

Next, we tested whether the WT and the PAF receptor–deficient macrophages differed in their intrinsic capacities to kill the parasite. No significant differences were observed either in the percentage of infected or in the number of amastigotes for untreated WT or PAF receptor–deficient macrophages (Fig. 5). However, the PAF receptor–deficient macrophages showed consistently higher parasite loads than did the WT macrophages after miltefosine treatment (Fig. 5). Miltefosine executed its leishmanicidal function in BALB/c macrophages as early as 4 h post infection, but such activity was significantly compromised in PAF receptor–deficient macrophages. This early antileishmanial function is perhaps mediated through oxidative burst, as this early oxidative...
burst was inhibited by a PAF antagonist (P.R. Gangalum, R. Dey, S. Majumdar, and B. Saha, unpublished observations). Nonetheless, these observations substantiated the requirement of PAF receptor expression in the macrophage for full anti-leishmanial activity of miltefosine.

PAF receptor deficiency significantly reduces the antileishmanial efficacy of miltefosine in mice

Once we substantiated the feasibility of miltefosine as ligand for PAF receptor, we tested whether this was functionally linked to the final outcome of Leishmania infection in a mouse model. To this end, BALB/c mice (WT) and PAF receptor–deficient mice on the BALB/c background were infected with L. donovani followed by miltefosine treatment. The kinetics of the hepatic and splenic parasite burden in untreated BALB/c was comparable to that in PAF receptor–deficient mice (Fig. 6A), but not after miltefosine treatment (20 mg/kg body weight). A substantial reduction of parasite load in the spleen and full clearance of Leishmania from the liver were observed in WT BALB/c mice, but not in PAF receptor–deficient mice (Fig. 6B). A lower dose of miltefosine (10 mg/kg body weight) also diminished parasite burdens in both mouse strains, but with much lower efficiency in PAF receptor–deficient mice (W. de Castro and L.Q. Vieira, unpublished observations). These data prove the importance of PAF receptor for miltefosine-dependent leishmanicidal activity.

Discussion

The antimony-based drugs used for the treatment of VL have toxic side effects. Their prolonged use has led not only to the emergence of antimony-resistant parasites (2) but also to reduced leishmanicidal efficacy (4). For their optimal antileishmanial effects, these drugs require a robust host immune response (4), but L. donovani infection impairs the effective orchestration of the immune response. Therefore, finding less toxic drugs capable of restoring host-protective immune response to the parasite but maintaining an intrinsic activity on the parasite has been a pressing need. The quest for new drugs led to amphotericin B and paromomycin (4), both not orally bioavailable. By contrast, because of its oral bioavailability, miltefosine became the drug of choice for treating VL patients (27).

The mechanism of antileishmanial function of miltefosine is not fully known. Reportedly, miltefosine interferes with lipid synthesis
or impairs the bioenergetic homeostasis of the parasite to cause parasite death by an apoptosis-like mechanism (5, 6). Miltefosine mandatorily required interactions with the plasma membrane of the parasite. Driven by its long fatty acyl chain, this drug inserts and accumulates into the phospholipid matrix, leading either to altered membrane dynamics through a detergent-like action (28) or to interference with cellular signaling, similar to the mechanism described in cancer cells (6). Furthermore, miltefosine insertion into the membrane is required for its recognition by the amino-phospholipid translocase involved in the translocation of this drug into the cytoplasmic leaflet of the plasma membrane, facilitating its interaction with the intracellular targets (29). The incorporation of miltefosine into the lipid membrane may alter ligand-bound receptor diffusion and signaling intermediate recruitments to the receptor, causing interference with cellular signaling in cancer cells (7, 30). Although these observations supported the direct action of the drug on the parasite, some reports suggest that miltefosine’s immunomodulatory functions (9–12) contribute to the elimination of intracellular amastigotes, although the underlying mechanism for macrophage activation by miltefosine remains unknown. The studies reported in this article indicate that a significant component of the antileishmanial function of miltefosine is executed through PAF receptor.

This observation was first supported by the structural semblances between miltefosine and PAF, affording HPC to work as a PAF receptor ligand, a possibility that was further suggested by MD simulation of miltefosine docking to PAF receptor. From a functional perspective, the reduced TNF-α production by BALB/c-derived macrophages after the blockade of PAF receptor with an anti-PAF receptor Ab and the inability of PAF receptor–deficient macrophages to synthesize TNF-α in response to miltefosine emphasized miltefosine’s requirement for PAF receptor to exhibit its leishmanicidal functions. The importance of TNF-α in miltefosine-induced antileishmanial effects was stressed, as miltefosine failed to reduce the parasite load in the presence of the anti-TNF-α Ab. It is quite possible that being a G-protein–coupled receptor, PAF receptor is able to activate the TNF-α converting enzyme, which can cleave membrane-bound pro–TNF-α to release TNF-α (P.R. Gangalum and B. Saha, unpublished observations). Although TNF-α may contribute to inducible NO synthase expression, other works addressing the antileishmanial mechanism of PAF on *L. amazonensis* do not substantiate a straightforward relationship between NO production driven by PAF and the elimination of *Leishmania* (13–16). These results lead to an important conclusion that an effective synergy emerges from the dual effects of miltefosine: direct killing of the parasite and restoration of a host-protective response (31). Miltefosine was active, apparently through the PAF receptor, as early as 4 h after macrophage infection. We observed that 3 h after the addition of miltefosine to *L. donovani*–infected macrophages (IM), substantial amount of reactive oxygen species is produced and that production of the reactive oxygen species by these macrophages was inhibited by a PAF antagonist (P.R. Gangalum, R. Dey, S. Majumdar, and B. Saha, unpublished observations). Hence, it is possible that *L. donovani* is being killed by macrophages exposed to miltefosine by the oxidative burst, triggered by miltefosine via PAF receptor. The involvement of the PAF receptor in triggering this effector mechanism is further corroborated by the fact that this early leishmanicidal activity was not observed in PAF receptor–deficient macrophages. As *L. donovani* is highly susceptible to reactive oxygen species (32), the observations indicate that the miltefosine-induced oxidative burst is perhaps responsible for its PAF receptor–mediated leishmanicidal function. We also found evidence for a direct effect of miltefosine on parasites inside macrophages at later time points of in vitro infection, when miltefosine promoted parasite death in PAF receptor–knockout (KO) macrophages. Hence, miltefosine would act both via PAF receptor, as early as 4 h after macrophage infection.

FIGURE 6. Antileishmanial activity of miltefosine is compromised in PAF receptor–deficient mice. (A) BALB/c and PAF receptor–KO mice were infected with *L donovani* as described above. At the indicated times, mice were sacrificed, and quantification of parasites in liver and spleen was performed by limiting dilution. Results are the mean of five mice per time point in each group (±SD). Results are of one experiment of two performed with similar results. *Statistical difference.

(B) WT BALB/c and PAF receptor–KO mice were infected with *L donovani* as described in Materials and Methods. At 14 d post infection, daily treatment with vehicle (PBS) or 20 mg/kg body weight of miltefosine (HPC) was initiated and proceeded for 2 wk, when mice were sacrificed. Quantification of parasites was performed by limiting dilution (1:4 serial dilutions) in livers, spleens, and bone marrow. Results are the mean of five mice per time point in each group (mean ± SD). Results are from one of three experiments performed independently and with similar results. *Statistical difference.
STAT-1 inactivation by SHP-1 (12, 33). As a result, a diminished IFN-γ responsiveness ensues. As the anti-PAF receptor Ab reduced miltefosine-induced IFN-γ production while increasing IL-4 production and parasite growth in a macrophage and T cell coculture setup, we cannot rule out that PAF receptor also plays a role in miltefosine’s antileishmanial function through T cells, as well. It is an intriguing observation, as PAF receptor modulation of T cell response is not reported to date. Therefore, the role of PAF receptor in miltefosine modulation of T cell responses requires an independent and detailed investigation.

An alternative explanation for our observations could be an autocrine antileishmanial function of the miltefosine-induced PAF in macrophages. However, as the parasite loads both in untreated macrophages from BALB/c mice and in PAF receptor–deficient mice were comparable, the role of intrinsic PAF in antileishmanial function appears unlikely. If miltefosine, as demonstrated in this study, can act as a ligand to PAF receptor, this may lead to the activation of platelets, cells with a decreased density under progressive leishmaniasis and with high PAF receptor expression. In fact, for L. major, activation of platelets is required for the migration of a specific set of monocytes highly effective in the elimination of Leishmania (34). At the same tune, miltefosine-dependent activation of platelets, known to be highly involved in inflammation, may underlie some limitations and contra-indications concerning the use of miltefosine in pregnant women. Altogether, our results provide an account of the possible role of PAF receptor in leishmanicial activity and pathophysiology in miltefosine-treated VL patients. These findings may contribute to a more rational therapy for this disease as well as to improving the design for a new generation of analogs.

Disclosures

The authors have no financial conflicts of interest.

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