Miltefosine represses HIV-1 replication in human dendritic cell/T-cell cocultures partially by inducing secretion of type-I interferon

Ravendra Garg a, Michel J. Tremblay a,b,∗

a Centre de Recherche en Infectiologie, Centre Hospitalier Universitaire de Québec, CHUL, Canada
b Département de Microbiologie-Infectiologie et Immunologie, Faculté de médecine, Université Laval, Québec, Canada

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A B S T R A C T
Miltefosine (Milt) was originally synthesized as an antineoplastic agent but this phospholipid drug is now clinically used as an antipROTOzoal compound. We demonstrate here that Milt reduces replication of HIV-1 in cocultures of human dendritic cells (DCs) and CD4+ T cells. This phenomenon is due to a rapid secretion of soluble factors by DCs. We present evidence that the Milt-mediated repression in virus production is associated with induction of type-I interferon (IFN) in DCs. The Milt-dependent diminution in HIV-1 production was not totally abrogated by B18R, a vaccinia virus-encoded neutralizing type-I IFN receptor, which suggests the involvement of another yet to be identified soluble factor. Altogether, these results suggest that a therapy with Milt when used to control protozoan infections in individuals also carrying HIV-1 might also help to limit viral load. Additional studies are warranted to estimate the exact therapeutic potential of Milt as an anti-HIV-1 agent.

Introduction

The emergence of the acquired immunodeficiency syndrome (AIDS) epidemic in the early 1980s has changed dramatically the disease pattern caused by Leishmania parasites. For example, species that traditionally caused one type of disease have been shown to be capable of causing other types of diseases. The protozoan parasite Leishmania is highly prevalent in many areas of the world and in particular visceral leishmaniasis (VL) is now becoming of higher clinical importance in individuals infected with human immunodeficiency virus type-1 (HIV-1) because the distribution of both human pathogens overlaps in various parts of the world (Alvar et al., 2008). Furthermore, leishmaniasis’ classification as a childhood disease has been drastically altered due to HIV-1 and Leishmania co-infections. There is also a growing consensus that VL has emerged as an important opportunistic infection in AIDS patients (Alvar et al., 1997; Tremblay et al., 1996; Wolday et al., 1999).

The major challenge in dually infected patients is the administration of a successful treatment regimen during the acute phase of the disease. The situation is further complicated by the fact that HIV-1–positive individuals show higher rates of drug toxicity and relapses, as well as lower cure rates with initial treatment as comparison to seronegative patients. First-line drugs for the treatment of VL in both HIV-1–infected and non-infected patients are the lipid formulations of amphotericin B, pentavalent antimonials and miltefosine (Alvar et al., 2008). However, several studies in co-infected individuals have shown only moderate success rates with regard to initial cure and relapses.

Miltefosine (Milt) is a phosphorylcholine ester of hexadecanol which is acting as a membrane-active synthetic ether-lipid analog. This compound was originally discovered and synthesized as an antineoplastic, particularly active against breast cancer metastases. Milt has been recently found to be highly active against Leishmania species both in in vitro and in vivo studies (Croft et al., 1987; Murray and Delph-Etienne, 2000; Sundar et al., 1998). The expanded use of Milt for treating VL is due to its convenient oral administration route, the increasing leishmanial resistance to antimonials and the unaffordable cost of liposomal amphotericin B. It has been reported that Milt is effective at controlling Leishmania parasitemia in both immunodeficient BALB/c and nude mice (Le Fichoux et al., 1998; Murray and Delph-Etienne, 2000). This pre-clinical information has supported its general use in individuals infected also with HIV-1 because these are known to be immunodeficient. Indeed, Milt has been used for the treatment of VL in AIDS patients and has also been employed with some success in cases with recurrent leishmaniasis in HIV-1–infected individuals (Rihl et al., 2006; Schrainer et al., 2005). Interestingly, a number of reports have demonstrated that Milt exerts significant immunomodulatory properties in different cell types (Eue, 2002; Hochhuth et al., 1992).
It is now well accepted that HIV-1 mainly infects CD4+ T cells but can also replicate in other cell subpopulations such as macrophages and dendritic cells (DCs) (Coleman and Wu, 2009; Wu and KewalRamani, 2006). DCs are potent and versatile antigen-presenting cells and recent evidence suggests that they play a pivotal role in establishment and dissemination of HIV-1 infection. The infection process seems to be facilitated by virtue of DCs being the initial target cell type of HIV-1 and of their unique capacity to migrate from virus entry sites in the periphery to the T cell-rich areas in human lymphoid tissues. Moreover, an intimate contact between DCs and CD4+ T cells has been shown to promote a very efficient transmission of HIV-1 by locally concentrating virus, viral receptor and coreceptors, as well as certain adhesion molecules.

It is of interest to note that it has been shown that Milt reduces HIV-1 production in macrophages via downstream activation of Akt kinase (Chugh et al., 2008). Since DCs are crucial actors in HIV-1 mucosal transmission and the overall pathogenesis of the disease and given that CD4+ T cells act as a major cellular reservoir for HIV-1, we analyzed the capacity of Milt to affect HIV-1 propagation in cocultures made of human immature monocyte-derived DCs and autologous CD4+ T cells. We report here that Milt diminishes HIV-1 production in such cocultured cells. Furthermore, we provide evidence that the Milt-dependent reduction in virus replication is mainly affecting virus production in CD4+ T cells and is at least partly linked with secretion of type-I interferon by DCs.

Results

Milt reduces HIV-1 replication in cocultures of iDCs and CD4+ T cells. Milt is currently one of the first-line drug for the treatment of VL in HIV-1-infected and non-infected patients. In an attempt to define whether Milt can possibly affect the life cycle of HIV-1, we studied its effect on virus replication in the context of cocultures made of iDCs and autologous CD4+ T cells. To this end, iDCs were initially pulsed with fully competent R5-tropic virions (i.e. NL4-3) and next exposed to increasing doses of Milt. Finally, immature monocyte-derived DCs (iDCs) were cocultured with autologous CD4+ T cells. Results showed a statistically significant dose-dependent inhibition of HIV-1 replication in such cocultured cells (Fig. 1). It should be noted that Milt and B18R have no effect on HIV-1 replication in CD4+ T cells in absence of iDCs (data not shown). The colorimetric MTS cell proliferation assay revealed that the observed reduction in virus production is not due to cell toxicity since cellular viability remains the same in the presence of all tested concentrations of Milt (data not shown). Moreover, similar observations were made when using a CFSE-based viability and proliferation test (data not shown).

Milt represses virus replication in CD4+ T cells. We next tried to shed light on the mechanism(s) by which Milt can reduce HIV-1 replication in human DCs/T-cell cocultures. First, iDCs were pretreated with the HIV-1 reverse transcriptase inhibitor efavirenz (EFV) during the virus pulsing period. This treatment will abrogate de novo virus production in iDCs without altering transfer of viruses located on their surface or within their endosomal apparatus and the subsequent replication in CD4+ T cells. This treatment completely abolished virus production in iDCs cultured alone and a reduction in HIV-1 replication was also seen in cocultured cells (data not shown). Interestingly, a comparable diminution in virus production was detected in both untreated and EFV-treated cocultures exposed to Milt (Fig. 2A). Therefore, it can be proposed that Milt is not modulating susceptibility of iDCs to virus infection and is thus most likely exerting its inhibitory effect in CD4+ T cells. To provide more direct evidence on the mechanism of action of Milt, iDCs were initially exposed to fully infectious X4-tropic virions (i.e. NL4-3) and next cocultured with autologous CD4+ T cells in presence of various concentrations of Milt. The rationale for this experimental setup is based on the...
previous report showing that iDCs are refractory to productive infection with X4-using variants (Pion et al., 2007). A dose-dependent reduction in HIV-1 replication was still seen in cocultures treated with Milt (Fig. 2B), which supports the idea that Milt is affecting HIV-1 replication in CD4⁺ T cells. To validate that HIV-1 production in CD4⁺ T cells is decreased upon a coculture step with Milt-treated iDCs, we performed another set of experiments where autologous CD4⁺ T cells were first pulsed with HIV-1 and then cocultured with iDCs either left untreated or treated with Milt. Moreover, this set of experiments was also aimed at assessing whether a cell-to-cell contact is needed to see the Milt-mediated diminution in virus production. To do so, we used permeable cell supports with a membrane pore size of 0.4 μm, which allows the crossing of virions and soluble factors but not that of cells (Ramirez-Pineda et al., 2004). Results depicted in Fig. 3 demonstrate that viral replication in CD4⁺ T cells is similarly decreased by a coculture step with Milt-loaded iDCs when cells are in a close physical contact. When iDCs were physically separated from CD4⁺ T cells by a permeable membrane, virus production was still diminished by a Milt treatment. Thus, these results corroborate that the inhibitory effect of Milt in cocultured cells is due to a repression of virus production in CD4⁺ T cells and suggest that this process is caused by a Milt-mediated release of a soluble factor by iDCs.

**Fig. 3.** Milt-dependent decrease in virus production seen in cocultured cells is due to a soluble factor. First, CD4⁺ T cells were incubated for 2 h with NL4-3. Next, CD4⁺ T cells were cocultured with iDCs in the presence of the listed concentration of Milt for 4 day either in cell-to-cell contact or non-contact conditions. Virus production was estimated by measuring p24 levels in the culture supernatants at the indicated time points. Data shown represent the means ± SEM of triplicate samples and are representative of five independent experiments. Asterisks denote statistically significant differences from the cells infected with HIV-1 only (**P < 0.01**).

Milt-induced diminution of virus replication is partially mediated by type-I IFN. It has been reported that Milt drives secretion of IFN-γ, TNF-α and IL-12 production from macrophages (Wadhone et al., 2009). Consequently, we hypothesized that the observed decrease in viral replication might be due to production by iDCs of a soluble factor displaying an anti-HIV-1 activity in CD4⁺ T cells. Given that type-I IFN is recognized as a powerful inhibitor of in vitro and in vivo HIV-1 infection, our next series of investigations was aimed at assessing the capacity of Milt to induce secretion of type-I IFN in iDCs with the use of the HEK-Blue™ IFN-α/β indicator cell line. We observed that Milt acts indeed as a good inducer of type-I IFN following treatment of human iDCs (Fig. 4A). To validate the putative role of type-I IFN in the observed Milt-dependent diminution of HIV-1 production in cocultured cells, virus transfer experiments were performed with the soluble vaccinia virus-encoded protein B18R that has been demonstrated to inhibit the antiviral activity and cellular binding of the type-I IFN of different mammalian species (e.g. IFN-α, IFN-β and IFN-ω) (Alcami et al., 2000; Symons et al., 1995). B18R was added simultaneously with Milt in iDCs inoculated with HIV-1 prior to initiation of the coculture step with autologous CD4⁺ T cells. Data from Fig. 4B indicate that the Milt-mediated reduction in *de novo* virus production seen in cocultured cells was associated with secretion of type-I IFN. A higher concentration of B18R did not further increase virus production (data not shown), thus suggesting that the Milt-directed inhibition of virus production in cocultured cells is not only due to production of type-I IFN by iDCs but also to another unknown soluble factor.

**Fig. 4.** Milt-induced inhibition of virus replication in cocultured cells is partially mediated by type-I IFN. (A) Cocultured cells were either left untreated or treated with Milt for 6 h. Next, supernatants were collected and the levels of type-I IFN were quantitated through the use the HEK-Blue™ IFN-α/β cells. Data shown represent the means ± SEM of triplicate samples and are representative of five independent experiments performed with different donors. (B) First, iDCs were pulsed for 60 min with NL4-3Balenv and either left unexposed or exposed to Milt. Next, iDCs were cocultured with autologous CD4⁺ T cells and either left untreated or treated with B18R. Virus production was estimated by measuring p24 levels in the culture supernatants at 4 day following initiation of the coculture. The results shown represent the means ± SEM of triplicate samples and are representative of four separate experiments performed with different donors and are expressed as relative viral production compared to cocultured cells exposed to HIV-1 but left untreated with Milt and B18R. Asterisks denote statistically significant data (**P < 0.05; **P < 0.01).
protease inhibitors significantly inhibit the intracellular survival of *Leishmania* parasites (Trudel et al., 2008; Valdivieso et al., 2010; White et al., 2011). Milt tolerability and efficacy in immunocompetent individuals have already been proven in earlier studies (Jha et al., 1999; Le Fichoux et al., 1998; Murray and Delph-Etienne, 2000) but there is still many unanswered questions in immunocompromised persons. These reports together with the idea that the efficacy of drugs aimed at controlling each pathogen remains largely undefined in dually infected persons underlined the need for further investigation.

DCs are central for the development of pathogen-specific immune responses and are well equipped for activation of both the innate and adaptive immune response (Mellman and Steinman, 2001). It has been demonstrated that human pathogens targeting DCs, such as HIV-1, have evolved strategies to impair DC functions, thereby enhancing their capacity to persist and evade immune surveillance. DCs have been reported as playing an important role in the pathogenesis of AIDS, favoring both the initial establishment and spread of the infection and the development of antiviral immunity. In the present study, we investigated whether Milt treatment can modulate HIV-1 replication when iDCs are cocultured with autologous CD4⁺ T cells.

We provide the first evidence showing that Milt represses HIV-1 replication in iDCs-T-cell cocultures of human origin. To define the precise contribution of *de novo* virus production from iDCs in the Milt-dependent down-regulatory effect on HIV-1 replication, coculture experiments were performed in presence of the anti-retroviral compound EFV. Results with EFV-treated cells and experiments performed with X4-tropic virions led us to conclude that the Milt-directed effect in virus production is independent of productive infection of iDCs. Interestingly, our data indicate also that a close physical contact between Milt-treated iDCs and CD4⁺ T cells is not necessary to diminish HIV-1 production, therefore indicating that the observed reduction in virus replication is achieved through the secretion of a soluble factor. This postulate is supported by the previous observation that Milt induces production of numerous cytokines such as TNF-α, GM-CSF, IL-12 and IFN-γ (Beckers et al., 1994; Hochhuth et al., 1992; Wadhone et al., 2009; Zeisig et al., 1995). The human IFN network is known as one of the fastest responding branch of our immune defense to control an invading pathogen. The IFN system is an extremely powerful antiviral response that is capable of controlling most, if not all, virus infections in the absence of adaptive immunity. Type-I IFN (e.g. α and β) are produced by many cell types, while type-II IFN (i.e. γ) is more restricted since it is produced by immune cells. Type-I IFN comprises a large group of molecules that includes IFN-α and -β as well as IFN-ω, -ε, -τ, -δ and -κ. The IFN-α and -β genes are induced directly in response to viral infection, whereas IFN-ω, -ε, -τ, -δ and -κ play less well-defined roles, such as regulators of maternal recognition in pregnancy. We show here using HEK-Blue™ IFN-α/β cells that the Milt-mediated restriction of HIV-1 replication is due in part to an endogenous production of type-I IFN. Indeed, this indicator cell line has allowed us to determine that treatment of iDCs with Milt induces a rapid production of type-I IFN (i.e. as early as 3 h following exposure to the drug) reaching a peak at 6 h (data not shown).

Previous studies revealed that treatment of human cells with type-I IFN restricts HIV-1 replication via blocking both early and late stages of the virus replicative cycle (Agy et al., 1995; Bacar-Regen et al., 1994; Coccia et al., 1994; Shirazi and Pitha, 1992). Treatment of iDCs with the type-I IFN-binding receptor B18R confirms the importance of such a soluble factor in the Milt-dependent inhibitory effect on HIV-1 gene expression. However, the Milt-mediated diminution of virus production was not totally abrogated in the presence of the recombinant protein B18R, therefore indicating that another soluble factor in addition to type-I IFN is involved in the inhibitory effect mediated by this drug. Altogether our data suggest a partial role of type-I IFN in the Milt-mediated inhibition of HIV-1 replication seen in a coculture system consisting of iDCs and autologous CD4⁺ T cells.

It was shown previously that patients dually infected with HIV-1 and *Leishmania*, who had experienced failure of standard leishmaniasis treatment, displayed an initial response rate of 64% and a cure rate of 43% during the first treatment cycle with Milt (Sindermann et al., 2004). Milt is now recognized as the first oral agent that appears to be highly effective and well tolerated for the treatment of co-infected patients. The current work demonstrates for the first time that Milt can, in addition to its already known anti-leishmanicidal properties, restrict also HIV-1 propagation and shed light on the mechanisms by which this compound can modulate virus replication. These results add new and interesting in vitro insights into the possible inhibitory activity of Milt against HIV-1 and suggest studying its efficacy on others cell targets (e.g. macrophages) to validate its use on a larger scale in individuals co-infected with HIV-1 and *Leishmania*. However, the precise clinical relevance of our present findings remains to be determined.

**Material and methods**

**Reagents.** Milt was obtained from Alexis Biochemicals (San Diego, CA) while B18R was purchased from ebioscience (San Diego, CA). Recombinant human interleukin-2 (rhl-2), IFV and azidothymidine (AZT) were obtained from the NIH AIDS Repository Reagent Program (Germantown, MD). Interferon-gamma (IFN-γ) and IL-4 were both purchased from R&D systems (Minneapolis, MN), whereas granulocyte macrophage-colony stimulating factor (GM-CSF) was a generous gift from Cangene (Winnipeg, MB). Lipopolysaccharide (LPS) and phytohemagglutinin-L (PHA-L) were obtained from Sigma (St-Louis, MO). The culture medium consisted of RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 U/ml) and glutamine (2 mM), which were all purchased from Wisent (St-Bruno, QC), and primocine (Amaca Biosystems, Gaithersburg, MD).

**Cells.** Human embryonic kidney 293 T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (Wisent, St-Bruno, QC). DCs were generated from monocytes obtained from the blood of healthy donors. Briefly, CD14⁺ cells (i.e. monocytes) were isolated from peripheral blood mononuclear cells, using a monococyte-positive selection kit according to the manufacturer’s instructions (CD14-positive selection kit; StemCell Technologies Inc., Vancouver, BC). Purified CD14⁺ cells were cultured in RPMI-1640 medium supplemented with 10% FBS, GM-CSF (1000 U/ml) and IL-4 (200 U/ml) for 7 day to obtain iDCs as previously described (Gilbert et al., 2007). Autologous CD4⁺ T cells were isolated with a negative selection kit (Stem Cell Technologies) and activated with the mitogenic agent PHA-L (1 µg/ml) and IL-2 (30 U/ml) for 48 h prior to their use. HEK-Blue™ IFN-α/β cells (InvivoGen, San Diego, CA) were maintained in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% FBS, glutamine (2 mM), penicillin G (100 U/ml) and streptomycin (100 mg/ml). Culture media used for HEK-Blue™ IFN-α/β cells was supplemented with 30 µg/ml of chloramphenicol and 100 µg/ml of Zeocin.

**Viral preparations.** Virus stocks were produced upon transient calcium phosphate transfection of 293 T cells with full-length infectious molecular clones NL4-3 (X4) (NIH AIDS Repository Reagent Program) and NL4-3Balenv (R5) (Dornadula et al., 1999). Virus stocks were normalized by using a sensitive in-house, double-antibody sandwich enzyme-linked immunosorbent assay (EIA) assay.
specific for the major viral core p24 protein (Bounou et al., 2002). Viral preparations underwent a single freeze-thaw cycle before being their use.

**Virus transmission assays.** First, iDCs were inoculated with NL4-3 or NL4-3BalEnv virions (10 ng of p24 per10^6 cells) for 60 min at 37 °C and unbound virus was eliminated by extensive washes with phosphate-buffered saline (PBS). Next, iDCs were either left untreated (used as a control) or treated with different concentrations of Milt. Cells were then incubated with activated CD4^+ T cells at a 1:3 ratio (iDCs:CD4^+ T cells). Viral production was assayed by measuring the cell-free p24 content at different time intervals. In some experiments, iDCs were treated with the antiretroviral drug EFV (50 nM) for 30 min before pulsing with fully competent HIV-1 particles. In some HIV-1 transfer studies, permeable cell inserts with polycarbonate membranes (Transwell™, Corning Inc., Lowell, MA) (pore size: 1 μm) were used to separate iDCs and CD4^+ T cells. Briefly, iDCs were either left untreated or treated with Milt. Next, PHA-treated autologous CD4^+ T cells were pulsed with HIV-1 for 2 h, washed extensively with PBS and transferred into permeable cell inserts and cocultured with iDCs for 3 day. Virus production was assessed by estimating the p24 content.

**Quantification of type-I IFN and blocking experiments.** iDCs were either left untreated or treated for 6 h with different doses of Milt. Thereafter, levels of type-I IFN (i.e. IFN-α and -β) in cell-free supernatants were determined through the use of HEK-Blue™ IFNα/β cells according to the manufacturer's protocol (InvivoGen). These cells allow the detection of bioactive IFN-α and -β by monitoring the activation of the ISGF3 pathway. HEK-Blue™ IFNα/β cells are stably transfected with a SEAP promoter gene under the control of the IFNb/α-inducible ISGF34 promoter. Stimulation of these cells with type-I IFN activates the JAK/STAT/ISGF3 pathway and induces subsequently the secretion of SEAP in the supernatant. A standard curve of IFN-α ranging from 1 to 250 Units/ml was used to quantify the amounts of type-I IFN released in the culture medium. The type-I IFN inhibitor B18R (200 ng/ml), a vaccinia virus-derived soluble receptor that blocks the effect of biologically functional type-I IFN, was added to the culture medium when initiating virus transmission experiment with Milt.

**Statistical analysis.** The statistical significance of the results was defined by performing a one-way ANOVA analysis of variance with Bonferroni’s multiple comparison tests and Dunnett’s post-tests to compare treated versus untreated control samples. All analyses were performed on raw data (i.e. p24 concentrations). P values lower than 0.05 were considered statistically significant. InStat software (GraphPad Software version 5.04) was used for all analyses.

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