Miltefosine is fungicidal to *Paracoccidioides* spp. yeast cells but subinhibitory concentrations induce melanisation

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A B S T R A C T

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the dimorphic fungi *Paracoccidioides* spp. The duration of antifungal treatment ranges from months to years and relapses may nevertheless occur despite protracted therapy. Thus, there remains an urgent need for new therapeutic options. Miltefosine (MLT), an analogue of alkylphospholipids, has antifungal activity against species of yeast and filamentous fungi. The aim of this study was to evaluate the antifungal effects of MLT on the yeast forms of *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*. MLT demonstrated inhibitory activity from 0.12 to 1 μg/ml, which was similar to amphotericin B or the combination trimethoprim/sulfamethoxazole but was not more effective than itraconazole. The fungicidal activity of MLT occurred at concentrations ≥1 μg/ml. Ultrastructural alterations were observed following exposure of the fungus to a subinhibitory concentration of MLT, such as cytoplasmic membrane alteration, mitochondrial swelling, electron-lucent vacuole accumulation and increasing melanosome-like structures. Melanin production by yeasts following MLT exposure was confirmed by labelling with antibodies to melanin. In addition, the combination of a subinhibitory concentration of MLT and tricyclazole, an inhibitor of DHN-melanin biosynthesis, drastically reduced yeast viability. In conclusion, MLT had a fungicidal effect against both *Paracoccidioides* spp., and a subinhibitory concentration impacted melanogenesis. These findings suggest that additional investigations should be pursued to establish a role for MLT in the treatment of PCM.

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1. Introduction

*Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* are dimorphic fungi that are the causative agents of paracoccidioidomycosis (PCM), which is primarily distributed in Latin America with high prevalences in Brazil, Colombia, Venezuela and Argentina [1]. This systemic mycosis is initiated by the inhalation of airborne propagules from the mycelium phase of *Paracoccidioides* spp., which rapidly undergo phase transition into the pathogenic yeast form [1]. PCM is considered a serious public health problem in endemic regions, where it is the leading cause of death due to systemic mycoses in immunocompetent patients [2]. Furthermore, PCM is the eighth most common cause of mortality from chronic infectious diseases in Latin America, with reaching rates of 1.65 deaths per million inhabitants [3].

Treatment of PCM is usually long-term, typically ranging from 6 to 24 months; moreover, there are few drugs available with proven efficacy against *Paracoccidioides* spp. The most commonly used therapeutics are itraconazole (ITC), amphotericin B (AmB) and trimethoprim/sulfamethoxazole (TMP/SMX) [1]. However, clinical failures or relapses are well documented during and after treatment with these drugs [3]. Thus, there is an urgent need to identify alternative antifungals for treatment of PCM.

Miltefosine (MLT) is an alkylphospholipid analogue [4–6]. Originally developed as an antitumour drug, MLT has also shown inhibitory activities against *Leishmania* spp. and *Trypanosoma cruzi* [4–6]. In many countries of Latin America, India, Germany and others, MLT is frequently used for the treatment of leishmaniasis and breast cancer. Recently, MLT was approved by the US Food and Drug Administration (FDA) for cutaneous and visceral leishmaniasis [7].

In addition to its antiparasitic activity, investigators have demonstrated in vitro antifungal activities of MLT against dimorphic fungi such as *Coccidioides posadasii* [8], *Histoplasma capsulatum* and *Sporothrix* spp., [8–10] clinically relevant moulds such as...
azole-susceptible and -resistant Aspergillus, Fusarium solani and Fusarium oxysporum [11], dermatophytes [12], Scedosporium spp. [13] and yeasts including Candida spp., Cryptococcus neoformans and Cryptococcus gattii [14]. In addition, MLT has displayed antibiofilm action against Candida albicans and non-albicans Candida spp. [15–18] and F. oxysporum [18]. In vivo studies have also demonstrated the efficacy of MLT in murine models of disseminated cryptococcosis [14] and oral candidiasis [16].

Although the antifungal activity of MLT has been reported for many fungi, there are no studies showing the effects of MLT against Paracoccidioides spp. Hence, the aim of this study was to evaluate the in vitro antifungal effects of MLT on the yeast forms of *P. brasiliensis* and *P. lutzii*.

2. Materials and methods

2.1. Paracoccidioides spp. isolates

*Paracoccidioides brasiliensis* strains were isolated from human PCM in the Brazilian Southeast (Phb18 and Phb339) and Amazonas State (Phb113) [19], and the Pb dog strain was isolated from a dog in Paraná State [19]. All strains of *P. lutzii* (Phb01, Ed01, Phb8334, Phb1578 and Phb66) were isolated from human PCM in the Brazilian Midwest [20]. Isolates were obtained from the fungal collection of the Institute of Tropical Medicine, University of São Paulo (São Paulo/SP, Brazil). Maintenance of the yeast phase of *Paracoccidioides* spp. was achieved by weekly passaging of isolates in Fava–Netto agar medium at 37 °C.

2.2. Antifungal drugs

Miltefosine (MLT) (Cayman Chemical Co., Ann Arbor, MI) was dissolved in sterile distilled water; Amphotericin B (AmB), itraconazole (ITC), sulfamethoxazole (SMX), trimethoprim (TMP) (all from Sigma-Aldrich, St Louis, MO) and tricyclazole (TCZ) (Dow AgroSciences, Indianapolis, IN) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). TMP/SMX was used in its commercial formulation (Bactrim; Roche, São Paulo/SP, Brazil). Maintenance of the yeast phase of *Paracoccidioides* spp. was achieved by weekly passaging of isolates in Fava–Netto agar medium at 37 °C.

2.3. Broth microdilution assay

Minimum inhibitory concentrations (MICs) of the antifungal drugs were determined by a previously standardised and validated broth microdilution method [20]. Yeasts from *Paracoccidioides* spp. were cultivated in McVeigh & Morton (MVM) medium [21] at 37 °C under orbital agitation for 7–days. Following incubation, yeast suspensions were held stationary for 3–5 min to allow large cell aggregates in the culture to settle.

Subsequently, the supernatants were collected, the yeasts were counted using a Neubauer chamber and a yeast suspension was standardized to 1 × 10⁵ CFU/mL. Microscopy was used to confirm single-cell suspensions and the viability was demonstrated to be ≥90% by Trypan blue staining. Antifungal drugs were serially diluted (1:2) in MVM medium in a flat-bottomed 96-well microdilution plate and a 100 μL yeast suspension was added to each well to obtain a final concentration of 0.5–2.5 × 10⁴ CFU/mL and the following antifungal drug concentrations: 0.03–16 μg/mL for MLT, AmB and ITC; 0.25–128 μg/mL for SMX, TMP and TCZ; and 0.03–16 μg/mL SMX combined with 0.005–2.56 μg/mL TMP (TMP/SMX combination). Microplates were incubated at 37 °C for 14 days in a humid dark chamber. The MIC was defined as the lowest concentration inhibiting 80–100% of fungal growth compared with untreated yeasts by visual observation, which readily identifies yeast aggregates. This assay was performed in duplicate at least twice for each condition tested.

2.4. Chequerboard assay

Combinations between MLT with ITC, TMP/SMX or TCZ were performed using a chequerboard microlution assay [22]. Yeast inocula of *P. brasiliensis* (Phb18) or *P. lutzii* (Phb01) and several concentrations of antifungals were used ranging from 0.12 × 8 × MIC as described above. After 14 days of incubation at 37 °C, MICs were determined for drugs alone and for their combinations, and the fractional inhibitory concentration index (FICI) was calculated as follows: FICI = (MICdrug a in combination/MICdrug a tested alone) + (MICdrug b in combination/MICdrug b tested alone), where MIC corresponds to drug a, and ITC, TMP, SMX or TCZ correspond to drug b. A synergistic effect was considered at FICI ≤ 0.5, indifference at FICI > 0.5 and ≤4, and antagonistic at FICI > 4 [22].

2.5. Time–kill assays

Yeasts of *P. brasiliensis* (Phb18) or *P. lutzii* (Phb01) (1 × 10⁵ CFU/mL) were treated with different concentrations of MLT (0.5, 1, 2, 4 and 8 μg/mL) in MVM medium for 1, 3, 5, 7 and 14 days at 37 °C. At each time, treated and untreated yeasts were diluted in phosphate-buffered saline (PBS) and 100 μL was plated on brain–heart infusion agar medium supplemented with 4% foetal calf serum and 5% *P. brasiliensis* (strain 192) culture filtrate [23] at 37 °C for 14 days before CFU counting. Fungicidal activity was defined as a reduction of ≤99.9% in the number of CFU relative to that found in the starting inoculum; otherwise, the activity was considered to be fungistatic [24].

2.6. Transmission electron microscopy (TEM)

Yeast cells of *P. brasiliensis* (Phb18) or *P. lutzii* (Phb01) (1 × 10⁵ CFU/mL) treated with a subinhibitory concentration of MLT (0.5 μg/mL) and incubated for 14 days at 37 °C under orbital agitation were collected by centrifugation and were washed three times in PBS. Yeasts were then fixed for 2 h at room temperature with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Post-fixation was carried out in 1% osmium tetroxide in cacodylate buffer containing 1.25% potassium ferrocyanide and 5 mM CaCl₂ for 2 h at room temperature. Next, the yeasts were dehydrated in increasing ethanol concentrations and were then plated into 100% propylene oxide and were embedded in Spurr’s resin. Ultrathin sections were stained with 0.5% uranyl acetate and 0.5% lead citrate and were observed in a JEOL 100CX transmission electron microscope (JEOL, Tokyo, Japan).

2.7. Viability of yeasts treated with miltefosine combined with tricyclazole, an inhibitor of 1,8-dihydroxyquinaphthalene (DHN) melanin synthesis

Structures observed in TEM images led us to suspect that subinhibitory concentrations of MLT interfered with melanin production. Thus, the effect of an inhibitor of DHN-melanin biosynthesis (i.e. TCZ) on the viability of *Paracoccidioides* spp. yeasts was evaluated. First, broth microdilution assays and chequerboard testing were performed to evaluate the antifungal activity of TCZ alone or in combination with MLT as describe above. Then, the viabilities of *P. brasiliensis* (Phb18) or *P. lutzii* (Phb01) strains were also evaluated by CFU determination after 7 days of treatment with subinhibitory concentrations of MLT (0.25 μg/mL and 0.5 μg/mL) combined with a subinhibitory concentration of TCZ (32 μg/mL) in MVM medium at 37 °C. CFU counting was performed as described for the time–kill assay.
128 whereas TCZ failed to inhibit events (3.1. Miltefosine exhibits fungicidal activity on Paracoccidioides yeasts

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (μg/mL)</th>
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<tbody>
<tr>
<td>Pb339</td>
<td>0.5</td>
</tr>
<tr>
<td>Pb18</td>
<td>1</td>
</tr>
<tr>
<td>Pb113</td>
<td>0.12</td>
</tr>
<tr>
<td>P. lutzii</td>
<td>0.12</td>
</tr>
<tr>
<td>Pbdog</td>
<td>0.25</td>
</tr>
<tr>
<td>Pb01</td>
<td>0.5</td>
</tr>
<tr>
<td>ED 01</td>
<td>0.5</td>
</tr>
<tr>
<td>Pb3334</td>
<td>1</td>
</tr>
<tr>
<td>Pb1758</td>
<td>0.5</td>
</tr>
<tr>
<td>Pb66</td>
<td>1</td>
</tr>
</tbody>
</table>

MLT, miltefosine; AmB, amphotericin B; ITC, itraconazole; SMX, sulfamethoxazole; TMP, trimethoprim.

3. Results

3.1. Miltefosine exhibits fungicidal activity on Paracoccidioides spp. yeasts

MLT inhibited the yeast growth of Paracoccidioides spp. isolates at concentrations from 0.12 to 1 μg/mL, which was similar to that observed for AmB and TMP/SMX. SMX alone showed a wide range of MICs (0.25–16 μg/mL) and TMP alone required high concentrations to produce an inhibitory effect on the Paracoccidioides isolates (Table 1). ITC displayed the lowest MICs (≤0.12 μg/mL) (Table 1), whereas TCZ failed to inhibit Paracoccidioides growth at up to 128 μg/mL (data not shown).

Time–kill assays of P. brasiliensis (Pb18) and P. lutzii (Pb01) were used to determine the fungicidal activity of MLT (Fig. 1). For both species, concentrations above the MIC of MLT (i.e. >1 μg/mL) produced fungicidal effects at 1 day of exposure. Treatment with 1 μg/mL of MLT sterilised the cultures of Pb18 by Day 5 and of Pb01 by Day 7 (Fig. 1). A subinhibitory concentration of MLT (0.5 μg/mL) inhibited ca. 50% of yeast growth of P. lutzii (Pb01) by 14 days of incubation in relation to the untreated yeasts (Fig. 1B); in contrast, P. brasiliensis (Pb18) growth was not inhibited by the same MLT concentration (Fig. 1A).

3.2. Miltefosine induces morphological alterations on Paracoccidioides yeasts

The normal morphology of untreated yeasts from P. brasiliensis (Pb18) and P. lutzii (Pb01) is characterised by an electron-dense cytoplasm containing one or more nuclei, mitochondria and some electron-lucent vacuoles as well as a continuous cellular membrane and compact cell wall (Fig. 2A,D). Notably, yeasts treated with a subinhibitory concentration of MLT (0.5 μg/mL) showed ultrastructural alterations in the cytoplasmic membrane (insets in Fig. 2B,E), mitochondrial swelling (Fig. 2B,C,F) and an accumulation of electron-lucent vacuoles (Fig. 2B,C,E) as well as the presence of electron-dense particles inside vacuoles (black arrows in Fig. 2B,C,E).

3.3. Melanosome-like structures in Paracoccidioides yeasts

Further analyses of TEM images at high magnification allowed for a more detailed characterisation of the electron-dense particles inside vacuoles that were present in large numbers in the P. brasiliensis (Pb18) and P. lutzii (Pb01) yeasts treated with a subinhibitory concentration of MLT (0.5 μg/mL) (Fig. 2). These structures were observed in different electron-density stages (Fig. 3) and they presented a similar organisation to the melanosome-like structures described in Fonsecaea pedrosoi conidia [25]. Melanosome-like matrix formation can be observed (Fig. 3A) and maturation of granules was represented by an increase in electron density (Fig. 3B,C), which was presumably followed by granule deposition in the fungal cell wall (Fig. 3D).

The melanosome-like structures were quantified using several TEM images (100–200 yeasts were analysed for each sample). Untreated yeasts had lower numbers of melanosome-like structures (0.12 structures/yeast of Pb18 and 0.13 structures/yeast of Pb01). In comparison, MLT treatment led to a significant increase in the production of melanosome-like structures inside the yeast cells of P. brasiliensis (Pb18) (1.3 structures/yeast or 10.8 times higher than untreated group) and P. lutzii (Pb01) (1.13 structures/yeast or 8.7 times higher than untreated group).

3.4. Miltefosine combined with tricyclazole reduces Paracoccidioides spp. viability

Although the combination of MLT with TCZ showed an indifferent interaction by the checkerboard technique, the combination of subinhibitory concentrations of MLT and TCZ nevertheless affected the viability of these fungi, especially for P. lutzii (Pb01) (Table 2). Paracoccidioides lutzii (Pb01) had a 100% reduction of yeast cell viability following treatment with the combination of MLT 0.5 μg/mL with TCZ 32 μg/mL (P < 0.01) compared with MLT alone, and a 30% reduction after exposure to 0.25 μg/mL MLT with 32 μg/mL TCZ (P > 0.05) (Table 2). For P. brasiliensis, combinations of 0.25 μg/mL or 0.5 μg/mL MLT with 32 μg/mL TCZ decreased the viability compared with MLT alone, but the reductions were not significant.

3.5. Yeasts of Paracoccidioides spp. produce melanin during treatment with a subinhibitory concentration of miltefosine

Yeasts exposed to a subinhibitory concentration of MLT (0.5 μg/mL) were labelled with melanin-binding antibody 6D2 and...
were then submitted to flow cytometry assay and were compared with untreated yeasts to evaluate melanin production. Treatment with MLT resulted in a significant increase in the melanin present in the yeast cells of *P. brasiliensis* (Pb18) (47%; *P* < 0.01) and *P. lutzii* (Pb01) (267%; *P* < 0.001) (Fig. 4). On the other hand, the combination of subinhibitory concentrations of MLT (0.5 μg/mL) with TCZ (32 μg/mL) significantly decreased melanin production compared with MLT alone, resulting in melanin levels similar to that of untreated yeasts (Fig. 4).

4. Discussion

Current treatment of PCM is inadequate because the drugs that compose therapy require administration over months to years resulting in toxicities to the patient, and the rates of recurrence and relapse remain high [1]. MLT is an alkylphospholipid drug, a phosphaocholine analogue, which has activity against *Leishmania* spp., *T. cruzi* [5] as well as some yeasts, moulds and dimorphic fungi [8–18]. The present study is the first to explore the antifungal activity of MLT against the yeast form of *Paracoccidioides* spp.

MLT proved to be effective in inhibiting the growth of *Paracoccidioides* spp. isolates (0.12–1 μg/mL), which is better than or similar to MIC data for other fungi such as *Cryptococcus* spp. and *Candida* spp. (0.25–8 μg/mL) [14], dermatophytes of the genera *Trichophyton*, *Epidermophyton* and *Microsporum* (0.25–2 μg/mL) [12], *Aspergillus* spp., *Rhizopus* spp., *Mucor* spp., *Fusarium* spp. and *Scedosporium* spp. (2–64 μg/mL) [26] and *Sporothrix* spp. (0.25–2 μg/mL) [9,10]. Moreover, MLT MICs were similar to the MICs exhibited by standard antifungal drugs such as AmB and TMP/SMX; however it was not more effective than ITC. In addition, MLT was fungicidal to yeasts of *Paracoccidioides* spp., which has also been described for *Candida* spp., *Cryptococcus* spp., *Aspergillus* spp., *Sporothrix* spp., *H. capsulatum* and *Coccidioides immitis* [8–10,14]. The current approach did not detect synergistic interactions between MLT and standard drugs used for PCM treatment (ITC or TMP/SMX), which is consistent with the lack of synergy reported between azoles and MLT for *Sporothrix brasiliensis* [10]. However, synergism of MLT has been demonstrated with voriconazole and posaconazole against *F. oxysporum* and mucormycetes [26] as well as for *F. solani*, *Scedosporium* spp. and *Aspergillus* spp. [11].

A subinhibitory concentration of MLT (0.5 μg/mL) inhibited ca. 50% of fungal growth of *P. lutzii* (Pb01) and did not have any effect in growth inhibition of *P. brasiliensis* (Pb18). Despite the lack of inhibition of *P. brasiliensis* (Pb18), MLT (0.5 μg/mL) caused ultrastructural alterations both of *P. brasiliensis* (Pb18) and *P. lutzii* (Pb01), including the accumulation of electron-lucent vacuoles, morphological changes in the cytoplasmic membrane and mitochondrial swelling. The presence of electron-lucent vacuoles may indicate lipid accumulation in the cytoplasm of *Paracoccidioides* spp. yeasts as also seen in *C. albicans* and *S. brasiliensis* yeasts treated with MLT [10,15] and in *Candida* spp. treated with ergosterol biosynthesis inhibitors [27,28]. Recently, MLT was found to reduce membrane ergosterol in the dimorphic fungi *C. posadasii* and *H. capsulatum* [8] as well as altering the permeability of the cytoplasmic membrane of *C. posadasii*, *H. capsulatum* and *S. brasiliensis*, which could result in morphological changes in the membrane similar to those found in the current study and in previous publications [8,16]. Confirming our findings, some studies have described the interaction of MLT with lipid components of erythrocytes [29] and the interference of protozoan membrane composition altering its permeability [30].
MLT has been associated with apoptosis in *Saccharomyces cerevisiae*. MLT is quickly absorbed and penetrates the internal mitochondrial membrane of *S. cerevisiae*, where it interrupts the membrane potential and causes dose-dependent inhibition of cytochrome c oxidase, leading to cell death by apoptosis [31]. In the current work, the mitochondrial ultrastructure swelling in *Paracoccidioides* spp. yeasts treated with MLT suggests that it affects mitochondrial function, which can also lead to loss of fungal viability. Nevertheless, the mechanism of action of MLT on eukaryotic cells is not well understood. Studies on tumour cells and protozoans suggest that MLT has more than one target and have identified disturbances of lipid-dependent cell signalling pathways in addition to apoptotic processes [31]. Hence, these studies suggest that MLT functions concomitantly or sequentially via one or more of the following mechanisms: (i) a detergent-like mechanism of action that leads to alteration of cytoplasmic membrane permeability; (ii) interference in lipid biosynthesis; and (iii) induction of cell death by apoptosis (mitochondrial pathway).

Notably, the current results suggest that a subinhibitory concentration of MLT (0.5 μg/mL) promotes an increase in melanin production in the yeast form of *Paracoccidioides* spp. Flow cytometry analysis using the melanin-binding antibody 6D2 further supported the finding that yeasts treated with MLT had increased melanin content compared with untreated cells, which is consistent with the results recently observed in *S. brasiliensis* [10]. Also, *Paracoccidioides* yeasts exposed to MLT (0.5 μg/mL) displayed a significant increase in the number of electron-dense particles inside vacuoles (melanosome-like structures) (black arrows in B,C,E,F). Bar = 1 μm; bar in insets = 0.1 μm.

![Fig. 2. Transmission electron microscopy images of (A–C) *Paracoccidioides brasiliensis* (Pb18) and (D–F) *Paracoccidioides lutzii* (Pb01) yeast cells untreated or treated with a subinhibitory concentration of miltefosine (0.5 μg/mL) for 14 days at 37 °C. Untreated yeasts (A,D) displayed an electron-dense cytoplasm containing one or more nuclei (n), mitochondria (m) and electron-lucent vacuoles (v) as well as a compact cell wall and cytoplasmic membrane (insets in A,D). Yeasts treated with miltefosine (B,C,E,F) had alterations of their cytoplasmic membranes (insets in B,E), accumulation of electron-lucent vacuoles (B,C,E), mitochondrial swelling (B,C,F) and increasing numbers of electron-dense particles inside vacuoles (melanosome-like structures) (black arrows in B,C,E,F). Bar = 1 μm; bar in insets = 0.1 μm.](image-url)
Melans are multifunctional polymers found throughout nature and can be synthesised from DHN using precursors of metabolites of the acetate–malonate pathway or formed by the DHN pathway by microorganisms. Melans can protect from toxic effects of MLT, where the subinhibitory concentrations of MLT modulate melanin production in the yeast culture medium.

Table 2
Yeast cell viability of Paracoccidioides brasiliensis (Pb18) and Paracoccidioides lutzii (Pb01) after treatment with miltefosine (MLT) (0.5 μg/mL or 0.25 μg/mL) alone or combined with a subinhibitory concentration of tricyclazole (TCZ) (32 μg/mL) for 7 days at 37 °C.

<table>
<thead>
<tr>
<th>Treatment/concentration</th>
<th>Log CFU/mL (mean ± S.D.)</th>
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<tbody>
<tr>
<td></td>
<td>P. brasiliensis (Pb18)</td>
</tr>
<tr>
<td>Untreated</td>
<td>5.99 ± 0.01</td>
</tr>
<tr>
<td>TCZ 32</td>
<td>5.77 ± 0.07</td>
</tr>
<tr>
<td>MLT 0.5</td>
<td>5.68 ± 1.22</td>
</tr>
<tr>
<td>MLT 0.5/TCZ 32</td>
<td>3.81 ± 1.71</td>
</tr>
<tr>
<td>MLT 0.25</td>
<td>5.95 ± 0.83</td>
</tr>
<tr>
<td>MLT 0.25/TCZ 32</td>
<td>4.58 ± 1.04</td>
</tr>
</tbody>
</table>

S.D., standard deviation; N/D, not detected (<50 CFU/mL).

In summary, this study clearly demonstrates that MLT has low toxicities; however, severe nephrotoxicity caused by MLT is rare. The major drawbacks of MLT are its effect on the mucosa of the gastrointestinal tract when orally administered as well as hepatic and renal toxicities; however, severe nephrotoxicity caused by MLT is rare.

In summary, this study clearly demonstrates that MLT has low toxicities; however, severe nephrotoxicity caused by MLT is rare. The major drawbacks of MLT are its effect on the mucosa of the gastrointestinal tract when orally administered as well as hepatic and renal toxicities; however, severe nephrotoxicity caused by MLT is rare.
References


