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### Permalink

<https://escholarship.org/uc/item/4xf130r2>

### Journal

Journal of Antimicrobial Chemotherapy, 78(4)

### ISSN

0305-7453

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### Publication Date


2023-04-03

### DOI

10.1093/jac/dkad053

Peer reviewed

# Oral delivery of brain-targeted miltefosine-loaded alginate nanoparticles functionalized with polysorbate 80 for the treatment of cryptococcal meningitis

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Received 29 July 2022; accepted 15 February 2023

**Objectives:** To develop alginate nanoparticles functionalized with polysorbate 80 (P80) as miltefosine carriers for brain targeting in the oral treatment of cryptococcal meningitis.

**Methods:** Miltefosine-loaded alginate nanoparticles functionalized or not with P80 were produced by an emulsification/external gelation method and the physicochemical characteristics were determined. The haemolytic activity and cytotoxic and antifungal effects of nanoparticles were assessed in an *in vitro* model of the blood–brain barrier (BBB). A murine model of disseminated cryptococcosis was used for testing the efficacy of oral treatment with the nanoparticles. In addition, serum biomarkers were measured for toxicity evaluation and the nanoparticle biodistribution was analysed.

**Results:** P80-functionalized nanoparticles had a mean size of ~300 nm, a polydispersity index of ~0.4 and zeta potential around –50 mV, and they promoted a sustained drug release. Both nanoparticles were effective in decreasing the infection process across the BBB model and reduced drug cytotoxicity and haemolysis. In *in vivo* cryptococcosis, the oral treatment with two doses of P80 nanoparticles reduced the fungal burden in the brain and lungs, while the non-functionalized nanoparticles reduced fungal amount only in the lungs, and the free miltefosine was not effective. In addition, the P80-functionalization improved the nanoparticle distribution in several organs, especially in the brain. Finally, treatment with nanoparticles did not cause any toxicity in animals.

**Conclusions:** These results support the potential use of P80-functionalized alginate nanoparticles as miltefosine carriers for non-toxic and effective alternative oral treatment, enabling BBB translocation and reduction of fungal infection in the brain.

## Introduction

Cryptococcal meningitis (CM) is an opportunistic fungal infection mainly caused by *Cryptococcus neoformans* that occurs commonly in immunocompromised patients, such as those with advanced HIV infection.<sup>1,2</sup> Inadequate or no treatment is associated with 81% mortality; however, the mortality rate of patients who have received appropriate antifungal treatment reduces to 20%–30%.<sup>1</sup> The infection begins in the lung through inhalation of yeasts or spores present in the environment. Following the establishment of pulmonary infection, cryptococci disseminate primarily to the CNS upon crossing the blood–brain barrier (BBB).<sup>3–5</sup>

The recommended therapy for CM is the combination of amphotericin B with flucytosine in the induction phase followed by

fluconazole in the maintenance phase.<sup>6,7</sup> Besides antifungal therapy being limited to a few drugs, there are problems of significant toxicity and increased resistance.<sup>8</sup> Furthermore, treatment of CNS infections is often difficult because the BBB limits the diffusion of molecules to the CNS and efflux pumps can reduce drug concentrations in the tissue, resulting in therapeutic failure.<sup>9</sup> Thus, the need for new treatment options for CM is evident.

Previous *in vitro* and *in vivo* studies showed that miltefosine, an oral FDA-approved treatment of leishmaniasis (2014),<sup>10</sup> also has broad-spectrum activity and fungicidal effects against dimorphic, filamentous and yeast fungi.<sup>11–21</sup> In female mice with disseminated cryptococcosis, oral miltefosine increased animal survival and reduced the fungal burden;<sup>21</sup> however, a limited effect was observed in male mice models of CM and disseminated

cryptococcosis.<sup>22</sup> Indeed, data on miltefosine antifungal activity in *in vivo* models are scarce and still inconclusive.

Recently, miltefosine was granted 'Orphan Drug' designation by the FDA for the treatments of invasive candidiasis (2021) and primary amoebic meningoencephalitis (2016).<sup>23</sup> However, this drug has some disadvantages, such as limited penetration across the BBB in humans (only 2%–4% of plasma concentration) and high affinity for serum proteins, thus limiting tissue distribution.<sup>24,25</sup> In addition, miltefosine causes gastrointestinal adverse effects when orally administered and presents renal and hepatic toxicities, a teratogenic effect and high haemolytic activity.<sup>24</sup>

The use of drug delivery systems has increased in the last few decades as alternative treatments for many diseases, mainly to overcome drug toxicity and pharmacokinetic limitations. Nanocarriers have been increasingly investigated to deliver drugs and macromolecules to the brain as a non-invasive approach to promote transport through the BBB.<sup>26</sup> In this regard, nanocarriers can undergo surface modifications to improve drug delivery, and the use of surfactants, such as polysorbate 80 (P80), can increase the ability of nanocarriers to cross the BBB.<sup>27</sup> Recently, our research group demonstrated standardized alginate-based nanoparticles as miltefosine carriers as an alternative treatment of cryptococcosis, candidiasis and aspergillosis in a larval model of infection and a murine model of vaginal candidiasis.<sup>12,28,29</sup> Furthermore, the alginate nanoparticles released miltefosine in a sustained manner, decreased drug toxicity in the larval model, and no haemolytic effect was observed compared with free miltefosine.<sup>29</sup> Building upon this work, we functionalized miltefosine-loaded alginate nanoparticles with P80 for brain targeting, and assessed their toxicity, biodistribution and antifungal activity in a human *in vitro* BBB model and in a murine model of systemic cryptococcosis.

## Materials and methods

### Alginate nanoparticle production

Alginate nanoparticles were produced by emulsification using the external gelation method. The unloaded alginate nanoparticles (AN) and miltefosine-loaded alginate nanoparticles (MFS-AN) were produced according to the protocol previously described.<sup>29</sup> The P80-functionalized alginate nanoparticles (P80-AN and P80-MFS-AN) were standardized using the same protocol, except that P80 was included. Briefly, an emulsion with 1.35 g of 1% alginate (MP Biomedicals, USA) and 2.04 g of sunflower oil containing 3% SPAN 80 (Sigma-Aldrich, USA) was prepared, homogenized for 1 min and probe sonicated for 10 min (50 s on—10 s off). Under stirring, 0.2 M calcium chloride with 0.5% P80 (Synth, Brazil) was added and sonicated for 5 min (50 s on—10 s off). After 30 min of stirring, the emulsion was centrifuged for 10 min at 3000 g and the supernatant was removed. Then, 10% trehalose was added to the samples for freeze-drying for 24 h. To obtain P80-MFS-AN, 3 mg of miltefosine (Cayman Chemical, USA) was added to the alginate dispersion.

P80-MFS-AN and MFS-AN nanoparticles were stored at  $-22^{\circ}\text{C}$  for 1 to 90 days to evaluate the stability by determination of average diameter (Dz), polydispersity index (Pdi) and zeta potential. Nanoparticles were diluted in distilled water (1:1000, v/v) to determine Dz and Pdi by dynamic light scattering (DLS), while the zeta potential was measured by electrophoresis using a Nano ZS (Malvern Instruments, UK).

### Encapsulation efficiency and *in vitro* miltefosine release assay

The encapsulation efficiency of miltefosine in the P80-functionalized alginate nanoparticles was obtained by quantification of miltefosine

remaining in the supernatant. To assess drug release, P80-MFS-AN nanoparticles were dispersed in 1 mL of sterile distilled water and incubated at  $37^{\circ}\text{C}$  under constant agitation (200 rpm). At 6, 12 and 24 h, the samples were centrifuged for 5 min at 3000 g and the supernatants were collected for miltefosine quantification. Miltefosine was quantified colorimetrically at 460 nm (Epoch 2, BioTek, USA) based on a miltefosine calibration curve (15.62 to 2000 mg/L).<sup>30</sup> The encapsulation percentage was calculated using the formula:  $100 - (\text{miltefosine in supernatant} \times 100 / \text{amount of initial miltefosine})$ .<sup>29</sup>

### Antifungal susceptibility test

The MIC values of miltefosine, in its free form and loaded in nanoparticles (P80-MFS-AN) were determined by the broth microdilution technique<sup>31</sup> against *C. neoformans* H99. MIC was defined as the lowest concentration that inhibited 90% of fungal growth by visual inspection.

### Haemolytic activity

A 4% suspension of RBCs (v/v, in 5% glucose-PBS) was subjected to treatment with various concentrations of miltefosine and P80-MFS-AN nanoparticles for 2 h in a bath at  $37^{\circ}\text{C}$ . The negative (no treatment) and positive (0.1% Triton X-100) controls were included in the test for haemolytic activity (HA) determination,<sup>29</sup> and 50% HA ( $\text{HA}_{50}$ ) was obtained by linear prediction.

### *In vitro* BBB model

#### Antifungal effect

Endothelial cells (hCMEC/D3 passages 25–30) were seeded at 50% confluent density on collagen-coated permeable transwell inserts (Corning, USA; 8  $\mu\text{m}$  pore diameter) in endothelial basal media (Lonza, USA) supplemented with human fibroblast growth factor (Gibco, USA; 1 ng/mL), 2.5% FBS and antibiotics. After reaching confluence at 4 days, growth factors were reduced first to 50% for 24 h, then to 25% 24 h prior to treatment. Transwells were treated with miltefosine (2 mg/L) or miltefosine-loaded alginate nanoparticles (MFS-AN or P80-MFS-AN; 100 mg/L miltefosine) added to the top of inserts, along with  $3.3 \times 10^4$  *C. neoformans* H99. After 12 h incubation at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ , cfu count beneath the transwell inserts were determined by plating on Sabouraud dextrose agar.

#### Cytotoxicity

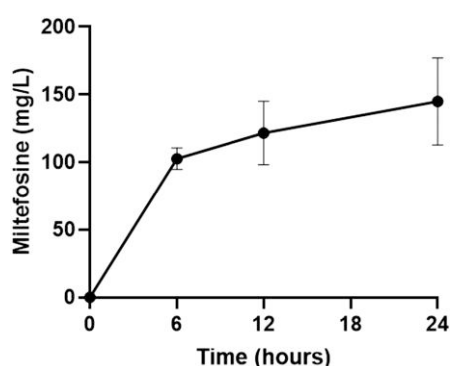
Endothelial cells were grown in 96-well, opaque-wall, clear-bottom plates under the conditions described above. Miltefosine (1.56 to 25 mg/L), MFS-AN or P80-MFS-AN (50 to 800 mg/L miltefosine) were diluted in serum-free media and introduced to triplicate wells. After 24 h incubation at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , cytotoxicity was evaluated with an MTT assay kit (Abcam, USA), and calculated as  $\% \text{cytotoxicity} = 100 \times [(\text{absorbance of control}) - (\text{absorbance of treatments})] / (\text{control absorbance})$ .

### *In vivo* experiments

Male BALB/c mice aged 6–8 weeks with an average weight of 25 g were kept in pathogen-free conditions with water and food *ad libitum* in the Animal Experimentation Vivarium of the Departments of Parasitology and Microbiology (ICB/USP). All experimental protocols were previously approved by the Ethics Committee for Animal Use of the Institute of Biomedical Sciences (CEUA-ICB/USP, Reg 68/2014).

### Antifungal activity and toxicity of alginate nanoparticles in a murine model of systemic cryptococcosis

*C. neoformans* H99 yeast was cultivated twice in Sabouraud dextrose broth for 72 h at  $35^{\circ}\text{C}$ . A yeast suspension was adjusted to  $1 \times 10^7$  cfu/mL of PBS



**Figure 1.** *In vitro* miltefosine release from polysorbate 80-functionalized alginate nanoparticles. The miltefosine quantification, in the supernatant, was performed by the colorimetric method with ammonium ferri-thiocyanate.<sup>30</sup> The experiment was performed three times in triplicate.

for inoculation of 100  $\mu$ L into the tail vein of the mice. After 1 h of infection, the animals (five per group) were orally treated by gavage with 100  $\mu$ L of three treatments: (i) 10 mg/kg miltefosine, in water, once a day for 5 days (miltefosine group); (ii) 20 mg/kg miltefosine-loaded alginate nanoparticles, in PBS, after 1 and 72 h of infection (MFS-AN group); and (iii) 20 mg/kg miltefosine-loaded P80-functionalized alginate nanoparticles, in PBS, after 1 and 72 h of infection (P80-MFS-AN group).

On the sixth day after infection, the animals were euthanized with a lethal dose of anaesthetics (xylazine 30 mg/kg and ketamine 900 mg/kg). The organs (lung and brain) were excised, weighed, macerated in PBS, and plated on Sabouraud dextrose agar with 50 mg/L chloramphenicol and incubated at 35°C for 72 h for cfu count. During organ removal, a fraction of each tissue was separated, fixed in 10% formalin and processed for histopathological analysis using Gomori-Grocott staining.<sup>28</sup>

Additionally, after euthanasia the blood was collected and the serum obtained was used for measurement of glucose, triglycerides, cholesterol, creatinine, urea, alkaline phosphatase (ALP), AST and ALT. In addition, a non-infected and non-treated group (NINT), i.e. mice that did not suffer any procedure ( $n=5$ ), was included in this assay. The analyses were carried out by the wet biochemistry method (Equipment Urit 8210, URIT Medical, China).

## Biodistribution of alginate nanoparticles

### Production of fluorescent nanoparticles

Alginate nanoparticles were produced according to the protocols described above, except that 5 mg of rhodamine B (Rod) (Sigma-Aldrich, USA) was added to the alginate dispersion to obtain the fluorescent nanoparticles, modified or not with polysorbate 80 (P80-AN-Rod and AN-Rod, respectively).

### Biodistribution assay

One hundred microlitres of P80-AN-Rod or AN-Rod, dispersed in PBS, were orally administered by gavage. Animals that received PBS-Rod solution (Rod group) or only PBS (PBS group) were included as control groups. After 12 and 24 h, the animals were euthanized with a lethal dose of anaesthetics. The organs were removed for evaluation of fluorescence in a bioimaging system (IVIS Spectrum System, Perkin-Elmer Life Sciences, USA) using an exposure time of 1 s and excitation/emission wavelengths of 535/580 nm. After that, the organs were macerated with 1 mL of PBS and 100  $\mu$ L added to the wells of the 96-well polystyrene flat-bottom plate for fluorescence quantification in the plate reader (Synergy/

H1, BioTek, USA) at excitation/emission wavelengths of 535/580 nm, resulting in fluorescence measured in relative fluorescence units (RFU).

## Statistical analyses

The statistical analyses were performed using the GraphPad Prism version 8.0 (GraphPad Software, USA) and a  $P$  value less than 0.05 was considered significant.

## Results

### P80-functionalization does not alter the average size and polydispersity of alginate nanoparticles and increases stability

Both nanoparticles P80-MFS-AN and MFS-AN had similar  $D_z$  ( $\sim 300$  nm) and  $P_{di}$  ( $\sim 0.4$ ), and the P80 functionalization increased the absolute value of zeta potential. In addition, the freeze-drying step did not interfere with these parameters (Table S1, available as [Supplementary data](#) at JAC Online).

The size and  $P_{di}$  values of P80-MFS-AN nanoparticles did not change during 60 days of storage, but after 75 and 90 days, the size increased 1.2-fold ( $P < 0.05$ ) although no changes to the dispersion were noticed (Figure S1). Compared with P80-functionalized nanoparticles, the MFS-AN displayed more pronounced increases in size and  $P_{di}$  at shorter periods of time. More specifically, 1.8- and 1.92-fold increases in size and  $P_{di}$ , respectively, at 60 days, and 1.92- and 2.28-fold in size and  $P_{di}$ , at 90 days, respectively, were observed ( $P < 0.001$ ) (Figure S1).

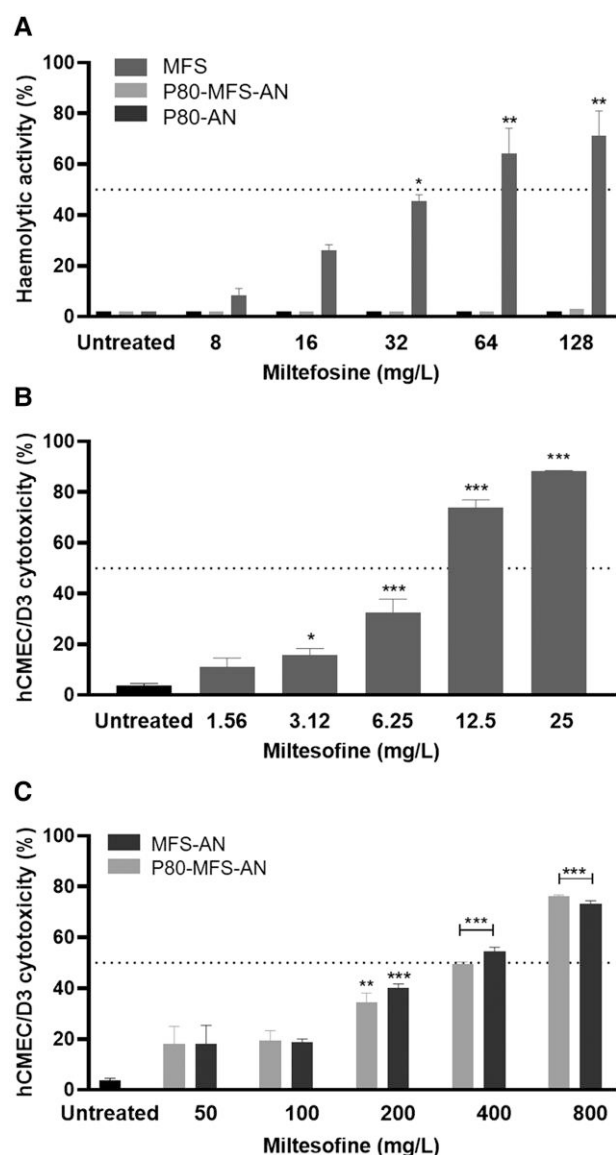
### P80-alginate nanoparticles encapsulate miltefosine and promote its sustained in vitro release

The encapsulation efficiency of miltefosine in P80-functionalized alginate nanoparticles was  $73.20\% \pm 9.89\%$ . P80-MFS-AN nanoparticles promoted a sustained release of miltefosine with an increase in concentration over the first 6 h, obtaining a peak release of approximately 102.65 mg/L in 6 h. After this period, the drug release was slow and prolonged with a peak value ( $\sim 144.87$  mg/L) at 24 h (Figure 1).

### Alginate nanoparticles reduce miltefosine haemolytic activity and cytotoxicity on endothelial cells

The haemolytic effect of miltefosine was concentration dependent (Figure 2a) and its  $HA_{50}$  value was  $\sim 43.6$  mg/L. Unloaded and miltefosine-loaded nanoparticles (P80-MFS-AN) did not cause any haemolytic effect, even at the highest tested miltefosine concentration (128 mg/L), which is consistent with previously obtained results for MFS-AN.<sup>29</sup>

Increases in miltefosine concentration also increased its cytotoxic effects in an *in vitro* BBB model with hCMEC/D3 endothelial cells (Figure 2b and c). Compared with miltefosine, which presented a  $CC_{50}$  of 8.67 mg/L, drug encapsulation in nanoparticles drastically reduced its cytotoxic effect and increased the  $CC_{50}$  by 38.4- and 45.12-fold for MFS-AN and P80-MFS-AN, respectively. In addition, concentrations less than or equal to 3.12 mg/L miltefosine and 100 mg/L miltefosine-loaded nanoparticles (P80-MFS-AN and MFS-AN) resulted in low and insignificant cell damage ( $P > 0.05$ , Figure 2b and c).



**Figure 2.** Cytotoxicity of miltefosine (MFS) in its free form or loaded on alginate nanoparticles. (a) Haemolytic activity of MFS and MFS-loaded alginate nanoparticles, functionalized or not with polysorbate 80 (P80-MFS-AN and MFS-AN, respectively). (b) Cytotoxicity of MFS and (c) P80-MFS-AN and MFS-AN on hCMEC/D3 endothelial cells. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus untreated group (one-way ANOVA with Dunnett's post-test). The assays were performed three times in triplicate.

### ***In vitro* antifungal activity using a BBB model**

The MIC values of miltefosine, in its free form and loaded in nanoparticles (P80-MFS-AN) were 2 and 100 mg/L for *C. neoformans*. Unloaded P80-AN nanoparticles were evaluated as a control and no inhibitory effect was observed.

After cytotoxicity assays and MIC determination, the antifungal activity of 2 mg/L miltefosine and 100 mg/L miltefosine-loaded nanoparticles (MFS-AN and P80-MFS-AN) was evaluated in the *in vitro* BBB model infected with *C. neoformans*. After 12 h, all treatments were significantly effective in reducing fungal viability compared with the untreated group ( $P < 0.001$ , Figure 3).

### ***P80-functionalized alginate nanoparticles decrease fungal burden in the brain and lung in a murine model of systemic cryptococcosis***

The oral treatment with P80-MFS-AN nanoparticles significantly decreased the fungal burden in the brain ( $\sim 1.8$  log,  $P < 0.05$ ) and lung ( $\sim 1$  log,  $P < 0.05$ ) when compared with the untreated group (Figure 4a and b). MFS-AN nanoparticles significantly decreased the fungal burden in the lungs ( $\sim 1$  log,  $P < 0.05$ ), but failed to significantly reduce it in the brain when compared with the untreated group (Figure 4a and b). On the other hand, the oral miltefosine treatment was the least effective, reducing only  $\sim 0.5$  log of fungal burden in the lung and brain of animals (Figure 4a and b). These cfu results corroborated the semi-quantitative data obtained from histopathological analysis, which demonstrated that P80-MFS-AN treatment led to a lower amount of yeast in the brain and lung of the animals, highlighting it as the best antifungal therapy in the murine model of systemic cryptococcosis (Figure 4c–j). In addition, no significant difference was observed in hepatic (AST, ALT and ALP) and renal (creatinine and urea) toxicity biomarkers as well as glucose, triglycerides and cholesterol levels when compared with the NINT group (Table 1).

### ***P80-functionalization increases the biodistribution of alginate nanoparticles and enhances their presence in the brain, suggesting improved translocation across the BBB***

To evaluate the biodistribution of alginate nanoparticles, P80 functionalized or not, we encapsulated a fluorescent marker rhodamine. As expected, little or no fluorescence was detected in the organs of animals that received only PBS. While rhodamine, *per se*, was absorbed and distributed to some organs, the fluorescence intensity was much lower than for animals that received alginate nanoparticles (Figure 5).

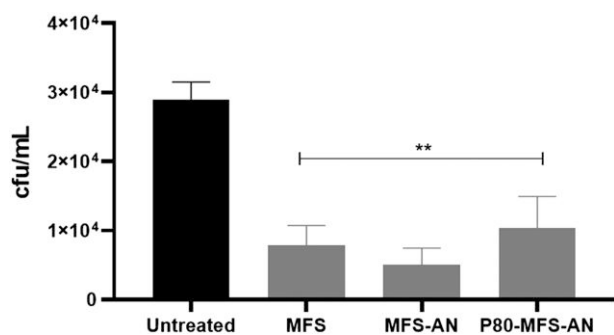
The qualitative analysis of the organs' fluorescence was performed in a bioimaging system at 12 h after oral administration of nanoparticles (Figure 5a). P80 functionalization of alginate nanoparticles (P80-AN-Rod) had a larger biodistribution, observed in almost all analysed organs (heart, lungs, kidneys, liver, pancreas, stomach, testicle, intestine and brain) (Figure 5a). On the other hand, alginate nanoparticles (AN-Rod) were detected in the liver, pancreas, stomach, testicle and intestine (Figure 5a). At 24 h, the organs' fluorescence reduced to control PBS and rhodamine levels.

Importantly, the fluorescence results were confirmed in a plate reader as the method is more sensitive for quantification of fluorescence (Figure 5b). Indeed, P80-functionalized alginate nanoparticles were detected in all organs, with higher amounts in the brain, while non-functionalized alginate nanoparticles were found in the kidneys, spleen and liver; in the lungs and pancreas, they were found in greater amounts than P80 functionalized nanoparticles (Figure 5b).

## **Discussion**

The use of nanoparticle-mediated drug delivery systems functionalized with ligands targeting the CNS for the treatment of brain diseases has been increasing.<sup>26</sup> For fungal brain diseases, few antifungals have suitable CNS penetration with adequate





**Figure 3.** Antifungal activity of miltefosine (MFS), in its free form or loaded on alginate nanoparticles, during *C. neoformans* infection across the BBB transwell model (hCMEC/D3 cells). Fungal burden (cfu/mL) was recovered from the basal compartment of the transwell after 12 h of infection and treatment with free MFS (2 mg/L) and MFS-loaded alginate nanoparticles, functionalized or not with polysorbate 80 (P80-MFS-AN and MFS-AN, respectively) (100 mg/L MFS). \*\* $P < 0.001$  versus untreated group (one-way ANOVA with Dunnett's post-test). The experiment was performed three times in triplicate.

concentrations.<sup>9</sup> Here, we chose P80, due to its low cost, commercial availability and ease of handling.<sup>32</sup> We produced alginate nanoparticles that were P80 functionalized as miltefosine carriers (P80-MFS-AN) that improved antifungal activity in CM treatment when compared with unfunctionalized alginate nanoparticles (MFS-AN).

The physicochemical characteristics of the nanoparticles are important parameters that have an impact on the systemic activities of the nanocarriers,<sup>33</sup> and the P80-MFS-AN nanoparticles had a Dz and Pdi similar to those previously observed for MFS-AN.<sup>29</sup> The P80-MFS-AN showed the absolute value of zeta potential increased to approximately  $-50$  mV. This is an important feature since zeta potential higher than  $30$  mV (in module) has been associated with greater electrical stability and lower particle aggregation.<sup>34,35</sup> The higher zeta potential of P80-MFS-AN compared with MFS-AN may have contributed to its enhanced stability.

The toxicity of miltefosine has been described in previous reports;<sup>24</sup> the haemolytic effect and cytotoxicity on hCMEC/D3 endothelial cells at low concentrations corroborate previous observations. In contrast, the use of alginate nanoparticles as miltefosine carriers reduced its cytotoxicity considerably, abolishing the haemolytic effect and increasing the  $CC_{50}$  values in hCMEC/D3 cells 38–45 times. Drug delivery systems usually reduce the toxic effect of drugs, as observed in this work and by other authors who incorporated miltefosine in delivery systems.<sup>36–38</sup>

The *in vitro* antifungal activity of P80-MFS-AN was similar to that observed for MFS-AN with high values compared with miltefosine.<sup>29</sup> This is expected in *in vitro* assays and can be explained by the slow and sustained release of the drug, also observed in other studies that tested antifungal nanocarriers.<sup>39,40</sup> Additionally, in an *in vitro* BBB model, the miltefosine and miltefosine-loaded nanoparticles were effective in decreasing *C. neoformans*' viability during infection. The hCMEC/D3 immortalized brain endothelial cell line is an alternative to *in vivo* studies of the BBB, which has already been used for research of *C. neoformans* invasion in the CNS.<sup>41</sup> In this model, it was possible to observe that miltefosine and miltefosine-loaded alginate nanoparticles decreased the

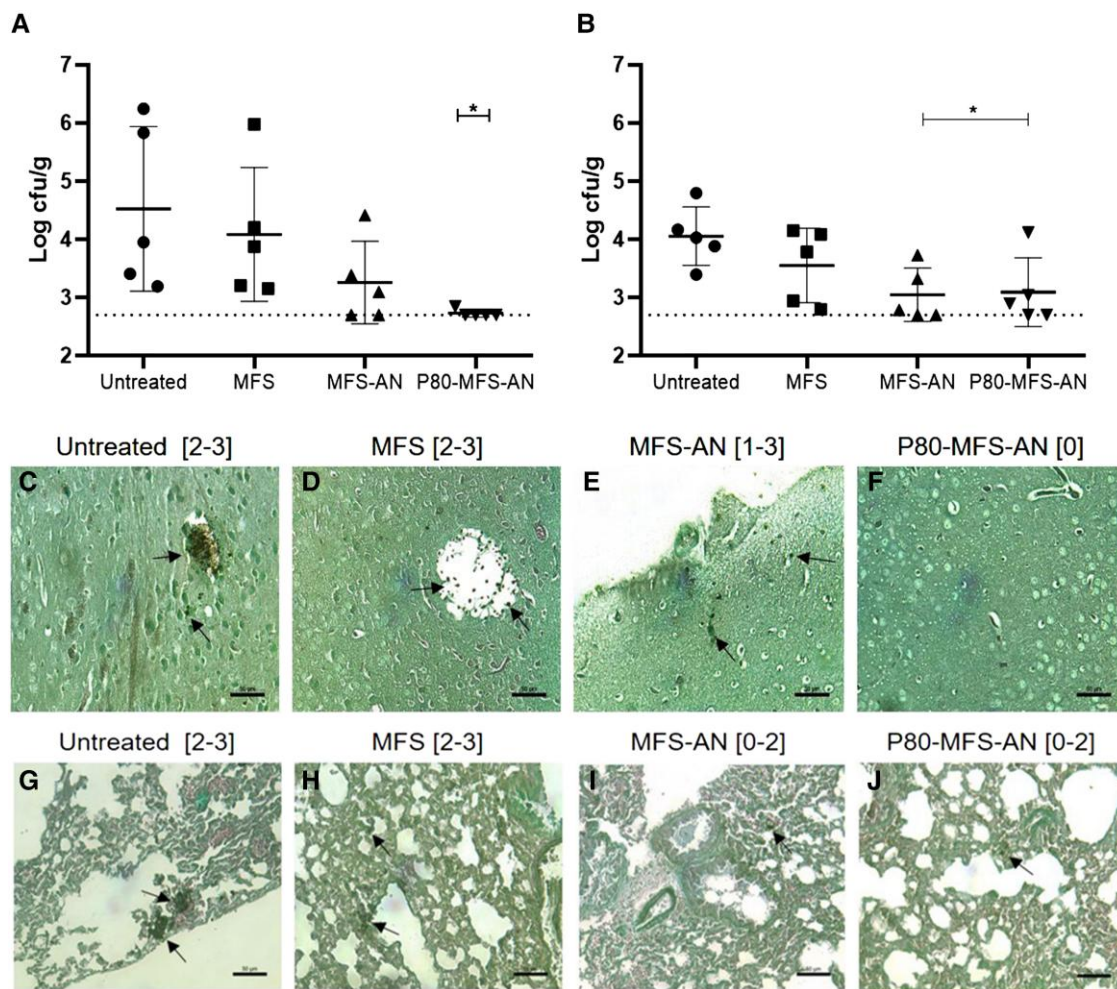
passage of fungus through the BBB, a very important step in the disease and that, for reasons not yet well known, this fungus has a special tropism for the brain and can invade the BBB by different and concomitant mechanisms.<sup>5</sup>

The use of nanoparticles for drug delivery to the brain is a promising alternative due to the possibility of surface multifunctionalization that can promote the targeting or/and crossing enhancement to the BBB.<sup>42</sup> Among functionalization options is the use of surfactants, with polysorbates being the most efficient for targeting to the brain when compared with poloxamers, poloxamine 908, Cremophor<sup>®</sup> EZ, Cremophor<sup>®</sup> RH 40, polyoxyethylene-(23)-lauryl ether (Brij<sup>®</sup> 35).<sup>43</sup> Consistent with previous reports, MFS-AN nanoparticles, which were prepared with poloxamer 407,<sup>29</sup> did not reach the brain in adequate amounts to control the cerebral infection. In contrast, P80-MFS-AN nanoparticles, which were functionalized with P80, showed greater biodistribution and crossed the BBB, reaching the brain in higher amounts and resulting in a significant reduction in fungal burden to undetectable levels.

Our findings corroborated other studies that used P80 for functionalization of amphotericin B-loaded polymeric nanoparticles, enabling the nanoparticles to cross the BBB and increase drug concentrations in brains of mice,<sup>44</sup> whereas free amphotericin B was not detected. As a result, treatment of cryptococcosis with these nanoparticles decreased the fungal burden and increased the survival rate of animals to 80%.<sup>45</sup> In addition, a nanoemulsion with 3% surfactant blend containing Tween<sup>®</sup> 80 and Soluplus<sup>®</sup> and incorporating flubendazole (an anthelmintic reported to have antifungal activity) was effective when orally administered in murine systemic cryptococcosis, decreasing the fungal burden in the brain by 30%.<sup>46</sup>

Several mechanisms have been proposed to explain the efficiency of nanoparticles to pass through the BBB.<sup>47,48</sup> For surfactant-functionalized nanoparticles, e.g. polysorbate 80, the previously proposed mechanism is based on the solubilization of lipids in the endothelial cell membrane that would lead to fluidization and destabilization of the membrane and greater permeability of the nanoparticles through the BBB.<sup>47,48</sup> Additionally, this general effect of surfactants might contribute to the wider biodistribution of P80-functionalized alginate nanoparticles in mice compared with the non-functionalized nanoparticles. This finding might open up new possibilities for therapeutic applications of P80-MFS-AN nanoparticles, such as for the treatment of leishmaniasis and other systemic fungal infections. On the other hand, because wider distribution might lead to more side effects, we also addressed toxicological concerns by assessing markers of hepatic and renal damage. Biochemical analysis of serum after treatment with miltefosine and miltefosine-loaded alginate nanoparticles indicated that hepatic and renal markers, as well as markers of carbohydrate and lipid metabolism were not altered after treatments. These data support the idea that nanoparticles, in the therapeutic scheme used here, are safe to treat CM.

It is important to highlight that oral administration of P80-MFS-AN nanoparticles (two doses, 1 and 72 h after infection) were the best therapy to reduce fungal burden in the brain and lungs while the oral free miltefosine (five doses, 24/24 h) was ineffective to control the infection in systemic cryptococcosis as well as observed previously in CM and disseminated cryptococcosis models treated orally with miltefosine.<sup>22</sup> Our data differ

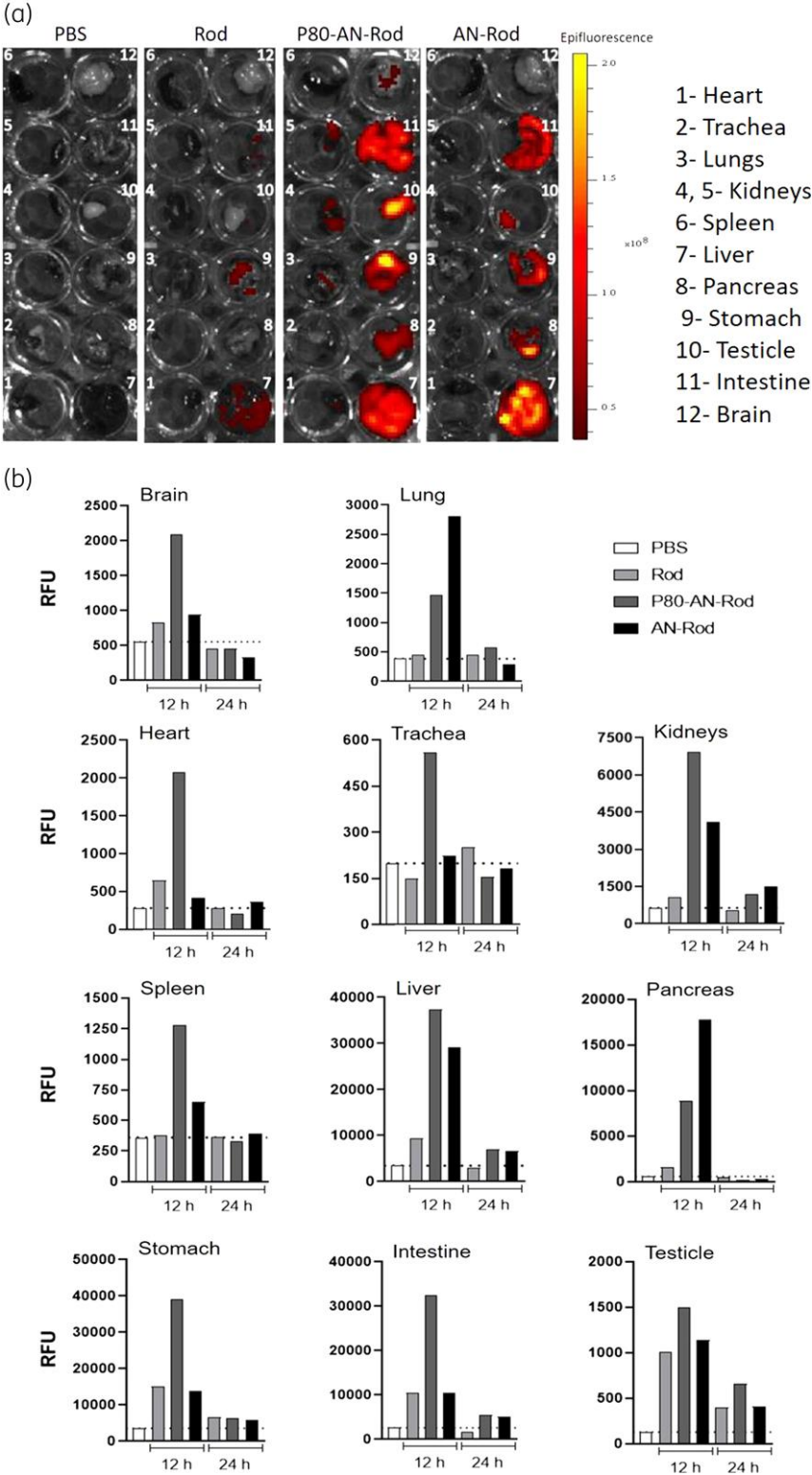


**Figure 4.** Antifungal effect of miltefosine (MFS), in its free form or loaded on alginate nanoparticles, in the murine model of systemic cryptococcosis. Fungal burden in the brain (a) and lungs (b) of male mice infected with *C. neoformans* H99 untreated and treated with MFS or MFS-loaded alginate nanoparticles (MFS-AN and P80-MFS-AN). \* $P < 0.05$  versus untreated group (one-way ANOVA with Dunnett's post-test). Gomori-Grocott-stained histopathological sections of brain (c-f) and lungs (g-j) of the animals untreated and treated with MFS, MFS-AN or P80-MFS-AN. Results of semi-quantitative fungal load analysis from brain and lung tissue sections are indicated in brackets according to the scale: 0, no fungal load; 1, up to 5 fungal elements per section; 2,  $\geq 6$  fungal elements per section; 3, from 6 to 50 fungal elements per field; and 4, more than 50 fungal elements per field.<sup>28</sup> Bars = 50  $\mu\text{m}$ . Arrows indicate the fungal cells. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

**Table 1.** Serum biomarkers of mice infected by *C. neoformans* and treated with miltefosine (MFS) or MFS-loaded alginate nanoparticles functionalized or not with polysorbate 80 (P80-MFS-AN and MFS-AN, respectively)

Biomarkers	NINT	Untreated	MFS	MFS-AN	P80-MFS-AN
Urea (mg/dL)	56.76 $\pm$ 7.77	49.84 $\pm$ 2.70	64.32 $\pm$ 18.56	49.76 $\pm$ 9.35	52.70 $\pm$ 9.66
Creatinine (mg/dL)	0.69 $\pm$ 0.08	0.68 $\pm$ 0.15	0.80 $\pm$ 0.06	0.78 $\pm$ 0.12	0.67 $\pm$ 0.09
AST (U/L)	268 $\pm$ 102.9	263.2 $\pm$ 249.7	265.8 $\pm$ 222.4	329.2 $\pm$ 283.8	197.2 $\pm$ 128.5
ALT (U/L)	49.50 $\pm$ 6.6	44.40 $\pm$ 18.62	52 $\pm$ 25.14	62.20 $\pm$ 23.44	43.20 $\pm$ 17.92
ALP (U/L)	518.4 $\pm$ 55.61	390.8 $\pm$ 83.79	361.6 $\pm$ 48.63	424.8 $\pm$ 114	375.2 $\pm$ 73.02
Glucose (mg/dL)	367.2 $\pm$ 94.28	258.8 $\pm$ 79.26	209.8 $\pm$ 52.96	271 $\pm$ 37.87	293.8 $\pm$ 32.15
Triglycerides (mg/dL)	211.4 $\pm$ 52.86	176 $\pm$ 19.52	208.3 $\pm$ 60.42	186.7 $\pm$ 44.83	194.7 $\pm$ 87.50
Cholesterol (mg/dL)	109.7 $\pm$ 8.57	106.6 $\pm$ 11.56	138.7 $\pm$ 47.13	121.6 $\pm$ 13.59	119.3 $\pm$ 6.43

All values are given as mean  $\pm$  SD.



**Figure 5.** Biodistribution of alginate nanoparticles, functionalized or not with polysorbate 80. (a) Fluorescent images obtained in a bioimaging system of the organs of mice treated with rhodamine-loaded alginate nanoparticles without (AN-Rod) and with polysorbate 80 (P80-AN-Rod) at 12 h. (b) RFU of organs from mice treated with AN-Rod or P80-AN-Rod obtained at excitation/emission wavelengths of 535/580 nm at 12 and 24 h. Rhodamine B (Rod) and PBS were used as controls. These data are representative of an experiment carried out twice. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.



from results reported by Widmer and co-workers,<sup>21</sup> although the murine model used here was similar, except the mice gender (they used female mice). It was reported that steroid hormones affect *C. neoformans* virulence,<sup>49</sup> and oestradiol, a female hormone, is associated with a protective factor against *Cryptococcus* infection.<sup>50</sup>

Notably, the alginate nanoparticles, especially P80-functionalized ones, improved the *in vivo* antifungal effect of miltefosine when compared with miltefosine in solution. Moreover, the increase in dose interval used here resulted from the nanoparticle ability to modify and slow down the release of miltefosine. This is consistent with the drug release profile observed from non-functionalized nanoparticles (MFS-AN)<sup>29</sup> and other alginate-based carriers.<sup>51,52</sup> This feature visibly was relevant at decreasing *Candida albicans* infection, and a single dose of MFS-AN was sufficient to treat vaginal candidiasis in a murine model.<sup>28</sup>

In conclusion, P80-MFS-AN represents a safe oral delivery system to improve the brain delivery of miltefosine in CM treatment. In addition, the nanoparticles promoted sustained release of miltefosine, were more widely biodistributed, including in the brain, and decreased brain fungal burden without producing detectable damage to other organs. This study demonstrates that it is possible to obtain new management strategies for treatment of CM. Finally, our results open many other questions regarding the efficacy of combined treatment of P80-MFS-AN with standard antifungals (amphotericin B, flucytosine and/or fluconazole) and pharmacokinetic/pharmacodynamic data. Further studies must be conducted to bring promising results to improve CNS treatments and support the clinical trials.

## Acknowledgements

We would like to thank Marcela Gonçalves for her assistance in preparation of mice samples for histopathological analysis. IVIS analysis was conducted at the CEFAP core facility (Institute of Biomedical Sciences, University of São Paulo).

## Funding

This study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP, Brazil, 2021/01279-5 and 2018/13877-1), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) and National Institute of Health (NINDS, USA, NS110800). C.C.S. was FAPESP fellow (2018/12149-2) and D.F.F.J. was CAPES fellow (finance code 001). K.I. and L.B.L. are research fellows of the CNPQ (303373/2019-9 and 306866/2020-0, respectively).

## Transparency declarations

None to declare.

## Author contributions

C.C.S. performed the experiments, analysed the results, and wrote the manuscript. M.V.A. and D.F.F.J. contributed with murine model assays. D.M.L. did the blood-brain barrier experiments. K.I., A.G. and L.B.L. designed the experiments, performed data critical review, and wrote and edited the manuscript. All authors have read and approved the manuscript before publication.

## Data availability

All data will be made available from the corresponding author upon request.

## Supplementary data

Figure S1 and Table S1 are available as [Supplementary data](#) at JAC Online.

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