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Amphotericin B: an antifungal drug in nanoformulations for the treatment of paracoccidioidomycosis

La anfotericina B: una droga antifúngica en nanoformulaciones para el tratamiento de la paracoccidioidomicosis

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ABSTRACT

The use of magnetic nanoparticles (MNPs) in drug delivery vehicles must address issues such as drug-loading capacity, desired release profile, aqueous dispersion stability, biocompatibility with cells and tissue, and retention of magnetic properties after interaction with macromolecules or modification via chemical reactions. Amphotericin B (AmB) is still the first choice for the treatment of severe paracoccidioidomycosis, an important systemic fungal infection caused by *Paracoccidoides brasiliensis*. Unfortunately, AmB causes acute side effects (mainly urinary problems) following intravenous administration, which limits its clinical use. The use of magnetic nanoparticles stabilized with biocompatible substances, together with the possibility of their conjugation with drugs has become a new nanotechnological strategy in the treatment of diseases for drug delivery to specific locations, such as the lungs in paracoccidoidiodomycosis. This review provides an overview of the disease, its etiologic agent and treatment with emphasis on the main strategies to improve the use of AmB in nanoformulations.

Keywords: *Paracoccidioides brasiliensis*; amphotericin B; magnetite nanoparticles; magnetic fluid; drug delivery complex

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RESUMEN

El uso de nanopartículas magnéticas (MNPS) en los vehículos de suministro de fármacos debe abordar cuestiones como la capacidad de carga de las drogas, el perfil deseado de liberación, estabilidad de la dispersión acuosa, biocompatibilidad con las células, tejidos y la conservación o la modificación de las propiedades magnéticas después de la interacción con macromoléculas y/o reacciones químicas. La anfotericina B (AnB) continua siendo la primera opción para el tratamiento de la paracoccidioidomicosis grave, una importante infección sistémica causada por el hongo *Paracoccidoides brasiliensis*. Sin embargo, la AnB causa efectos secundarios agudos (principalmente problemas urinarios) tras la administración intravenosa, limitando su uso clínico. El uso de nanopartículas magnéticas estabilizadas con sustancias biocompatibles y conjugadas con fármacos, se ha convertido en una nueva estrategia nanotecnológica para el tratamiento de enfermedades en sitios específicos, como los pulmones en paracoccidioidiodomycosis. En esta revisión se hace una descripción general de la enfermedad, su agente etiológico y su tratamiento con énfasis en la principales estrategias para mejorar el uso de AnB en nanoformulaciones.

Palabras clave: *Paracoccidioides brasiliensis*, anfotericina B, nanoparticulas de magnetita; fluido magnético; entrega controlada de medicamentos

Introduction

The paracoccidioidomycosis (PCM) is a systemic mycosis autochthonous from South and Central America, endemic in rural populations, but with heterogeneous distribution (with low and high endemicity).

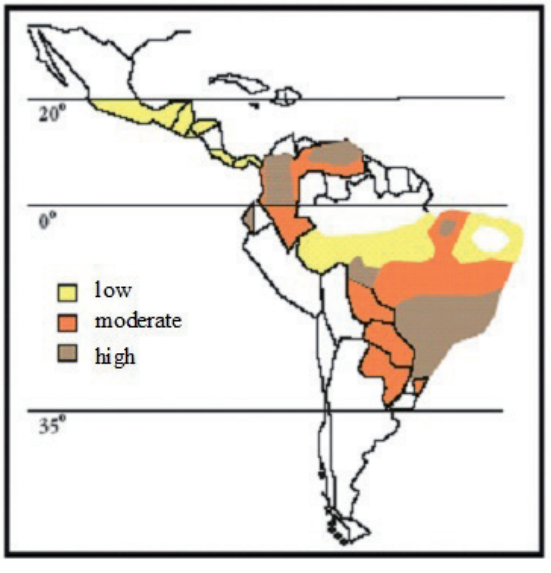

Figure 1. Geographical distribution of paracoccidioidomycosis (adapted from Shikanai-Yasuda et al., 2006)

This mycosis affects mainly men between 30 and 60 years old. It is believed that 50% of the inhabitants of endemic areas in countries such as Venezuela, Colombia, Argentina and Brazil have been exposed to the etiologic agent of this mycosis. However, only 2% of individuals develop some clinical manifestation of the disease [1,2]. It is noteworthy that when not diagnosed and treated appropriately, the PCM can disseminate affecting progressively the lungs, and may reach other organs. Even if the patient presents improvements due to the treatment, it can develop pulmonary fibrosis, the most serious sequel resulting from lung granulomatous processes, leading to limitations of the individual activities [3].

The etiologic agent of the PCM is *Paracoccidioides brasiliensis*, a saprobiotic fungus that in nature presents itself as filamentous between 25 ºC and 30 ºC, *i.e*. a multicellular mycelium containing propagules called conidia infectors. Once inhaled by mammals, the propagules turn into yeast forms of fungus that will be their parasitic form in host tissues, thus behaving as a thermo-dimorphic fungus [1,4].

Treatment of paracoccidioidomycosis

Unlike other pathogenic fungi, the *P. brasiliensis* is sensitive to most antifungal drugs therefore various antifungal agents are used in the treatment of PCM [5]. The choice of drug to be used is in accordance with the state of the patient, and requires, besides a long period of treatment, patient monitoring in order to evaluate the efficacy and his tolerance to the antifungal [6]. Among the most popular drugs, stand out Itraconazole and Amphotericin B [1].

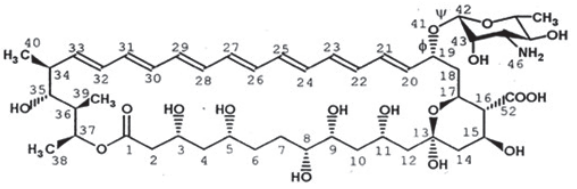

Figure 2. Chemical structure of the drug amphotericin B. Adapted from Carrillo-Muñoz et al., 2006.

Amphotericin B is a polyene antibiotic and is produced by the actinomycete *Streptomyces nodosus*. It has been the drug of choice for the treatment of most systemic mycoses [7,8].

AmB mechanism of action occurs by its hydrophobic interaction with ergosterol, the most abundant sterol in *P. brasiliensis* cell membranes. These interactions induce the formation of aqueous pores in the fungi membranes and thus triggering death [9,10]. Although Amphotericin B preferentially binds to the ergosterol, it can also bind to cholesterol[11], the most abundant sterol founded in mammals' cells membranes [9,12], and for that reason its administration in humans must be controlled. This drug has broad-spectrum antifungal action, potent fungicidal activity and rare episodes of resistance, which contributes to the clinical success of this drug[13]. Nevertheless, AmB induces severe side effects in humans and is considered one of the most toxic antibiotics for humans[7,13], often leading to patient hospitalization during the administration of this drug for adequate monitoring [1,14,15]. The most frequent side effects are nausea, vomiting, fever, headache, hypotension, liver damage, anemia and especially nephrotoxicity [7,13].

The incidence of nephrotoxicity induced by AmB is very high, affecting 49 to 65% of the patients[16]. Nephrotoxicity is due to the binding of AmB to cholesterol in the nephron convoluted tubules cell membranes[14,15,17], creating an ion channel in these membranes that allows the flow of ions and small intracellular molecules. When these ions exit, especially potassium, impairment of cell metabolism occurs [18]. The accumulation of potassium ions in the blood, hyperkalemia, can induce severe cardiac arrhythmia [19]. The serum levels of the drug contribute to increase such toxicological effects, since the greater the amount of free flowing drug, more damage will cause to the kidneys. Nephrotoxicity is the cause of prolonged hospitalization and mortality rates, especially in patients requiring hemodialysis.

In order to reduce the adverse effects of Amphotericin B in treatment of PCM, new strategies are being developed in the formulation of this drug, specially using nanotechnology.

Nanotechnology as a tool for drug delivery

Nanotechnology is referred as the manipulation of matter with at least one dimension sized from 1 to 100 nanometers. Nanobiotechnology is the science that investigates the interactions between those nanoscale materials and biological systems. Thus, nanostructured materials, nanoparticles in particular, exhibit new thermal, mechanical, magnetic and optical properties such as small size, large surface area to mass ratio, and high reactivity delivery, that allow for their widespread application in biomedicine and many industrial sectors [20].

A major contribution of nanobiotechnology for new formulations of conventional drugs is the possibility of creating functional systems of drug delivery at the nanoscale so that their kinetic properties and dynamics can be modified to optimize its pharmacological response [21]. Among the benefits of a nanoscale system for drug delivery is the bioavailability enhancement of associated drugs and/or improvement of drugs distribution and targeting in tissues. This controlled drug delivery allows decreasing in the number of drug applications in patients, and also reduces nanoparticle uptake by the reticuloendothelial system (RES).

Nanoparticles are typically defined as solids with less than 100 nm in all three dimensions. Most often, they are particles having diameters about 10 nm or less and this size is similar to most biological molecules and structures [22]. Thereby, nanoparticles can be useful in biomedical research and applications. Some nanoparticles commonly consist of magnetic elements such as iron, nickel and cobalt and their chemical compounds. These nanoparticles can be synthesized and modified with various chemical functional groups and conjugated with biological molecules or structures, such as drugs of interest, opening a wide range of potential applications in biomedicine [20]. Metal and magnetic nanoparticles have been continuously used and modified to enable their use as a drug delivery system.

It is worth mentioning that liposomes also are used in drug delivery. Liposomes are spherical vesicles made from phospholipids bilayer. Thus lipid-soluble drugs can be incorporated into their lipid phase, whereas water-soluble drugs can be entrapped into their aqueous phase. Their advantage is the easily manufacture

process, they are no covalent aggregates, have almost no toxicity, biodegradable, among others. Therefore, nanoparticles and liposomes can be applied to facilitate the administration of antimicrobial drugs, thereby overcoming some of the limitations in traditional antimicrobial therapeutics. According to Zhang et al, antimicrobial drugs encapsulation enhances therapeutic effectiveness and minimizes side effects of the drugs [23].

Antimicrobial drug associated to metal nanoparticles

Metal nanoparticles exhibit excellent bactericidal action against Gram-positive and Gram-negative bacteria. This effect has been attributed to small size and high surface to volume ratio, which allows them to interact closely with microbial membranes and is not merely due to the release of metal ions in solution [24]. Silver nanoparticles (AgNPs), for example, have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes, like the permeability, in the membrane and cell death [25]. Thus, AgNPs can kill antibiotic-resistant microbes. Several studies have demonstrated antimicrobial effects of silver nanoparticles against *Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Pseudomonas aeruginosa, Bacillus subtilis* and yeast strains [26-29]. It is noteworthy that AgNPs exhibited no antibacterial activity in the presence of serum proteins. However, as demonstrated by Gnanadhas and co-workers AgNPs capped with citrate or poly (vinylpyrrolidone) exhibited antibacterial activities *in vivo* against Salmonella infection compared to uncapped AgNPs [30]. It is worth mentioning that there are numerous consumer products utilizing the antimicrobial properties of AgNPs, such as cosmetics, water filters, and food packaging containers. Although the most common metal nanoparticles used as the antimicrobial agent are AgNPs, gold nanoparticles (AuNPs) are being used effectively against strains of Gram-positive and Gram-negative bacteria like *Micrococcus luteus, Pseudomonas aeruginosa Escherichia coli* and *Staphylococcus aureus.* It also exhibit antifungal activity against *Aspergillus niger* and *Fusarium oxysporum* [31]. Additionally AuNPs dispersed on zeolites eliminate *Escherichia coli* and *Salmonella typhi* in 90 minutes [32].The authors showed that AgNP were intrinsically antibacterial, whereas AuNP were antimicrobial only when ampicillin was bound to their surface. The antimicrobial activity of AuNPs can be attributed to the ability of interaction with functional groups on bacterial cell and inactive bacteria; it cause structural changes, degradation and cell death [33]. Furthermore AuNPs also may act as drug carriers[34].

Antimicrobial tests have also shown that copper nanoparticles can have antimicrobial activity; surfaces of the copper nanoparticles interact directly with the outer bacterial membrane causing it to rupture and thus killing bacteria [24]. The authors clearly demonstrated that copper nanoparticles synthesized in green synthesis method exhibit more antibacterial activity against *Escherichia coli* than copper sulphate solution and pure ginger extract. The encapsulation of antimicrobial drugs in nanoparticle systems enhances therapeutic effectiveness and minimizes side effects of antimicrobial agents.

Magnetic nanoparticles and antimicrobial activity

Magnetic nanoparticles (MNPs) commonly consist of magnetic elements such as iron, nickel, cobalt and manganese or zinc, and most often by ferrite as magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) [35]. Such nanoparticles when dispersed in colloidal solutions constituted magnetic fluids (MFs), stable suspensions of magnetic nanoparticles with a diameter generally ranging between 5 and 15 nm, in inorganic or organic solvent carrier. In these solutions, the particle-liquid interactions are strong enough that their magnetic behaviors are transmitted to the liquid as a whole [36.] Magnetic nanoparticles can bind to drugs, proteins, enzymes, antibodies, or nucleotides and can be directed to an organ. Although MNPs are mostly used on cancer research and treatment, new antibiotic coated magnetic nanoparticles intended as magnetically controllable pharmaceutical agents for the recovery of bacteria loaded tissues and organs. It is reasonable to assume that the conjugation of antimicrobial drugs to magnetic nanoparticles can combine the best properties of both, generating an improved antimicrobial nanoparticle, and enhancing therapeutic effectiveness of antimicrobial drugs in the treatment of infectious diseases.

Antimicrobial activity of MNPs was described by Grumezescu and collaborators, they reveal the synergistic effect of the synthesized water dispersible magnetic nanocomposites on the activity of different antibiotics against Gram-positive and Gram-negative bacterial strains[37]. Similarly, Dong and co-workers have shown that the combination of barbituric acid-based N-halamine with magnetic nanoparticles exhibit higher biocidal activity than the bulk powder barbituric acid-based N-halamine besides facilitates the repeated antibacterial applications [38]. Nevertheless, magnetic nanoparticles are recognized by macrophages of the mononuclear phagocyte system and are eliminated

from the body. In order to improve biocompatibility, to reduce toxicity and to ensure non-immuno-genicity, particles have been encapsulated (e.g., with chitosan, dextran, lactic acid).

Another important factor is that it consists of iron, which acts to maintain the operation of essential metabolic pathways present in living organisms in general [39,40]. For pathogenic microorganisms, especially *P. brasiliensis*, the ability to acquire iron is crucial for the establishment of infection, so that the ability to capture this element from the host is considered a virulence factor [41]. Moreover, studies show that *P. brasiliensis,* both the yeast and mycelial forms, has a metabolic demand for iron[42.] Cano and colleagues demonstrated that the restriction of iron was one of the mechanisms by which inhibit the transformation of yeast in the form of conidia in activated macrophages, subsequently yeast growth within macrophages [43]. Similarly, studies with chloroquine, a drug which affects the metabolism of iron in macrophages decreases the intracellular survival of the yeast *P. brasiliensis* in macrophages by interfering with the acquisition of iron by the fungus [44-46].

Other studies also demonstrate antimicrobial effect of zinc oxide (ZnO) nanoparticles. Vani et al [47] reports that they can be applied effectively for the control of microorganisms and the prevention of infections caused by *Staphylococcus aureus*. The antibacterial activity of ZnO nanoparticles also has been studied by Jones et al [48]. According to the authors these nanoparticles have a potential application as bacteriostatic agent in visible light and may be used to control the spread and infection of a variety of bacterial strains.

Anfotericin formulations

The burden of invasive fungal infections (IFIs) has increased in the last years, especially from the increasing prevalence of individuals with immunosuppression, causing high morbidity and mortality, partly among ill patients [49,50]. Amphotericin B deoxycholate (AmB-D) has been the cornerstone for the treatment of IFIs over the past four decades. Its broad-spectrum fungicidal activity has been showing efficient in candidiasis, cryptococcosis, aspergillosis, histoplasmosis, blastomycosis, coccidioidomycosis, zygomycosis, sporotrichosis, fusariosis, and phaeohyphomycosis. Fungi resistant to AmB are rare, including *Trichosporon* spp., *Aspergillus terreus*, *Scedosporium* spp. and *Malassezia furfur* [51].

However, as already written, conventional AmB-D is associated with adverse effects in 50-90% of cases, including acute infusion reactions, electrolyte imbalances, and dose-dependent nephrotoxicity [46]. The infusion reactions are probably linked with the induction of pro-inflammatory cytokines demonstrated to be produced by AmB and the release of TNF-alpha from macrophages [52]. Nephrotoxicity is defined in most studies as duplication of baseline creatinine levels. It is associated with vasoconstriction causing ischemic injury and direct interaction with epithelial cell membranes causing tubular dysfunction [53].

Given the above, extensive efforts were made to reformulate AmB in the last 15 years. AmB has strong lipophilic properties that led to the encapsulation of the drug into lipossomes or binding to lipid complexes. These lipid formulations of AmB are an attempt to enhance efficacy by increased dosing and to improve the safe profile, reducing the adverse effects [52].

Three lipid formulations of AmB are licensed and available. They are: 1) amphotericin B lipid complex (ABLC), composed of amphotericin B complexed with two phospholipids in a 1:1 drug-to-lipid molar ratio. The two phospholipids, l-α-dimyristoylphosphatidylcholine and l-α-dimyristoylphosphatidylglycerol, are present in a 7:3 molar ratio. ABLC has a ribbons-shaped complex with length range from 1.6 to 11.1 nm. The commercial product is Albelcet®. 2) amphotericin B colloidal dispersion (ABCD), consists of a 1:1 (molar ratio) complex of amphotericinB and cholesteryl sulphate. Upon reconstitution it forms a colloidal dispersion of microscopic uniform disc-shaped particles with diameter range from 120 to 140 nm and thickness of 4 nm. The commercial product is Amphotec® and Amphocil®. 3) Liposomal amphotericin B (L-AmB), consists of a 1:9 (drug-to-lipid molar ratio) of amphotericin B with hydrogenated soy phosphatidylcholine, distearoyl, hosphatidylglycerol, cholesterol, sucrose, and disodium succinate hexahydrate as buffer. It consists of unilamellar bilayer liposomes with amphotericin B intercalated within the membrane. Due to the nature and quantity of amphophilic substances used, and the lipophilic moiety in the amphotericin B molecule, the drug is an integral part of the overall structure of the liposomes. L-AmB is sphere-shaped with diameter range from 45 to 80 nm. The commercial product is Ambisome® [54,55].

These lipid formulations differ in several aspects, in their lipid composition, shape, physicochemical

properties and pharmacokinetic parameters. They share different accumulation rates to various tissue components.

ABLC, because of its size, is taken up rapidly by macrophages and becomes sequestered in tissues of the mononuclear phagocyte system such as the liver and spleen, so it has lower circulating amphotericin B serum concentrations when compared to AmB-D [56]. Lung levels are considerably higher than those achieved with other lipid-associated preparations, suggesting a potential formulation for the treatment of fungal respiratory infections, such as paracoccidioidomycosis. The recommended therapeutic dose of ABLC is 5 mg/kg/day [57].

ABCD complexes remain largely intact, after intravenous injection, and are rapidly removed from the circulation by macrophage. The peak plasma level (C_{max}) achieved is lower than that attained by AmB-D. ABCD exhibits dose-limiting, infusion-related toxicities; consequently, the administered dosages should not exceed 3–4 mg/kg/day [56].

L-AmB avoids substantial recognition and uptake by the reticuloendothelial system due to its small size and negative charge. Therefore, a single dose of L-AmB results in a much higher Cmax/MIC value than AmB-D and a much larger area under the concentration–time curve. Tissue concentrations in patients receiving L-AmB tend to be lower in kidneys and lung and highest in the liver and spleen. Recommended therapeutic dosages are 3–6 mg/kg/day [56-58].

Wade and coworkers, in a well-designed head-to-head observational study, compared the nephrotoxicity and other adverse events among patients receiving liposomal amphotericin B or amphotericin B lipid complex. A total of 327 hospitalized patients were analyzed, they differed in terms of age, gender, race, and urgent/emergent admission status, but all of them were infected with *Aspergillus*, *Candida*, and/or *Cryptococcus*, were older than 18 years, with evidence of renal impairment or with increase risk of nephrotoxicity from AmB. They observed that those receiving ABLC demonstrated approximately threefold greater odds of developing nephrotoxicity compared to patients who received L-AMB, as well as the ABLC therapy was associated with significantly higher rates of infusion reactions [50].

In clinical studies it has been proved that the cost of treatment with lipid formulations of AmB can be overweighed by the cost of nephrotoxicity. Interpreting the findings of all studies is further complicated by differences in study populations with respect to age, disease state, infectious organism, risk factors for IFIs, exposure to nephrotoxic agents, and different definitions of nephrotoxicity [58].

In summary, all of the lipid formulations have demonstrated equivalent efficacy and reduced toxicity compared to AmB-D, but by a mechanism that is not yet exactly known. It is assumed that they offer less free drug that is able to bind to the kidney epithelial cells as mammalian cells are affected only by high free amphotericin B concentrations.

A formulation of poly (lactic-co-glycolic acid) (PLGA) and dimercaptosuccinic acid (DMSA) polymeric nanoparticles loaded with AmB-D (Nano-AmB) was tested in mice infected with *P. brasiliensis*. At 30 days post-infection, the animals were treated with Nano-AmB (6 mg/kg of encapsulated AmB-D, intraperitoneally (ip), interval of 72 h) or AmB-D (2 mg/kg, ip, interval of 24 h) during 30 days. Nano-AmB showed a marked antifungal efficacy. No renal or hepatic biochemical abnormalities, as well as no genotoxicity and cytotoxicity effects, were found in the animals treated with Nano-AmB. Thus, Nano-AmB comprises an AmB formulation able to lessen the number of drug administrations, once it showed a favorable extended dosing interval [59].

Alternatively, our group have been developed a new formulation of AmB associated with maghemite-based magnetic fluid stabilized with bilayer of lauric acid (BCL-AmB).

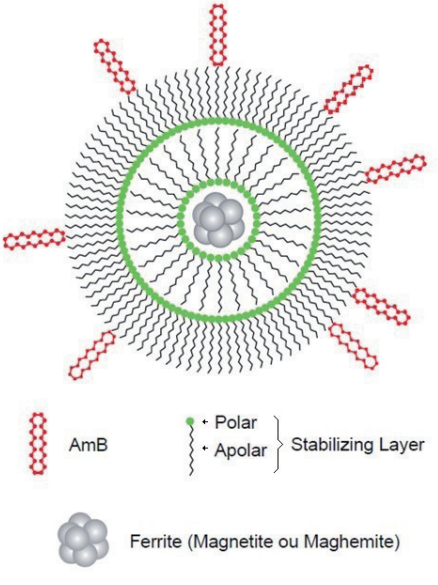

Figure 3. Schematic structure of amphotericin B conjugated with a magnetic nanoparticle stabilized with bilayer of lauric acid.

It is a very stable nanomaterial (over 240 days) with average size value of 13 nm. BCL-AmB presented antifungal activity against *P. brasiliensis* with a higher MIC value compared to AmB-D, and presented no cytotoxicity to the human urinary cells while inducing low cytotoxicity to the peritoneal macrophages. *In vivo* studies showed that BCL-AmB was effective against the acute form of PCM experimental, but not the chronic infection, and did not induce clinical, biochemical and histolopathological alterations (Paper in preparation).

Lung tissue samples removed from *P. brasiliensis*-infected and BCL-AmB treated mice were deposited onto Surface Enhanced Raman Scattering (SERS) active substrates for recording the Raman spectra. The results revealed spectral changes in relative intensities which are associated to the oxidation state of both the protein b_{588} and the myeloperoxidase enzyme, and so consistent with the oxygenation process of neutrophils's heme groups triggered by fungal infection [60].

It is possible to consider that the fungus infection in animals treated with Free AmB and BCL-AmB is much less than in animals treated with PBS, and both treatments led to similar therapeutic outcomes. We claim that the therapeutic approach using BCL-AmB has advantages over the conventional one, since the AmB content administered in BCL-AmB is 40% lower than the content administered in Free AmB, and it is well know that adverse effects reduce as the AmB doses reduce also. In addition, the magnetic drug carrier (BCL-AmB) administration was performed in intervals three-times (72 hours) longer than free AmB (24 hours) [60].

Therefore, it is reasonable to believe that AmB when coupled to magnetic nanoparticles stabilized with bilayer lauric acid, by having similar antifungal activity and do not induce adverse effects at therapeutic doses in acute infection and also allows reduction of the number of applications, can be an alternative nanotool to the treatment of acute form of PCM, but further studies must be done to improve effectiveness in chronic infection.

CONCLUSION

In conclusion, the use of magnetic nanoparticles stabilized with biocompatible substances, together with the possibility of their conjugation with drugs has become a new nanotechnological strategy in the treatment of diseases for drug delivery to specific locations, such as the lungs in paracoccidoidiodomycosis.

CONFLICT OF INTEREST

The authors of this paper have no conflict of interest.

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