Targeted Delivery of Antifungal Liposomes to *Rhizopus delemar*

Subjects: Mycology

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Mucormycosis (a.k.a. zygomycosis) is an often-life-threatening disease caused by fungi from the ancient fungal division Mucoromycota. Globally, there are nearly a million people with the disease. *Rhizopus* spp., and *R. delemar* (*R. oryzae, R. arrhizus*) in particular, are responsible for most of the diagnosed cases. Pulmonary, rhino-orbito-cerebral, and invasive mucormycosis are most effectively treated with amphotericin B (AmB) and particularly with liposomal formulations (e.g., AmBisome®). However, even after antifungal therapy, there is still a 50% mortality rate. Hence, there is a critical need to improve therapeutics for mucormycosis. Targeting AmB-loaded liposomes (AmB-LLs) with the pathogen receptor Dectin-1 (DEC1-AmB-LLs) to the beta-glucans expressed on the surface of *Aspergillus fumigatus* and *Candida albicans* lowers the effective dose required to kill cells relative to untargeted AmB-LLs. Because Dectin-1 is an immune receptor for *R. delemar* infections and may bind it directly, the researchers explored the Dectin-1-mediated delivery of liposomal AmB to *R. delemar*. DEC1-AmB-LLs bound 100- to 1000-fold more efficiently to the exopolysaccharide matrix of *R. delemar* germlings and mature hyphae relative to AmB-LLs. DEC1-AmB-LLs delivering sub-micromolar concentrations of AmB were an order of magnitude more efficient at inhibiting and/or killing *R. delemar* than AmB-LLs. Targeted antifungal drug-loaded liposomes have the potential to improve the treatment of mucormycosis.

Keywords: mucormycosis ; Rhizopus delemar ; C-type lectin receptors ; Dectin-1 ; amphotericin B ; liposomes ; DectiSomes ; oligoglucans ; beta-glucan

1. Introduction

Globally, there are approximately 900,000 individuals with mucormycosis, mostly in India [1][2]. Among those at particular risk are patients with lung diseases; neutropenic patients, such as those receiving prolonged immunosuppression for hematopoietic stem cell transplants; patients receiving long-term treatment for inflammatory diseases; and patients with diabetic ketoacidosis, COVID-19, or AIDS [3][4][5][6][7][8][9][10]. The number of reported cases of mucormycosis has increased 6- to 7-fold in the last four decades [7], paralleling the increasing numbers of individuals on immunosuppressants and very recently COVID-19. Among the diverse Mucoromycota [11], the genus *Rhizopus* and one species in particular, *Rhizopus delemar* (*R. oryzae, R. arrhizus*), are responsible for 50% or more of all diagnosed cases [7][12][13]. *R. delemar* is an opportunistic pathogen living in soil on rotting vegetation. The primary infection route is via inhalation of its sporangiospores, which leads most commonly to pulmonary and rhino-orbito-cerebral infections [14]. Liposomal amphotericin B (AmB) followed by isavuconazole (ISZ) and/or posaconazole (POS) are the most commonly prescribed antifungals [15]. The surgical removal of infected tissue prior to antifungal therapy significantly improves the outcome [16][17]. However, even with antifungal therapy and surgery, there is still approximately a 50% to 99% mortality rate within several months of diagnosis depending upon the level of dissemination at the time of accurate diagnosis and treatment [7][14][16][18]. Clearly, there is a critical need for improved antifungal therapies for mucormycosis.

The immune response to infections caused by *Rhizopus* spp. is mediated by signaling from the C-type lectin pathogen receptor Dectin-1 (CLEC7A) [12]. Dectin-1 is expressed on the surface of some classes of leukocytes, including dendritic cells and neutrophils. Indirect evidence suggests Dectin-1 may bind directly to oligoglucans expressed by *Rhizopus* [19][20]. Two Dectin-1 monomers float together such that their extracellular carbohydrate recognition domains (CRDs) form homo-dimers that bind with high affinity to beta-glucans in the cell wall and/or the exopolysaccharide matrices of pathogens [21]. The researchers have been developing DectiSomes as anti-infective agents, using C-type lectin pathogen receptors to target liposomes loaded with antifungal drugs to pathogenic fungi [22][23][24]. The researchers have shown that the CRD and stalk region of Dectin-1 may be tethered to liposomes loaded with antifungal drugs, targeting these liposomes specifically to beta-glucans on the surface of fungal pathogens [23][25]. As designed, Dectin-1 CRD monomers float in the liposomal membrane and form the functional homo-dimers necessary for beta-glucan binding. Dectin-1-coated, AmB-loaded liposomes (DEC1-AmB-LLs) bind to the cell walls and exopolysaccharides of Aspergillus fumigatus and Candida

albicans orders of magnitude more strongly than untargeted AmBisome®-like AmB-LLs. DEC1-AmB-LLs also inhibit and/or kill in vitro-grown A. fumigatus 100-fold more efficiently than AmB-LLs, reducing the in vitro effective dose for 90% killing more than 10-fold. Considering that Dectin-1 might bind directly to *R. delemar*, the researchers explored the binding of DEC1-AmB-LLs to *R. delemar* and their potential to enhance the efficacy of antifungal liposome treatment.

2. Current Insights

Dectin-1 recognizes beta-glucans that are present in fungal cell walls and exopolysaccharide matrices but are sometimes masked by other molecular components. The researchers showed that Dectin-1 was extremely efficient at targeting AmB-loaded liposomes, DEC1-AmB-LLs, to *R. delemar* swollen sporangiospores, germlings, and mature hyphae. The researchers observed DEC1-AmB-LLs bound primarily to the exopolysaccharide matrix and less so to the cell wall or to exopolysaccharide deposited close to the cell wall. Rhodamine-tagged Dectin-1 protein bound with the same specificity to *R. delemar*'s exopolysaccharide. Hence, it appears that the 100 nm-size of DEC1-AmB-LLs did not significantly limit liposome access to its cognate ligands. DEC1-AmB-LLs were significantly and dramatically more effective at inhibiting and/or killing *Rhizopus* in vitro than untargeted AmB-LLs or BSA-AmB-LLs. Using both cell growth and metabolic assays, the researchers observed that DEC1-AmB-LLs delivering sub-micromolar concentrations of AmB were significantly more efficient at inhibiting and/or killing *R. delemar* than untargeted AmB-LLs. The researchers were able to detect significant loss of metabolic activity within three hours of treatment.

AmB has several partially validated antifungal activities related to its affinity for ergosterol (Erg) in the fungal bilipid membrane, including opening ion channels in the membrane to cause lethal ion leakage and extracting Erg from the lipid bilayer to the membrane surface, which also compromises the membrane [26]. The results do not distinguish among the various mechanisms of AmB's activity. Yet, the data robustly demonstrate that that Dectin-1-targeted DEC1-AmB-LLs were more efficiently associated with *R. delemar*'s exopolysaccharides and had greater antifungal activity than either AmB delivered in AmB-LLs or the protein-coated control BSA-AmB-LLs. Therefore, it does not appear that AmB itself plays a measurable role in the enhanced efficacy of targeted liposomes.

Each DEC1-AmB-LL DectiSome contains several thousand molecules of rhodamine B that enhance signal intensity and more than a thousand Dectin-1 receptor molecules on its surface, enabling multimer formation that enhances the avidity of binding to cognate oligoglycans [25]. If a C-type lectin receptor protein was used alone in a fungal cell binding study and assayed by immunofluorescence, signal intensities might be reduced by orders of magnitude relative to that achieved by a fluorescent DectiSome. This makes DectiSomes excellent reagents for examining the direct binding of different C-type lectins to various pathogens [22][23][24][25][27].

The Mucoromycota is an ancient division of the fungal kingdom that contains a large number of morphologically diverse human pathogens that cause mucormycosis [28][29]. They are estimated to have diverged from a common ancestor in the fungal tree of life nearly 1.3 billion years ago [30][31]. Hence, it is not surprising that the glycan composition of the Mucoromycota cell wall and exopolysaccharide matrix [19][32][33][34] appear to be distinct from other pathogenic fungi [35][36][37][38]. The sporangiospore and hyphal cell wall [33] and the exopolysaccharide matrix [34] each are composed of approximately 43% glucose; other components include lower amounts of N-acetyl-glucosamine, mannose, fructose, lipids, proteins, and phosphate [33][34]. Considering that Dectin-1 recognizes oligo-beta-glucans, it is not surprising that Dectin-1-targeted liposomes bound to *Rhizopus.* The weaker binding the researchers observed to the cell wall relative to the exopolysaccharide of *Rhizopus* suggests that most of the cell wall oligoglucans were masked from DEC1-AmB-LL binding. Experiments with DectiSomes targeted by the oligo-mannan-specific C-type lectin Dectin-2 are ongoing.

While liposomal AmB formulations such as AmBisome® delivering as much as 10 mg/kg/day are significantly less toxic than alternative AmB therapies, the several-months-long therapies needed to clear mucormycosis still result in infusion-related reactions and nephrotoxicity [39][40][41]. If DEC1-AmB-LLs can reduce the effective dose of liposomal AmB and/or reduce the duration of treatment in the clinic, this should reduce the risk of patients developing toxic effects from AmB. Salvage therapies after patients become intolerant to AmB include very high doses of posaconazole (POS) or isavuconazole (ISZ) on the order of hundreds of mg/kg/day [42][43][44][45][46]. Even if Dectin-1-targeted liposomes improve the performance of POS or ISZ by 10-fold in the clinic, it may not be cost-effective to prepare targeted liposomes that deliver tens of mg/kg/day doses of these drugs. However, the data also suggest that DEC1-AmB-LLs kill *Rhizopus* faster than untargeted therapies. Enhanced speed of killing may enable patients to clear *Rhizopus* infections with drug regimens of shorter duration or with fewer treatments, reducing the risk of AmB toxicity.

Immunoliposomes have been used in the clinic for some time to target anti-cancer drugs to cancer cells and tumors. They generally improve drug efficacy several-fold over untargeted drugs [47][48][49]. Although conceptually DectiSomes function

similarly to immunoliposomes by targeting drugs to pathogenic cells, DectiSomes have some distinct advantages [24]. C-type lectin receptors such as Dectin-1 generally recognize a much wider variety of target ligands than monoclonal antibodies, which supports their development as pan-antifungal reagents. Dectin-1 in particular recognizes the beta-glucans produced by nineteen of the twenty genera of pathogenic fungi [12]. Once developed for one fungal pathogen in the clinic, it should not be difficult to broaden their application to other pathogens. In addition, C-type lectins are much less expensive to produce than monoclonal antibodies, which will favor their development as reagents to treat fungal diseases in less wealthy countries [50][51][52][53]. Finally, low production costs may encourage the pharmaceutical industry to expend the large amounts of capital needed to develop DectiSomes.

In conclusion, there is a pressing demand for more effective therapeutics to treat mucormycosis because even after surgery and drug treatment, there is still a high mortality rate. The researchers have shown order of magnitude improvements in the in vitro performance of AmB against *R. delemar* when delivered by Dectin-1-targeted liposomes. It appears that targeting liposomal AmB to the exopolysaccharide matrix of *Rhizopus* is sufficient to significantly improve liposomal drug performance. Future experiments will focus on mouse models of mucormycosis, including determining if Dectin-1-targeted liposomes bind to *R. delemar* at infection sites in the lung, reduce fungal burden in the lungs, and improve mouse survival. The researchers also will need to confirm that Dectin-1-targeted liposomes work effectively against other clinically relevant members of Mucoromycota [54] in light of their ancient diversity [29].

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