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Nanodisks protect amphotericin B from ultraviolet light and oxidation-induced damage

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Abstract

BACKGROUND—Macrolide polyene antibiotics possess potent broad-spectrum antifungal properties. Use of these agents in the field or in controlled environments is impeded by their poor water solubility and susceptibility to oxidation- and/or light-induced degradation. While typically used for human disease therapy, there is potential to expand the utility of polyene macrolide antibiotics, such as amphotericin B, for control of fungal disease infestation in agricultural settings. Thus, the susceptibility of this antibiotic to exposure-induced activity loss was evaluated.

RESULTS—Incubation of the prototype polyene amphotericin B (AMB) with phospholipid vesicles and apolipoprotein A-I results in the formation of nanoscale complexes, termed nanodisks (NDs), capable of solubilizing significant quantities of AMB. To evaluate whether AMB incorporation into NDs conferred protection against light- or oxidation-induced damage, yeast growth inhibition assays were conducted. Compared with AMB solubilized in detergent micelles, AMB incorporated into NDs was protected from damage caused by exposure to UV light as well as by KMnO₄-induced oxidation. Furthermore, AMB-NDs inhibited growth of the turfgrass fungus *Marasmius oreades* Fr.

CONCLUSION—Results suggest that this water-soluble formulation of a natural, biodegradable, antifungal agent represents a potential cost-effective, non-toxic and environmentally friendly substitute for chemical agents currently employed to control a range of fungal infestations.

Keywords

apolipoprotein A-I; nanodisks; amphotericin B; *Saccharomyces cerevisiae*; *Marasmius oreades*

1 INTRODUCTION

The macrolide polyene antibiotics comprise a large family of naturally occurring compounds produced by several species of *Streptomyces*. Common structural features shared by polyenes include a macrocyclic lactone ring with a series of conjugated double bonds (Fig. 1A). Approximately 100 different polyene antibiotics have been described, and these differ in the size of the macrolide ring, the number of double bonds and the presence or absence of mycosamine and/or aromatic ring structures.¹ In general, polyene antibiotics display potent antifungal activity while having little or no activity against bacteria. The mechanism of action of these compounds involves membrane pore formation which results in ion leakage and,

ultimately, cell death. Interaction of polyenes with membrane sterols, including the fungal sterol ergosterol is important for pore formation.^{2,3} Characteristic properties of these compounds include insolubility or very poor solubility in aqueous solvents and extreme susceptibility to oxidation and light-induced damage, causing loss of biological activity.⁴

Amphotericin B (AMB) has gained considerable clinical attention as a therapy for systemic fungal infections. Indeed, this antibiotic has been used to treat human disease for nearly 50 years.⁵ Likewise, the related polyene nystatin is commonly used to treat oral thrush,⁶ while pimarinin (also known as natamycin) is used in the food and beverage industry to prevent fungal contamination.⁷ Advantages of the polyene antibiotics include their broad specificity, their fungicidal activity profile and the lack of development of resistance by susceptible strains. Recently, the authors developed a novel AMB formulation that effectively solubilizes high concentrations of AMB into nanoscale ternary complexes of AMB, phospholipid and scaffold protein,⁸ termed nanodisks (NDs) (Fig. 1B).

Based on the water solubility, nanoscale size and retention of potent antibiotic activity, the authors explored whether incorporation of AMB into NDs permits applications beyond human disease therapy. The present study reports that integration into NDs protects AMB from UV irradiation and oxidation-induced loss of activity and shows that AMB-NDs inhibit growth of the common turfgrass disease fungus *Marasmius oreades* Fr.

2 MATERIALS AND METHODS

2.1 Materials

Recombinant apoA-I was produced as previously described.⁹ Amphotericin B lipid complex (ABLC) and AMB deoxycholate were obtained from the In-patient Pharmacy at the Children's Hospital and Research Center, Oakland. AMB deoxycholate was reconstituted in sterile deionized water according to the manufacturer's instructions.

2.2 Analytical procedures

ApoA-I concentration was determined by the bicinchoninic acid assay (Pierce Chemical Co.) with bovine serum albumin as standard. AMB levels were determined using an extinction coefficient at 416 nm = $1.214 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ in dimethylsulfoxide (DMSO).¹⁰

2.3 AMB-ND preparation

Unless otherwise indicated, AMB-NDs were prepared as described by Tufteland *et al.*¹¹ Briefly, ABLC was gently agitated to disperse the settled mixture. Quantities of 1 mL ABLC suspension (5 mg phospholipid, 5 mg AMB) and 0.4 mL apoA-I (5 mg mL⁻¹) in phosphate-buffered saline (PBS; 20 mM sodium phosphate, pH 7.4, 150 mM sodium chloride) were combined, and the vessel was flushed with nitrogen gas, capped and subjected to bath sonication at 22 °C for 40 min. The resulting solution was sterile filtered (0.22 µm) and stored at 4 °C until use. Empty NDs lacking AMB, as well as AMB-NDs used in *M. oreades* growth inhibition studies, were prepared according to Oda *et al.*⁸

2.4 UV irradiation exposure studies

Samples (in quartz cuvettes) were placed 5 cm from a 6W UV lamp (UVGL-58; UVP, Upland CA) and irradiated for 0–12 h at a wavelength of 254 nm. Control samples were kept in the dark at 4 °C. The effect of UV exposure on AMB biological activity was monitored in yeast growth inhibition assays (Section 2.7) conducted using a fixed AMB concentration of 0.3 µg mL⁻¹.

2.5 UV/V is absorbance spectroscopy

Aliquots of AMB-NDs or AMB-deoxycholate (7.5 µg AMB; volume <10 µL) were transferred to DMSO (1 mL), and the sample was scanned from 300 to 450 nm in a Perkin Elmer Lambda 20 spectrophotometer.

2.6 Chemical oxidation

Specified AMB formulations (260 µM AMB) were incubated in solutions containing increasing amounts of potassium permanganate (0–600 µM) for 24 h at 20 °C. Subsequently, AMB biological activity was determined in yeast growth inhibition assays (Section 2.7) using a fixed AMB concentration of 0.15 µg mL⁻¹.

2.7 Yeast growth inhibition assays

Cultures of the yeast *Saccharomyces cerevisiae* Meyer ex Hansen were grown in yeast extract peptone glucose broth medium (YEPD; Teknova, Hollister, CA). A quantity of 20 µL of a saturated overnight culture was used to inoculate 5 mL YEPD in the absence or presence of indicated amounts of a given AMB formulation. Cultures were grown for 16 h at 28 °C with rotation, and the extent of culture growth was monitored by measuring sample turbidity at 600 nm.

2.8 *Marasmius oreades* growth inhibition assays

Marasmius oreades (ATCC No. 4019) was cultured in GMY medium (4 g L⁻¹ glucose; 10 g L⁻¹ malt extract; 4 g L⁻¹ yeast extract) for 1–3 weeks with shaking at room temperature. During this time, the fungus grew slowly, forming a collection of 2–4 mm balls. A quantity of 10 mL of the medium was removed, and the hyphal masses were disrupted by vortexing with glass shards for 30–45 s. This procedure yielded a heterogeneous suspension of material. The sample was diluted (1 : 4), and 600 µL was transferred to incubation tubes containing various amounts of empty NDs or AMB-NDs. The tubes were shaken at room temperature in the dark, and the amount of fungal growth was examined after 3 days of incubation at room temperature (~22 °C).

3 RESULTS

3.1 Rationale and design

In an effort to expand the utility of polyene macrolide antibiotics for the control of fungal disease, the authors evaluated the effect of incorporation of AMB into NDs on the susceptibility of this antibiotic to exposure-induced activity loss. AMB-NDs prepared using established methods confer water solubility to the antibiotic at concentrations of >10 mg mL⁻¹. Since AMB itself is virtually insoluble in aqueous media, control incubations were conducted using AMB solubilized in deoxycholate micelles.

3.2 Effect of UV irradiation on AMB biological activity

In preliminary experiments, AMB-NDs and AMB deoxycholate micelles were exposed to sunlight for 4 h. Following this, the effect of sunlight exposure on the ability of AMB to inhibit *S. cerevisiae* growth was monitored and compared with control samples incubated in the dark. Whereas AMB-NDs and AMB deoxycholate retained full biological activity on incubation in the dark, the AMB deoxycholate formulation lost activity rapidly on exposure to sunlight. By contrast, however, AMB formulated into NDs was protected from light-induced loss of biological activity under these conditions (data not shown). In an effort to extend these observations and control for variability in sunlight intensity and ambient temperature, experiments were conducted using a UV lamp. Samples were exposed to a constant level of UV irradiation for 0–12 h at 20 °C, followed by assessment of yeast growth inhibition activity.

In the case of AMB deoxycholate micelles, after 6 h of exposure to UV irradiation, biological activity was lost (Fig. 2). On the other hand, AMB incorporated into NDs was largely unaffected by UV irradiation, retaining its biological activity under these conditions.

3.3 Absorbance spectroscopy of AMB before and after exposure to UV radiation

AMB dissolved in DMSO displays a characteristic spectral profile with well-resolved maxima at 416, 392 and 372 nm.⁸ At time 0, aliquots of AMB-NDs and AMB deoxycholate in DMSO gave rise to similar spectra. After 12 h UV exposure, however, absorption peaks for AMB were abolished in the AMB deoxycholate sample. On the other hand, the AMB-ND sample retained its characteristic spectral signature (Fig. 3), although the maxima were decreased in intensity (37% decline at 416 nm). The observation that, in spite of the decline in spectral maxima, there was no apparent loss in biological activity (see Fig. 2) may be explained by the fact that the AMB concentration employed in these assays ($0.3 \mu\text{g mL}^{-1}$) was far enough above the IC_{90} ($0.15 \mu\text{g mL}^{-1}$) for a minor change in biological activity not to have been detected.

3.4 Effect of AMB formulation on antibiotic susceptibility to oxidation

Another major environmental factor that can potentially limit expanded use of polyene antibiotics is oxidation. The conjugated double bonds characteristic of this family of compounds represent a target for oxidation-induced damage and subsequent loss of biological activity. To determine if incorporation into NDs protects AMB from oxidation-induced loss of biological activity, AMB solubilized in NDs or deoxycholate micelles were incubated in buffer containing increasing concentrations of potassium permanganate for 24 h, followed by determination of yeast growth inhibition activity (Fig. 4). The results showed that, whereas AMB deoxycholate was highly susceptible to oxidation-induced loss of activity, AMB incorporated into NDs was protected, retaining biological activity after exposure to potassium permanganate.

3.5 Effect of temperature and solution pH on AMB biological activity

Other factors that could potentially limit the use of polyene antibiotics in field applications include susceptibility to temperature and/or pH extremes. To examine this, AMB deoxycholate and AMB-NDs were incubated at various temperatures (up to 43°C) or pH solutions (range $\text{pH} = 3\text{--}9$) for 24 h, followed by determination of the yeast growth inhibition activity. No loss of biological activity was detected with either formulation (data not shown).

3.6 Effect of lyophilization on the biological activity of AMB-NDs

Long-term storage is a concern for an aqueous formulation of AMB, and this concern is exacerbated by knowledge that liquid formulations of AMB are reported to have a relatively short shelf life of approximately 30 days.¹² Indeed, for optimal versatility in environmental settings, it is essential that a given polyene antibiotic formulation be able to withstand lyophilization and subsequent reconstitution with retention of biological activity. To examine this, a solution of AMB-NDs in PBS was subjected to lyophilization. Following reconstitution of the dry sample to its original volume with water, sample clarity was restored with no loss of material as a precipitate. Furthermore, yeast growth inhibition assays (Fig. 5) revealed that control and reconstituted AMB-NDs displayed identical antifungal activities.

3.7 Effect of AMB-NDs on *Marasmius oreades* growth

To assess the ability of AMB-NDs to inhibit growth of a fungal species that impacts upon agriculture, studies were conducted with the fairy ring mushroom, *M. oreades*. This species of saprophytic fungi is probably the best known of all turfgrass diseases and is economically important. Whereas chemical control methods are usually employed, the authors determined whether AMB-NDs could inhibit the growth of this fungus. Incubations of *M. oreades* with

control empty NDs had no effect on fungal growth when compared with untreated control cultures. In the case of AMB-NDs, however, high sensitivity to AMB was observed (Table 1), with an apparent minimal inhibitory concentration of $0.3 \mu\text{g mL}^{-1}$ AMB.

4 DISCUSSION

Fungal infestations in agricultural crops, ornamental plants and turfgrass cause significant damage every year.¹³ Currently, the predominant way in which such diseases are treated is with chemical agents that, in many cases, are toxic to the environment. Thus, it is generally agreed that there is a need for environmentally friendly, biodegradable yet effective antifungal agents. Among naturally occurring compounds, the family of polyene antibiotics possesses broad-spectrum and potent antifungal properties.¹⁴ Factors that prevent their application in many cases relate to their very poor solubility in water combined with their high sensitivity to sunlight- or oxidation-induced damage, with corresponding loss of biological activity. Thus, whereas the polyene antibiotic AMB has been used to treat systemic fungal infections in humans for 50 years, to the authors' knowledge this broad-spectrum antibiotic has not been successfully employed in field applications.

In 2006, Oda *et al.*⁸ reported the incorporation of AMB into nanoscale ternary complexes of phospholipids, apolipoprotein and AMB. These disk-shaped particles (i.e. NDs) can be prepared at high AMB concentration, conferring considerable solubility in aqueous media. When considered in light of the potent antibiotic activity of AMB, ND particles may provide a useful means to deliver this drug. The present study was conducted in an effort to expand the use of AMB-NDs beyond the treatment of systemic human fungal infections. To do this, the authors evaluated several parameters related to AMB stability and biological activity. Whereas AMB bioactivity is reportedly destroyed by pH extremes, heat, light or air,¹⁵ the data presented here show that incorporation of AMB into NDs confers considerable protection against light- and oxidation-induced damage, in addition to withstanding potentially damaging effects of pH and temperature extremes. These findings, together with the fact that highly concentrated solutions of AMB-NDs can be generated and the antibiotic is potent at very low concentrations, suggest that the complexes may be useful for field application.

In an effort to evaluate the growth inhibition properties of AMB-NDs against an economically important fungal pest, the ability of AMB-NDs to inhibit growth of the 'fairy ring' mushroom, *M. oreades*, was investigated. Fairy ring is a common disease of turfgrasses worldwide, and necrotic and severely injured turfgrasses are frequently observed in those sites exhibiting fairy rings.¹⁶ More fungicides are used in the treatment of turfgrass than on any other crop in the United States.¹³ However, fungicides have achieved only marginal success in the control of fairy ring. Many fail because they are not water soluble and are therefore difficult to distribute uniformly to the fungi-colonized soil.¹⁷ As such, there is a need for a water-soluble and biodegradable fungicide formulation that can withstand environmental exposure. The finding that AMB-NDs show strong growth inhibition activity against this species suggests that AMB packaged in this way may be a suitable, cost-effective means to control or prevent fungal infestations of turfgrass.

In considering the broad range of polyene antibiotics characterized to date, their potent broad-spectrum antifungal properties and their naturally occurring, non-toxic, fully biodegradable chemical composition, combined with their solubilization in ND complexes, suggest broad potential for new field applications. Indeed, fungal diseases of plants are a major concern for ornamentals, agricultural crops and turfgrass, with significant annual losses occurring. Further research on ND particles harboring other members of the family of polyene antibiotics will likely reveal new applications.

Acknowledgments

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Figure 1.
(A) AMB chemical structure and (B) AMB-ND schematic diagram.

Figure 2.

Effect of UV irradiation on AMB-mediated *Saccharomyces cerevisiae* growth inhibition. AMB deoxycholate (○) or AMB-NDs (●) were exposed to UV light (254 nm) for 0–12 h. Following UV exposure, AMB (1.5 µg AMB) was added to 5 mL YEPD medium inoculated with *S. cerevisiae*. After culturing for 16 h, yeast growth was determined by absorbance at 600 nm. Values reported are the mean ± SD ($n = 3$).

Figure 3.

Effect of UV exposure of AMB on spectral properties. AMB deoxycholate or AMB-NDs in buffer were incubated in the presence or absence of UV light. Following incubation, sample aliquots (7.5 μ g AMB) were transferred to 1 mL DMSO and scanned from 300 to 450 nm. (a) control AMB-NDs; (b) control AMB deoxycholate; (c) AMB-NDs exposed to UV light for 12 h; (d) AMB deoxycholate exposed to UV light for 12 h.

Figure 4.

Effect of potassium permanganate on AMB-mediated inhibition of *Saccharomyces cerevisiae* growth. AMB deoxycholate (○) or AMB-NDs (●) were incubated in buffer containing indicated amounts of potassium permanganate for 24 h. Following incubation, aliquots of each formulation (0.75 μg AMB) were added to 5 mL YEPD medium inoculated with *S. cerevisiae*. After culturing for 16 h, yeast growth was determined by absorbance at 600 nm. Values reported are the mean ± SD ($n = 3$).

Figure 5.

Effect of lyophilization on AMB-ND-mediated *Saccharomyces cerevisiae* growth inhibition. AMB-NDs in PBS (pH = 7.4) were stored in solution at 4 °C or quick frozen and lyophilized to dryness followed by reconstitution in water. Increasing amounts of lyophilized/reconstituted (○) and control (●) AMB-NDs were added to YEPD medium inoculated with *S. cerevisiae*. After culturing for 16 h, yeast growth was determined by absorbance at 600 nm. Values reported are the mean ± SD ($n = 3$).

Table 1Effect of AMB-NDs on *Marasmius oreades* growth

AMB-ND concentration ($\mu\text{g mL}^{-1}$)	<i>M. oreades</i> growth ^a
0.12	++
0.3	±
0.6	–
1.2	–

^a ++, non-inhibitory, 100% growth; ±, minimum inhibitory concentration (MIC); –, no growth observed.