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Differential modulation of the antifungal activity of amphotericin B by natural and *ent*-cholesterol

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Abstract—The addition of exogenous *ent*-cholesterol suppressed the antifungal activity of the amphotericin B when added to cultures of *Candida albicans*, but to a lesser extent than natural cholesterol. There were no detectable differences between added **2a** or **2b** on the antifungal activities of jaspamide or bengazole A, two unrelated antifungal natural products.

1. Introduction

The polyene antibiotic amphotericin B (AmB, **1**) has been the drug of choice for over three decades for treatment of systemic, life-threatening fungal infections including opportunistic infections in AIDS patients.[1] Although effective azole drugs such as Fluconazole and Voriconazole[2] have risen in clinical importance and bypass some of the serious side effects of polyene antibiotics, AmB exhibits two properties that are highly valued: very low rates of resistance, and true fungicidal activity rather than the fungistatic properties of azole drugs.[3] AmB manifests activity against cells by binding to sterol present in fungal cell-membranes, inducing membrane permeability and eventually cell lysis. AmB forms a 1:1 complex with sterol that undergoes spontaneous self-assembly into a barrel-shaped pore than spans the width of the lipid bilayer and induces ion-permeability, especially towards K^+.[4] The association of AmB with cholesterol is weaker than that with ergosterol (**3**), the major phytosterol found in fungal cell membranes.[5] This difference in AmB binding between mammalian and fungal sterols is thought to be the basis of the therapeutic index of AmB and related polyene antibiotics.

The nature of the AmB/sterol complex and its self-assembly has been studied intensively but hampered by the amphiphilic properties of AmB and the lack of consistent models to explore the biological and physicochemical properties of AmB-induced membrane channels. Recent CD and ion-channel measurements of phospholipid liposomes containing synthetic covalently linked AmB dimers provided evidence for enhanced sterol-dependent ion-permeability.[6] It is known that addition of exogenous **2** or **3** to fungal cell cultures prior to treatment with **1** resulted in suppression of the antifungal activity. This phenomenon was ascribed to competition for **1** with exogenous sterol versus sterol present in the bilayer.[5b] Structure–activity studies showed a strong dependence of the residual antifungal activity of AmB with the structures of added exogenous sterol. New studies by Murata and co-workers show that addition of cholesterol to prepared phospholipid vesicles, *after* the incorporation of AmB into unilamellar phospholipid membranes, results in suppression of ion conductance, possibly as a result of 'membrane thickening'.[7] Collectively, these model studies reprise the pivotal role of sterol in AmB interactions with phospholipid membrane in relevant physicochemical models although they do not illuminate the molecular basis for activity.

An important question arises—does the *biological activity* of **1** depend *solely* on changes in the physical properties of the phospholipid membrane induced by sterol or are specific molecular contacts between molecules of sterol and **1** important? We sought to address this question by examining the antifungal activity of **1** in *Candida albicans* cultures in the presence of the two enantiomers of cholesterol. We now report that *ent*-cholesterol

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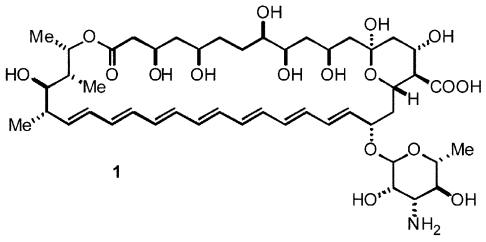
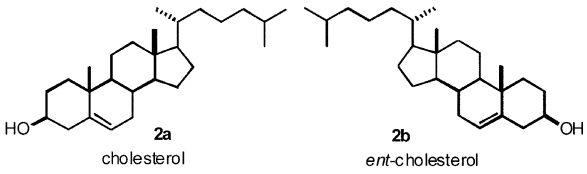
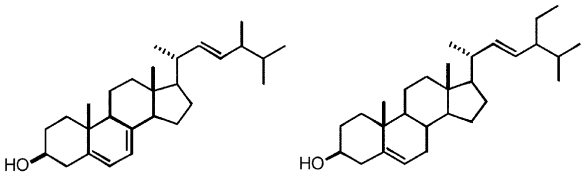


1



2a
cholesterol

2b
ent-cholesterol



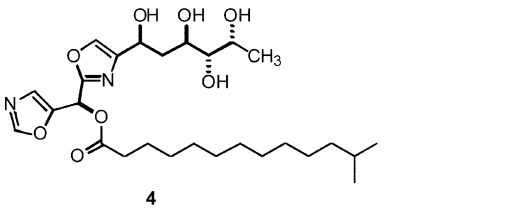
3
ergosterol

7
stigmasterol

(**2a**) and cholesterol (**2b**) manifest differential suppression of antifungal activity with **1**, a property that is not shared by two non-polyene antifungal compounds (bengazole B, **4**,[8] jaspamide, **5**)[9] that were tested under the same conditions. These results suggest that stereospecific molecular interactions in the complexes **1**:**2a** and **1**:**2b** may elicit different biological responses for AmB antibiosis and the key role of AmB–sterol interactions.

2. Results and discussion

Enantiomers can be used as specific probes of the chiral environment of their cognate receptors. It is expected the enantiomer of cholesterol (*ent*-cholesterol, **2b**) will associate differently with **1** due to different molecular contacts between the diastereomeric **1**:**2b** and **1**:**2a** complexes.[10] If the binding of **1** to **2a** affects the biological activity of **1**, we expected the effect to be manifested as altered antifungal activity upon addition of exogenous sterol. *ent*-Cholesterol (**2b**) was synthesized in 17 steps from (−)-(7a*R*)-7,7a-dihydro-7a-methyl-l,5(6*H*)-indandione (**6**) according to our published procedure[11] using a modification of the Hoffmann La Roche protocol,[12] via *ent*-testestosterone, and employing the Hajos–Parrish annulation.[13,14] Bengazole A (**4**) and jaspamide (=jasplakinolide, **5**) were isolated from samples of the marine sponges *Jaspis splendens* and *Jaspis* sp., respectively, using a modification of literature procedures.[15]



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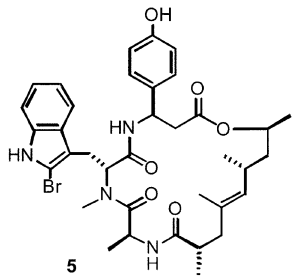

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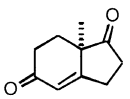

(−)-6

Sterile agar plates were prepared according to standard procedure[16] except the hot aqueous agar solution was treated with ethanol solutions of sterols (**2a**, **2b**, **3** or stigmasterol, **7**) to give media containing final sterol concentrations ranging from 0 to 30 ppm and 1% EtOH. Control plates contained only media and EtOH. Antifungal assays were carried out using the disk diffusion method.[17] Cultures of *C. albicans* were applied evenly over the surface of the each plate and the surface of treated plates were planted with standard 6.5 mm paper disks containing measured aliquots of antifungal agents at concentrations sufficient to achieve 14–24 mm zones of inhibition after overnight incubation (**1**, 4 μg/disk; **4**, 2 μg/disk; **5**, 10 μg/disk). After incubation overnight at 37° C, the circular zones of inhibition were measured to within 0.5 mm. Treatments of **1** with **2a** and **2b** were measured in triplicate and the average zones of inhibition as percentages of the control were plotted against sterol concentrations (Fig. 1).

As expected, addition of sterol to yeast cultures suppressed antifungal activity of **1** down to 62–82% of the control at the highest sterol concentration tested (60 ppm). Ergosterol **3** was the most effective (62% of control) while incorporations of exogenous cholesterol (**2a**) or stigmasterol (**7**) into the agar media were less effective

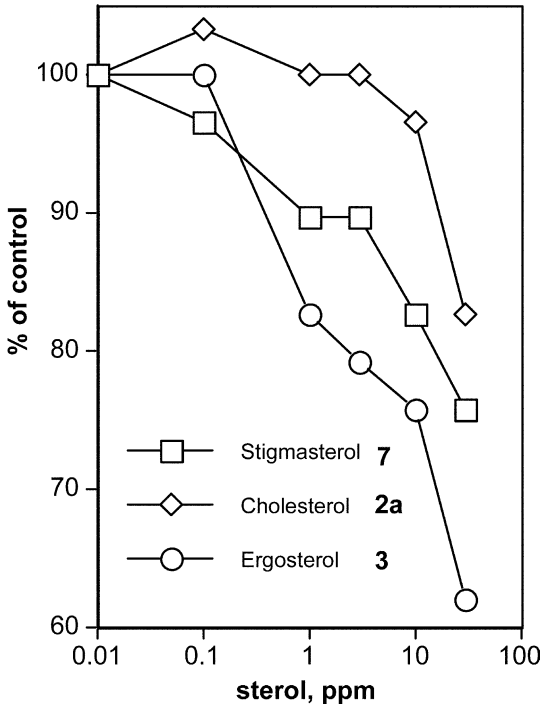

Figure 1. Antifungal activity of **1** (4 μg/disk) against *C. albicans* in the presence of sterol (control = no sterol).

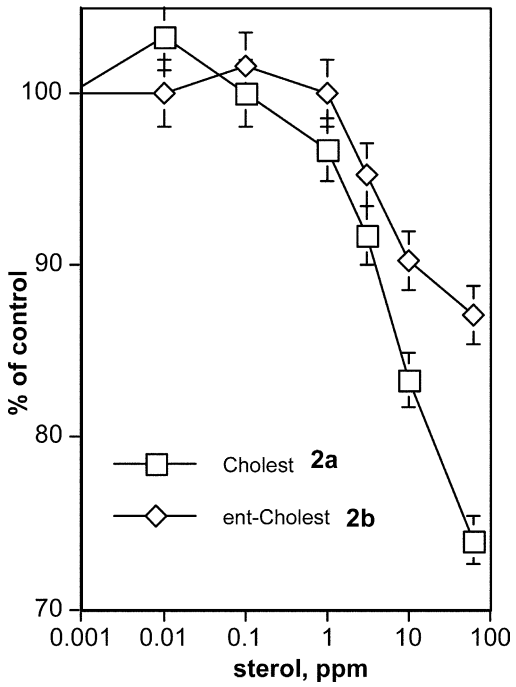

Figure 2. Antifungal activity of **1** (4 μg/disk) against *C. albicans* in the presence of cholesterol (**2a**) or *ent*-cholesterol (**2b**) (control = no sterol).

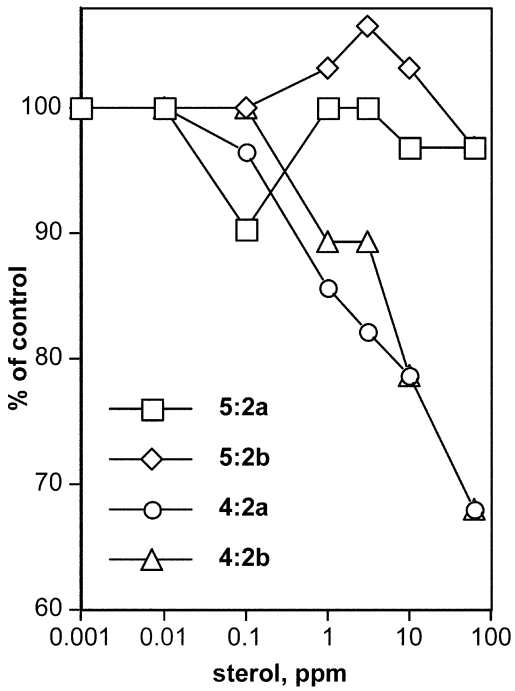

Figure 3. Antifungal activity of **4** (2 μg/disk) and **5** (10 μg) against *C. albicans* in the presence of cholesterol (**2a**) or *ent*-cholesterol (**2b**) (control = no sterol).

in reducing activity (82 and 76% residual activity of the control, respectively). When *ent*-cholesterol (**2b**) was substituted for cholesterol (**2a**) at the same concentrations, the attenuation of AmB activity was diminished (Fig. 2). Amphotericin B retained only 73% of its activity in the presence of 60 ppm of **2a** but 87% of activity in the presence of 30 ppm **2b**. In contrast, neither **4** nor **5** suffered differential suppression of activity against *C. albicans* in the presence of **2a** and **2b** (Fig. 3).

Jaspamide (**5**) is known to disrupt microfilament organization,[18] and thereby exerts general toxicity towards susceptible eukaryote cells, including *Candida* spp., through mechanisms unrelated to membrane permeability.

Bengazole A, a fatty acid ester homologue of **4**,[8] shares, with **1**, the property of sterol-dependent antifungal activity, but **5** does not.[15] The structural similarities between **1** and **4** are not immediately obvious except that both molecules have hydrophilic and hydrophobic regions. Bengazole B has a hydrophobic fatty acid tail that is essential for activity and a hydrophilic polyol side chain, but is neutral at physiological pH. AmB is a zwitterionic macrolide that has an elongated rod-like shape with opposing hydrophobic and hydrophilic faces formed by the linear polyene and polyol segments, respectively. Unlike, **4**, AmB exhibits complex solution-structure behavior. While it appears that **4** does not exhibit differential activity in the presence of the enantiomers of cholesterol, as does **1**, the possibility that **4** binds to sterols in some different manner cannot be discounted.

The first report of enantiospecific properties of **2b** with amphotericin B demonstrated differential behavior of single-channel ion-conductance in bilayers formed from azolecithin or racemic glycerol monooleate with 5% of **2a** or **2b**. *ent*-Cholesterol induced formation of ion-channels with lower conductance than those formed by using natural cholesterol.[19] Other recent reports also demonstrate enantiospecificity of **2b** in the physical properties of membranes.[20] We have now ascribed the enantiospecific effect of cholesterol to a biological property–antifungal activity.

3. Conclusion

The results of this study show a clear enantiospecificity of the interaction with AmB with enantiomers of cholesterol in a biologically-relevant assay. While the unusual behavior of *ent*-cholesterol does not exclude the possibility that it alters membrane fluid or dimensional properties as a result of subtle molecular interactions with the chiral phospholipid molecules, it seems unlikely that such subtle effects would not be averaged out within the highly mobile fluid bilayer. The results described above and earlier work favor a model that ascribes specific molecular contacts between AmB and **2** as important factors in determining sterol-mediated pore formation by AmB, ion-mobilization across fungal phospholipid membranes and cell death.

Acknowledgements

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15. *Jaspis splendens* was collected from Pohnpei, Federated States of Micronesia, and *Jaspis*. sp. (bengazoles) was obtained from the Great Barrier Reef, Australia. In each case, the lyophilized sponge was extracted exhaustively with methanol (MeOH) and the extract partitioned sequentially against hexanes, $CHCl_3$, and *n*-BuOH after first adjusting the H_2O content of the MeOH for each partition to achieve immiscible phases. Bengazoles A–G and jaspamide were found in the CCl_4- and $CHCl_3$-soluble fractions of each respective sample. Final purification was achieved by silica chromatography (silica, MeOH: $CHCl_3$ gradient) followed by HPLC (RP C18 reversed phase, MeOH/H_2O).
16. A simple agar disc diffusion assay for antifungal susceptibility was adapted in the following way. Tryptic soy agar (TSA) plates were prepared by mixing bactoagar (Difco, 4% w/v), tryptic soy digest (Difco, 3% w/v, sterol free by 1H NMR and TLC), hot deionized water with stirred until dissolved. Aliquots of sterol solutions in ethanol were to directly to the hot agar solution and dispersed by vortexing or vigorous stirring prior to autoclaving and pouring into Petri plates. The final EtOH concentration was 1% v/v before autoclaving. Control plates were prepared containing 1% EtOH and no sterol.
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