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Temperature-dependent transfer of amphotericin B from liposomal membrane of AmBisome to fungal cell membrane

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ABSTRACT

Liposomal amphotericin B (AMPH-B), also known as AmBisome, exhibits a potent antifungal effect through its binding to ergosterol contained within the fungal cell membrane. However, the mechanism responsible for the movement of AmBisome-derived AMPH-B to the fungal cell membrane through the cell wall is not yet clear. Therefore, in the present study we aimed at elucidating this mechanism operating in *Saccharomyces cerevisiae*. AmBisome showed its antifungal effect against *S. cerevisiae* at 35 °C but not at 4 °C, whereas free AMPH-B was effective at both temperatures. A significant difference in the amount of AMPH-B transferred to the fungal cells between incubation at 4 and 35 °C was also observed when AmBisome was used. Confocal microscopic study, however, indicated that NBD-labeled AmBisome was localized on the surface of the fungal cells at either temperature. To decrease the affinity of AMPH-B for the liposomal membrane, we entrapped AMPH-B in fluid liposomes containing egg yolk phosphatidylcholine (EPC) instead of hydrogenated soy PC (HSPC). These liposomes showed the antifungal effect even at 4 °C. On the contrary, AMPH-B in liposomes containing ergosterol (Erg-AmB) instead of cholesterol showed a significantly weaker antifungal effect at 35 °C with reduced transfer of AMPH-B to the fungal cells. These results suggest that not the binding of AmBisome to target cells but the transfer of AMPH-B from liposomal membrane of AmBisome to the cell membrane is critical for the antifungal activity of AmBisome. This transfer is dependent on the temperature, fluidity of the liposomal membrane, and the affinity of AMPH-B for the fungal cell membrane.

1. Introduction

Since patients with infectious diseases caused by certain bacteria or fungi are at a high risk for death, the development of effective drugs for these diseases is of great importance. Mycosis is an infectious disease caused by fungal invasion, and it is divided into 3 classes based on the infection site. Among them, infection by fungi at deep internal organ such as lung and brain is the most serious and is known as deep mycosis. Amphotericin B (AMPH-B), a drug with strong antifungal activity, is effective against deep mycosis and also has a wide antibacterial spectrum [1,2]. As to its mechanism of action, AMPH-B is known to bind to ergosterol contained in the fungal cell membrane, which binding induces a permeability change in the membrane [3,4]. Since AMPH-B also has the ability to bind to cholesterol to some extent [5], it causes side effects such as nephrotoxicity [6]. In order to reduce the side effects of AMPH-B, a liposomal formulation of AMPH-B, known as AmBisome, has been developed. AmBisome is a small unilamellar vesicle containing AMPH-B that is stably retained in the hydrophobic part of the liposomal membrane by complexing with liposomal cholesterol. Since liposomalization of AMPH-B prolongs the circulation of AMPH-B in the bloodstream and decreases the transfer of AMPH-B to cell-membrane cholesterol, the use of AmBisome reduces the side effects of AMPH-B [7–11]. The mechanism of transfer of AMPH-B from AmBisome to the fungal membrane is still unclear, since AmBisome retains the drug in the liposomal membrane rather tightly. By electron microscopy Adler-Moore et al. previously examined the localization of liposomal lipids of AmBisome after exposure to fungal cells such as *Candida glabrata* and *Aspergillus fumigatus* and observed that AmBisome-derived lipids were distributed throughout the cytoplasm of the fungal cells after long-term incubation [12,13]. They also speculated that AmBisome has an affinity for the fungal cell wall and initially binds to it and that the liposomal lipids from AmBisome become dispersed throughout the cytoplasm after damage to the fungal cell membrane caused by AMPH-B released from disrupted AmBisome [13]. Their report indicates that the transfer of AmBisome-derived AMPH-B to the fungal cell membrane is a key step in the action of AmBisome against fungi.

In the present study, we aimed at elucidating the mechanism underlying the transfer of AMPH-B from AmBisome to the fungal cell membrane by using *Saccharomyces cerevisiae* as a model fungal cell. *S. cerevisiae* has a thick cell wall similar to that of other fungal cells [14].

Abbreviations: AMPH-B, amphotericin B; EPC, egg yolk phosphatidylcholine; EPG, egg yolk phosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; HSPC, hydrogenated soy phosphatidylcholine; NBD-PE, *N*-4-nitrobenzo-2-oxa-13-diazol phosphatidylethanolamine.

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At first, we examined the temperature dependence of AmBisome activity against *S. cerevisiae*, and observed that, unlike AMPH-B, the antifungal activity of AmBisome was drastically suppressed at a low temperature. Then, we examined the localization of AmBisome in yeast cells at different temperatures by confocal laser-scanning microscopy and measured the amount of AMPH-B taken up into the cells. The transfer of AMPH-B from AmBisome to the cell and the antifungal activity of the liposomes were increased by raising the incubation temperature. Interestingly, AMPH-B in a fluid liposomal membrane was taken up into the cells more easily and was fungicidal at a low temperature. On the contrary, AMPH-B in ergosterol-containing liposomes was taken up into the cells in less amount; and the cytotoxic action was suppressed even at a high temperature. The results indicate the importance of translocation of AMPH-B from AmBisome to the cells in its antifungal activity.

2. Materials and methods

2.1. Reagents

AmBisome was the product of Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan) Hydrogenated soy phosphatidylcholine (HSPC), distearoylphosphatidylglycerol (DSPG), egg yolk phosphatidylcholine (EPC), egg yolk phosphatidylglycerol (EPG), and cholesterol were gifts from Nippon Fine Chemical Co., Ltd. (Hyogo, Japan). Amphotericin B (AMPH-B), ergosterol, and *N*-4-nitrobenzo-2-oxa-13-diazol phosphatidylethanolamine (NBD-PE) were purchased from The United States Pharmacopeial Convention, Inc. (Rockville, MD, U.S.A.), Sigma-Aldrich Co. (St Louis, MO, U.S.A.), and Avanti Polar Lipids, Inc. (Alabaster, AL, U.S.A.), respectively. Rhodamine-dextran (10 kDa) was purchased from Wako Pure Chemical Industries, Ltd.

2.2. Yeast cell culture

Yeast cells of the *S. cerevisiae* ATCC 9763 (ATCC, U.S.A.) were used as a model of fungal cells in this experiment. They were colonized on YPD/agar medium and kept at 4 °C. Before experimental use, a single colony was picked up and grown in YPD medium with shaking at 30 °C for at least 12 h; and the OD_{600} of the cell suspension was adjusted to 0.1 using RPMI 1640 medium (Sigma-Aldrich Co.) buffered to pH 7.0 with 0.165 M MOPS (Dojindo Laboratories, Kumamoto, Japan).

2.3. Preparation of liposomes

AmBisome (liposomal AMPH-B) was composed of HSPC, DSPG, cholesterol, and AMPH-B (10:4:5:2 as a molar ratio). AmBisome solution was prepared by hydration of lyophilized AmBisomal components with ultrapure water. For preparation of NBD-labeled AmBisome, the lyophilized AmBisomal components were firstly dissolved in chloroform, after which NBD-PE solution was added to them. Then, they were lyophilized again with *t*-butanol and rehydrated with succinate-buffered solution (pH 5.5). Unincorporated NBD-PE was removed by gel filtration chromatography with a PD-10 column. For preparation of AmBisome encapsulating rhodamine-dextran, the lyophilized AmBisomal components were hydrated with rhodamine-dextran solution; and then free rhodamine-dextran was removed by column chromatography with Sepharose™ 4 Fast Flow (GE Healthcare UK Ltd., England). AMPH-B in liposomes containing ergosterol (Erg-AmB) was prepared with HSPC, DSPG, ergosterol, and AMPH-B (10:4:5:2 as a molar ratio). The thin lipid film containing AMPH-B was hydrated with succinate-buffered solution with 9% sucrose at 60 °C and freeze-thawed with liquid nitrogen for 3 cycles. The obtained liposomal solution was sized by sonication. Unincorporated AMPH-B was removed by column chromatography with Sepharose™ 4 Fast Flow. Liposomal AMPH-B composed of highly fluid phospholipids (Egg-AmB) was prepared with EPC, EPG, cholesterol, and AMPH-B (10:4:5:2 as a molar ratio) in the similar manner as used to prepare Erg-AmB. The particle size of each liposome was measured by dynamic light scattering analysis with a Zetasizer Nano (Malvern Instruments, Malvern, U.K.) and was about 100 nm in diameter.

2.4. Colony formation assay

Each liposomal AMPH-B diluted in succinate-buffered solution with 9% sucrose or free AMPH-B dissolved in 0.1% DMSO (final conc.) at a concentration of 20 μM as AMPH-B was added to a yeast cell suspension and incubated in MOPS-buffered RPMI 1640 medium at 4 or 35 °C for 0.5 or 3 h. Then, the cell suspension was centrifuged and washed twice with PBS. The cell pellet was resuspended in PBS and plated on YPD/agar medium. After 24 h of incubation at 30 °C, the number of colonies formed was counted.

2.5. Confocal microscopy

NBD-labeled liposomal AMPH-B (20 μM) was added to a yeast cell suspension, which was then incubated in MOPS-buffered RPMI 1640 medium at 4 or 35 °C for 3 or 24 h with shaking. After having been washed 3 times with PBS, the cells were fixed with 4% paraformaldehyde and stained with Fluorescent Brightener 28 (Sigma-Aldrich Co., U.S.A.) for cell-wall imaging. After another 3 washes with PBS, the cells were attached to MAS-coated glass slides (Matsunami Glass Ind., Ltd., Japan) by centrifugation, and then localization of liposomes in yeast cells was observed under an LSM510 META confocal laser-scanning microscope (Carl Zeiss, Inc., Germany).

2.6. Measurement of the amount of AMPH-B transferred to yeast cells

Liposomal AMPH-B (20 μM) was added to a yeast cell suspension, which was then incubated in MOPS-buffered RPMI 1640 medium with shaking at 4 or 35 °C for 0.5 or 3 h. After having been washed with PBS, the cells were disrupted by using glass beads with shaking and sonication, and AMPH-B in the cells was extracted with methanol. After centrifugation, the methanol extract was evaporated; and the residual AMPH-B was dissolved in the mobile phase for HPLC analysis. The HPLC conditions were the following: Column, TSKgel ODS-100Z 4.6×250 mm (Tosoh Co., Japan); mobile phase, acetonitrile and 2.5 mM EDTA (pH 5.0), 4:6 (v/v); temperature, 35 °C; injection volume, 50 μL; flow rate, 1.0 mL/min; and UV detection, 405 nm. Smart Chrom software was used for control of the HPLC system and data processing.

In the inhibition experiment, AmBisome was added to a yeast cell suspension, which was subsequently incubated in the presence or absence of drug free cholesterol liposomes (Cho-Lip) or vacant ergosterol liposomes (Erg-Lip) in MOPS-buffered RPMI 1640 medium. After a 3-h incubation, the amount of AMPH-B transferred to the cells was measured.

2.7. Investigation of AmBisome disruption

Rhodamine-encapsulating AmBisome was added to different numbers of yeast cells ($OD_{600}=0.1$ or 0.3), and the cells were incubated in MOPS-buffered RPMI 1640 medium for 0.5 or 3 h at 4 or 35 °C. After centrifugation for washing, the supernatant medium was collected and ultracentrifuged to separate the intact AmBisome into the pellet. The resulting supernatant was then collected, and the fluorescence intensity of the rhodamine-dextran that had been released into the medium from AmBisome was measured.

2.8. FRAP experiment

NBD-labeled AmBisome and Egg-AmB were similarly prepared as described above and then gradually frozen to make giant-sized liposomes. Then, these liposomes were applied on MAS-coated slide glasses and dried in the dark at room temperature for overnight. FRAP experiments were performed with LSM510 META confocal laser-