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Bioorganic & Medicinal Chemistry 16 (2008) 3084–3090
Bioorganic & Medicinal Chemistry

Effects of lipid constituents on membrane-permeabilizing activity of amphidinols

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Received 15 November 2007; revised 14 December 2007; accepted 15 December 2007
Available online 23 December 2007

Abstract—Amphidinols (AMs) are a new class of polyhydroxyl polyene compounds with potent antifungal activity. Membrane-permeabilizing activities of AM2, AM3, and AM6 were examined using fluorescent-dye leakage experiments with various phosphatidylcholines (PCs) and sterols. All the AMs tested showed the potent activity to cholesterol-containing liposomes. In the absence of the sterol, AM2, AM3, and AM6 had no membrane-permeabilizing activities to membranes of saturated PC. In liposomes consisting of unsaturated PC, AM2, which possesses an additional ether ring in a polyhydroxyl chain, showed membrane-permeabilizing activities with a moderate efficacy, while AM3 or AM6 did not. The potentiation by sterols was prominent even at 0.5% (wt/wt) and structure-dependent, which ruled out the possibility that alteration of the membrane physical properties induced by sterol was chiefly responsible for this sterol effect. The finding that their activity was not affected by membrane thickness implies that AMs permeabilized membrane by a different mechanism from that of polyene macrolide antibiotics.

1. Introduction

Marine dinoflagellates are a rich source of biologically and structurally unique secondary metabolites.[1–5] Amphidinols (AMs) are a class of polyhydroxyl polyene compounds isolated from *Amphidinium klebsii*.[6–13] Closely related compounds have been isolated from the same genus of the dinoflagellate.[14–16] AMs possess very potent antifungal activity, which substantially exceeds that of commercial drugs such as amphotericin B.[9,13,17] The structure of AMs is characterized by a linear polyene-polyhydroxyl chain and, unlike other natural or synthetic antifungal agents, neither nitrogenous polycycles nor macrocyclic structures are present in the structure. These unique features make AMs an intriguing model to gain a better understanding of the mechanism of antifungal activities.

In the previous studies,[8,9,18] we have shown that AMs increase membrane permeability by binding to lipid bilayers. The molecular mode of action has been proposed on the basis of the conformation of AM3 in sodium dodecyl sulfate (SDS) micelles as follows: (a) AMs bind to bilayer membrane chiefly with the polyene part (C52–C67 of AM3); (b) the polyhydroxyl chain (C1–C20 of AM3) is responsible for formation of the pore/lesion across membrane; (c) the central region (C20–C51 of AM3) takes hairpin-shaped conformation that is stabilized by hydrogen bonds under amphipathic environments. The structure–activity relationship of amphidinols has been reported for naturally occurring and chemically modified AMs.[13] First, hydrophobicity of the polyene chain of AMs dramatically affects the membrane activities. Second, the polyhydroxyl chain moderately modulates the biological activities. Third, substitution of the sulfate group is generally inhibitory to antifungal and hemolytic activities, while giving rise to an insignificant effect on the size of pores/lesions. Finally, the pore size is not greatly affected by length of the polyhydroxyl chain, which suggests that the difference in the potency among AMs is not accounted for by the size of membrane pore/lesion.

The apparent partition coefficients to multilamellar vesicle (MLV) membrane were reported for AMs[18]; the K'_m values of AM2, AM3, and AM4 in eggPC preparations were 0.77×10^3, 22.2×10^3, and 2.24×10^3, respectively.

Keywords: Amphidinols; Antifungal; Hemolysis; Mode of action; Phospholipid; Sterol.
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doi:10.1016/j.bmc.2007.12.029

Their order in K'_m values agrees with that of the membrane-permeabilizing activities, in which AM3 with a butadiene terminus binds most efficiently to eggPC membrane. The pore/lesion formed by AMs are supposed to induce the passage of small molecules which is presumably responsible for their antifungal activities as is the case with polyene macrolide antibiotics.[19–25] The effect of sterols on the membrane activity reported for these antibiotics[26–33] may be attributable either to the change in the properties of the membrane[34,35] or to direct participation in a molecular assembly such as an ion channel.[36–39] A similar sterol effect has also been reported for AMs.[9,10]

In this report, we examined the effects of lipid constituents on the membrane-permeabilizing activities of AMs using fluorescent-dye leakage experiments. Concomitantly, we determined the efficacies of AM2, AM3, and AM6 by the same experiments with changing membrane thickness, sterol contents, liposome size, and temperature, which led to some new findings and confirmation of previous results.

2. Results

2.1. Membrane permeabilization by AMs to PC–cholesterol membrane

It is reported that AMs possess different partition coefficients with egg yolk phosphatidylcholine (eggPC),[18] which mainly consists of PC, phosphatidylethanolamine, and cholesterol. The membrane-permeabilizing activities of AMs were evaluated in terms of AM-lipid ratios using calcein leakage experiments with eggPC liposomes containing 2% (wt/wt) cholesterol (Fig. 2). The results reveal that as compared with filipin, a sterol-dependent membrane disrupting agent, AMs possess much greater efficacy. Among the AMs tested, their efficacies are not markedly dependent on structure and those at 50% leakage fall within the range of 0.1–0.5 AM/PC ratios. Effects of a polyhydroxyl chain of AMs can be compared between AM6 and AM7 since they share the same core/polyene structures but possess the very different polyhydroxyl chains, particularly in their length. AM6 and AM7 revealed the similar efficacy, thereby suggesting that the polyhydroxyl chain is not a critical factor for the activity (Figs. 1 and 2). In addition, AM3 with a medium-length chain permeabilized the membrane at the lowest concentration among them.

2.2. Effect of PC composition and acyl-chain on AM activities

In the previous study,[8,9,18] AMs showed different affinities to eggPC, which prompted us to further examine the effect of lipid composition on the permeabilizing activities of AMs. The calcein leakage experiments were carried out with various lengths of saturated and unsaturated PCs. To compare the effect of saturation and length of fatty-acyl chains on membrane activities, LUVs composed of C_{14}–C_{20} acyl PCs with and without one double bond (C_{16} and C_{18} acyls) were subjected to the experiments. As shown in Table 1, AM2, AM3 and AM6 showed no or weak activity for cholesterol-free liposomes. By adding 10% cholesterol to each preparation of liposomes, the activities of AMs were dramatically recovered (Fig. 3), while sphingomyelin (SM, 10%) gave rise to no significant effect on POPC liposomes (Table 1). Another notable finding in Table 1 is that AM2 shows a moderate activity to unsaturated DPOPC, POPC, and DOPC liposomes even in the absence of sterol. AM7 which possesses moderate activity to eggPC LUV (Fig. 1) showed similar features to those of AM3 and AM6; in sterol-free liposomes composed of POPC, AM7 induced no membrane permeabilization up to 20 μM (data not shown).

2.3. Effect of sterol content and structure on AM activities

Cholesterol causes some physical changes in the lipid membrane, which may affect membrane-permeabilizing activity of AMs. Thus, we next determined the efficacies of AM2 and AM3 in the calcein leakage experiments for POPC liposomes with various cholesterol and ergosterol contents. Figure 4 clearly demonstrates that, as the sterol amount increases, AMs' efficacies are enhanced up to 5–10% (wt/wt) cholesterol. Greater content of sterols leads to a significant loss of AM2's activity in contrast to slight changes with AM3.[17] These results may partly account for the relatively high activity of AM3 in hemolysis because erythrocytes contain around 25% of cholesterol.[40] Next, we examined structure–activity relationship for sterols. Figure 5 shows the EC_{50} values of AM2 and AM3 in the calcein leakage experiments with 10% sterol-containing POPC liposomes, and reveals that cholesterol and ergosterol have a similar efficacy in potentiation of the AM activities. In contrast, dihydrocholesterol showed significantly weaker effects; the efficacy of AM2 was roughly equal to that in the absence of sterol. 25-Hydroxycholesterol has approximately the same effect as cholesterol and ergosterol.

3. Discussion

In the present study, we are able to obtain several new findings for membrane-permeabilizing actions by AMs. Among those, it may be notable that AM2 shows the different activity from that of AM3, AM6 (or AM7) in the responses to unsaturated PC liposomes. All of them induced no activity with bilayer membranes consisting of pure saturated lipids such as DPPC, DSPC, and DAPC (Table 1). In the absence of sterol, AM2 revealed calcein leakage (Table 1) in liposomes consisting of unsaturated DPOPC, POPC or DOPC (Fig. 3), while AM3 and AM6, elicited no activity up to 40 μM in these PC membranes. AM2 activity with unsaturated PCs was, however, enhanced in the presence of sterol more than five times (Table 1). The sterol effect on the efficacy of AM2 was similar to those of AM3 and AM6 while the effects of unsaturated PC were different among them. Therefore, it is postulated that, under sterol-dependent and independent mechanisms, AM2 can interact with sterol and with the carbon–carbon double bond of PC acyl chains, respectively. Another possible account for

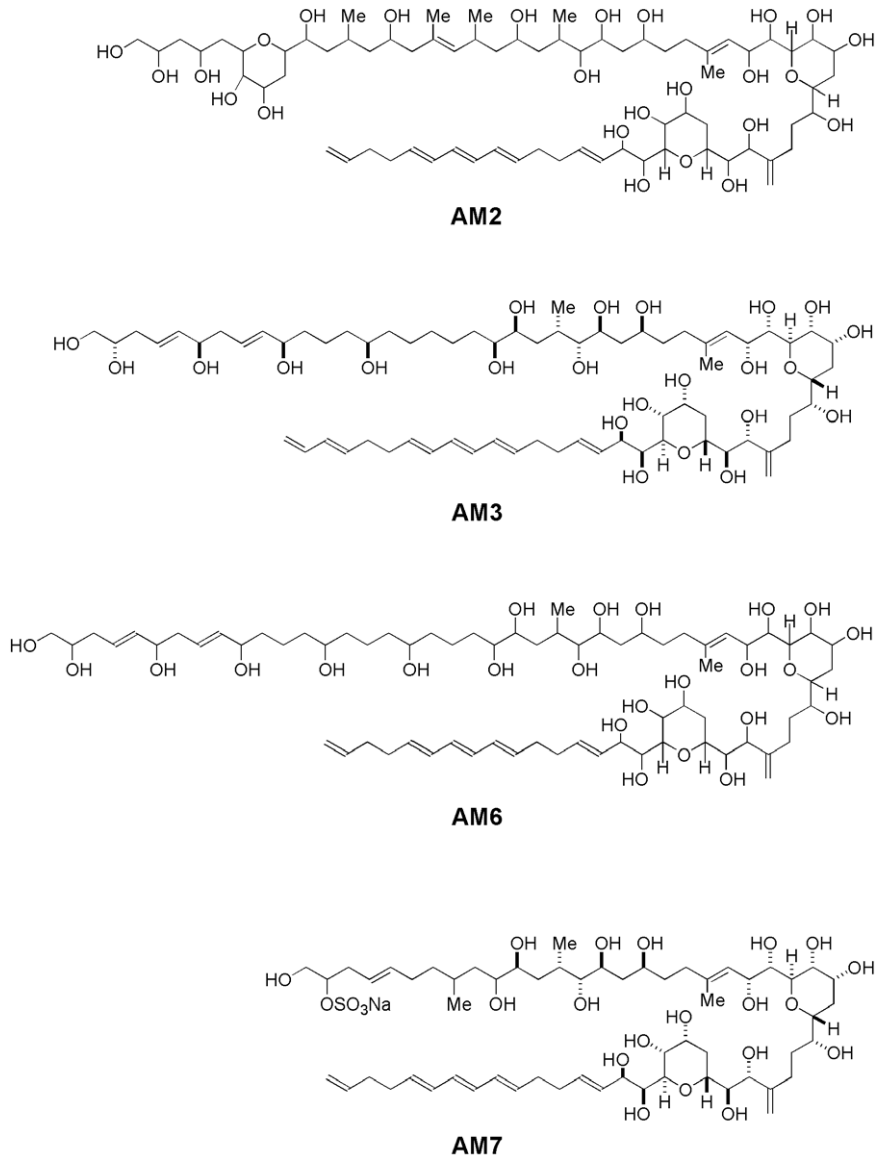

Figure 1. Structures of AM2, AM3, AM6, and AM7.

the AM2 activity to unsaturated PC liposomes is that physical properties of membranes more significantly influence the efficacy of AM2 than those of the other AMs; in particular, ordering of acyl chains, which is higher in saturated PC membranes, possibly affects the orientation and dynamics of membrane-bound entities.[32,41] The importance of the unsaturated bond is shown in the sterol effects (Fig. 5), where unsaturated sterol (cholesterol) enhances the AM activity more efficiently than saturated sterol (dihydrocholesterol). The order of efficacy in the presence of sterol agrees well with those of hemolytic and antifungal activities (AM3 > AM2 > AM6),[13] which further ascertains an important role of sterol in the antifungal activity of AMs.

In the previous report,[17] we demonstrated that AM3 hardly showed the sterol-dependency in the Na^+ flux activity across LUV membranes. In those experiments, we also used membrane preparations composed of commercial eggPC labeled as cholesterol free. The apparent contradiction to the present results may be due to the small amount of cholesterol (a few %) contaminated in the eggPC used in the previous study. Figure 4b depicts that a small amount of cholesterol greatly enhances the activity of AM3 and the potency is virtually unchanged above 5%.

Sterols control the physicochemical properties of bilayer membranes, which include the ordering effect on PC acyl chains.[42,43] On the other hand, sterols maintain the liquid crystal phases and hence undergo lateral diffusion by itself. One of the possible explanations for the enhancement of the AM activities by cholesterol (Figs. 3 and 4) is the change in ordering of the acyl chains.[42,43] It is revealed that the activities of AM2, AM3, and AM6 are enhanced as temperature decreases to 10 °C (Supporting information), which suggests that the ordering effect of lipid acyl chains is an important factor. However, the other results give a negative connotation to this hypothesis; as shown in Figure 4, no more than 0.5% of

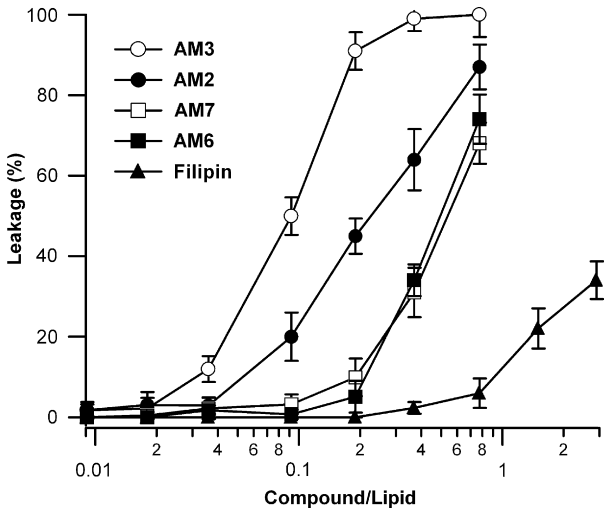

Figure 2. Lipid/AM ratio dependence of membrane-permeabilizing activities. LUV composed of eggPC (2% cholesterol) was used with the same concentration (27 μM) throughout the experiments. Five percent or more leakage is considered as significant activity.

sterol in PC, which hardly influenced the physical properties of membrane, markedly enhanced the activity of AM2 and AM3. In addition, dihydrocholesterol, which shows virtually no potentiation for AM3, is known to possess very similar features to those of cholesterol on the membrane properties such as phase-transfer temperature, mean molecular areas, elasticity, and surface pressure.[41–46] The other possible account is the direct involvement of sterol in a molecular assembly of AMs, which facilitates membrane-permeabilization.

Polyene macrolide antibiotics including some clinical drugs are known to exert their pharmacological actions by forming an ion-permeable complex across fungal membranes. These drugs generally show marked dependence on membrane thickness; for example, amphotericin B elicited virtually no permeabilization with the bilayer membranes consisting of C_{20} or longer acyl-PCs.[37,41] In order to explore the properties of a possible ion channel complex of AMs, the efficacies of AM2 and AM3 were measured by changing acyl chains of PC–cholesterol liposomes. As shown in Figure 6, the acyl

Table 1. EC_{50} of membrane-permeabilizing activities of AM2, AM3, and AM6 for liposomes with different lipid compositions

Lipid[a]	Liposomes	EC_{50} (μM)		
		AM2	AM3	AM6
14:0[b]	DMPC + 10% cholesterol	12.4	8.2	>40
16:0	DPPC	>40	>40	>40
	DPPC + 10% cholesterol	13.5	10	>40
16:1	DPOPC	22	>40	>40
	DPOPC + 10% cholesterol	3.5	1.7	6.5
16:0, 18:1	POPC	16	>40	>40
	POPC + 10% sphingomyelin	17.8	>40	>40
	POPC + 10% cholesterol	3	2.5	20
18:0	DSPC	>40	>40	>40
	DSPC + 10% cholesterol	12.6	8.8	>40
18:1	DOPC	30	>40	>40
	DOPC + 10% cholesterol	3.8	2.5	8.1
20:0	DAPC	>40	>40	>40
	DAPC + 10% cholesterol	12.4	8.5	40

[a] In all cases, the lipid concentration was 27 μM.
[b] Pure DMPC liposomes suitable for this experiments could not be prepared.

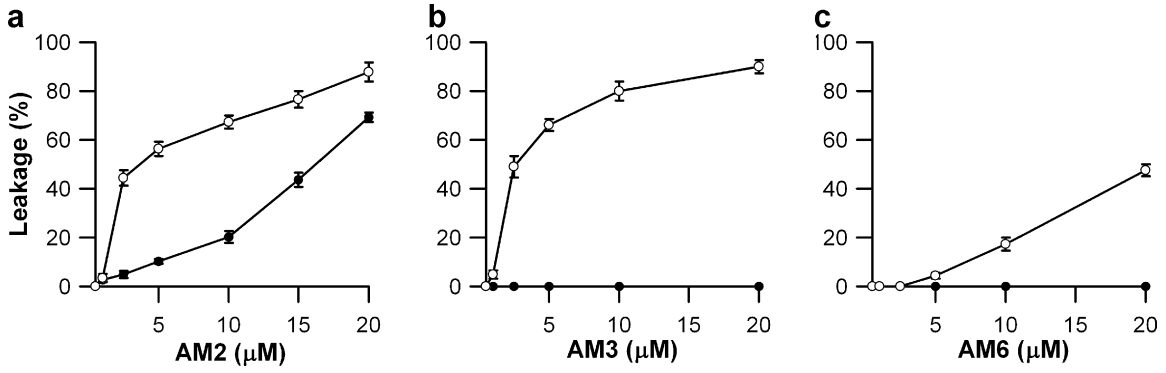

Figure 3. Effects of cholesterol and unsaturated PC on membrane-permeabilizing activities of AM2, AM3, and AM6. Open circles (○) depict the leakage of cholesterol-containing liposomes and closed circles (•) are those of sterol-free liposomes. LUV composed of unsaturated POPC was used with the same concentration (27 μM). Five percent or more leakage is considered as significant activity.

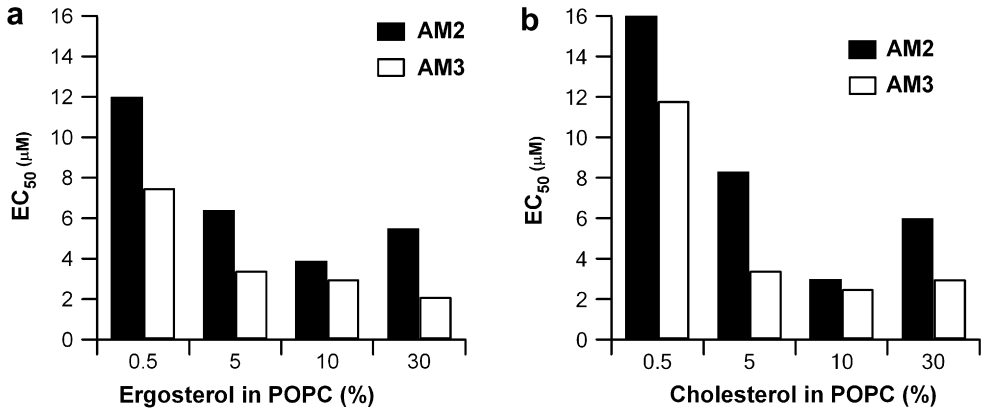

Figure 4. Effect of sterol content in POPC liposomes on membrane-permeabilizing activities induced by AM2 and AM3: (a) ergosterol; (b) cholesterol. In all cases, LUV liposomes were used and the phospholipid concentration was 27 μM. EC_{50} values were determined from dose–response curves as those in Figure 3.

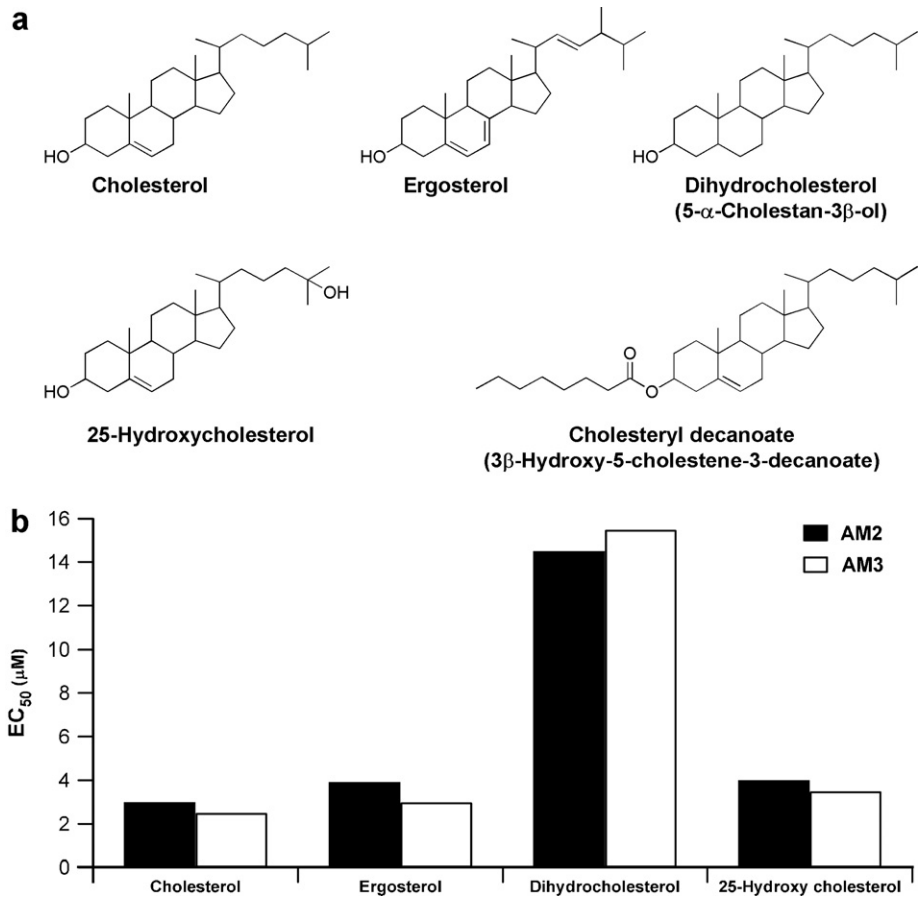

Figure 5. (a) Structures of sterols used; (b) membrane-permeabilizing activities of sterol-containing liposomes induced by AM2 and AM3. Cholesteryl decanoate was inhibitory to the activities of AM2 and AM3, and EC_{50} was not determined up to 40 μM (data not shown, see Supporting information for further results). Ten percent cholesterol–POPC (27 μM) was used for all the experiments. EC_{50} values were determined from dose–response curves as those in Figure 3.

chain length of PC, which usually corresponds to thickness of the hydrophobic interior of membrane, gave rise to no significant influence on their activities. Moreover, phase-transition temperatures, T_m, are greatly varied among PCs tested. As long-chained PCs have higher T_m, these PC membranes tend to take the gel phase in the experimental temperature, which makes their membranes even thicker. The results in Figure 6 can be explained by two different ways. One explanation is that the mechanism of AM activity follows the 'barrel-stave model'[47] where the hydrophobic chain of AM is so long with respect to membrane thickness that the chain length of PCs has no significant effect. The other account is that AM forms a toroidal[48] or carpet-type

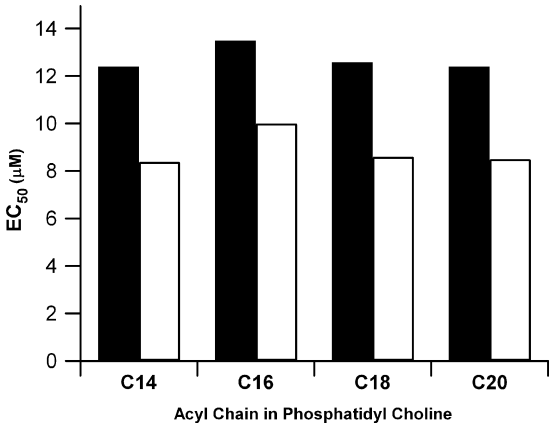

Figure 6. Effect of acyl chain length of PC on membrane-permeabilizing activities induced by AM2 (filled bars) and AM3 (open bars). Ten percent cholesterol–PC (27 μM) was used for all the experiments. EC_{50} values are listed in Table 1.

pore,[49] whose permeability is not much affected by membrane thickness as reported for amphipathic peptides.[50] In the toroidal model, a channel complex has a surface extending from one side to the other and PC head groups partly form a hydrophilic channel lining. In both the toroidal and carpet models, AM molecules closely interact with the head groups of PC. In our hypothesis, the barrel-stave model may not be likely under the conditions of this study because the reported properties of AMs, particularly in their hair-pin conformation, make it difficult to span a bilayer membrane with a single hydrophobic polyene chain of C_{16} length. The previous NMR study of mixed micelles of AM and SDS, however, revealed that the polyene chains deeply penetrate into micelle interior and closely interact in an intermolecular fashion,[17,18] supporting the 'barrel-stave' rather than the other. The hydrophilic head group of AM, upon taking the hair-pin conformation,[18] becomes disproportionally large as compared with the hydrophobic polyene chain, which leads to destabilization of PC packing in membrane. This process is thought to be necessary for formation of toroidal pores.[49,50] With the present results, we cannot speculate on whether the 'barrel-state' or 'toroidal/carbet' model is suitable for AM activity. To gain a better understanding of the molecular mode of action of these unique antifungals, structure-based studies are essential for AM–AM, AM–PC, and AM–sterol interactions.

4. Conclusions

Membrane-permeabilizing activities were examined for AM2, AM3, and AM6 by fluorescent-dye leakage experiments using liposomes composed of various PCs and sterols. The results reveal that AMs tested have no membrane-permeabilizing activities with saturated lipids. AM2, however, showed the moderate activities for liposomes of unsaturated PC, while AM3 and AM6 did not. The present study confirms the marked sterol dependency of AM activity. The potentiation by sterols is prominent even at 0.5% (wt/wt) and strictly structure-dependent, which rule out the possibility that sterol-induced changes in general membrane properties account for this sterol potentiation effect. Direct involvement of sterol in an ion channel complex of AMs may provide a more plausible mechanism.

5. Experimental

5.1. Chemical and instruments

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (DPOPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine (DAPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol, ergosterol, and sphingomyelin from bovine brain were purchased from Sigma–Aldrich (St. Louis, MO). Calcein (Fluorexone) and egg yolk lecithin (70% phospholipids) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All the other chemicals were standard and analytical quality reagents.

5.2. Preparation of liposome loaded with fluorescent dye of calcein

Large unilamellar vesicles (LUVs) were prepared as follows. Lipid (20 mg) with or without sterol was dissolved in 3 mL of $CHCl_3$. After evaporation of $CHCl_3$ at 30 °C under vacuum for 2 h, it was dried in vacuo overnight. The lipid film was suspended in 3 mL of 10 mM Tris–HCl (pH 7.5) containing 60 mM calcein and agitated for 30 min. A freeze-thaw cycle was repeated three times to obtain multilamellar vesicles (MLV). Subsequently, the suspension was passed through a polycarbonate membrane filter (pore size, 200 nm) 19 times using a Liposofast® extruder (Avestin Inc., Ottawa, Canada) above 5 degree of T_m. The resultant calcein-entrapping LUVs were separated from the excess amount of calcein by gel filtration using Sepharose 4B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with 10 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA and 150 mM NaCl. The lipid and cholesterol concentration in the LUV fraction were measured using a phospholipid C-Test® (Wako Pure Chemical Industries, Ltd, Osaka, Japan)[36] and cholesterol E-test Wako® (Wako Pure Chemical Industries, Ltd, Osaka, Japan), respectively. The resulting stock solution was stored at 4 °C under nitrogen gas.

5.3. Calcein leakage assay with LUV

To monitor calcein leakage from LUV, 20 μL of the LUV suspension in a cuvette was diluted with 980 μL of the same buffer. A 20-μL aliquot of AM in MeOH was added to the LUV suspension. The liposome leakage measurements were performed on a Jasco FP 6500 spectrofluorometer (JASCO® Corporation, Tokyo, Japan) with excitation at 490 nm (slit 1.5 nm), emission

at 517 nm (slit 5 nm) at 20 °C in a final volume of 1 mL buffer with vesicles. Triton X-100 (10%, 20 μL) was added to obtain the condition corresponding to the 100% leakage.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research (A) (No. 15201048), (S) (No. 18101010), and Priority Area (A) (No. 16073211) from MEXT, Japan, Egyptian government, and Naito Science Foundation. The authors are grateful to Tetsuro Takano for assistance in this study.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.12.029.

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