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Synthesis and Investigation of Tryptophan–Amphotericin B Conjugates

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Amphotericin B (**1**) is a powerful antifungal agent used in the clinic to treat serious fungal infections.[1] The mechanism of action of amphotericin B (AmB) remains enigmatic and its most prominent feature—pore formation in the fungal membrane—could represent only a part of a more complex mechanism.[2] In the context of our interest in this molecule,[3] we have synthesized a series of tryptophan–amphotericin B conjugates with the aim of generating compounds with enhanced membrane localization properties, based on intriguing effects that have been reported for tryptophan at the interfacial regions of proteins and small oligopeptides. Herein, we demonstrate that such conjugates can exhibit greater selectivity for ergosterol-containing vesicles over cholesterol-containing counterparts when compared to the parent molecule amphotericin B. Additionally, the effect of the tryptophans is manifest in the observation of faster K^+ efflux from sterol-free liposomes; this indicates that the suggested anchoring effect due to the tryptophans can compensate for the sterol requirement that has traditionally been invoked in understanding channel formation in amphotericin. The lack of correlation between K^+ efflux in liposome assays and activity in yeast is striking and suggests that the biological activity of AmB and its derivatives might be more complex than the simple models discussed to date.

Naturally occurring tryptophan-rich peptides, such as tritrpticin,[4] indolicidin,[5] and lactoferricin B[6] have been shown to possess important antimicrobial activity. These interesting biomolecules are conformationally ill-defined in solution, and require lipophillic–hydrophilic interfaces to undergo proper folding. Consequently, in membranes, including biological and abiological ensembles, such as vesicles, these peptides display well-defined tertiary structures facilitated by localization at interfacial domains.[7] In studies of these peptides, the tryptophan residues have largely been found on the extracellular side of the biological membrane,[8] and make what are believed to be crucial interactions at the membrane–water interface region.[9] Moreover, indole and other tryptophan congeners are found in proximity to the glycerol and bridge the tail and head groups in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipids. This interesting feature of tryptophans has been recently employed as a convenient means of sizing a population of vesicles with random diameter into a set of unisized liposomes.[10]

We have recently reported the synthesis of an aminohexanoyl piperazinyl–amphotericin B conjugate (**2**) with a readily accessible synthetic handle to attach groups to amphotericin B.[3c] We reasoned that it would be fundamentally interesting to investigate the properties of tryptophan–amphotericin compounds as a means to explore the effect of such conjugates on activity. For such molecules, one might hypothesize that the indole moiety would enhance the membrane binding and anchoring of amphotericin B (**1**) and lead to channel formation in the absence of sterols. In such a scenario, it was anticipated that tryptophan oligomers would be more effective than a single and/or no tryptophan.

The synthesis depicted in Scheme 1 commences with 6-aminohexanoyl piperazinyl derivatized amphotericin B (**2**), which was prepared on the multigram scale (2 g) from **1**.[3c] Fmoc-protected tryptophan hydroxysuccinimide active ester was condensed with **2** (pyridine, DMF) and deprotection was conducted with piperidine in DMSO to afford **3**. The bi- and tritryptophan conjugates **4** and **5**, respectively, were then prepared in an iterative fashion.

To assess the pore-inducing potency of newly synthesized amphotericin B conjugates we employed an assay in which vesicles are prepared with K^+ inside and a solution of Na^+ ions (outside).[3c] The vesicles were prepared from POPC, a synthetic mimic of the egg yolk phosphocholine,[11] with or without admixed ergosterol (5 mol%) or cholesterol (5 mol%). Pore formation was measured by the observation of an induced K^+ efflux from the vesicles—an event that can be recorded by using K^+-selective electrodes. Each conjugate was dissolved in a minimal amount of DMSO and added to a vesicle suspension. As shown in Figure 1, K^+ efflux induced by the different compounds was recorded at two different concentrations (10 μM: solid line; 1 μM: dashed line).[12]

As anticipated on the basis of prior work,[13] in our assay amphotericin B induced only slow K^+ efflux in nonsterol containing POPC vesicles (Figure 1A) when compared to cholesterol (Figure 1E) and ergosterol (Figure 1I) containing membranes. All of the tryptophan–amphotericin B conjugates were as active as amphotericin B (**1**) in sterol free POPC vesicles at the highest concentrations (10 μM; Figure 1B–D). At 1 μM, the beneficial effects of the tryptophan conjugates become apparent. The bi- and tritryptophan–amphotericin B conjugates **4** (Figure 1C) and **5** (Figure 1D) displayed K^+ release that clearly surpassed the efflux induced by native amphotericin B. This suggests that the effects of **1** and tryptophan, vis-à-vis its suggested role in biological membranes, can indeed be combined,

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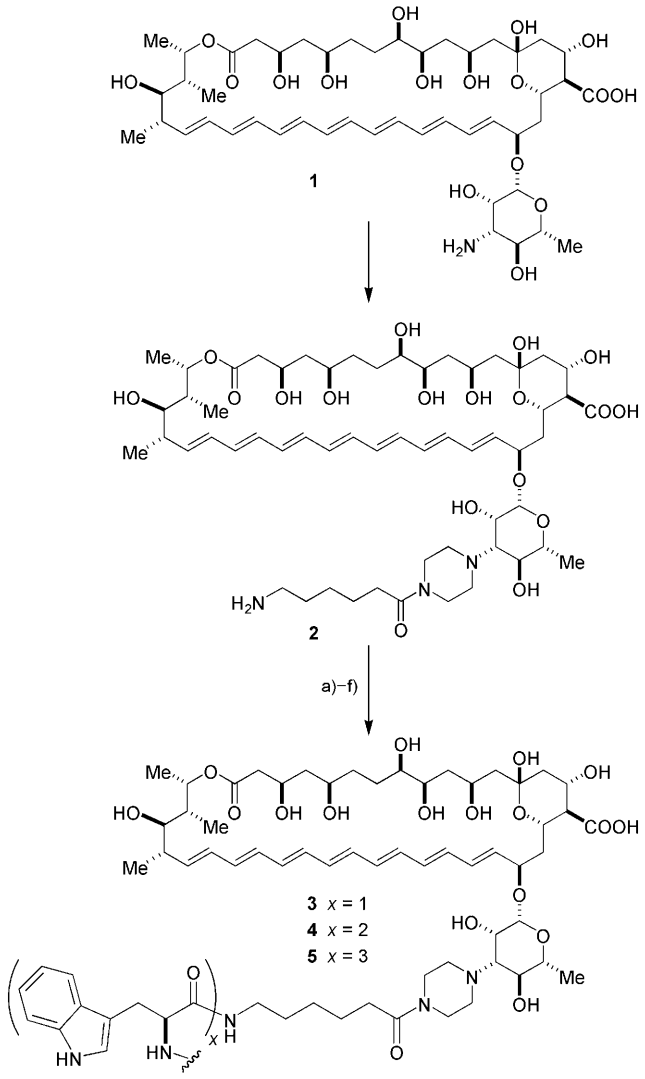

Scheme 1. A) Fmoc-Trp-OSu, pyr, DMF, 1 h, 56%; B) pip, DMSO, 1 h, 90%; C) Fmoc-Trp-OSu, pyr, DMF, 0.75 h, 71%; D) pip, DMSO, 0.5 h, 85%; E) Fmoc-Trp-OSu, pyr, DMF, 0.5 h, 91%; F) pip, DMSO, 0.5 h, 37%.

and that the tryptophan effect can be potentiated by using tryptophan oligomers.

In cholesterol-containing vesicles amphotericin B induced rapid K^+ release at 10 and 1 μM (Figure 1E). This trend was found again for the tritryptophan–amphotericin B conjugate **5** (Figure 1H). The mono- and bitryptophan–amphotericin B conjugates **3** and **4** were less active (Figure 1F and G).

In the case of ergosterol-containing membranes all compounds tested induced immediate full release of trapped K^+ at both 10 and 1 μM (Figure 1I–L). At the lower concentration used, the monotryptophan–amphotericin B conjugate (**3**) showed high selectivity for ergosterol- over cholesterol-containing membranes or sterol-free membranes.

We then tested all compounds for inhibition of growth of the yeast *Saccharomyces cerevisiae*. Only **3** was active at a minimal inhibitory concentration of 20 μM (native amphotericin B was active at 1 μM). None of the other compounds was toxic up to a concentration of 100 μM, as was the tripeptide (W_3), which was used as a control.[14] These results are particularly interesting given the fact that at 1 μM all tryptophan–amphotericin B conjugates (**3**, **4**, and **5**) display K^+ efflux from vesicles containing the fungal sterol, ergosterol, similar to amphotericin B. It is therefore noteworthy that **3** is the only active conjugate that is, at the same time, selective for the ergosterol-containing membrane of *S. cerevisiae* (compare Figure 1F, J).[15]

In conclusion, we have prepared tryptophan–amphotericin B conjugates and have shown that they posses unexpected properties; the results suggest that the membrane-anchoring effect of tryptophan and the pore-inducing effect of amphotericin B (**1**) can act synergistically in K^+ efflux assays involving liposomes. It is particularly noteworthy that in sterol-free vesicles the effect increases with each tryptophan attached, which gives compounds that display greater differentiation for ergosterol- over cholesterol-containing membranes. In pure POPC vesicles the tritryptophan–amphotericin B (**5**) has even a higher pore-inducing activity than native **1**. Most strikingly, however, is the lack of correlation between K^+ efflux in liposome assays and minimum inhibitory concentration (MIC) studies in yeast. These observations suggest that the biological activity of AmB and their derivatives might be more complex than indicated by the simple models discussed to date.

Experimental Section

Liposome preparation: The appropriate lipids (POPC, POPC/cholesterol 95:5, mol%/mol%, POPC/ergosterol 95:5, mol%/mol%) were dissolved in CH_2Cl_2 in a round-bottom flask (250 mL) and the solvent was removed under reduced pressure (ca. 40 kPa) in a Rotavapor (Büchi, Switzerland). The thin lipid film was dried, overnight, at high vacuum, then hydrated with a KCl (150 mM), HEPES (5 mM, pH 7.4) buffer, in order to obtain a liposome suspension with an approximate overall lipid concentration of 3 mM in the case of POPC and 5 mM in the cases of POPC/cholesterol and POPC/ergosterol. The suspension was sonicated under nitrogen atmosphere for 30 min in a bath sonicator (Bandelin Sonorex RK100H, 140 W, 35 kHz). Then the liposomes were sized by extrusion (The Extruder®, Lipex Biomembranes, Inc., Vancouver, Canada), that is, the suspension was passed through two (stacked) polycarbonate membranes (Nuclepore® Whatman) of 400 nm, 200 nm, and finally 100 nm pore size (ten times for each pore size). The resultant "100 nm" unilamellar liposomes (generally about 30 mL) were dialyzed (Spectra/Por® Membranes MWCO 3500; Spectrum) three times against NaCl (150 mM; 600 mL), HEPES (5 mM, pH 7.4) buffer. The actual phospholipid concentration was determined by measuring the inorganic phosphate, and then the suspension was diluted with NaCl (150 mM), HEPES (5 mM, pH 7.4) buffer to 1 mM overall lipid concentration (phospholipid + sterols). For each efflux measurement this liposome suspension (10 mL) was placed in a small beaker.

After recording the amphotericin-induced potassium efflux, liposomes were lysed by adding sodium cholate (172 mg). The resulting reading, which was taken after 0.5 h, was used to quantify the 100% K^+ release.

Ion-selective electrode (ISE) membrane preparation and potentiometric measurement: The membrane components (typically 300 mg of total mass) were dissolved in THF (3.0 mL) during approximately 2 h and poured into a glass ring (37 mm inner diame-

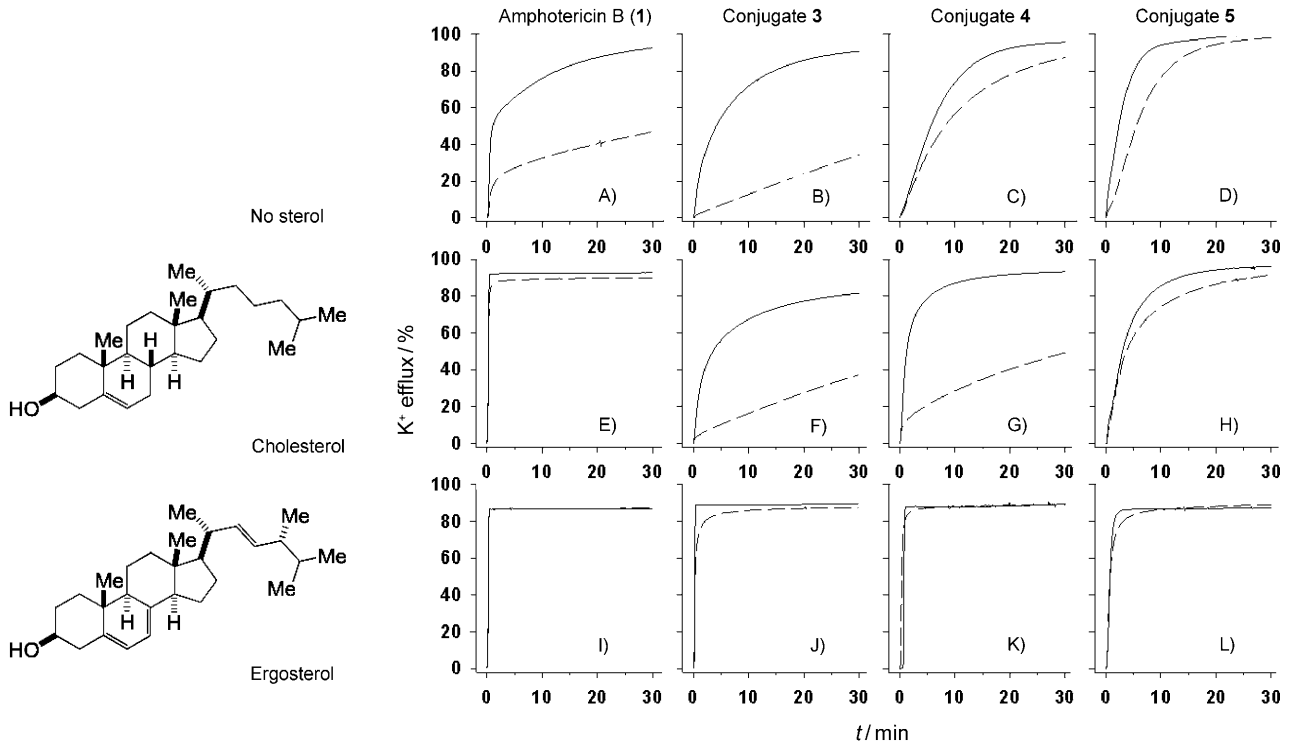

Figure 1. K^+ efflux from vesicles prepared from pure POPC induced by amphotericin B (**1**), tryptophanyl aminohexanoyl piperazinyl–amphotericin B (**3**), bi-tryptophanyl aminohexanoyl piperazinyl–amphotericin B (**4**), and tritryptophanyl aminohexanoyl piperazinyl–amphotericin B (**5**). Concentrations of the compounds after external addition (as DMSO solutions) to the stirred vesicle suspension: high concentration: 10 μM (solid lines); medium concentration: 1 μM (dash lines). The total concentration of lipids (POPC + 5 mol% sterol) was 1 mM in HEPES buffer (5 mM, pH 7.4). Injections of pure DMSO gave baseline traces only (data not shown; identical to data reported in refs. [3c, d]).

ter) fixed on a glass support. After overnight evaporation of the solvent at room temperature, 5 mm disks were punched from the mother membrane (thickness, about 240 μm) and glued with THF to a plasticized PVC tubing (inner diameter 4 mm), which was mechanically fixed to a 1000 μL pipette tip. The membranes contained valinomycin (about 3.1 mg, 9.2 mmol kg^{-1}), DOS (about 150 mg), PVC (about 150 mg), KTFPB (about 1.1 mg, 4.2 mmol kg^{-1}), ETH 500 (about 3.1 mg, 8.9 mmol kg^{-1}). The inner solution consisted of KCl (10^{-3} M). The ISEs were conditioned, overnight, in KCl (10^{-3} M). Potentiometric measurements were performed with a 16-channel electrode monitor (Lawson Labs, Inc., Malvern, PA, USA) in magnetically stirred solutions at ambient temperature (20–22 °C). Activity coefficients were obtained from the Debye–Hückel approximation and electromotive force (EMF) values were corrected for liquid-junction potentials with the Henderson equation. The reference electrode was a Metrohm double junction Ag/AgCl reference electrode (Metrohm AG, CH-9010 Herisau, Switzerland; No. 6.0729.100) with KCl (3 M) as reference electrolyte and LiOAc (1 M) as bridge electrolyte. After each efflux measurement the reference electrode was washed twice with H_2O and twice with LiOAc (1 M) then refilled with LiOAc (1 M). This introduced an error in the absolute value measured, but reduced the drift to approximately 1 mV min^{-1}.

Prior to selectivity measurements, the ISEs were conditioned, overnight, in NaCl (10^{-2} M). The sequence of the investigated ions was: Na^+, H^+, K^+. For each of these ions, the membrane was conditioned in the corresponding chloride solution (10^{-2} M; during 0.5 h) after which calibration curves were determined. The logarithmic selectivity coefficients were: -4.11 ± 0.04 and -4.35 ± 0.1 (SD, $n = 3$), for Na^+ and H^+, respectively.

MIC assays: We determined the minimal drug concentration required to prevent the growth of *S. cerevisiae* as follows. For each amphotericin conjugate, several stock solutions (100x) were prepared in DMSO. Each stock solution was then diluted 1:100 in YPD agar medium that had cooled to 50 °C before 3 mL of the mixture was poured into small Petri dishes. Wild-type yeast cells (BY4741, a derivative of S288C) were grown in YPD medium, and approximately 10^4, 10^3, 10^2, and 10 cells were spotted on each plate. Colony formation was scored after incubation at 30 °C for 36–48 h.

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[14] The tripeptide also failed to induce K^+ efflux in the vesicle assay.

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