Bioorganic Chemistry

An Amphotericin B–Fluorescein Conjugate as a Powerful Probe for Biochemical Studies of the Membrane**

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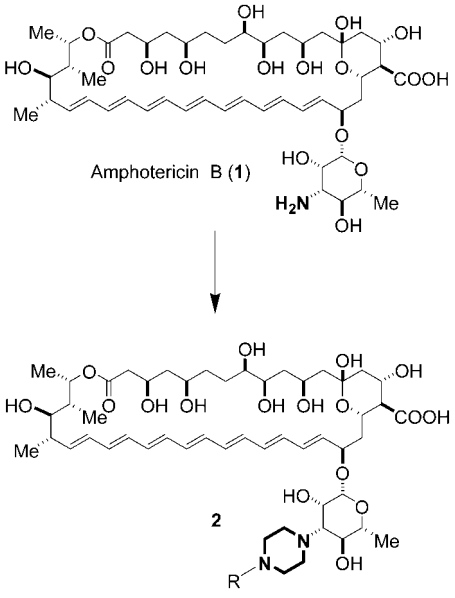
Dedicated to Professor Peter B. Dervan

Amphotericin B is an antimycotic agent extracted and isolated from a soil streptomycete[1] and is currently in clinical use against chronic fungal infections. An enormous amount of data on the biological activity of this polyketide has been accumulated, which underlines the potency and effectiveness of the medicament, as well as its importance in membrane research.[2] Even so, after five decades of intensive research the details of the mechanism of action of amphotericin B remain far from completely elucidated.[3] It is generally accepted that amphotericin B induces leakage of electrolytes and small molecules from exposed cells. However, a definitive answer to the question of whether such electrolyte efflux is the primary cause of amphotericin-B-induced cell death remains elusive.[4] Recent attempts at mechanistic inquiry have involved use of the tools of synthesis[5] and metabolic engineering.[6] There is a need for the design and synthesis of amphotericin B probes with which to test the various existing hypotheses.[7] Herein, we describe the use of a readily accessible piperazine linker as a synthetic anchor for the introduction of reporter groups such as fluorescein to amphotericin B. We used the resulting probe to conduct several in vivo and in vitro comparative studies involving mammalian and fungal cells, as well as vesicles (Liposomes). Several of the observations documented herein are of particular significance: 1) the amphotericin–fluorescein conjugate is rapidly internalized in mammalian cells, but no such uptake occurs in fungal cells; 2) in contrast, the conjugate is localized in the fungal membrane, as observed by epifluorescence microscopy; 3) despite the fact that liposome studies show the amphotericin conjugate to lead to rapid K^+ efflux, the compound is not lethal to yeast over a wide range of concentrations; 4) the conjugate was found to be uniformly distributed throughout the membrane in exponentially growing and budding yeast. The various intriguing properties of this probe may facilitate further studies aimed at gaining an understanding of the difference between the behavior of amphotericin towards mammalian cells and that towards fungal cells, and may already provide an insight into the origins of the severe side-effects of the drug.

Despite the density of functionality of amphotericin B, it only has two addressable reactive centers that allow selective conjugation: the primary amine function found in the pendant mycosamine moiety and the carboxylic acid group in the amphoteronolide fragment (Scheme 1). Analysis of prior work in this area[8] suggests that conjugates anchored through the mycosamine moiety must contain a linker that preserves a protonatable amine function to allow observation of the fast kinetics of K^+ release in liposomes. Murata and co-workers[5c] recently published a study in which a linker was introduced



Scheme 1.

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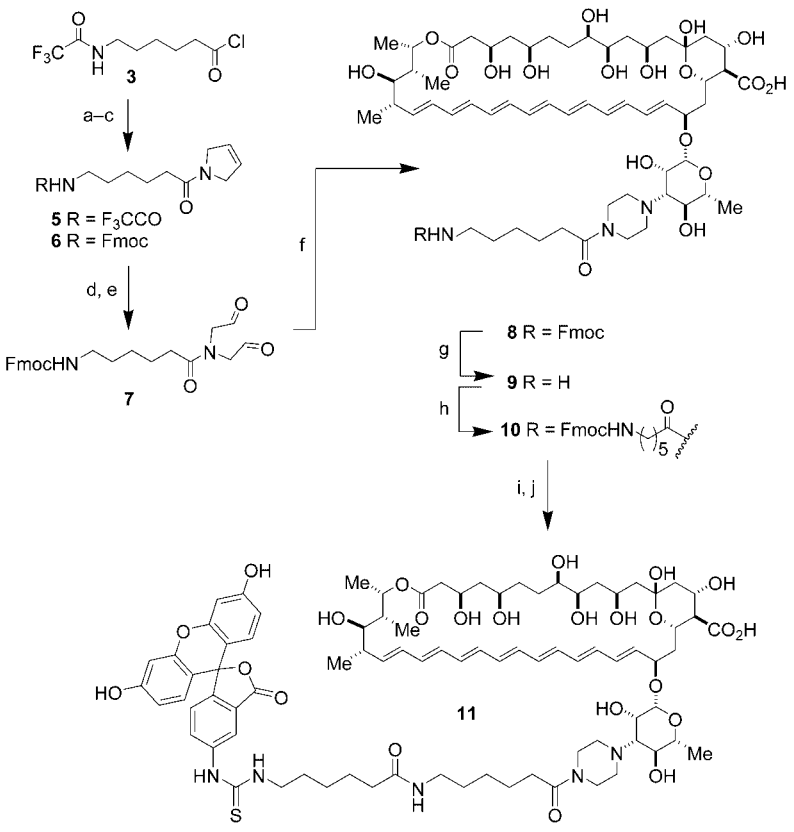
onto amphotericin B (**1**) through reductive amination of an aldehyde. Such a strategy suffers from the disadvantage that a second reductive alkylation can ensue, a process that can be difficult to control.[9] Our attempts to carry out reductive monoalkylation at the amphotericin B mycosamine function proved the process to be capricious; production of a mixture of secondary and tertiary amines could not be avoided, and separation of these compounds was far from simple. We sought a novel alternative conjugation strategy and reasoned that a linker programmed to undergo double reductive alkylation would completely obviate the problems outlined above. Moreover, the tertiary amines **2** so produced would retain their basicity and could be protonated under physiological conditions.[8]

There is no precedence for the use of such a linker with amphotericin B, or any other biomolecule. We developed an easy, straightforward synthetic route to attach a piperazine linker to amphotericin B, which allowed subsequent conjugation (Scheme 2). Coupling of 6-*N*-trifluoroacetamide-hexanoyl chloride (**3**)[10] with 3-pyrroline (**4**)[11] gave **5**, which was converted into the corresponding Fmoc-protected amide **6**. Oxidative cleavage of **6** afforded dialdehyde **7**[12]. This aldehyde readily underwent double reductive amination with the primary amine group of amphotericin B to form **8** in 63% yield.



Scheme 2. a) 3-pyrroline (**4**), pyr, CH_2Cl_2, 3 h, 69%; b) K_2CO_3, MeOH, H_2O, 13 h, 82%; c) FmocOSu, DMF, pyr, 12 h, 77%; d) $K_2OsO_3{\cdot}2H_2O$, acetone, H_2O, *t*BuOH, 16 h, 66%; e) $NaIO_4$, Si gel, $CHCl_3$, H_2O, 0.5 h; f) amphotericin B, DMF, $NaCNBH_3$, HCl, 13 h, 63% over 2 steps; g) piperidine, DMSO, quantitative; h) *N*-Fmoc-6-NH-$(CH_2)_5CO_2Su$, *i*Pr_2NEt, DMF, 38%; i) piperidine, DMSO, quantitative; j) fluorescein isothiocyanate, *i*Pr_2NEt, DMF, 66%. Pyr, pyridine. DMF = dimethylformamide, DMSO = dimethyl sulfoxide, Fmoc = 9-fluorenylmethoxycarbonyl, Su = succinimide.

Quantitative deprotection (piperidine/DMSO) of **8** to form the free amine **9** prepared the compound for conjugation.[13] Treatment of **9** with the Fmoc-protected 6-aminohexanoic acid *N*-hydroxysuccinimidyl ester gave **10**, which was deprotected (piperidine, DMSO, quantitative yield) then allowed to react with fluorescein isothiocyanate (Hünig's base, DMF, 66% yield) to form the amphotericin B–fluorescein conjugate **11**.[14–16]

To evaluate the new linker system, the free amine **9** and the amphotericin B–fluorescein conjugate **11** were examined thoroughly in both liposomal and cellular assays. For each of the two compounds we determined the minimal inhibitory concentration (MIC) required to inhibit growth of *Saccharomyces cerevisiae*. Native amphotericin B (**1**; MIC = 1 μM) and the aminohexanoyl piperazinyl conjugate **9** (MIC = 1.6 μM) are equally active, which indicates the usefulness of the new linker system. The fluorescein–amphotericin B conjugate **11** is not toxic in the measured range of up to 500 μM.

We adapted a vesicle assay to our purposes.[17,18] Large unilamellar vesicles (LUVs) made from POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) or from POPC admixed with sterols (mimicking conditions in natural biomembranes[19]) were prepared from a thin film of the lipid(s) in a mixture containing KCl (150 mM) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (5 mM) at pH 7.4.[20] The unilamellar vesicles were dialyzed against NaCl to create an ion gradient (K^+ inside, Na^+ outside). The amphotericin B conjugates were added to the vesicle suspension to form ion channels leading to K^+ efflux, which was measured with freshly prepared valinomycin-based K^+-selective electrodes.[21,22]

Native amphotericin B (**1**), the conjugate with the piperazine linker **9**, and the fluorescein conjugate **11** all induced immediate total release of trapped K^+ from ergosterol-containing vesicles (Figure 1). Treatment of cholesterol-containing vesicles with the new conjugates **9** and **11** resulted in a slower release of ions than treatment with amphotericin B (**1**), which indicates that the piperazine-linked conjugates have a preference for ergosterol-containing rather than cholesterol-containing vesicles.[23] K^+ release from sterol-free vesicles was slow, as we expected based on literature data for amphotericin B.[2] The fluorescein–amphotericin B conjugate **11** induced rapid K^+ release from vesicles while having no toxic effect on yeast cell growth. This observation is consistent with previous proposals that channel formation is not sufficient to induce cell death.[4]

We tested fluorescein–amphotericin B conjugate **11** in a variety of other in vivo experiments. A cholesterol-derived synthetic membrane anchor has recently been developed that allows the uptake of antifluorescein antibodies and the associated Protein A into mammalian cells.[24] Since amphotericin B is known to insert into membranes,[1] we tested the fluorescein–amphotericin B conjugate **11** in the same assay.

To investigate the recruitment of **11** both by *Saccharomyces cerevisiae* FY250 and by Jurkat human T cells, the cells were incubated with **11** for 1 h at 37 °C

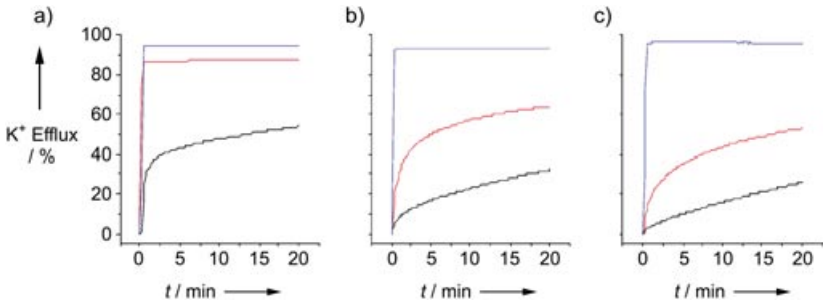


Figure 1. K^+ efflux from vesicles prepared from pure POPC (black), POPC admixed with 30 mol% cholesterol (red), and POPC admixed with 13 mol% ergosterol (blue). The concentration of amphotericin B (**1**, a) and its conjugates **9** (b) or **11** (c) after addition (as a DMSO solution) to the stirred vesicle suspension was 5 μM. The total concentration of lipids (POPC + sterol) was 1 mM in 5 mM HEPES buffer at pH 7.4.

(Jurkat cells) or 30 °C (*S. cerevisiae*) and the cultures were washed with media (Phosphate–buffered saline (PBS) for the yeast cells and RPMI media for the Jurkat cells, see Supporting Information) and resuspended. Subsequent analysis by flow cytometry revealed that both yeast and Jurkat lymphocytes recruit **11**, but the compound has a slight preference for the mammalian cell line.[25]

We next conducted experiments aimed at establishing the avidity of antifluorescein IgG for cellular plasma membranes incubated with **11**. We employed nonfluorescent rabbit IgG complexed with red fluorescent conjugates of commercially available Protein A (PrA) from *Staphylococcus aureus* in a previously described assay.[26,27] Flow cytometry allowed quantification of the recruitment of antifluorescein IgG bound to Alexa Fluor 633-conjugated PrA by Jurkat lymphocytes and yeast.[25] Jurkat cells were treated with **11** for 1 h and then with IgG for 4 h at 37 °C. The cells were washed with 5-aminofluorescein prior to measurement to ensure that all the noninternalized IgG was removed. Yeast cells were treated similarly but were not washed with 5-aminofluorescein. The results of these experiments lead to the conclusion that **11** associates with both yeast and Jurkat human T cells in a dose-dependent manner, which is in line with the results of the studies described above. More importantly, treatment with the amphotericin conjugate leads to a significanct increase in the uptake of IgG by Jurkat lymphocytes. No internalization of **11** or its complex with antifluorescein IgG by yeast cells was observed.

To investigate the nature of the interaction of **11** with the cellular targets in the presence of IgG in greater detail, cells incubated with **11** were examined by epifluorescence microscopy (see Figure 2). Uptake into Jurkat lymphocytes and IgG recruitment by yeast were assayed as described above, except that Alexa Fluor 594-conjugated Protein A was substituted for Alexa Fluor 633-conjugated Protein A. Both cell lines showed red and green fluorescence. It is particularly interesting that the amphotericin B–conjugate **11** is localized at the membrane in yeast, while the micrograph of the mammalian cell lines reveals that the amphotericin conjugate has been taken up by the cells. The fact that the fluorescein–amphotericin B conjugate **11** is taken up into mammalian cells leads us to hypothesize about the mechanism of action of amphotericin B with reference to its reported side-effects on mammalian cells. The generally held view is that, despite the inherent preference of amphotericin B for channel formation in yeast membranes as a result of ergosterol stabilization, the compound retains the ability to form channels competitively in mammalian membranes. Model studies with vesicles have shown that approximately 16 amphotericin B molecules are required for channel formation.[28] In the light of the observations described above for Jurkat cells, we propose that transport of the molecules across mammalian cell membranes into the interior depletes amphotericin B at the membrane and thus diminishes channel formation. Oxidative (free radical) activity inside the cell may be the source of the observed side-effects of the drug.[2e,f]

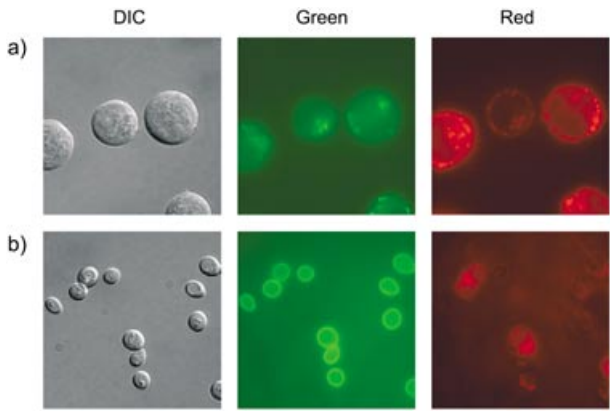


Figure 2. Epifluorescence and differential interference contrast (DIC) microscopy of compound-treated Jurkat lymphocytes (a) and *Saccharomyces cerevisiae* (b) 30 min after IgG addition. Green fluorescence: $\lambda_{ex} = 480$ nm, $\lambda_{em} = 535$ nm; red fluorescence: $\lambda_{ex} = 560$ nm, $\lambda_{em} = 645$ nm.

We carried out additional in vivo experiments on yeast cells to demonstrate the utility of conjugate **11** as a probe for investigation of biological phenomena at the membrane. Previous work has suggested that ergosterol-rich plasma membrane domains localize in areas of cell polarization. Polyene macrolide filipin was used as a probe because it is known to associate with ergosterol.[29,30] Localized fluorescence regions were observed in *S. cerevisiae* at the tip of mating projections formed in response to pheromones.[31] In previous studies with the fission yeast *S. pombe*, ergosterol appeared to accumulate at cell-division-associated regions and at the growing poles.[32] We therefore wanted to investigate whether **11** distributes uniformly throughout the plasma membrane of *S. cerevisiae* or accumulates specifically in polarized regions of the cells. We analyzed cells during exponential growth, when the cells polarize towards the newly forming daughter bud (Figure 3a and c), and in the course of the formation of mating projections in response to pheromones (Figure 3b and d). In both cases, fluorescein–amphotericin conjugate **11** appeared to be distributed uniformly throughout the plasma membrane of each yeast cell. Ampho-

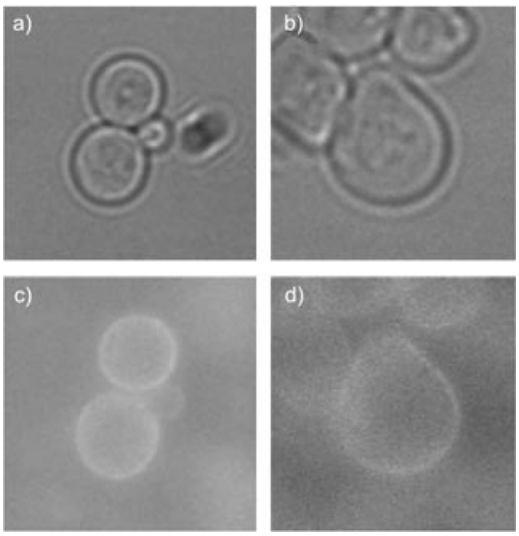


Figure 3. Localization of **11** in the plasma membrane of yeast cells. The cell outlines were observed by phase contrast microscopy (a, b), and the localization of **11** by fluorescence microscopy (c, d). The figure shows exponentially growing cells (a, c) and cells forming mating projections after exposure to mating pheromone (b, d).

tericin B is expected to undergo pronounced complex formation with ergosterol[33] so these observations suggest that ergosterol-rich domains might not accumulate significantly in distinct regions of the plasma membrane. In support of this hypothesis, recent data suggests that filipin itself might perturb the local composition of cellular membranes.[34] Further work is required to determine whether filipin and amphotericin B bind to the same molecular structures in vivo, and to answer the question of whether ergosterol-rich membrane domains are polarized during yeast cell growth.

In conclusion, we have established a new piperazine linker system for bioconjugation through amines that allows the synthesis of amphotericin B conjugates on a multigram scale. This linking strategy is suitable for bioconjugation in a number of other biologically important systems for which functionalization through a primary amine is required and may be used to obtain molecules with interesting new properties. A fluorescein conjugate was prepared as a benchmark case. This conjugate displayed preserved K^+ efflux-inducing properties in an LUV assay but completely lacks toxic effects on yeast cell growth. These results imply that ion-channel formation in artificial vesicles and the lethal effect induced by amphotericin B are separate phenomena. We also demonstrated that amphotericin B–fluorescein conjugate **11** can function as a useful probe of biological activity. Our studies revealed that **11** associates with both yeast (*Saccharomyces cerevisiae* FY250) and Jurkat human T cells in a dose-dependent manner but that it is taken up by mammalian cells whilst it remains localized in the yeast membrane. Additional investigations with this probe in yeast cells revealed a uniform distribution of the compound throughout the plasma membrane, which opens up the possibility that ergosterol might not accumulate in polarized regions of the cells as suggested previously. Additional applications can be envisioned for this new fluorescein–amphotericin B conjugate, for example, it could be used as a means for studying transmembrane transport or as an anchor for selective mediation of the delivery of toxins to fungal cells.

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