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 antymycotic agent extracted and isolated from a soil streptomycete 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. Even so, after five decades of intensive research the details of the mechanism of action of amphotericin B remain far from completely elucidated. 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. Recent attempts at mechanistic inquiry have involved use of the tools of synthesis and metabolic engineering. There is a need for the design and synthesis of amphotericin B probes with which to test the various existing hypotheses. 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 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 recently published a study in which a linker was introduced...
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. 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 so produced would retain their basicity and could be protonated under physiological conditions.

There is no precedent for the use of such a linker with amphotericin B, or any other biomolecule. We developed an easy, straightforward synthetic route to attach a piperazinyl linker to amphotericin B, which allowed subsequent conjugation (Scheme 2). Coupling of 6-N-trifluoroacetamide-hexanoyl chloride (3) with 3-pyrroline (4) gave 5, which was converted into the corresponding Fmoc-protected amide 6. Oxidative cleavage of 6 afforded dialehyde 7. 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, CH₂Cl₂, 3 h, 69%; b) K₂CO₃, MeOH, H₂O, 13 h, 82%; c) FmocOSu, DMF, pyr, 12 h, 77%; d) K₂OsO₃·2H₂O, acetone, H₂O, 12 h, 77%; e) K₂CO₃, MeOH, H₂O, 13 h, 82%; f) iPr₂NEt, DMF, 66%. Pyr, pyridine. DMF

d, e) FmocOSu, DMF, pyr, CH₂Cl₂, 3 h, 69%; g) FmocOSu, DMF, pyr, CH₂Cl₂, 3 h, 69%; h) FmocOSu, DMF, pyr, CH₂Cl₂, 3 h, 69%; i) piperidine, DMSO, quantitative; j) fluorescein isothiocyanate, iPr₂NET, 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. Treatment of 9 with the Fmoc-protected 6-amino-hexanoic acid N-hydroxysuccinimidy ester gave 10, which was deprotected (piperidine, DMSO, quantitative yield) then allowed to react with fluorescein isothiocyanate (Hüning’s base, DMF, 66% yield) to form the amphotericin B–fluorescein conjugate 11.

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. 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) were prepared from a thin film of the lipid(s) in a mixture containing KCl (150 mM) and 2-[(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (5 mM) at pH 7.4. The large 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.

Native amphotericin B (1), the conjugate with the piperazine linker 9, and the fluorescein–amphotericin B 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. K⁺ release from sterol-free vesicles was slow, as we expected based on literature data for amphotericin B. 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.

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. Since amphotericin B is known to insert into membranes, 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.
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.[20] 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.[24]

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.[22] 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 toward 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. Amphotericin B with reference to its reported side-effects on mammalian cells.

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.

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: λ<sub>em</sub> = 535 nm; red fluorescence: λ<sub>em</sub> = 645 nm.
perturb the local composition of cellular membranes. Hypothesis, recent data suggests that filipin itself might
distinct regions of the plasma membrane. In support of this
ergosterol-rich domains might not accumulate significantly in
different 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 piperazinyl 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 bench-
mark 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 (Saccha-
rornycyces 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 conju-
gate, 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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The importance of structural assignment for any subsequent mechanistic investigation led us to confirm the success of the conjugation reaction by using a spectral aliasing technique to acquire high-resolution HSQC and HMBC spectra (see refs. [15, 16]). All but a few signals in the double-bond regions of such 'H, 13C, DEPT, COSY, HSQC, and HMBC spectra were unambiguously assigned to 8.

For the synthesis of an NBD (7-Nitro-2-oxa-1,3-diazol-4-yl)–amphotericin conjugate with an amide linker, see: N.O. Petersen Spectros. Int. J. 1983, 2, 408–414. No characterization data were given for this conjugate; to the best of our knowledge it has not been used for biological studies.

For improved ruggedness, ion-selective membranes were used that had a higher than usual (see ref. [22]) content of PVC and a lipophilic salt was added to compensate for the increased resistance.

The use of external Ca2+ instead of Na+ ions (as reported previously) seemed to block the pores and yielded no results.

This result may imply a beneficial increase in the therapeutic effect of amphotericin B.

For flow cytometry data, see the Supporting Information.

PrA is a 57-kDa protein that binds to the invariant Fc fragment of rabbit-derived IgG proteins with a dissociation constant of approximately 60 nM. See ref [24].