A novel linker strategy is presented based on a double reductive amination of a dialdehyde to the amine of the amphotericin B mycosamine sugar and the biological activity of a series of conjugates is compared to the native amphotericin B.

Despite the fact that it was discovered well over 40 years ago, the mode of action of the polyene macrolide antimycotic agent amphotericin B remains enigmatic, puzzling biochemists and pharmacologists alike.1,2 Although there is clear evidence that its incorporation into biological and abiological membranes results in electrolyte efflux, there persists an ongoing debate as to the causal relationship of this observed effect on cell death. Indeed, numerous investigations reveal contradicting behaviour between model studies and those in vivo.3

We have been interested in developing biochemical probes based on amphotericin B (1, Fig. 1) as a means of studying several hypotheses concerning the mode of action of this important antifungal agent and to facilitate the study of processes believed to occur at the membrane. We have initiated a program in the design and synthesis of tailor-made amphotericin B conjugates bearing reporter groups that permit insight into the fate of amphotericin in cells and liposomes.4,5 In our preliminary disclosure, we documented the preparation of an amphotericin B–fluorescein conjugate which exhibited interesting properties when employed as a probe in the fungal membrane. In this communication we report the synthesis of additional conjugates using a piperazine as a point of attachment, and we introduce a related conjugation strategy involving a 4-carboxypiperidinyl linker, which is
prepared through a short convenient sequence of reactions from commercially available 3-cyclopentene-1-carboxylic acid and 1-Fmoc-1,6-diaminohexane. Furthermore a preliminary validation on the potential utility of one of these conjugates is carried in yeast; we also document ion-flux studies in POPC vesicles (liposomes).

Earlier structure–activity relationship studies on amphotericin B underlined the importance of the basic amine for the mechanism of action. Simple acylations of this functional group have led to compounds with significantly lowered biological activity. As such, this delimits the types of functionality that can be relied upon to prepare conjugates. Two different solutions to this
problem have appeared recently. Murata and co-workers have reported the monoalkylation of amphotericin mycosamine sugar through reductive amination of an aldehyde.\(^7\) We have
documented the use of dialdehydes in a double reductive
alkylation reaction of amphotericin mycosamine to afford novel
piperazinyl linked structures (Scheme 1). This latter approach
is convenient in that the formation of dialkylated products
produced when monoaldehydes are employed are avoided, thus
giving products that are easier to purify.

In order to evaluate the new linker we determined the minimal
inhibitory concentration (MIC) required to inhibit growth of
Saccharomyces cerevisiae. Amphotericin B (1) completely stopped
cell growth when added at a concentration of 1 \(\mu\)M. Conju-
gate 4 retained activity and was toxic above a threshold of
1.6 \(\mu\)M.

We also prepared large (100 nm) unilamellar vesicles (LUVs)
and directly measured the induced \(K^+\) permeability of the LUVs
with a \(K^+\)-selective electrode. This assay contrasts with that
recently employed, which monitors the pH-sensitive shift corre-
sponding to the \(^{31}\)P NMR signal of a membrane probe.\(^6\) For such a
commonly employed assay, amphotericin B-induced leakage of \(K^+
ions from liposomes triggers a counter \(H^+\) ion influx, levelling out a
transmembrane pH gradient. We reasoned that the direct analysis
of \(K^+\) efflux via a \(K^+\)-selective electrode would have the benefit of
fast on-line measurements with increased accuracy as well as have
the advantage of maintaining the same physiologically-relevant
pH throughout the efflux experiment. This is especially important
since amphotericin B–liposome interactions are known to be very
sensitive to pH change.\(^8\)

In the vesicle assay that we have adapted for our purposes,\(^9\)
LUVs from POPC or from POPC with admixed sterols (mimicking
conditions in natural biomembranes\(^{w}\)) were prepared in a KCl
solution and the vesicles were dialyzed against NaCl in order to
create an ion gradient (\(K^+\) inside, \(Na^+\) outside). Freshly prepared
valinomycin-based \(K^+\)-selective electrodes were characterized and
employed as described earlier.\(^6\)

The parent amphotericin B (1) triggered an immediate release of
\(K^+\) ions from liposomes containing cholesterol or ergosterol
(Fig. 2). Conjugate 4 showed an efflux pattern that was very similar
to native amphotericin B in the case of ergosterol containing
vesicles. However, a difference was found when comparing the
efflux from cholesterol and non-sterol containing liposomes. The
new conjugate 4 induced a clear distinction between the
three vesicle systems, a fact that may show beneficial influence
on the selectivity of the drug for fungal versus mammalian
cells.

We set out to establish the utility of 4 by preparation of
several amphotericin B conjugates incorporating typical affinity-
(5), fluorescent- (6), photolabelling- (8) groups (Fig. 3) as well as
amphotericin–cholesterol conjugate 7 and dimer 9. Piperazinyl-
linked amphotericin analog 4 was allowed to react with biotin \(N-
hydroxysuccinimide ester to give 5, with fluorescein isothiocyanate
to furnish 6, with cholesterol chloroformate to provide 7, and
with the \(N\)-hydroxysuccinimide ester of 4-(3-trifluoromethyl-3\(H\)
diazen-3-yl)-benzoate ester to afford diazirane 8. When 4 was
condensed with an activated form of glutaric acid dimer 9 was
isolated in 24% yield from amphotericin B, which is a considerable
improvement over the yield that had been previously described for
related dimeric compounds.\(^7\)

Biotin conjugate 5 was selected for thorough examination in
both liposomal (\(K^+\) efflux) and cellular assays. For comparison
purposes, amide-linked conjugate 10 was prepared (Fig. 4).

Both biotin-amphotericin B conjugates showed a \(K^+\) release pattern
similar to native amphotericin B (1) (Fig. 5). The piperazinyl-
linked conjugate 5 however, retained a high toxicity against fungal
cells (MIC = 20 \(\mu\)M) whereas the amide linked conjugate 10
showed a fivefold drop in activity (MIC = 100 \(\mu\)M) compared to 5.
This feature of probe 5 permits its use for subsequent investigations
of channel-forming properties in the membrane.\(^11\)

As an alternative approach we have examined the use of a 3-
cyclopentene-1-carboxylic acid derivative, providing a diverse set
of building blocks for the construction of amphotericin conjugates.
The new linker strategy also allows for simple synthetic variations
of the binding motive. The commercially available cyclopentene

carboxylic acid is converted into the corresponding acid chloride
(oxalyl chloride, DMF) and treated with monoprotected 1,6-
diaminohexane in pyridine to afford a cyclopentenyl amide
which following dihydroxylation furnishes 11. Oxidation of this
diol to the dialdehyde and double reductive amination with
amphotericin B (1) installed the new linker. After deprotection
the diaminohexany1 pyridyl amphotericin B 12 was isolated in
91% yield (Scheme 2).

![Scheme 2](image_url)
of other biologically important systems wherein functionalization through a primary amine is required yielding molecules with interesting new properties. A biotin conjugate was prepared as a benchmark case which displayed comparable biological activity to the parent compound and preserved $K^+$ efflux-inducing properties in a LUV assay. The new linker strategy and various prepared amphothericin B conjugates will be useful in shedding some more light on the mechanism of action of this important antifungal drug.

Acknowledgements

We would like to thank Professor Dr Josef Brunner for kindly providing us with the active ester of the diazirine photo-crosslinker. We are grateful for generous support from ETH Zürich in the form of a TH-Gesuch.

Notes and references


11 However, we believe that although LUVs can serve as excellent model systems in a narrowly defined area we suggest caution in drawing a direct correlation between liposomal and cell studies. In fact, in a previous paper we documented a case with a conjugate where potassium efflux from LUV is induced by a non-toxic compound up to 500 µM+.