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Water-soluble amphotericin B–polyvinylpyrrolidone complexes with maintained antifungal activity against *Candida* spp. and *Aspergillus* spp. and reduced haemolytic and cytotoxic effects

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***Objectives*: Poor solubility and toxicity severely hinder the clinical use of amphotericin B (AmB), in spite of its attractive chemotherapeutic properties. Water-soluble complexes of AmB and polyvinylpyrrolidone (AmB–PVP) could display lower cytotoxicity while maintaining antifungal activity.**

***Methods*: AmB–PVP [with PVP of 10, 24 and 40 kDa (AC1, AC2 and AC4)] were compared with free AmB for (i) activity against *Candida* spp. (five albicans; nine non-albicans) and *Aspergillus* spp. (four strains), (ii) haemolysis of sheep red blood cells, and (iii) release of lactate dehydrogenase from J774 macrophages [with further comparison with free PVP and a liposomal formulation of amphotericin (AmBisome®)].**

***Results*: MICs and MFCs of AC1, AC2 and AC4 against *Candida* spp. and of AC2 and AC4 against *Aspergillus* spp. were similar to those of AmB (and even lower for some *Candida* strains). Killing kinetics (24 h) were also similar. Haemolytic activity of AC2 and AC4 was 2-fold lower than that of free AmB. Cytotoxicity of AC2 towards J774 macrophages was 8-fold lower, and that of AC4 5-fold lower than that of AmB and not significantly different from that of AmBisome®. The lower cytotoxicity of AC2, AC4 was correlated with a lower cellular accumulation of amphotericin. Spectroscopic analysis shows that the lower toxicity of AmB–PVP was not owing to significant change in the monomeric/polymeric forms ratio of the drug.**

***Conclusions*: AmB–PVP complexes compared favourably with AmB for antifungal activity, were less haemolytic and cytotoxic than AmB, and show a similar cytotoxicity profile to AmBisome®.**

Keywords: J774 macrophages, red blood cells, accumulation, lactate dehydrogenase, AmBisome®

Introduction

Opportunistic fungal infections have now emerged as an important cause of morbidity and mortality in immunocompromised and severely ill patients.[1,2] They also represent a major therapeutic challenge owing to the increasing prevalence of organisms resistant to commonly used azoles.[3,4] Developing novel drugs and/or treatment strategies to fight these infections is therefore critical. This has led to the development of azoles with enlarged spectrum[5] and to the discovery of novel, broad-spectrum fungicidal drugs such as the echinocandins.[6] Their cost, however, represents a severe limitation in their use for many countries or communities. In contrast, amphotericin B (AmB) is inexpensive while being highly fungicidal against most pathogenic fungi, and remains free of clinically-meaningful resistance so far.[4,7,8] The use of AmB, however, is hampered by its side effects, the frequency of which may be as high as 80%,[9,10] and also by a lack of solubility in injectable aqueous media.[11,12] To circumvent the latter disadvantage, and to partly alleviate toxicities, AmB is now often administered as a micellar dispersion in sodium deoxycholate,[13,14] or as a lipid formulation[15,16] such as liposomes,[17,18] nanospheres[19] or cochleates.[20] These strategies, however, also have their limitations

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that include a narrow therapeutic index for micellar dispersions,[21] or high cost for lipid formulations.[14] The development of other types of water-stable and well-dispersed aqueous solutions of AmB with low intrinsic toxicity and low manufacturing prices remains therefore highly desirable. Attempts have included so far the design of complexes of AmB with calcium[22] or succinic acid,[23] the synthesis of *N*-acyl derivatives,[24] *N*-methyl-*N*-D-fructosyl methyl esters,[25] polyethyleneglycol-[26] or arabinogalactan-conjugates[27], the entrapment of AmB in amphiphilic micelles,[28] or its association with microemulsions and monoglyceride-water systems.[29] Polyvinylpyrrolidone (PVP) has a long, proven history of pharmaceutical applications as delivery systems of poorly soluble drugs.[30,31] It also possesses a high degree of biocompatibility,[32,33] with the possibility to control the rate of drug release so as to improve the *in vivo* pharmacokinetics.[31] PVP has also been shown to inhibit drug crystallization in solution as well as in the solid state,[34] and protects against drug degradation in solution.[35] The formation of AmB–PVP complexes in non-aqueous solvents has been originally described in the mid-1970s,[36] and these complexes can now be manufactured as water-dispersible preparations.[37] In the present study, we have investigated their *in vitro* antifungal activities and evaluated their cytotoxicity in comparison with free AmB and with a liposomal formulation of AmB (AmBisome®).[38] Our results show that AmB–PVP complexes fully retain their activity against *Aspergillus* spp. and *Candida* spp. They are less haemolytic and also less cytotoxic (in relation to their reduced cellular accumulation). They compare favourably with a liposomal formulation (AmBisome®) for cytotoxicity.

Materials and methods

Reagents

PVP (molecular weight 24 kDa) was provided by Fluka/Sigma–Aldrich (Buchs, Switzerland), and PVP (molecular weights 10 and 40 kDa) and AmB (AmB) by Sigma–Aldrich (Steinheim, Germany). AmB was diluted and stored in DMSO (5 mg/mL). Cell culture media and fetal calf serum (FCS) were purchased from Gibco Biocult (Paisley, Scotland). AmBisome® (a liposomal formulation of AmB/hydrogenated soy phosphatidylcholine/cholesterol/distearoylphosphatidylglycerol 50 : 213 : 52 : 84, w/w/w/w)[38,39] was procured from UCB-Pharma, Brussels, Belgium as the branded product registered for clinical use in Belgium. Azoles were provided as standards for microbiological investigations by Janssen-Cilag Pharmaceutical S.A.C.I. (Pefki, Greece; ketoconazole and itraconazole) and by Pfizer-Greece (Athens, Greece; voriconazole). Unless stated otherwise, all other reagents were of analytic grade and purchased from E. Merck AG (Darmstadt, Germany).

Preparation of AmB–PVP complexes (AmB–PVP)

We used the same procedure as described previously.[40] Briefly, 5 mg of AmB was added to 100 mL of methanol containing 2 g of PVP (10, 24 or 40 kDa). The mixture was stirred continuously for 20 min at 50°C, using a vertical cooler in order to keep the methanol volume constant. Methanol was subsequently removed by evaporation at 45°C under reduced pressure (using a rotary vacuum evaporator spinning at 125 rpm) and the residue dissolved in distilled water to a final volume of 10 mL. The content of the final preparations in AmB was 0.249% w/w for the three complexes investigated, which will be referred to as AC1, AC2 and AC4 for preparations made with 10, 24 and 40 kDa PVP, respectively. HPLC, UV and IR spectra analyses were performed to control the formation of AmB–PVP complexes.

MIC and MFC determinations

All strains were obtained from and characterized by the Laboratory of Infectious Diseases of the Athens University School of Medicine, Laikon Hospital, Goudi, Athens, Greece. Susceptibility testing was performed following the guidelines of the NCCLS (present name: CLSI) according to version 1997 (yeasts)[41] for *Candida* spp., and to version 1998 (filamentous fungi)[42] for *Aspergillus* spp. For *Candida* spp., cells were collected from a 24 h Sabouraud dextrose agar cultures in 5 mL of sterile saline and thoroughly mixed by vortexing for 15 s. Turbidimetric measurement was made at a wavelength of 530 nm and the suspension adjusted to 0.5 McFarland units (corresponding to $1–5 \times 10^6$ cfu/mL), and diluted in MOPS-buffered RPMI 1640 medium (pH 7.2) to a final density of $1–5 \times 10^3$ cfu/mL. Aliquots (100 µL) of this suspension were added to 100 µL of serial 2-fold drug dilutions (also in MOPS-buffered RPMI 1640 medium) in 96-well flat-bottomed microdilution plates (drug concentration range: 0.032–64 mg/L in amphotericin). After 24 h incubation at 35°C, growth was assessed by turbidimetry using a conventional ELISA reader at 630 nm. MICs were defined as the lowest drug concentrations at which the medium remained optically clear. MFCs were determined by subculturing 100 µL from the wells with no visible growth onto Sabouraud dextrose agar plates. After 24 h incubation at 35°C, colonies were counted and the MFC was defined as the lowest concentration of drug that yielded less than five colonies. For *Aspergillus* spp., conidia developing after 4–5 days of culture were collected in sterile saline, washed in the same medium, counted with a haemocytometer and the suspensions adjusted to a density of 6×10^3 cells/mL. MICs and MFCs were then determined as for *Candida* spp.

Determination of time–killing activities

For *Candida albicans*, cells were collected from cultures made on Sabouraud dextrose agar maintained at 35°C. Five millilitres of suspension ($1–5 \times 10^5$ cfu/mL) in Sabouraud broth was shaken at 37°C in the presence of AmB or the AmB–PVP at a concentration of 4× the MIC (4, 1 and 2 mg/L for AmB, AC2 and AC4, respectively). At appropriate times, aliquots were removed and plated in duplicate on Sabouraud agar after serial dilutions. Plates were incubated at 35°C for 24 h and the numbers of cfu were counted. For *Aspergillus fumigatus*, 5-day-old conidia were collected in sterile water, washed once, diluted with MOPS-buffered RPMI 1640 medium at a final concentration of $\sim 5 \times 10^3$ cfu/mL and incubated in the same medium for different periods of time (2, 4, 6 and 24 h) in the presence of AmB or the AmB–PVP at a concentration of 5× the MIC (5 mg/L). Killing activity was thereafter determined as for *C. albicans*.

Determination of haemolytic activity

The haemolytic activity of the AmB–PVP versus AmB was determined using sheep red blood cells (SRBCs). SRBC suspensions (50%) were obtained from BioMérieux sa (Marcy l'Etoile, France), washed twice with isoosmotic veronal buffer (pH 7.2) and diluted 10-fold with the same buffer. One hundred microlitres of serial 2-fold dilutions of AmB–PVP or AmB were added to 900 µL of SRBCs suspensions. After 1 h of incubation at 37°C, samples were centrifuged for 15 min at 3000 rpm, the supernatant diluted twice with veronal buffer and its absorbance measured at 570 nm. The percentage of haemolysis was defined as $[(Abs_t - Abs_C)/Abs_{tot} - Abs_C)] \times 100$, where Abs_t is the absorbance of the supernatant from samples incubated with the drugs, Abs_C the absorbance of the supernatant from controls (no drug added) and Abs_{tot} is the absorbance of the supernatant of controls (no drug added) incubated in the presence of 1% Triton X-100, a non-ionic detergent causing full disruption of the pericellular membrane, to obtain complete haemolysis.

Determination of cell toxicity

J774 cells, a continuous reticulosarcoma cell line of murine origin,[43] were grown at 37°C in a 95% air/5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% FCS. Cultures were initiated at a density of $\sim 10^5$ cells/cm^2, and cells grown to apparent confluence (2–3 days) before exposure to PVP, AmB, AmB–PVP or AmBisome®. Cytotoxicity was assessed by the measurement of the release of the cytosolic enzyme lactate dehydrogenase (EC 1.1.1.27), with activity being measured by the method of Vassault.[44] Results are expressed as the activity found in the medium in percentage of the total amount found in cells and in the corresponding culture medium (corrected for by the activity found in a sample of the same batch of culture medium unexposed to cells and corresponding to the activity of lactate dehydrogenase present in the bovine fetal serum; this correction amounted to approximately two-thirds of the activity measured in the medium of control cells). We checked that addition of Triton X-100 (0.1%) yielded a complete release of lactate dehydrogenase (100.0 ± 0.3%).

Uptake and efflux studies

For influx studies, cells were incubated in the presence of AmB–PVP or AmB for appropriate times. Dishes were then placed on crushed ice and the cell sheets washed three times with ice-cold saline. Cells were collected by scraping with a Teflon® policeman in distilled water and fully dispersed by sonication at 50 W for 15 s. For efflux studies, cells were incubated with AmB–PVP or free AmB for 4 h, washed three times in ice-cold saline, and then reincubated in AmB–PVP- or AmB-free medium for up to 4 h. Cells were then washed and collected as for the influx studies.

Assay of AmB and calculation of AmB apparent cell accumulation

The quantification of AmB accumulation by cells (whether incubated with free AmB, AmB–PVP or AmBisome®) was performed by HPLC analysis using a Spectra Physics High Performance liquid chromatograph (Thermo Electron, Mountain View, CA, USA) equipped with a SP8800 solvent delivery system, a SP8450 UV/Vis detector (connected to a model SP4270 integrator). A 250 µL aliquot of cell lysate was mixed with 250 µL of acetonitrile (containing tenoxicam as internal standard). The mixture was vortexed for 30 s and centrifuged for 5 min at 12 000 **g**. The recovery of AmB was 94%, based on samples from control cells spiked with AmB. A 50 µL aliquot of the supernatant was injected onto the column [25 mm × 4.6 mm I.D., packed with Nucleosil 100 C18 (particle size, 5 µm), obtained from RigasLab, Thessaloniki, Greece]. Elution was made with acetonitrile/water/acetic acid (51 : 26 : 23, v/v/v) at a flow rate of 1.0 mL/min, and detection made at 382 nm. Typical retention times of tenoxicam and of AmB were 3.3 and 8.2 min, respectively. The detection limit of AmB was 0.125 µg/mL for lysates from control cells spiked with AmB. Standards were routinely prepared at a final concentration of 2 µg/mL for AmB and 1 µg/mL for tenoxicam. The cell content in AmB was systematically expressed by reference to the sample protein content, and the apparent cellular to extracellular concentration ratio calculated determined by using a conversion factor of 3.08 µL of cell volume per mg of cell protein.[45]

Spectroscopic measurements

UV-VIS spectra of AmB–PVP and AmB in water were recorded in the 280–500 nm range with a Jasco UV/VIS spectrophotometer (model 7800; Jasco Inc., Easton, MD, USA) equipped with a Jasco PTL-396 integrator.

Data analyses

Curve fittings were made, and correlations calculated with Graph Pad Prism® (V4.02 for Windows, GraphPad Software, San Diego, CA, USA). Statistical analyses [ANOVA for group comparisons; ANCOVA (analysis of covariance) for testing interaction effects of categorical variables, including residuals analysis and multiple comparisons tests] were made with the XLSTAT© software version 6.0 (Addinsoft SARL, Paris, France). Unless stated otherwise, all results are shown as means ± SD.

Results

Antifungal activities of AmB–PVP

Table 1 shows the MICs and MFCs of AmB–PVP and AmB against various strains of *Candida*. Except for one strain of *Candida tropicalis*, all values for the three types of complexes were equal or lower (1–4 dilutions) than those of AmB, with MFCs equal or only two dilutions higher than the corresponding MICs. For *Aspergillus* spp. (Table 2), activities of AmB–PVP were essentially similar or better than those of AmB, except for AC2 against *Aspergillus niger* (where the activity was 2-fold lower than that of AmB). Activities of both AmB–PVP and AmB were, however, globally weaker than those of voriconazole and itraconazole.

AC2 and AC4 were then used for further evaluation of antifungal activity by time–kill curve approaches (AC1 was discarded at this stage, based on preliminary studies showing considerably more cytotoxicity than AC2 or AC4). Figure 1 shows that both AmB and AmB–PVP (AC2 and AC4) caused a rapid decrease in the inoculum of *C. albicans*, yielding an ~4 log decrease in cfu within 24 h in all cases. Small differences were observed at the intermediate time points in favour of AC2, but these did not achieve statistical significance when analysed globally by ANCOVA or by paired group comparison. We checked in parallel experiments that PVP, up to 20% for 24 h, had no antifungal activity against *C. albicans* (data not shown). For *A. fumigatus*, the decrease in cfu proceeded slowly during the first 6 h but eventually reached a 2 log decrease (or more) at 24 h. AmB–PVP (AC2 and AC4) and AmB had similar activities at all times.

Haemolytic and cytotoxic activities

The haemolytic effect of AmB–PVP was thereafter examined in comparison with AmB using similar concentrations of the complexed and the free forms of amphotericin in the 0–40 mg/L range (chosen as to cover the ranges of MICs observed for AmB–PVP against *Aspergillus* spp.). Figure 2 shows that the haemolytic activity of AmB was concentration-dependent within that range, reaching almost 100% at 40 mg/L. AmB–PVP (AC2 and AC4) caused systematically about half of the haemolysis seen with AmB in the same range.

Figure 3 shows the release of lactate dehydrogenase from J774 macrophages over a 24 h incubation time when exposed to the same concentrations of amphotericin (in a 0–40 mg/L range) given as free drug (AmB), complexes with PVP (AmB–PVP AC2 or AC4) or a liposomal formulation (AmBisome®). We also included in this experiment cells incubated with PVP (40 kDa) alone, at a concentration equivalent to that used for cells incubated with AmB–PVP, to evaluate the cytotoxicity of the polymer itself. AmB caused a concentration-dependent release of this enzyme that reached ~80% of the maximal releasable amount for an extracellular

Table 1. Antifungal activities [MICs and MFCs (mg/L)] of the AmB–PVP complexes AC1, AC2 and AC4[a] and of AmB against *Candida* spp.[b]

	AmB–PVP complexes							
	AC1		AC2		AC4		AmB	
Strains	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans ATCC 90028	0.5	0.5	0.25	0.25	0.5	0.5	1	1
C. albicans Const2	0.5	0.5	0.25	0.25	0.5	0.5	1	1
C. albicans 4021B	0.125	0.25	0.125	0.125	0.125	0.125	1	2
C. albicans 4121	0.125	0.125	0.125	0.125	0.25	0.25	2	2
C. albicans 4190	0.125	0.125	0.125	0.25	0.125	0.125	1	2
C. glabrata 4224A	0.125	0.25	0.125	0.25	0.125	0.25	0.25	1
C. glabrata 4176C	0.25	0.25	0.125	0.125	1	1	2	2
*C. parapsilo*sis ATCC 22019	0.125	0.125	0.125	0.125	0.125	0.25	1	1
C. parapsilosis 3958	0.06	0.06	0.125	0.125	0.125	0.250	0.5	0.5
C. parapsilosis 3944D	0.06	0.06	0.125	0.125	0.125	0.250	0.5	0.5
C. tropicalis 3998	0.006	0.012	0.006	0.006	0.006	0.012	0.12	0.12
C. tropicalis 4300	0.25	0.25	0.5	0.5	0.5	0.5	2	2
C. tropicalis TEIA1	0.25	0.25	0.5	0.5	0.5	0.5	1	1
C. tropicalis TEIA2	0.25	0.25	0.5	0.5	0.5	0.5	0.125	0.125

[a]AC1, complex with 10 kDa PVP; AC2, complex with 24 kDa PVP; AC4, complex with 40 kDa PVP.
[b]Results from a typical experiment. These experiments were repeated three times with results differing by no more than one dilution.

Table 2. Antifungal activities [MICs and MFCs (mg/L)] of the AmB–PVP complexes AC2 and AC4[a] , AmB and three antifungal azoles (MICs only) against *Aspergillus* spp.[b]

	AmB–PVP complexes								
	AC2		AC4		AmB		Voriconazole	Itraconazole	Ketoconazole
Strains	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MIC	MIC
A. fumigatus ATCC 9157	1	8	1	8	1	8	0.5	0.5	4
A. niger 242	2	16	0.5	4	1	8	1	1	8
A. flavus E222	8	64	8	64	64	>64	2	0.5	4
A. flavus 538	16	32	8	32	16	32	1	0.5	1

[a]AC2, complex with 24 kDa PVP; AC4, complex with 40 kDa PVP.
[b]Results from a typical experiment. These experiments were repeated three times with results differing by no more than one dilution.

concentration of 40 mg/L (DMSO alone, used to dissolve amphotericin caused no additional lactate dehydrogenase release compared with control cells when used at the same concentration as that carried out in the culture medium for the largest concentration of AmB used). In sharp contrast, AmB–PVP complexes (AC2 and AC4), and the liposomal formulation caused <10% of lactate dehydrogenase release up to a 40 mg/L concentration in amphotericin. PVP 40 kDa was without cytotoxicity (and actually caused less lactate dehydrogenase release than what was seen in control cells) up to a concentration of 20 mg/L. In subsequent experiments, we examined the time-dependence of these effects using cells incubated with AmB, AmB–PVP, AmBisome® and PVP alone for only 4 h. AmB caused a release of lactate dehydrogenase that was essentially similar at 4 and 24 h, indicating a rapid interaction with cell membranes. All other treatments (AmB–PVP, AmBisome® or PVP alone) caused not significant additional release of lactate dehydrogenase compared with control cells (3.2 ± 0.6%; data not shown).

Uptake and efflux of amphotericin in macrophages

In the next series of experiments, we studied the kinetics of uptake and efflux of amphotericin in J774 macrophages incubated with AmB or AmB–PVP, comparing similar concentrations of free and complexed amphotericin. The results of the influx studies are shown in Figure 4. The left panel illustrates a typical uptake kinetic study made at a concentration of 2.5 mg/L in amphotericin given as AmB or as AmB–PVP (AC2 or AC4), this concentration being

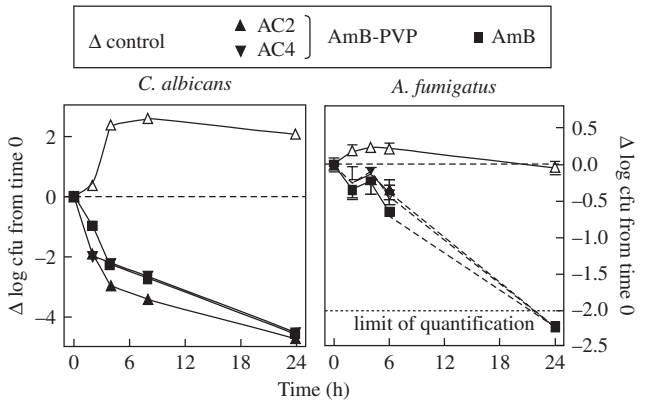

Figure 1. Time–killing activities of AmB–PVP [AC2 (filled triangles) and AC4 (filled inverted triangles)] in comparison with AmB (filled squares) against *C. albicans* TEI 184 [left panel, incubation at a concentration of 4× the MIC (1, 2 and 4 mg/L, respectively)] and against *A. fumigatus* ATCC 9197 [right panel, incubation at a concentration of 5× the MIC (5 mg/L)]. Open triangles, untreated cultures. The ordinate shows the change in cfu from time 0 (corresponding to the thick dotted line). Data are means ± SD ($n = 3$; error bars that are not visible are smaller than the symbols). For *Aspergillus*, cfu counted at 24 h were below the limit of quantification (50 cfu; thin dotted line), and data were arbitrarily set to a decrease of 2.25 log cfu. Statistical analysis (ANCOVA/Tukey's analysis of differences between groups with confidence interval at 95%): kinetics of growth in control conditions are significantly different from those obtained in the presence of AC2, AC4 or AmB ($P < 0.001$); differences between kinetics of growth obtained in the presence of AC2, AC4 and AmB are not significant. These experiments were performed twice with similar results.

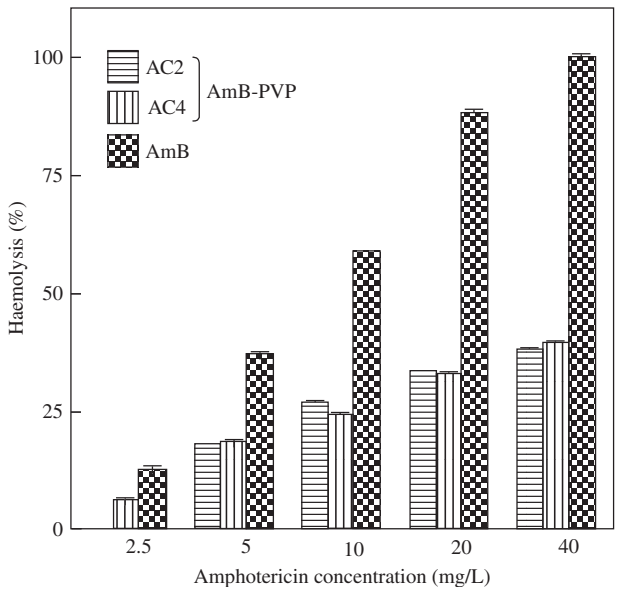

Figure 2. Haemolytic activity of AmB–PVP [AC2 (horizontally striped bars) and AC4 (vertically striped bars)] in comparison with AmB (chequered bars) towards SRBCs. Results are expressed as percentage of haemolysis. All concentrations (2.5, 5, 10, 20 and 40 mg/L) shown in the abscissa are expressed as amphotericin. Data are means ± SD ($n = 3$; error bars that are not visible are included in the histograms). Statistical analysis: haemolysis (i) two-way ANOVA on all data points: $P < 0.001$ for the effect of formulation (AC2 and AC4 versus AmB); $P < 0.001$ for the effect of concentration and $P < 0.001$ for the combined effect of formulation and concentration; (ii) Tukey–Kramer multiple comparison tests: for AmB, all data points are significantly different from those of AC2 or AC4 ($P < 0.001$); the differences between AC2 and AC4 are not significant except at 10 mg/L ($P < 0.001$).

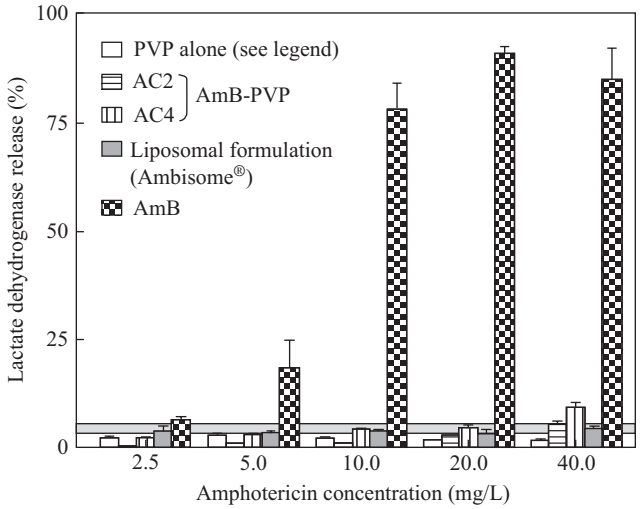

Figure 3. Cytotoxic activity of PVP alone (white bars), AmB–PVP [AC2 (horizontally striped bars); AC4 (vertically striped bars)], AmBisome® [a liposomal formulation of amphotericin (grey, dotted bars)] and AmB (chequered bars). The abscissa shows the concentration of amphotericin present in the culture fluid as AmB–PVP, AmBisome® or free AmB. For PVP alone, the concentrations of the polymer were adjusted to correspond to those created by adding the corresponding amounts of AmB–PVP (AC2 or AC4) (i.e. from 1 to 20 mg/L PVP), based on the ratio PVP/amphotericin used for preparing the complexes. Results are expressed as the release of lactate dehydrogenase observed after 24 h of incubation. The grey horizontal bar corresponds to the SD range of the value (5.28 ± 1.40) observed for controls (no addition). Data are means ± SD ($n = 3$; error bars that are not visible are included in the histograms). Statistical analysis: LDH release (i) two-way ANOVA on all data points: $P < 0.001$ for the effect of formulation; $P < 0.001$ for the effect of concentration and $P < 0.001$ for the combined effect of formulation and concentration; (ii) Tukey–Kramer multiple comparison tests: for AmB, all data points are significantly different from those of AC2 or AC4 except at 2.5 and 5 mg/L ($P < 0.001$); the differences between AC2 and AC4 are not significant.

chosen to remain in all cases below a threshold of 10% increase in lactate dehydrogenase release (see above). Uptake was found to proceed according to one-phase exponential association kinetics, reaching near-saturation at 24 h. At that point, the amphotericin cell content was ∼3 μg/mg protein, yielding an apparent cellular to extracellular drug concentration ratio of ∼300 (based on a cell volume to protein ratio of 3.08 μL/mg protein for control J774 macrophages).[45] In contrast, the accumulation of amphotericin in cells incubated with AmB–PVP (AC2 or AC4) reached a plateau at only one-sixth (AC4) or one-third (AC2) of the value observed for cells incubated with AmB. The right panel of Figure 4 shows the results of experiments in which we determined the cellular accumulation of amphotericin after 24 h incubation of the cells at increasing extracellular concentrations of amphotericin (0–40 mg/L) given either as AmB or as AmB–PVP (AC2 or AC4). In all cases, accumulation was linearly related to the extracellular concentration of amphotericin, but values were ∼5 times lower for cells incubated with AmB–PVP than for cells incubated with AmB. Accumulation of amphotericin was temperature-dependent, and values were ∼10 and 2.5 times lower in cells incubated at 4°C with AmB or AmB–PVP, respectively (data not shown).

In a distinct set of experiments, we examined the accumulation of amphotericin (40 mg/L) in cells incubated for 24 h with the free drug, the free drug mixed with 40 kDa PVP, a complex of amphotericin with 40 kDa PVP (AC4) or a liposomal formulation of

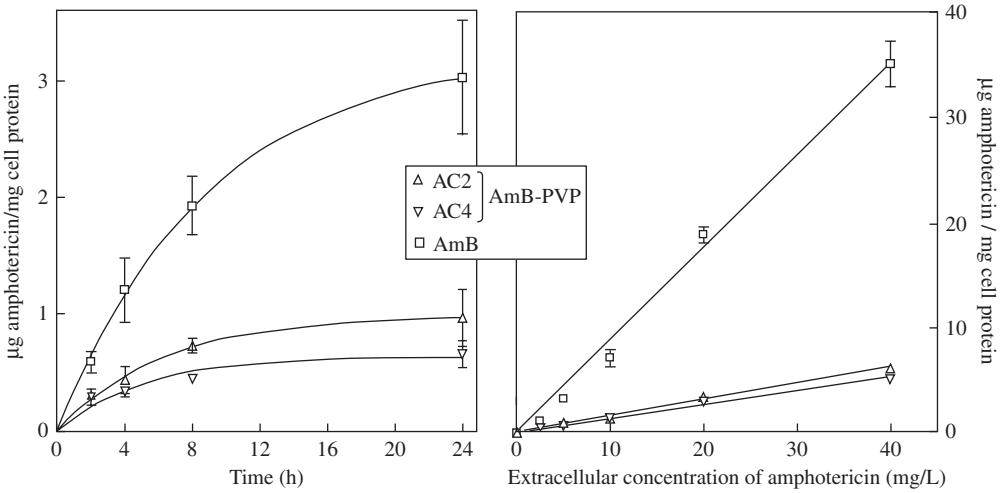

Figure 4. Accumulation of amphotericin in J774 macrophages incubated with AmB or AmB–PVP (AC2 and AC4) at 37°C. Left panel, cells were incubated at an extracellular concentration of 2.5 mg/L of AmB or its equivalent in amphotericin for AmB–PVP. Right panel, cells were incubated for 24 h with increasing concentrations of AmB or their equivalent in amphotericin for AmB–PVP. Results are given as means ± SD ($n = 3$). Statistical analysis: (i) one way ANOVA on all data points: $P < 0.001$; (ii) Tukey–Kramer multiple comparison tests: for AmB, all data points are significantly different from those of AC2 or AC4 ($P < 0.01$); the differences between AC2 and AC4 are not significant except at 8 h ($P < 0.01$).

Table 3. Accumulation of amphotericin in J774 macrophages exposed for 24 h at 37°C to 40 mg/L of AmB in the form of free drug, AmB mixed with PVP, AmB–PVP complex (AC4) or a liposomal formulation (AmBisome®)

	Accumulation[a] (µg/mg cell protein)
AmB	27.3 ± 4.3^{A}
AmB mixed with 40 kDa PVP	31.7 ± 2.1^{A}
AC4	3.56 ± 1.92^{B}
AmBisome®	1.07 ± 0.06^{C}

Results are presented as means ± SD ($n = 3$).
[a]Statistical analysis (paired *t*-test one tail): datasets with the same letter (A, B or C) are not significantly different from each other.

amphotericin (AmBisome®). Results shown in Table 3 demonstrate (i) that complexation with PVP (as AmB–PVP) reduced amphotericin uptake by macrophages to the same extent as for the liposomal formulation (AmBisome®) by comparison with free amphotericin; and (ii) that this effect did not result from the mere presence of PVP, since amphotericin mixed with PVP showed a similar accumulation to that of free amphotericin.

We then examined the release of amphotericin from cells incubated with AmB or AmB–PVP (AC2 or AC4) and thereafter transferred to drug-free medium. These experiments were conducted with a loading time of only 4 h to minimize the risk of cytotoxicity, but at an extracellular concentration of 5 mg/L of amphotericin [given as AmB or as AmB–PVP (AC2 or AC4)] to obtain enough sensitivity. In all cases, we observed a similar rate of release of amphotericin (fractional amount at 4 h, 71.0 ± 16.1, 70.2 ± 10.5 and 61.1 ± 7.9% of the original content of cell-associated amphotericin for cells incubated with AmB–PVP AC2, AmB–PVP AC4 and AmB, respectively). No significant loss of amphotericin was noted over 4 h if the experiment was performed at 4°C (data not shown).

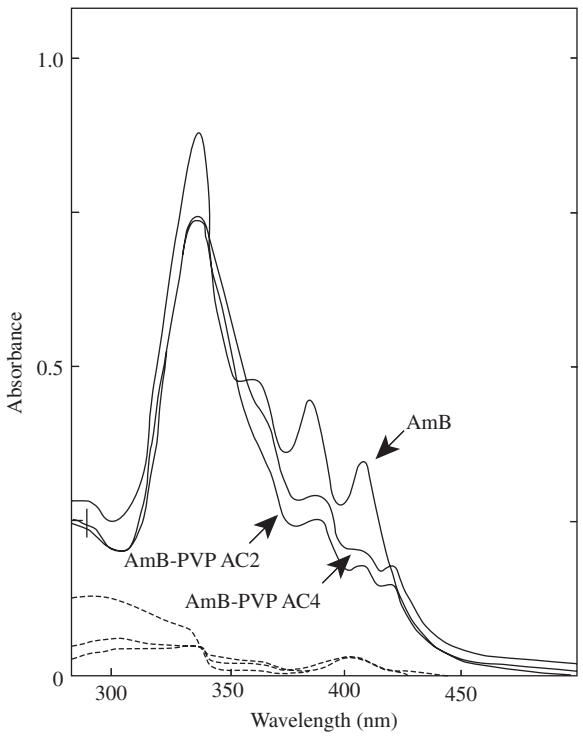

Figure 5. Absorption spectra of AmB–PVP (AC2 and AC4) and AmB in water at a concentration of 25 mg/L amphotericin (solid lines); the dotted lines show the absorption spectra of the corresponding drug-free media [water or water containing PVP (24 or 40 kDa)].

Spectroscopic analyses

AmB and AmB–PVP (AC2, AC4) were examined by IR and UV-VIS spectroscopy to detect the formation of the complexes, and to determine the state of aggregation of amphotericin. As shown in Figure 5, the differences in the UV-VIS spectra between AmB and

AC2 or AC4 confirmed the presence of complexes between AmB and PVP, which were also observed by IR spectroscopy (not shown). The UV-VIS spectra were also used to examine the state of aggregation of amphotericin. The UV-VIS absorption spectra of amphotericin are indeed characterized by four bands at 409, 385, 365 and 348 nm, and the ratio A_{348}/A_{409} is indicative of the monomeric/aggregated ratio, with a value of ~0.25 being characteristic of the monomeric form [checked for amphotericin in methanolic solution where this form predominates (data not shown)], whereas a value >2 is observed for drug in an aggregated form. Because only aggregates can form ion channels in cholesterol-containing membranes,[46,47] these are considered as being primarily responsible for toxicity towards mammalian cells. Figure 5 shows that the ratio A_{348}/A_{409} was in all cases >2, indicating that the drug was predominantly in its aggregated form in all cases (the differences between spectra were not considered significant as far as aggregation is concerned).

Discussion

The present report is a first systematic approach to the study of the potential usefulness of amphotericin–PVP complexes as antifungal agents. Activities have been determined against a limited number of strains, but these are representative of important fungal pathogens in humans, including, for *Candida*, several non-albicans strains. Our results show, quite unambiguously, that the activity of AmB was not impaired by its complexation with PVP (based on MIC, MFC and killing efficiency determinations). Conversely, the toxicity of the amphotericin–PVP complexes appeared markedly decreased compared with free amphotericin, based on the measurement of its lytic activity towards red blood cells and its capacity to cause leakage of the cytosolic enzyme lactate dehydrogenase from macrophages, two parameters used for several years to assess the toxic potential of amphotericin and its derivatives towards eukaryotic cells *in vitro*.[48,49]

The activity of amphotericin, as a polyene antifungal, involves the formation of complexes with ergosterol leading to the creation of transmembrane channels that disrupt the membrane permeability properties of the fungal cells.[50] This interaction relies on the availability of free amphotericin. The maintenance of antifungal activity when the drug is complexed with PVP may, therefore, seem surprising at first glance. A similar observation is, nevertheless, made for the liposomal forms of amphotericin,[51] and has been ascribed to the fact that these forms expose domains that allow a tight binding of the complex to the surface of the fungal cells and an interaction of amphotericin with ergosterol present in the fungal membrane. A similar mechanism may be operating here since PVP also can interact with lipids,[52] and could deliver amphotericin at the surface of the fungal cells in a similar way as it does for iodine with PVP-iodine complexes (povidone–iodine).[53]

Besides its interaction with ergosterol, amphotericin may, however, also interact with cholesterol, which is the most likely explanation for its toxicity towards eukaryotic cells. Amphotericin–cholesterol interaction occurs more easily when the drug is in an aggregated form as compared with monomers.[46,47] A reduction of aggregation by complexation with PVP could, therefore, have been critical to explain the lesser cytotoxicity of AmB–PVP compared with AmB. Spectroscopic analysis, however, failed to reveal important differences in the state of aggregation of AmB between the free and the complexed form. In contrast, our

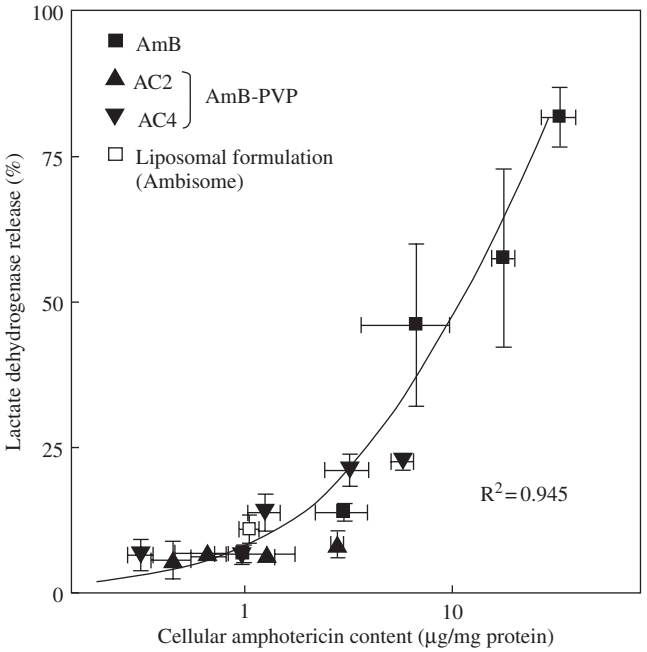

Figure 6. Correlation between the percentage of lactate dehydrogenase released and the apparent cell accumulation of amphotericin in cells incubated with AmB (filled squares), AmB–PVP [AC2 (filled triangles) and AC4 (filled inverted triangles)] and a liposomal formulation (AmBisome®; open squares). Data are from Figures 3 and 4 and Table 3, and are given as means ± SD (n = 3).

data show that the cellular accumulation of amphotericin was drastically reduced by its complexation with PVP, as was also found for its liposomal formulation (AmBisome®). Figure 6 shows that the cytotoxicity of amphotericin, whether used as free drug (AmB), as a complex with PVP (AC2; AC4) or as its liposomal formulation (AmBisome®), is in direct correlation with its cellular accumulation. One potential explanation for the lower accumulation of amphotericin when complexed with PVP is that its entry into mammalian cells will be restricted to the route of endocytosis as is the case for PVP in macrophages.[54] The complexes described here appear less cytotoxic than the AmB deoxycholate preparations presently marketed (Fungizone®), since the latter causes 50% haemolysis at 5 mg/L,[55] and was reported to be haemolytic at 8 mg/L.[56] We provide here direct evidence that AmB–PVP has a cytotoxic potential similar to that of a liposomal formulation of amphotericin (AmBisome®).

Our data cannot be extrapolated to the therapeutic arena without caution. The cellular concentrations of amphotericin observed in cells incubated with AmB are, indeed, considerably higher (20- to 200-fold) than what is measured in tissues of patients receiving conventional amphotericin therapy,[57,58] making, perhaps, the differences in cytotoxicity demonstrated here largely irrelevant. Yet, the therapeutic potential of AmB–PVP may deserve further investigated because (i) of the long history of pharmaceutical applications of PVP and its high degree of biocompatibility;[32,33] (ii) the maintenance of full *in vitro* activity compared with AmB, and a cytotoxicity similar to that of AmBisome® (for which extensive animal and clinical data demonstrating its decreased toxicity compared with AmB are available);[8,51] and (iii) the low acquisition cost of the constituents needed to manufacture these complexes.

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Transparency declarations

None of the authors has financial interests that could be perceived as having influenced the views they have expressed.

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