In-vitro antifungal activity, cytotoxicity and cellular accumulation of new water-soluble amphotericin B (AMB) - polyvinylpyrrolidone (PVP) formulations.

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ABSTRACT

Objectives: Amphotericin B, the drug of choice for the treatment of systemic fungal infections, is characterized by (i) a negligible solubility in water and therefore a poor bioavailability for host cells and (ii) a cellular toxicity. Liposomal or colloidal formulations have been developed to palliate these drawbacks. Conjugation of the aqueous-insoluble drug to water soluble biodegradable polymeric carriers also constitutes an attractive approach for improving AMB bioavailability. In the present work, we have investigated the in vitro antifungal activities of new water-soluble AMB-PVP complexes, as well as their accumulation in macrophages, in relation with their cytotoxicity.
Methods: Two new AMB-PVP complexes were synthesized as described earlier [Patent Certificate: 1003871, Greek Industrial Property Organization], namely AC2 (MW=24 kDa), and AC4 (MW=40 kDa). MICs and MFCs of *Candida spp* and *Aspergillus spp* were determined according to NCCLS guidelines. Killing activity at 4X MIC against *Candida* was also determined. Cytotoxicity was evaluated by hemolysis of sheep red blood cells and release of LDH from macrophages. Cellular accumulation in J774 macrophages was determined after measuring AMB concentration by HPLC.
Results: MIC of AMB-PVP complexes were up to 20 times lower as compared to AMB, (with AC2 showing the lowest MICs and MFCs) against *Candida spp*. and slighty lower to equal against *Aspergillus spp*. Killing activity after 2 hours exposure of *Candida albicans* to AMB and AMB-PVP complexes at 4XMIC, reached -2 log for complexes as compared to -1 log for AMB. Uptake in J774 macrophages after 24 hours was 3 times lower for complexes than for AMB, in direct relation with the LDH release. Hemolytic activity was considerably lower for complexes than for AMB.
Conclusions: AMB-PVP complexes showed improved antifungal activity as compared to AMB against *Candida spp*. and *Aspergillus spp*. They accumulate to lower levels than AMB in eucaryotic cells, which may explain their lower toxicity. These data demonstrate the potential benefits of these new formulations and suggest future applications.

INTRODUCTION

Opportunistic fungal infections have emerged as important causes of morbidity and mortality in immuno-compromised patients and remain a major challenge for infectious diseases clinicians. Beside their increasing frequency, invasive fungal infections are still associated with a very high mortality, up to 40% in bloodstream infections caused by *Candida albicans* and more than 50% in invasive aspergillosis.

In recent years, increased efforts have led to the discovery of new-engineered or reconsidered antifungal agents to allow for developing countries to benefit of efficacious, safe, and non expensive drugs. Among them, the polyene macrolide antibiotic amphotericin B, appears as a particularly interesting drug.

However, use of amphotericin B is hampered by its severe side effects, the frequency of which may be as high as 80 %, and also by its insolubility in injectable aqueous media.

The development of new drug delivery systems remains therefore a major task in antifungal drug development and conjugation of the aqueous-insoluble drug, amphotericin B to a water–soluble biodegradable polymeric carrier, like polyvinylpyrrolidone (PVP) constitutes an attractive approach for increasing water-solubility and bioavailability of Amphotericin B.

AIM OF THE STUDY

The aim of the present work is to compare polyvinylpyrrolidone (PVP) derivatives of amphotericin B to amphotericin B with respect to their
- ***in vitro* antifungal activities against *Candida spp.* and *Aspergillus spp*.**
- **Uptake and cellular accumulation in a macrophage cell line**
- **Toxicity as determined by hemolysis and LDH release from macrophages**

METHODS

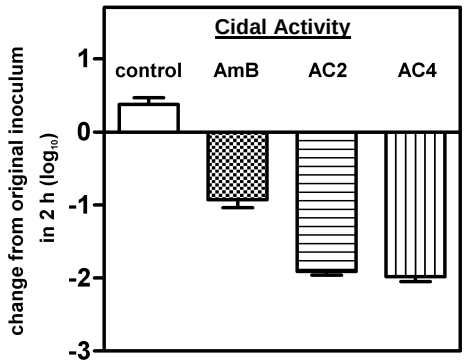
- Amphotericin B water-soluble PVP complexes (AC2: AmB-PVP [24 kDa]; AmB-PVP [40 kDa]) were prepared as previously described [1,2].
- Susceptibility testing for yeasts was performed according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS 1997).
- Killing activity at 4XMIC against *Candida* and 5XMIC against *Aspergillus* were obtained by determination of CFU/mg cell protein after plating cell lysates obtained after 24 hours.
- Cellular accumulation in J774 macrophages was determined after incubation with the compounds, washing in ice-cold PBS, harvesting by scraping and lysis by sonication. Cell lysates were used for determination of amphotericin B content by HPLC.
- Cytotoxicity was evaluated by hemolysis of sheep red blood cells and release of LDH from J774 macrophages. J744 cells were cultured at 37°C in 95% air/5%CO_2 atmosphere in RPMI 1640 medium supplemented with 10% foetal calf serum.

RESULTS

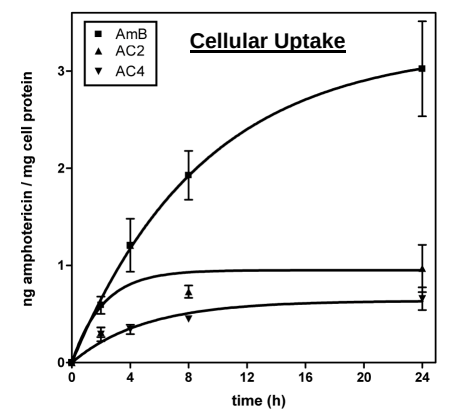
Antifungal activity (MIC/MFC) against *Candida spp.*

Strains	AmB		AC2		AC4	
	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans ATCC 90028	1	1	0.250	0.250	0.5	0.5
C. albicans 4121	2	2	0.125	0.125	0.250	0.250
C. glabrata 4176C	2	2	0.125	0.125	1	1
C. glabrata 4224A	0.250	1	0.125	0.250	0.125	0.250
C. parapsilosis ATCC22019	1	1	0.125	0.125	0.125	0.250
C parapsilosis 3958	0.5	0.5	0.125	0.125	0.125	0.125
C. tropicalis 3998	0.125	0.125	0.006	0.006	0.006	0.012
C. tropicalis 4300	2	2	0.5	0.5	0.5	0.5

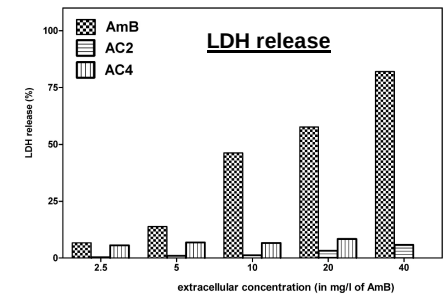
The MICs values against *Candida spp.* ranged from 0.125 to 2 µg/ml for amphotericin B and from 0.006 to 1 µg/ml for AmB-PVP complexes AC2 and AC4. The MICs of the AmB-PVP complexes were up to 20 times lower than those observed for amphotericin B. The MICs and MFCs of the two complexes investigated were comparable. The MFCs did not exceed twofold the MICs.
Against *Aspergillus*, the MICs values of AmB-PVP complexes were similar to that observed for amphotericin B.



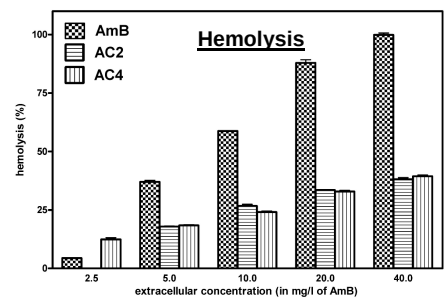
Exposure of *Candida albicans* TEI 184 to amphotericin B over a 2-hr period resulted, as compared to control, in a decrease of 1.5 log of the growth of *Candida albicans*. This activity was enhanced to 2.5 log decrease with AmB-PVP complexes.
Exposure of *Aspergillus* over a 24-hr period resulted for AmB and AmB-PVP complexes to a decrease of 2.5 log of the growth.



The uptake of amphotericin B and AmB-PVP complexes was examined as a function of time. The uptake proceeded according to a one phase exponential association process, reaching near the saturation after 24 hours for amphotericin B. For AmB-PVP complexes, the plateau value was reached after 4 to 6 hours and the kinetics for short times was more rapid for AC2 as compared to AC4.



For both amphotericin B and complexes, we observed a marked effect of the concentration in the range investigated (2.5 µg/ml to 40 µg/ml). The LDH release was however considerably lower for AC2 and AC4 complexes as compared to amphotericin B.



The hemolytic activity of amphotericin B and complexes increased with concentrations to reach a plateau value from 10 µg/ml for AC2 and AC4 and 20 µg/ml for amphotericin B. At 20 µg/ml, the hemolytic activity of AC2 and AC4 was one third of that of free amphotericin B.

CONCLUSIONS

The AmB-PVP complexes retain the broad spectrum of antifungal activity of the parent compound with a major increase of activity against *Candida spp.* They exhibit a two-orders of magnitude lower toxicity *in vitro* against red blood cells and macrophages. This major decrease of cytotoxicity may be related with a lower apparent cellular accumulation. These results suggest that AmB-PVP complexes may be a potent agent in the treatment of fungal infections.

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