Efficacy and Toxicity of Amphotericin B-Chitosan Nanoparticles in Mice with Induced Systemic Candidiasis

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Abstract

Amphotericin B (AmB) is one of the most widely used agents for treating systemic fungal infections. Although AmB is available as a colloidal dispersion with sodium deoxycholate (Fungizone®), its use is limited by renal toxicity. The goal of the present study was to evaluate the therapeutic activity and toxicity of a newly developed formulation, AmB chitosan nanoparticles (AmB-Chi nanoparticles), compared to Fungizone®. The *in vitro* antifungal activity was determined by measuring the growth inhibitory effect against *Candida albicans* and the *in vivo* antifungal activity was studied in mice with systemic candidiasis induced by *C. albicans*. The results demonstrate that both formulations showed no difference in their antifungal activity. The toxicity of both formulations was also investigated. In comparison with Fungizone® , AmB-Chi nanoparticles showed the same degree of toxicity but a slower progression of the acute toxicity and hence a reduced mortality rate. Amphotericin B chitosan particles showed less renal toxicity compared to the commercially available AmB formulation. The results suggest that AmB nanoparticles using chitosan as a carrier maintain their antifungal activity but are less toxic especially with respect to the renal toxicity compared to AmB-deoxycholate. Empty chitosan nanoparticles were also given to candidiasis compromised mice. They also showed a reduction of the mortality rate of the mice most probably due to binding of candida to the polymer resulting in a reduced activity and toxicity. The results of this study indicate that AmB-Chi nanoparticles have the potential to become a suitable drug delivery system for AmB, but more work is needed for reduction of the particle size of the carrier in order to increase its efficacy and decrease its side effects.

Keywords: Amphotericin B, Chitosan, Nanoparticle, Fungal infection, *Candida albicans*

Introduction

Candida albicans is one of the fungal pathogens that can cause systemic infection with a high mortality rate in human immunodeficiency virus (HIV)-infected and acquired immune deficiency syndrome (AIDS) patients. However, the epidemiology of systemic candidal infection nowadays shows the trend for serious infections of non-immonosuppressed patients (Eggimann et al., 2003; Richardson, 2005). In general, both amphotericin B (AmB) and the azoles play an important role in the treatment of candidiasis (Rex et al., 2000; Perfect, 2004). A conventional AmB formulation is available as a micellar solution with sodium deoxycholate as surfactant (Fungizone®). However, it is known that it has severe side effects and shows strong nephrotoxicity (Slain, 1999). Thus, there are several efforts to develop new AmB formulations with reduced nephrotoxicity. Three lipid-based liposome formulations of AmB (AmBisome®, Amphocil®, and Abelcet®) have been successfully tested and are now on the market (Gulati et al., 1998). Unfortunately, these products are costly and require higher doses than Fungizone®. Later, a lipid nano-sphere (LNS) AmB formulation was developed which can be used for a low-dose therapy with reduced side effects (Fukui, Koike, Saheki, Sonoke, & Seki, 2003; Fukui, Koike, Saheki, Sonoke, Tomii et al., 2003). Nanoparticles seem to be a good drug delivery system for AmB for intraveneous application with a controlled release of the active ingredient. Therefore, we were interested in the preparation of AmB-loaded nanoparticles using chitosan as a biocompatible carrier. Water-soluble chitosan is the deacetylated form of chitin. It is extracted primarily from shells of crustaceans such as shrimps and

crabs, and is widely used as a drug-delivery system (Ravi Kumar, 2000). The objective of this study was to investigate the therapeutic efficacy of amphotericin chitosan nanoparticles against systemic candidiasis in mice and to determine the toxicity of AmB-Chi nanoparticles in comparison with a conventional AmB formulation (Fungizone®).

Materials and Methods

Materials

Amphotericin B was purchased from Sigma, USA. Chitosan (molecular weight 30,000, 95% deacetylation) was purchased from Aquapremier, Bangkok, Thailand. Fungizone® and cyclophosphamide (Endoxan®) were obtained from Bristol-Myers Squibb Company and Baxter Oncology GmbH, respectively. Mice (ICR) were provided by National Animal Laboratory Centre, Bangkok, Thailand.

Preparation of the Amphotericin B nanoparticles

The AmB-Chi nanoparticles were prepared by phase separation technique at room temperature. The particles formed were induced by electrostatic interactions between the positively charged chitosan and negatively charged dextran sulfate. Briefly, twenty microliters of an AmB in dimethylsulfoxide (DMSO) solution (10 mg/ml) were added to 0.075 ml of dextran sulfate aqueous solution (1% w/v), mixed with 0.755 ml deionized water. The resulting solution was continuously stirred at 600 rpm. Then, 0.1 ml of aqueous chitosan solution (0.25 % w/v) was added dropwise and the resulting nanoparticles were stirred for 5 minutes. Fifty microliters of tripolyphosphate solution (5% w/v) were then added. The resulting stabilized AmB nanoparticles were dialyzed. Then mannitol was added to the purified, loaded nanoparticles to a final concentration of 5%w/v. The final suspension was then frozen and lyophilized at 0.4 mbar and -30^{o}C for 24 hours. The lyophilized nanoparticles were stored in a desiccator at 4^{o}C. The tested lyophilized AmB-chi nanoparticles possessed a mean particle size of 506 $\pm$ 9 nm with polydispersity index of 0.025.

In vitro antifungal activity

The *in vitro* efficacy of Fungizone® and AmB-Chi nanoparticle formulation was determined by measuring the growth inhibition of *C. albicans* ATCC 10230. Growth inhibition of *C. albicans* at 1x10^{3} cfu/ml was measured by the decrease in optical density at 600 nm in Saboraud dextrose broth using a microplate reader after incubation at 35^{o}C for 48 h.

In vivo antifungal activity in mice

Systemic candidiasis was induced in mice as previously described with minor modification (Fukui, Koike, Nakagawa et al., 2003). Briefly, mice (30-40 g) were intraperitoneally injected with immunosuppressive agent cyclophosphamide at a dose of 150 mg/kg. Systemic candidiasis was then induced by intravenous inoculation of *C. albicans* (5x10^{4} cells/mouse). Fungizone®, AmB-Chi nanoparticles and chitosan nanoparticles were intravenously injected at a dose of 1.0 mg/kg 4 h after fungal inoculation as single dose regimen. A multiple doses regimen was also conducted by administering these formulations via the tail vein for 4 consecutive days with the first dose started at 24 h after the fungal infection. The mice were observed for 14 days and *in vivo* systemic antifungal activity was evaluated by monitoring their survival and sickness status. Degrees of sickness from 0 to 5 were determined by visual observation; 0 = normal, 1 = slightly ill (slowly moving but quickly responding to stimulation), 2 = moderately ill (slowly moving and tardily responding to stimulation), 3 = very ill (no moving but still responding to stimulation), 4 = extremely ill (no moving and no responding to stimulation) and 5 = death. Animal experiments were approved by the Ethical Committee of Naresuan University.

Acute *in vivo* toxicity

Single dose injections of various doses of Fungizone® or AmB-Chi nanoparticle (1, 4, and 8 mg/kg) were given to groups of 5-8 male ICR mice, weighing 30-40 g, in the tail vein. Empty chitosan nanoparticles at equivalent amount of chitosan in AmB-Chi nanoparticles were also injected to the mice as negative control. Dead mice were counted daily for 14 days. In order to investigate the effects of these formulations on kidney function, blood urea nitrogen (BUN) and serum creatinine (Cr) were determined in the blood samples obtained from survivors at day 14.

Hemolysis test: toxicity in human red blood cell

Damage to red blood cell (RBC) was tested as previously described (Fukui, Koike, Saheki, Sonoke, Tomii et al., 2003). Human RBCs were washed three times with phosphate-buffered saline (PBS), and suspended in PBS at a hematocrit of 50%. Fungizone® or AmB-Chi nanoparticles in the range of 1-60 μg/ml were added to RBC at a final hematocrit of 1% in a glass vial. The mixtures were then incubated in a 37 ^{o}C for 20 min. Cell lysis was stopped by immersion of the vials in an ice bath, the unlysed cells were removed by centrifugation at 3000 rpm for 5 min, and the hemoglobin in the supernatant was determined spectrophotometrically at 541 nm.100% lysis was induced with distilled water.

Statistical analysis

The data of each group of treated mice were compared with the control groups using one-way analysis of variance (ANOVA). Significant level was defined as $p < 0.05$.

Results

In vitro antifungal activity

The *in vitro* antifungal activity against *C. albicans* of AmB-Chi nanoparticle was similar to that of Fungizone®. The minimum inhibitory concentrations (MIC) of Fungizone® and AmB-Chi nanoparticles were approximately 0.156 μg/ml and 0.150 μg/ml, respectively. Control chitosan nanoparticles showed no antifungal activity.

Treatment of induced systemic candidiasis mice

Systemic candidiasis was induced to cyclophosphamide-induced immunocompromised mice (CY-compromised mice). Thereafter, we recorded the curing process presented as numbers of sick animals and degree of sickness of each mouse to demonstrate the efficacy of the various treatments. Cyclophosphamide showed no effect on animal mortality (Table 1). Although the amount of *C. albicans* of $5x10^4$ cells/animal was reported to cause 100% mortality in CY-compromised mice (Shadkchan et al., 2001), this amount only caused 50% mortality in our experiments after three weeks observation (Table 1). Surprisingly, empty chitosan nanoparticles also reduced the mortality after fungal infection compared to the non-treated group. The results in figure 1 show that infected mice receiving Fungizone® or AmB-Chi nanoparticle returned to normal healthy conditions within one week after treatment whereas most animals receiving Chi nanoparticle were still sick throughout the three weeks observation period. The curing rate of AmB-Chi nanoparticle was similar to that of Fungizone®. Both therapy regimens, single (Figure 1A) and multiple administrations (Figure 1B), showed no marked difference in antifungal activity.

Table 1 Curing and mortality of fungal infected mice

Treatments	Numbers of sick/death		
	Day 7	Day 14	Day 21
Cyclophosphamide	0 / 0	0 / 0	0 / 0
C. albicans	8 / 0	7 / 1	4 / 4
C. albicans+Fungizone®	0 / 0	0 / 0	0 / 0
C. albicans+AmB-Chi nanoparticles	1 / 0	0 / 0	0 / 0
C. albicans+Chi nanoparticles	8 / 0	8 / 0	7 / 1

Note.
Each mice received treatment 4 h after fungal infection.

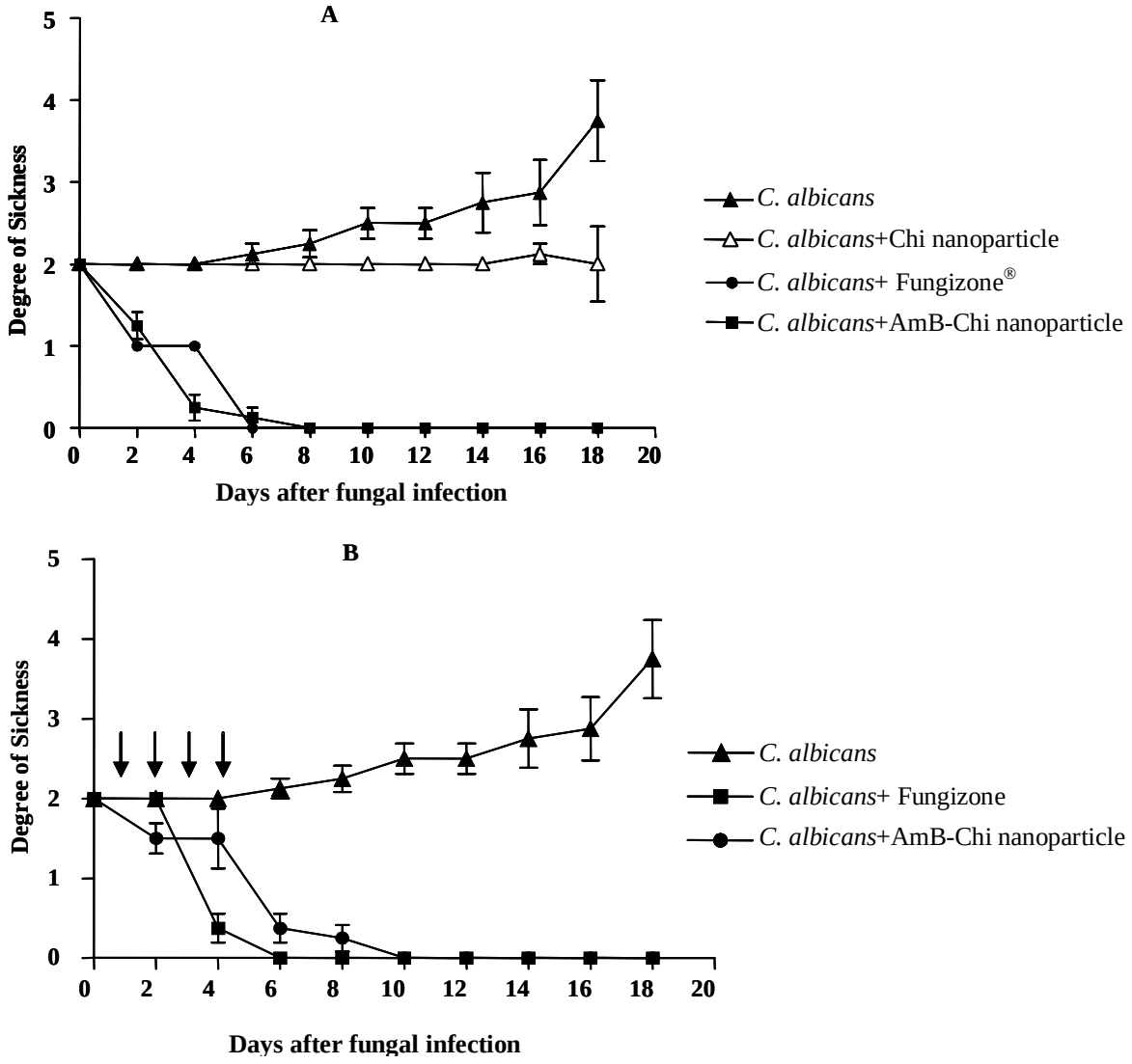

Figure 1 Antifungal activity in systemic candidiasis mice. Degree of sickness of each mouse was monitored after intravenous administration of single dose 4 h after fungal infection (**A**) or multiple doses (indicated by arrows) for 4 consecutive days (**B**) of Fungizone®, AmB-Chi nanoparticles or Chi nanoparticles. Degree of sickness scale 0 to 5 is normal to death and numbers in the between are different levels of sickness. Each point represents the mean±SEM of eight mice.

In vitro toxicity: effects on RBC membranes

Both Fungizone® and AmB-Chi nanoparticle were found to equally induce human erythrocyte lysis in a dose-dependent manner (Figure 2). 50% hemolysis was induced by Fungizone® and AmB-Chi nanoparticle at a dose of 5.66 μg/ml and 7.93 μg/ml, respectively. Chitosan nanoparticles without the active drug AmB were used as control and showed no effect on RBC membranes (data not shown).

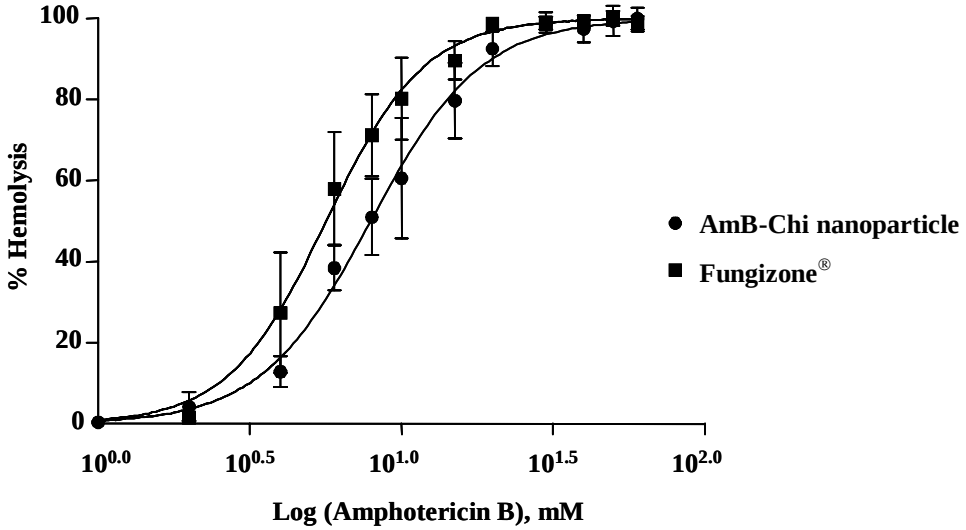

Figure 2 Toxicity to RBC of AmB formulations. Hemolysis tests of Fungizone® and AmB-Chi nanoparticles were conducted at 1-60 μg/ml concentrations of AmB. Log concentrations of AmB were plotted against % hemolysis. Each point represents the mean±SEM of three experiments.

In vivo toxicity: acute toxicity

In addition to the efficacy testing of AmB-Chi nanoparticle, we additionally tested the acute toxicity of this formulation compared to Fungizone®. Mice injected with AmB-Chi nanoparticle at doses of 1, 4 and 8 mg/kg exhibited the same acute toxicity as mice injected with the same doses of Fungizone® (Figure 3). The result showed that a low dose (1 mg/kg) had no lethal toxicity; however the higher dose of 4 mg/kg showed about 65-70% survival. No animal survived at the highest concentration of 8 mg/kg after 2 weeks period. There was only one animal that died after application of empty Chi nanoparticles at a dose of 4 mg/kg. However no animal died after an application of 8 mg/kg of empty chitosan nanoparticles.

Although the result showed the similarity in % survival of Fungizone® and AmB-Chi nanoparticles (Figure 3), the mortality produced by Fungizone was slightly faster than that of AmB-Chi nanoparticles, especially at high dose (Figure 4).

Renal toxicity of AmB formulations

Serum from mice surviving day 14^{th} of the acute toxicity test was collected and analyzed for blood urea nitrogen (BUN) and Cr levels (Table 2). Mice injected with all doses of Fungizone® had significantly increased serum creatinine concentrations. Although the BUN levels from these mice showed no statistically different from the normal group, there was a trend of increasing values at a concentration of 4 mg/kg. These findings indicate an increased renal toxicity with Fungizone® administration. On the contrary, serum BUN and Cr levels of mice receiving AmB-Chi or Chi nanoparticles showed no difference to the control group, except for the highest chitosan dose. Serum BUN was found to be significantly increased in mice receiving empty chitosan nanoparticles at a dose of approximately 7.2 mg/kg. It should be noted that the Fungizone® and AmB-Chi nanoparticle experiments were conducted in different sets of animals, therefore the control levels were slightly different.

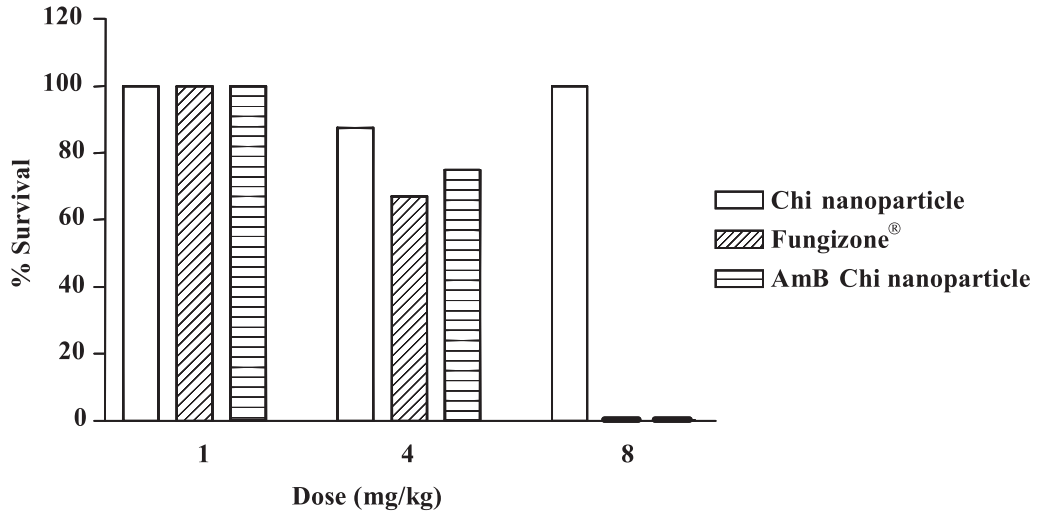

Figure 3 Acute toxicity of AmB formulations. Survival mice were counted at day 14th after Fungizone® or AmB-Chi nanoparticles administration. Chitosan nanoparticles were used as control by injecting chitosan at the equivalent amount of chitosan in each dose of AmB-Chi nanoparticles. Each treatment contains 6-8 mice.

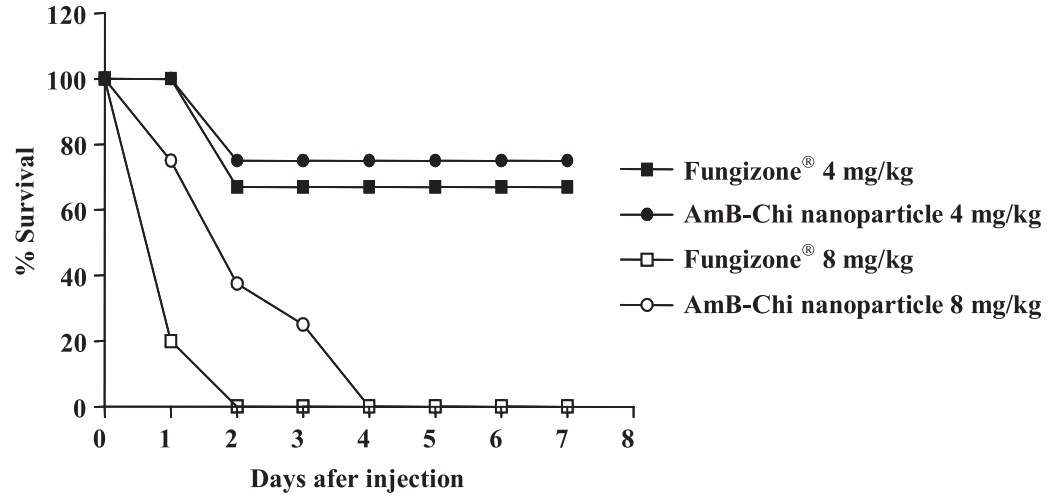

Figure 4 Mortality rate of AmB-Chi nanoparticles compared to that of Fungizone . Mice were injected with Fungizone® or AmB-Chi nanoparticles at 4 or 8 mg/kg. Each treatment contains 6-8 mice.

Table 2 Renal toxicity of AmB formulations

Dose (mg/kg)	Fungizone®		AmB-Chi nanoparticles		Chi nanoparticles	
	BUN (mg%)	Cr (mg%)	BUN (mg%)	Cr (mg%)	BUN (mg%)	Cr (mg%)
1	33.75±4.57	0.40±0.12*	28.67±0.02	0.28±0.08	35.83±4.07	0.25±0.05
4	39.50±2.08	0.48±0.05*	28.17±2.93	0.17±0.05	32.86±2.54	0.23±0.05
8	-	-	-	-	40.13±9.11*	0.26±0.07
control	32.00±5.72	0.10±0.08	30.44±6.67	0.21±0.08	30.44±6.67	0.21±0.08

Note

* $p < 0.05$ compared to the control group

There was no survival mice from 8 mg/kg Fungizone® and AmB-Chi nanoparticles.

Chitosan levels in Chi nanoparticles 1, 4 and 8 mg/kg were 0.9, 3.6 and 7.2 mg/kg, respectively.

Discussion

The main goal of this study was to evaluate the antifungal efficacy and toxicity of AmB-Chi nanoparticle in comparison with a conventional AmB formulation, Fungizone®. The data presented here show that the minimum inhibitory concentrations (MIC) against *C. albicans* of both formulations were the same. Therefore, it is likely that AmB incorporated into chitosan nanoparticles has the same activity to ergosterol in the membranes of the fungal cells.

To determine the *in vivo* antifungal activity, animals were compromised to systemic candidiasis by CY pretreatment. Since we observed an unexpected low mortality rate of only 50% after fungal inoculation, the curing rate was monitored in addition to the mortality rate to obtain better data analysis of the treatments. These experiments showed that survival and curing rates of AmB-Chi nanoparticles and conventional AmB formulation (Fungizone®) were not different in mice with induced systemic candidiasis. Either single or multiple drug administration did not affect the efficacy of treatments. Surprisingly, control animals treated with empty chitosan nanoparticles showed also a decreased mortality rate when they were injected 4 h after fungal inoculation. Chitosan was previously reported to promote cell adhesion to fungi and microorganisms in a dose-dependent manner (Wang et al., 2003). It is therefore possible that chitosan polymers are able to directly bind and deactivate *C. albicans* leading to a decreased availability of the harmful fungal cells in the blood circulation.

One of the purposes of formulating AmB-Chi nanoparticles was to reduce AmB toxicity. We therefore tested its acute toxicity compared to Fungizone®. Our data showed that Fungizone® and AmB-Chi nanoparticle had similar acute toxicity. The toxicity of AmB had been suggested to be the effect of the aggregation of AmB in the injected solution (Barwicz et al., 1992). We found that AmB in AmB-Chi nanoparticles as well as in Fungizone® formulations was predominantly present in the aggregated form (unpublished data). We also observed that the mortality rate induced by deoxycholate-AmB was faster than that induced by AmB chitosan nanoparticles. From these chitosan nanoparticles, AmB might be released more slowly and hence lower concentrations are less harmful to the kidney than the quick release of amphotericin from the deoxycholate micelles of Fungizone® leading to the slightly delayed toxicity. This hypothesis has to be further investigated and substantiated. This effect also could be the explanation for the reduced renal damage of AmB-Chi nanoparticles where BUN and Cr levels were found normal in all mice. Fungizone® especially showed nephrotoxicity when an increased of creatinine was observed. In this study we found that both AmB formulations resulted in dose-dependent human erythrocyte lysis, however the 50% hemolysis of AmB-Chi nanoparticles was slightly higher than that of Fungizone®. Chitosan was unexpectedly found to show some degree of renal toxicity due to the increased BUN, however it was observed only at high doses. It was previously demonstrated that low molecular weight chitosan was safe after intravenous administration (Richardson et al., 1999). However, further work has to be done to elucidate the effect of chitosan on the renal function. We believe that chitosan is a suitable drug delivery carrier due to its ability in controlling release of the incorporated drug, but we need to further improve the formulation by producing a smaller particle size. We speculate that the death of one mouse after the chitosan nanoparticle injection could be the result of local blockage of the blood circulation by large particles. The transient spasms occurring in some other animals might be the result of the blood circulation blockade as well.

Conclusions

AmB-Chi nanoparticles seem to be a promising delivery system for the treatment of systemic candidiasis. Although its acute toxicity and antifungal activity are similar to conventional AmB formulation, it showed a significant reduction of renal toxicity. We plan to further improve the formulation by producing smaller particle sizes in order to obtain optimal efficacy and minimal toxicity in comparison with other preparations.

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