LIPOSOMAL AMPHOTERICIN B FOR INVASIVE FUNGAL INFECTIONS

An experimental study in the leukopenic host

Els W.M. van Etten

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LIPOSOMAL AMPHOTERICIN B FOR INVASIVE FUNGAL INFECTIONS

An experimental study in the leukopenic host

LIPOSOMAAL AMPHOTERICINE B BIJ INVASIEVE MYCOSEN

Een experimentele studie in de leukopenische gastheer

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Cover photo: ©1995 Pfizer, Inc.; kindly provided by Pfizer B.V., The Netherlands. Electron micrograph showing a macrophage engulfing *Candida albicans*.

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Aan mijn ouders

Voor Ronald

Abbreviations

ABCD	amphotericin B colloidal dispersion
ABLC	amphotericin B lipid complex
AIDS	acquired immunodeficiency syndrome
ALAT	alanine aminotransferase
AMB	amphotericin B
AMB-DOC	amphotericin B-desoxycholate
ASAT	aspartate aminotransferase
BUN	blood urea nitrogen
CFU	colony forming units
Chol	cholesterol
CREAT	serum creatinine
DF	deferoxamine mesylate
DiI	1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanineperchlorate
DMEM	Dulbecco modified Eagle medium
DMPC	dimyristoylphosphatidylcholine
DMPG	dimyristoylphosphatidylglycerol
DMSO	dimethylsulfoxide
DOC	desoxycholate
DSPG	distearoylphosphatidylglycerol
EPC	egg phosphatidylcholine
FBS	fetal bovine serum
FITC	fluoresceinisothiocyanate
FLU	fluconazole
h	hours
HPLC	high-performance-liquid-chromatography
HSPC	hydrogenated soybean phosphatidylcholine
ip	intraperitoneally
iv	intravenously
LIP	liposomes
MIC	minimal inhibitory concentration
min	minutes
MFC	minimal fungicidal concentration
MLV	multilamellar vesicle
MPS	mononuclear phagocyte system
MTD	maximum tolerated dosage
PEG	containing PEG-DSPE
PEG-DSPE	polyethylene glycol(1900) derivatized distearoylphosphatidylethanolamine
PBS	phosphate-buffered saline
PHS	pooled human serum
SD	standard deviation
SDA	sabouraud dextrose agar
^{51}Cr	^{51}Chromium
^{67}Ga	^{67}Gallium
^{111}In	^{111}Indium

Contents

Chapter 1

General Introduction

THE EMERGING FUNGAL THREAT

Advances in medical treatment have improved the prognosis for patients with cancer. While significant progression has been made in eradicating certain malignant diseases, a growing concern for patients who receive cytotoxic chemotherapy is the development of fungal infections [14,135,156,183,186,202]. **Candidiasis** is the most common nosocomial mycosis. There are several predisposing factors for hematogenously disseminated candidal infections in patients with cancer. These factors include granulocytopenia, the use of extended-spectrum antibiotics, and the breakdown of anatomic barriers against infection caused by catheterization or the use of cytotoxic chemotherapy. Invasive **aspergillosis** is now the second most common mycosis encountered in patients with cancer, particularly in those with hematological malignancies. Patients are at high risk when their absolute neutrophil count is <500/mL and the duration of neutropenia exceeds 1 week. The diagnosis during life of both invasive candidiasis as well as invasive aspergillosis still remains a significant problem [204].

Invasive fungal infections are also a serious threat in non-granulocytopenic patients. Cryptococcosis, that is caused by *Cryptococcus neoformans*, is a life threatening infection in patients with acquired immunodeficiency syndrome (AIDS). Histoplasmosis, blastomycosis, or coccidioidomycosis are serious infections in AIDS patients who have resided in, or travelled through endemic regions for *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis*, respectively.

Candidiasis.

Candidiasis is a spectrum of infections that can be classified as cutaneous, mucosal, or deeply invasive [204]. Deeply invasive infection may be further classified as fungemia, tissue-proven disseminated candidiasis and single organ candidiasis. Disseminated candidiasis may be further classified as acute and chronic disseminated candidiasis, which constitute two ends of a clinical and pathologic spectrum. Among the conditions of mucosal candidiasis commonly encountered in granulocytopenic patients are oropharyngeal, esophageal, and urinary candidiasis. *Candida albicans* among the *Candida* species is the predominant cause of candidiasis. The gastrointestinal tract is the principal source of infection.

Aspergillosis.

Invasive pulmonary aspergillosis in granulocytopenic patients carries a mortality as high as 95% [204]. In its most serious form there is widespread growth of the fungus (mould) in the lungs and dissemination to other organs often follows. This condition occurs in immunocompromised individuals and is fatal if left untreated. In granulocytopenic patients this condition is often fatal, even if diagnosed during life and treated. *Aspergillus fumigatus* is widespread in the environment (soil, dust,

decomposing organic matter). Most infections follow inhalation by susceptible hosts of aspergillus spores that have been released into the air e.g. at sites of construction work or building sites [204].

TREATMENT OF INVASIVE FUNGAL INFECTIONS WITH AMPHOTERICIN B

The treatment of choice for most invasive fungal infections still is parenteral administration of the **fungicidal** agent **amphotericin B** (AMB) [204]. AMB is a macrolide polyene antibiotic derived from *Streptomyces nodosus*.

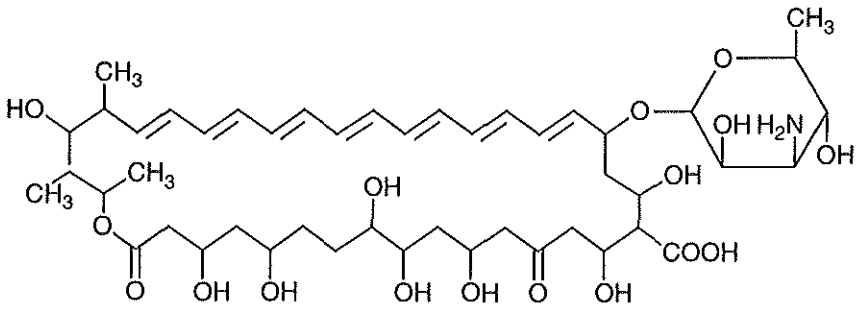

Figure 1. *Chemical structure of amphotericin B.*

AMB binds to ergosterol, the principal sterol in the membrane of susceptible fungal cells, causing impairment of membrane barrier function, loss of cell constituents, metabolic disruption and cell death. In addition to its membrane permeabilizing effects, the drug can cause oxidative damage to fungal cells possibly by formation of reactive forms of oxygen (free radicals) after auto-oxidation of AMB bound to membrane components [29]. AMB has a broad antifungal spectrum including *Candida albicans, Aspergillus fumigatus, Cryptococcus neoformans.*

AMB is administered as AMB-desoxycholate (AMB-DOC or Fungizone®). The clinical use of AMB-DOC is limited by toxic side effects [62,81,128]. This toxicity includes acute side effects during administration (chills, fever, myalgia), thrombophlebitis, nephrotoxicity, hypokalaemia, hypomagnesaemia, anaemia and more rarely thrombocytopenia. Although AMB-DOC is known to be highly fungicidal [62], clinical efficacy is often insufficient because toxicity limits the dose (maximum tolerated dose in patients is 0.7-1.5 mg/kg). High failure rates in

treatment of invasive fungal infections with AMB-DOC occur, especially in persistently granulocytopenic patients. Treatment failure attributable to the development of AMB resistance is rare, although resistant strains of *Candida* spp., with alterations in the cell membrane including reduced amounts of ergosterol, have been isolated during treatment [212].

SEARCH FOR NEW ANTIFUNGAL AGENTS

Ideally, new antifungal agents should possess the following characteristics: A rapid fungicidal action, a lack of clinically relevant toxicity, and a broad spectrum of activity including the major species of fungi pathogenic to man. Furthermore, it is important that both oral and parenteral formulations are available and that its pharmacokinetic profile allows it to reach extra- and intracellular sites of infection.

To improve antifungal chemotherapy two major lines of development have been initiated. On the one hand **new antifungal agents** [128] have been and are being produced, on the other hand **lipid formulations of AMB** [63,126] have been developed. The role of these agents in the treatment of invasive fungal infections in the granulocytopenic host is not yet fully defined nor well understood.

New (investigational) antifungal agents

Many new agents from different classes, with different mechanisms of action have been synthesized and studied.

Triazoles. The triazoles are **fungistatic** agents that inhibit fungal cytochrome P450-dependent biosynthesis of ergosterol, the principal sterol in the membrane of fungal cells. The consequent depletion of ergosterol and accumulation of methylated sterols leads to disturbances in a number of membrane-associated cell functions.

The triazole fluconazole has a broad antifungal spectrum including *Candida albicans* and *Cryptococcus neoformans*. It appears to be ineffective against *Aspergillus fumigatus*. Both oral and parenteral formulations of fluconazole are available. Fluconazole is well tolerated [71]. Acquired resistance is rare, but isogenic resistant strains of *Candida albicans* have been recovered from AIDS patients receiving long-term treatment with fluconazole for oropharyngeal or oesophageal candidiasis [28].

The antifungal efficacy of the triazole agents fluconazole, itraconazole, saperconazole, genaconazole (SCH 39304), ICI195739 or D0870, and BAY-R-3783 have been studied in experimental models of invasive candidiasis and aspergillosis (Table 1). In a number of studies immunosuppressed animals were used. From these animal studies it was concluded that these triazoles are more active when used for preventive and early treament than for treatment of fully established fungal disease.

Unfortunately, the carcinogenic potential of genaconazole has precluded its further development.

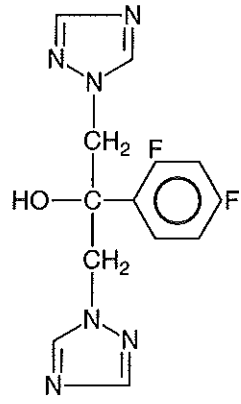

Figure 2. *Chemical structure of fluconazole.*

Echinocandins, pneumocandins, and papulacandins. These agents are **fungicidal** agents that inhibit 1,3-ß-glucan synthetase and consequently disturb fungal cell wall biosynthesis. Studies with cilofungin (LY-121019 or L-646,991) and the derivatives L-671,329, L-687,901, and L-687,781 in experimental models of invasive candidiasis (Table 1) are encouraging. Unfortunately, cilofungin (LY-121019) has been withdrawn from clinical trials because of related toxicities. Polyethylene glycol, which is used as carrier for cilofungin, has been shown to cause metabolic acidosis.

Nikkomycins. Nikkomycins are another group of **fungicidal** cell wall active agents; they are very specific inhibitors of chitin synthetase and consequently disturb fungal cell wall biosynthesis. Preliminary data in a mouse model of candidiasis (Table 1) showed only modest efficacy that may be due to their very short elimination half-lives (minutes) in plasma.

Lipid formulations of AMB

Amphiphilic molecules including detergents, such as desoxycholate, and phopholipids become arranged in various three-dimensional complexes when added to aqueous solutions.

Table 1. *Animal studies on antifungal efficacy of triazoles, and other investigational agents in models of invasive candidiasis or aspergillosis.*

antifungal agent	fungal infection	references
Triazoles:		
itraconazole	candidiasis	112[b],152,196[a],198[a],203[a]
	aspergillosis	111[b],137[a],151[a],196[a]
fluconazole	candidiasis	15[a],19[a],33[a],57,58,59, 107[a],152,164,195[a],198[a], 199[a],200[a],201[a],213,214
	aspergillosis	16[a]
saperconazole	aspergillosis	16[a], 150[a], 197[a]
genaconazole (SCH 39304)	candidiasis	33[a],153,188,201[a],203[a]
	aspergillosis	16[a],51[a],149[a],188
ICI195739 / D0870	candidiasis	217
	aspergillosis	217
BAY-R-3783	candidiasis	78
	aspergillosis	78
Echino- Pneumo- Papulacandins:		
cilofungin (LY-121019)	candidiasis	19[a],141,144,180
cilofungin (L-646,991)		21
L-671,329		21
L-687,901		21
L-687,781		21
Nikkomycins	candidiasis	22

[a]immunosuppressed animals
[b]antifungal agent entrapped in liposomes

Detergents with a hydrophilic polar headgroup and a single hydrophobic hydrocarbon tail are arranged into spherical structures, called micelles, in which the hydrophobic tails are arranged in the center of the structure. Phospholipids, containing a polar headgroup and two hydrophobic hydrocarbon tails, are

assembled into lipid-bilayers. The lipid bilayer can either enclose an internal aqueous compartment (liposomes) or can be arranged in a ribbon-like (lipid-ribbon) structure. Lipids other than phospholipids, such as cholesteryl sulphate, can be arranged in a disk-like structure (lipid-disks).

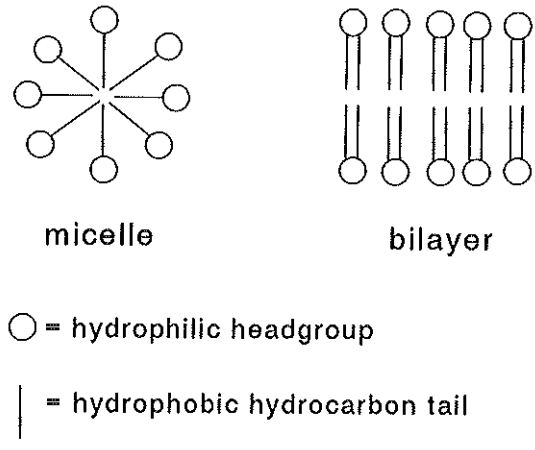

Figure 3. *Association patterns of amphiphilic molecules in aqueous solutions.*

Liposomes are vesicles consisting of one or more phospholipid bilayers surrounding aqueous compartments. Hydrophobic agents can be embedded in the lipid bilayer, whereas hydrophilic agents are entrapped in the internal aqueous spaces of the liposomes.

The charge, rigidity, size, and surface characteristics of liposomes can be controlled to a high degree by the choice of lipids and the method of preparation. The fate of liposomes in the body is primarily dependent on these parameters. Therefore, liposomes are considered to be versatile delivery systems [20,73]. The clinical applicability of liposomes, as opposed to other parenteral drug delivery systems, is based on the fact that the lipids mostly used for liposome preparation are those commonly found in cell membranes; in addition, liposomes are biodegradable, relatively nontoxic, and nonantigenic or weakly antigenic [184]. After intravenous administration liposomes circulate in blood and can either disintegrate in blood or can be taken up in intact form largely by cells of the mononuclear phagocyte system (MPS), such as macrophages in the liver (Kupffer cells) and spleen.

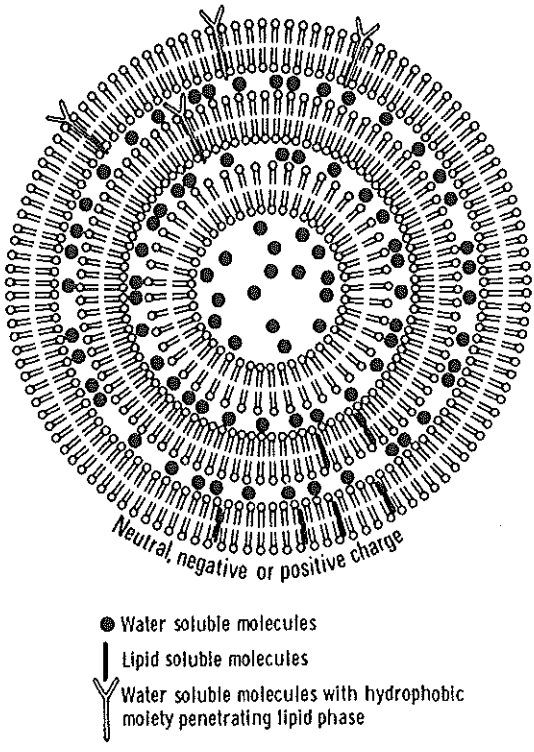

Figure 4. *Schematic representation of a multilamellar liposome, according to Gregoriadis [74].*

Recent progress in liposome design has yielded a new generation of liposomes characterized by long blood circulation half-lives and reduced hepatosplenic uptake. The development of these **MPS avoiding liposomes** opens new therapeutic ways to achieve improved delivery of antimicrobial agents to infected tissues including infections outside the MPS [17,18,26]. It was shown already many years ago that factors contributing to prolonged liposome circulation times include small liposome size and increased rigidity of the bilayer. Such liposomes show prolonged circulation but only at relatively high lipid doses. Further advances in liposome engineering have led to the formulation of sterically stabilized liposomes, with the polymer polyethyleneglycol (PEG) conjugated to phosphatidylethanolamine (PE) in the bilayer.

It was shown in many reports that PEG-PE incorporation into liposomes can effectively prolong their blood circulation time without the constraint of high lipid dose, small particle size, or rigid nature of the bilayer [8,9,10,11,18,61,104,105, 146,215,216].

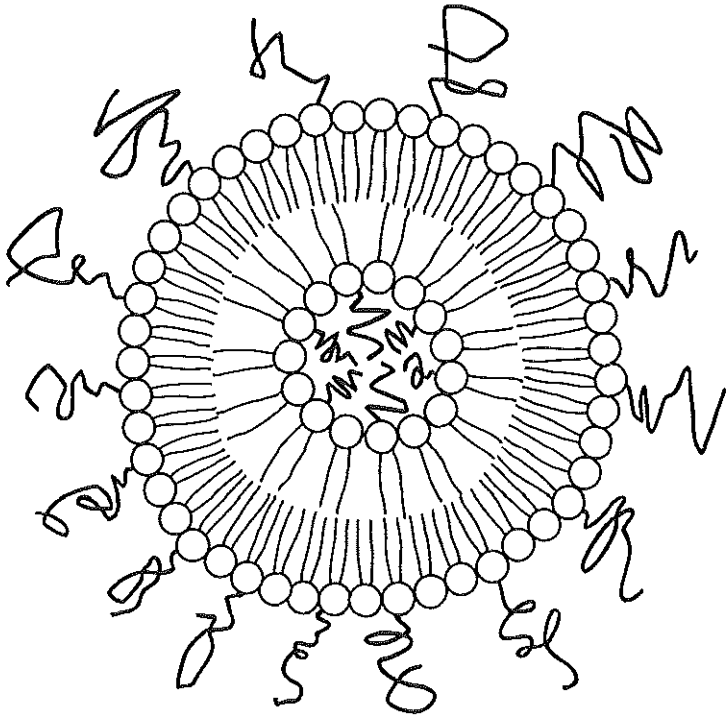

Figure 5. *Schematic representation of a PEG-PE containing MPS avoiding liposome, according to Lasic [110].*

Four **lipid-based intravenous formulations of AMB** are currently available for clinical use (Table 2). One is AMB-DOC prepared in a parenteral fat emulsion (Intralipid®), while the other three are industrially prepared AMB-lipid formulations. It is evident that these four AMB-lipid formulations have quite different structures

Table 2. *Intravenous formulations of amphotericin B.*

formulation	manufacturer	carrier	colloidal type
Fungizone®	Bristol Myers-Squibb	sodium-DOC	micelle
Fungizone® in 20% Intralipid®	Kabi Pharmacia	triglycerides/EPC/glycerol	undefined
AmBisome®	Vestar, Inc.	HSPC/DSPG/Chol	unilamellar liposome
Amphocil® (ABCD)	Liposome Technology, Inc.	cholesteryl sulphate	lipid disk
AMB Lipid Complex (ABLC)	The Liposome Company	DMPC/DMPG	lipid ribbon

DOC Desoxycholate
EPC Egg Phosphatidylcholine
HSPC Hydrogenated Soy Phosphatidylcholine
DSPG Distearoylphosphatidylglycerol
Chol Cholesterol
DMPC Dimyristoylphophatidylcholine
DMPG Dimyristoylphophatidylglycerol
ABCD AMB Colloidal Dispersion

and pharmaceutical characteristics. AmBisome® is a small unilamellar vesical (SUV) of about 80 nm in diameter with a lipid composition of hydrogenated soy phosphatidylcholine (HSPC) / distearoylphosphatidylglycerol (DSPG) / cholesterol (Chol) (10:5:4) containing 10 mol% AMB [158]. Amphocil® (Amphotericin B Colloidal Dispersion or ABCD) is a stable complex of AMB and cholesteryl sulphate in a 1:1 molar ratio. The particles have a disk-like structure with a diameter of 122 nm and a thickness of only 4 nm [75]. ABLC (AMB Lipid Complex) was derived from a liposomal formulation of AMB, developed by Lopez-Berestein *et al.* [118]. In the latter formulation AMB was entrapped into multilamellar vesicles (MLVs) consisting of dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) in a 7:3 molar ratio, containing 5 mol% AMB. The diameter of the MLVs ranged from 1-8 μm. ABLC is developed from a fraction of these original MLVs. The lipid composition of ABLC is similar to that of the MLVs, but this formulation consists of large ribbons (1-11 μm), not vesicles, containing 35 mol% AMB [90,91].

Toxicity was studied for a large number of different AMB-lipid formulations, including the formulations mentioned above. *In vitro* cellular toxicity was determined for a variety of target cells (Table 3). Various parameters for toxicity were used, indicative either for specific cell functions, for membrane permeability, or for viability of the cells. It is clear that when AMB is entrapped into liposomes or bound to other lipid carriers, its toxicity is greatly reduced. The *in vitro* antifungal activity of the various AMB-lipid formulations (Table 4) was shown to be similar to that of AMB-DOC after long term exposure of a great number of fungal pathogens. However, when determined after short term exposure the activity of the AMB-lipid formulations was less than than that of AMB-DOC. The reduction of toxicity and preserved antifungal activity of the AMB-lipid formulations was thought to result from differences in affinity of AMB to cholesterol in the human cell membrane (lowest affinity) as opposed to the lipids of the liposomal carrier (intermediate affinity) and to the ergosterol in the fungus membrane (highest affinity) [96,97].

The first animal studies were performed with AMB entrapped in MLVs and published in 1983 by Lopez-Berestein *et al.* [118]. From this moment, an increasing number of *in vivo* studies with a multitude of AMB-lipid formulations have been performed (Table 5 and Table 6). Toxicity was monitored in terms of impaired kidney and liver functioning or acute or subacute lethality in various animal species (Table 5), and was greatly reduced after administration of AMB entrapped in the various lipid formulations. With respect to the *in vivo* antifungal efficacy (Table 6), various animal models of fungal infections have been used. Studies have been performed in immunocompetent as well as in immunosuppressed animals. From these studies it can be concluded that, depending on the model of fungal infection, the immune status of the host, and the parameter for efficacy used, the antifungal activity of these AMB-lipid formulations is either somewhat less or equal to that of

Table 3. *Experimental studies on in vitro cellular toxicity of AMB-lipid formulations.*

target cell	AMB-lipid formulation	references
erythrocyte	AmBisome®	159
	ABLC	90,155
	AMB-MLV	96,97,130
	AMB-MLV-IgG	83
	AMB-IL	103
	other	1,30,32,98,99,157[a],182
renal cell	AmBisome®	3
	AMB-MLV	106,206,207
	AMB-IL	95
	other	93,94
fibroblast	other	30,32,187
macrophage	AmBisome®	3
	AMB-MLV	131
	other	187
blood neutrophil	AmBisome®	147
	ABLC	185
spleen lymphocyte	AmBisome®	173
	AMB-MLV	131

[a]AMB administered after incubation of target cells with empty liposomes

AmBisome®: HSPC/DSPG/Cholesterol 10:5:4, 10 mol% AMB
Amphocil®, Amphotericin B Colloidal Dispersion (ABCD): AMB/Cholesteryl sulphate 1:1
ABLC, Amphotericin B Lipid Complex: DMPC/DMPG 7:3, 35 mol% AMB
AMB-MLV: DMPC/DMPG 7:3, 5 mol% AMB
AMB-MLV-IgG: immunoliposome DMPC/DMPG 7:3, 5 mol% AMB, *α-C.albicans* IgG
AMB-IL: AMB-desoxycholate (Fungizone®) in Intralipid®
other: other AMB-lipid formulations

AMB-DOC at equivalent dosages. However, using liposomal or lipid-complexed AMB much higher dosages are tolerated, and these high doses result in improved antifungal efficacy, even in severe infection in immunocompromised animals.

The promising results of the animal studies along with technical advances in liposomal preparation techniques have led to their clinical application. Clinical studies with various AMB-lipid formulations have been in progress for several years [52,89]. The first studies were published in 1985 and 1889 by Lopez-Berestein *et al* [124, 127]. They treated patients with microbiologically or histologically proven invasive fungal infections and a variety of underlying diseases. The majority of the patients had received prior AMB in the form of AMB-DOC, and either had evidence

Table 4. *Experimental studies on in vitro antifungal activity of AMB-lipid formulations.*

fungal pathogen	AMB-lipid formulation	references
Candida albicans	AmBisome®	13,147[a]
	Amphocil®	77
	ABLC	155
	AMB-MLV	82,96,97, 130,160,206
	AMB-MLV-IgG	83
	other	30,32,80,94, 98,99,182
non-*albicans Candida spp.*	AmBisome®	13
	Amphocil®	77
	AMB-MLV	82,160
	other	187
Cryptococcus neoformans	AmBisome®	13
	Amphocil®	77
	AMB-MLV	82,160
	other	30,32,98
Aspergillus fumigatus	AmBisome®	13
	Amphocil®	77
non-*fumigatus Aspergillus spp.*	AmBisome®	13
	Amphocil®	77
Histoplasma capsulatum	Amphocil®	77
Blastomyces dermatitidis	Amphocil®	77
Coccidioides immitis	Amphocil®	77
Paracoccidioides brasiliensis	Amphocil®	77
Rhizopus spp.	Amphocil®	77
Sporothrix schenkii	Amphocil®	77
Fusarium spp.	AmBisome®	13
Saccharomyces cerevisiae	ABLC	155
	AMB-MLV	160
	other	187

[a]antifungal activity against intracellular *C.albicans* in blood neutrophils
Description of the various AMB-lipid formulations: see Table 3

Table 5. *Animal studies on in vivo toxicity and/or pharmacokinetics of AMB-lipid formulations.*

AMB-lipid formulation	animal species	references
AmBisome®	mouse	70,158,181
	rat	158
	rabbit	60,113
Amphocil®	rat	55,76
	rabbit	12
	dog	56
ABLC	mouse	90,143
	rat	143
AMB-MLV	mouse	120
	diabetic rat	205
AMB-IL	mouse	95
	rat	103
other	mouse	65,92,117,157[a],182, 187,189
	rat	208

[a]AMB administered shortly after administration of empty liposomes
Description of the various AMB-lipid formulations: see Table 3

of disease progression, or showed intolerance for AMB-DOC. The efficacy of liposomal AMB was good in patients with hepatosplenic candidiasis, a very protracted and difficult to treat multiorgan infection, and encouraging in patients with other fungal infections. The therapeutic efficacy of liposomal AMB in these and other 'compassionate treatment' studies is very impressive, as one should take into account the obvious selection bias towards more serious cases, as many patients had previously failed on AMB-DOC treatment. These studies were followed by many other publications (Table 7). Most published clinical data on efficacy are available for AmBisome®, showing encouraging results in a variety of fungal infections in immunocompromised patients.

It is evident that AmBisome®, Amphocil®, and ABLC have quite different structures and pharmaceutical characteristics. Their pharmacokinetics depend to a large extent on the composition and particle size of the liposomes or the lipid complexes (Table 5). Relatively large structures such as ABLC are rapidly taken up by the MPS. Due to uptake by the MPS, relatively high concentrations are obtained in the liver and the spleen without signs of major toxicity in these organs. The serum concentrationsare also highly variable between the formulations. The area under the serum concentration-time curve after a standardized dose is highest for

Table 6. *Animal studies on in vivo antifungal efficacy of AMB-lipid formulations.*

fungal pathogen	AMB-lipid formulation	animal species	references
Candida albicans	AmBisome®	mouse	2,70
		mouse[a]	145
	ABLC	mouse	40,90
		mouse[a]	40
		rabbit	154
	AMB-MLV	mouse	118,119,123,132
		mouse[a]	121,122
	AMB-MLV-IgG	mouse	84[b],85[b]
		mouse[a]	23[b],24[b]
	AMB-IL	mouse	103
	other	mouse	6,31,116,138,139, 194
non-*albicans Candida spp.*	AmBisome®	mouse[a]	101
	ABLC	mouse	40
Cryptococcus neoformans	AmBisome®	mouse	2
	Amphocil®	mouse	86
	ABLC	mouse	40
		rabbit[a]	154
	AMB-IL	mouse	95
	other	mouse	31,53[b],64,72,157[c]
Aspergillus fumigatus	AmBisome®	rabbit[a]	60
		mouse[a]	7
	Amphocil®	rabbit[a]	12,148
	ABLC	mouse[a]	40
	other	mouse	5,65
Histoplasma capsulatum	ABLC	mouse	40
		mouse[a]	40
	other	mouse[a]	189
Blastomyces dermatitidis	AmBisome®	mouse	45
	ABLC	mouse	42
Coccidioides immitis	Amphocil®	mouse	41
	ABLC	mouse	43
Paracoccidioides brasiliensis	AmBisome®	mouse	44

[a]immunosuppressed animals
[b]immunoliposome
[c]AMB administered shortly after administration of empty liposomes

Description of the various AMB-lipid formulations: see Table 3

Table 7. *Studies with AMB-lipid formulations in man.*

AMB-lipid formulation	extent of study	references
AmBisome®	case report	4,46,47,48,87,88,102,108,109,142, 163,174,177,178,218
	clinical study	25,38,39,49,54,79,134,136,165, 166,167,168,169,190,191,192,193
Amphocil®	healthy volunteers	172
ABLC	healthy volunteers	100
AMB-MLV	case report	114,161
	clinical study	124,125,127,162,179,210
AMB-IL	case report	129,209
	clinical study	34,35,36,37,140
other	case report	27,67,69,176
	clinical study	50,68,133,175

Description of the various AMB-lipid formulations: see Table 3

AmBisome®, and lowest for ABLC. The clinical pharmacokinetics and safety of AmBisome®, ABLC and Amphocil® have been reviewed [89]. It is not clear whether the differences in structure and pharmacokinetics may be of importance to tolerance and efficacy. Clinical trials comparing such data for the various liposomal and lipid-complexed AMB preparations are needed to assess the clinical value of these preparations and to determine the full spectrum of indications. Studies comparing two AMB-lipid formulations are still lacking.

AIM OF THE PRESENT STUDY

The **primary aim** of the work presented in this thesis was **to gain further insight into the therapeutic value of existing and newly developed liposomal formulations of amphotericin B in the treatment of invasive fungal infections.**

To this end, different animal models of invasive fungal infections were developed. Clinically relevant issues including persistent leukopenia and dissemination of infection, are addressed. One model was an invasive *Candida albicans* infection with dissemination to the kidney, liver, spleen, and lung in mice which were rendered persistently leukopenic. In addition, a model of invasive candidiasis restricted to the kidney in immunocompetent mice was used. Finally, a model of *Aspergillus fumigatus* one-sided pulmonary infection in persistently leukopenic rats was developed, in which the fungus disseminated to the right lung and liver during the course of the infection.

In this thesis the following issues were addressed:

- **fungistatic agent (fluconazole) versus fungicidal agent (AMB-DOC)**
  - *in vitro* antifungal activity against extracellular and intracellular *C.albicans* (Chapter 2)
  - *in vivo* antifungal efficacy in invasive candidiasis in immunocompetent mice versus persistently leukopenic mice (Chapter 3)
- **industrially prepared AMB-liposome formulation (AmBisome®) versus AMB-DOC**
  - *in vitro* antifungal activity, toxicity, *in vivo* antifungal efficacy in invasive candidiasis in immunocompetent mice versus persistently leukopenic mice (Chapter 3)
  - biodistribution in immunocompetent mice versus persistently leukopenic mice infected with *C.albicans* (Chapter 4)
  - interactions with extracellular and intracellular *C.albicans* (Chapter 5)
- **in-house prepared pegylated long-circulating AMB-liposome versus industrially prepared AmBisome®**
  - preparation, toxicity and biodistribution of pegylated long-circulating AMB-liposomes (Chapter 6)
  - *in vitro* antifungal activity, toxicity, *in vivo* antifungal efficacy in invasive candidiasis in persistently leukopenic mice (Chapter 7 and Chapter 8)
  - *in vivo* antifungal efficacy in pulmonary aspergillosis in persistently leukopenic rats (Chapter 9)

REFERENCES

1. Abu-Salah KM. Alteration of the permeability of the human erythrocyte membrane to cations by liposome encorporated amphotericin B. Eur J Clin Chem Clin Biochem 1992;30:737-44.
2. Adler-Moore JP, Chiang SM, Satorius A, Guerra D, McAndrews B, McManus EJ, Proffitt RT. Treatment of murine candidosis and cryptococcosis with a unilamellar liposomal amphotericin B formulation (AmBisome). J Antimicrob Chemother 1991; 28(Suppl.B):63-71.
3. Adler-Moore J. *In vivo* and *in vitro* evidence for reduced toxicity and mode of action of AmBisome®. Bone Marrow Transplant 1993;12(Suppl.4):S146.
4. Aguado JM, Hidalgo M, Moya I, Alcazar JM, Jimenez MJ, Noriega AR. Ventricular arrhythmias with conventional and liposomal amphotericin. Lancet 1993;342:1239.
5. Ahmad I, Sarkar AK, Bachhawat BK. Design of liposomes to improve delivery of amphotericin B in the treatment of aspergillosis. Mol Cell Biochem 1989;91:85-90.
6. Ahrens J, Graybill JR, Craven PC, Taylor RL. Treatment of experimental murine candidiasis with liposome-associated amphotericin B. Sabouraudia 1984;22:163-6.
7. Allen SD, Sorensen KN, Nejdl MJ, Proffitt RT. Efficacy of aerolsolized liposomal amphotericin B (AmBisome®) as a prophylactic treatment in an immune compromised murine model of pulmonary aspergillosis. J Control Release 1994;28: 348-9.
8. Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivaties of poly(ethylene glycol) show prolonged circulation half lives *in vivo*. Biochim Biophys Acta 1991;1066:29-36.
9. Allen TM, Hansen C. Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochim Biophys Acta 1991;1068:133-41.
10. Allen TM. Stealth liposomes: five years on. J Liposome Res 1992;2:289-305.
11. Allen TM. The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. Adv Drug Deliv Rev 1994;13: 285-309.
12. Allende MC, Lee JW, Francis P, Garrett K, Dollenberg H, Berenguer J, Lyman CA, Pizzo PA, Walsh TJ. Dose-dependent antifungal activity and nephrotoxicity of amphotericin B colloidal dispersion in experimental pulmonary aspergillosis. Antimicrob Agents Chemother 1994;38:518-22.
13. Anaissie E, Paetznick V, Proffitt R, Adler-Moore J, Bodey GP. Comparison of the *in vitro* antifungal activity of free and liposome-encapsulated amphotericin B. Eur J Clin Microbiol Infect Dis 1991;10:665-8.
14. Anaissie E. Opportunistic mycoses in the immunocompromised host: experience at a cancer center and review. Clin Infect Dis 1992;14(Suppl. 1):S43-S53.
15. Anaissie E, Karyotakis NC, Hachem R, Dignani MC, Rex JH, Paetznick V. Correlation between *in vitro* and *in vivo* activity of antifungal agents against *Candida* species. J Infect Dis 1994;170:384-9.
16. Andriole VT, Miniter P, George D, Kordick D, Patterson TF. Animal models: Usefuless for studies of fungal pathogenesis and drug efficacy in aspergillosis. Clin Infect Dis 1992;14(Suppl. 1):S134-8.
17. Bakker-Woudenberg IAJM, Lokerse AF, Ten Kate MT, Melissen PMB, Van Vianen W, Van Etten EWM. Liposomes as carriers of antimicrobial agents or immunomodulatory agents in the treatment of infections. Eur J Clin Microbiol Infect Dis 1993;12(Suppl. 1):S61-7.

18. Bakker-Woudenberg IAJM, Lokerse AF, Ten Kate MT, Mouton JW, Woodle MC, Storm G. Liposomes with prolonged blood circulation and selective localization in *Klebsiella pneumoniae*-infected lung tissue. J Infect Dis 1993;168:164-71.
19. Bannatyne RM, Cheng PC, Fong IW. Comparison of the efficacy of cilofungin, fluconazole, and amphotericin B in the treatment of systemic *Candida albicans* infection in the neutropenic mouse. Infection 1992;20:168-70.
20. Barenholz Y, Crommelin DJA. Liposomes as pharmaceutical dosage forms. In: Swarbrick J (Ed.) Encyclopedia of pharmaceutical technology, Volume 9. New York: Marcel Dekker, 1994:1-39.
21. Bartizal K, Abruzzo G, Trainor C, Krupa D, Nollstadt K, Schmatz D, Schwartz R, Hammond M, Balkovec J, Vanmiddlesworth F. *In vitro* antifungal activities and *in vivo* efficacies of 1,3-ß-D-glucan synthesis inhibors L-671,329, L-646,991, tetrahydro-echinocandin B, and L-687,781, a papulacandin. Antimicrob Agents Chemother 1992;36:1648-57.
22. Becker JM, Marcus S, Tallock J, Miller D, Krainer E, Khare RK, Naider F. Use of the chitin-synthesis inhibitor nikkomycin to treat disseminated candidiasis in mice. J Infect Dis 1988;157:212-4.
23. Belay T, Hospenthal DR, Rogers AL, Patterson MJ. Evaluation of antibody-bearing liposomal amphotericin B in the treatment of systemic candidiasis in a neutropenic murine model. J Med Vet Mycol 1991;29:419-21.
24. Belay T, Hospenthal DR, Rogers AL, Patterson MJ. Treatment of systemic candidiasis in a murine model using immunoglobulin G bearing liposomal amphotericin B. Mycopathologia 1993;123:9-17.
25. Berenguer J, Munoz P, Parras F, Fernández-Baca V, Hernández-Sampelayo T, Bouza E. Treatment of deep mycoses with liposomal amphotericin B. Eur J Clin Microbiol Infect Dis 1994;13:504-7.
26. Bergers JJ, Ten Hagen TLM, Van Etten EWM, Bakker-Woudenberg IAJM. Liposomes as delivery systems in the prevention and treatment of infectious diseases. Pharmacy World & Science 1995;17:1-11.
27. Björkholm M, Källberg N, Grimfors G, Eklund LH, Eksborg S, Juneskans OT, Udén M. Successful treatment of hepatosplenic candidiasis with a liposomal amphotericin B preparation. J Intern Med 1991;2:173-7.
28. Boken DJ, Swindells S, Rinaldi MG. Fluconazole-resistant *Candida albicans*. Clin Infect Dis 1993;17:1018-21.
29. Brajtburg J. Powderly WG, Kobayashi GS, Medoff G. Amphotericin B: current understanding of mechanism of action. Antimicrob Agents Chemother 1990;34:183-8.
30. Brajtburg J, Elberg S, Kobayashi G, Medoff G. Inhibition of amphotericin B (Fungizone) toxicity to cells by egg lecithin-glycocholic acid mixed micelles. Antimicrob Agents Chemother 1990;34:2415-6.
31. Brajtburg J, Elberg S, Travis SJ, Kobayashi G. Treatment of murine candidiasis and cryptococcosis with amphotericin B incorporated into egg lecithin-bile salt mixed micelles. Antimicrob Agents Chemother 1994;38:294-9.
32. Brajtburg J, Elberg S, Kobayashi G, Bolard J. Amphotericin B incorporated into egg lecithin-bile salt mixed micelles: molecular and cellular aspects relevant to therapeutic efficacy in experimental mycoses. Antimicrob Agents Chemother 1994; 38:300-6.
33. Cacciapuoti A, Loebenberg D, Parmegiani R, Antonacci B, Norris C, Moss EL, Menzel F, Yaros-Tomaine T, Hare RS, Miller GH. Comparison of SCH 39304,

fluconazole, and ketokonazole for treatment of systemic fungal infections in mice. Antimicrob Agents Chemother 1992;36:64-7.
34. Caillot D, Chavanet P, Casanovas O, Solary E, Zanetta G, Buisson M, Wagner O, Cuisenier B, Bonnin A, Camerlynck P, Portier H, Guy H. Clinical evaluation of a new lipid-based delivery system for intravenous administration of amphotericn B. Eur J Clin Microbiol Infect Dis 1992;11:722-5.
35. Caillot D, Casanovas O, Solary E, Chavanet P, Bonnotte B, Reny G, Entezam F, Lopez J, Bonnin A, Guy H. Efficacy and tolerance of an amphotericin B lipid (Intralipid) emulsion on the treatment of candidaemia in neutropenic patients. J Antimicrob Chemother 1993;31:161-9.
36. Caillot D, Reny G, Solary E, Casanovas O, Chavanet P, Bonnotte B, Perello L, Dumas M, Entezam F, Guy H. A controlled trial of the tolerance of amphotericin B infused in dextrose or in Intralipid in patients with haematological malignancies. J Antimicrob Chemother 1994;33:603-13.
37. Chavanet PY, Garry I, Charlier N, Caillot D, Kisterman JP, D'Athis M, Portier H. Trial of glucose versus fat emulsion in preparation of amphotericin for use in HIV infetced patients with candidiasis. Br Med J 1992;305:921-5.
38. Chopra R, Blair S, Strang J, Cervi P, Patterson KG, Goldstone AH. Liposomal amphotericin B (AmBisome) in the treatment of fungal infections in neutropenic patients. J Antimicrob Chemother 1991;28(Suppl.B):93-104.
39. Chopra R, Fielding A, Goldstone AH. Successful treatment of fungal infections in neutropenic patients with liposomal amphotericin (AmBisome)- a report on 40 cases from a single centre. Leukemia Lymphoma 1992;7(Suppl.):73-7.
40. Clark MJ, Whitney RR, Olsen SJ, George RJ, Swerdel MR, Kunselman R, Bonner DP. Amphotericin B lipid complex therapy of experimental fungal infections in mice. Antimicrob Agents Chemother 1991;35:615-21.
41. Clemons KV, Stevens DA. Comparative efficacy of amphotericin B colloidal dispersion and amphotericin B deoxycholate suspension in treatment of murine coccidioidomycosis. Antimicrob Agents Chemother 1991;35:1829-33.
42. Clemons KV, Stevens DA. Comparative efficacies of amphotericin B lipid complex and amphotericin B deoxycholate suspension against murine blastomycosis. Antimicrob Agents Chemother 1991;35:2144-6.
43. Clemons KV, Stevens DA. Efficacies of amphotericin B lipid complex (ABLC) and conventional amphotericin B against murine coccidioidomycosis. J Antimicrob Chemother 1992;30:353-63.
44. Clemons KV, Stevens DA. Comparison of a liposomal amphotericin B formulation (AmBisome) and deoxycholate amphotericin B (Fungizone) for the treatment of murine paracoccidioidomycosis. J Med Vet Mycol 1993;31:387-94.
45. Clemons KV, Stevens DA. Therapeutic efficacy of a liposomal formulation of amphotericin B (AmBisome) against murine blastomycosis. J Antimicrob Chemother 1993;32:465-72.
46. Coker R, Tomlinson D, Harris J. Successful treatment of cryptococcal meningitis with liposomal amphotericin B after failure of treatment with fluconazole and conventional amphotericin B. AIDS 1991;5:231-2.
47. Coker RJ, Murphy SM, Harris JRW. Experience with liposomal amphotericin B (AmBisome) in cryptococcal meningitis in AIDS. J Antimicrob Chemother 1991; 28(Suppl.B):105-9.
48. Coker RJ, Murphy SM, Harris JRW. Short-course treatment and response to liposomal amphotericin B in AIDS-associated cryptococcosis. J Infect Dis 1992;165:593.

49. Coker RJ, Viviani M, Gazzard BG, Du Pont B, Pohle HD, Murphy SM, Atouguia J, Champalimaud JL, Harris JRW. Treatment of cryptococcosis with liposomal amphotericin B (AmBisome) in 23 patients with AIDS. AIDS 1993;7:829-35.
50. Collette N, Van der Auwera P, Pasual Lopez A, Heymans C, Meunier F. Tissue distribution and bioactivity of amphotericin B administered in liposomes to cancer patients. J Antimicrob Chemother 1991;27:535-48.
51. Defaveri J, Salazar MH, Rinaldi MG, Graybill JR. Pulmonary aspergillosis in mice: treatment with a new triazole SCH39304. Am Rev Respir Dis 1990;142:512-5.
52. De Marie S, Janknegt R, Bakker-Woudenberg IAJM. Clinical use of liposomal and lipid-complexed amphotericin B. J Antimicrob Chemother 1994;33:907-16.
53. Dromer F, Barbet J, Bolard J, Charreire J, Yeni P. Improvement of amphotericin B activity during experimental cryptococcosos by incorporating into immunoliposomes. Antimicrob Agents Chemother 1990;34:2055-60.
54. Emminger W, Graninger W, Emminger-Schmidmeier, Zoubek A, Pillwein K, Susani M, Wasserer A, Gadner H. Tolerance of high doses of amphotericin B by infusion of a liposomal formulation in children with cancer. Ann Heatol 1994;68:27-31.
55. Fielding RM, Smith PC, Wang LH, Porter J, Guo LSS. Comparative pharmacokinetics of amphotericin B after administration of a novel colloidal delivery system, ABCD, and a conventional formulation to rats. Antimicrob Agents Chemother 1991;35:1208-13.
56. Fielding RM, Singer AW, Wang LH, Babbar S, Guo LSS. Relationship of pharmacokinetics and drug distribution in tissue to increased safety of amphotericin B colloidal dispersion in dogs. Antimicrob Agents Chemother 1992;36:299-307.
57. Filler SG, Crislip MA, Mayer CL, Edwards, JE (1991), Comparison of fluconazole and Amphotericin B for treatment of disseminated candidiasis and endolphthalmitis in rabbits. Antimicrob Agents Chemother 1991:35:288-92.
58. Fisher MA, Lee PG, Tarry WF. Fluconazole (UK-49,858) treatment of candidiasis in normal and diabetic rats. Antimicrob Agents Chemother 1989;33:1042-5.
59. Fisher MA, Shen SH, Haddad J, Tarry WF. Comparison of *in vivo* activity of Fluconazole with that of Amphotericin B against *Candida tropicalis, Candida glabrata,* and *Candida krusei*. Antimicrob Agents Chemother 1989;33:1443-6.
60. Francis P, Lee JW, Hoffman A, Peter J, Francesconi A, Bacher J, Shelhamer J, Pizzo A, Walsh TJ. Efficacy of unilamellar liposomal amphotericin B in treatment of pulmonary aspergillosis in persistently granulocytopenic rabbits: the potential role of bronchoalveolar D-mannitol and serum galactomannan as marker of infection. J Infect Dis 1994;169:356-8.
61. Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, Cohen R, Martin F, Huang A, Barenholz Y. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Res 1994;54:987-92.
62. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-29.
63. Gates C. Pinney RJ. Amphotericin B and its delivery by liposomal and lipid formulations. J Clin Pharm Ther 1993;18:147-53.
64. Gilbert BE, Wyde PR, Wilson SZ. Aerosolized liposomal amphotericin B for treatment of pulmonary and systemic *Cryptococcus neoformans* infections in mice. Antimicrob Agents Chemother 1992;36:1466-71.
65. Gokhale PC, Kshirsagar NA, Pandya SK. Preclinical and pharmaceutical testing of liposomal amphotericin B. Indian J Med Res[B] 1993;98:75-8.

66. Gokhale PC, Kshirsagar NA, Pandya SK. Development and therapeutic application of liposomal amphotericin B. Curr Sci 1993;65:448-54.
67. Gokhale PC, Barapatre RJ, Advani SH, Kshirsagar NA, Pandya SK. Successful treatment of disseminated candidiasis resitant to amphotericin B by liposomal amphotericin B- A case report. J Cancer Res Clin Oncol 1993;119:569-71.
68. Gokhale PC, Barapatre RJ, Advani SH, Kshirsagar NA, Pandya SK. Pharmacokinetics and tolerance of liposomal amphotericin B in patients. J Antimicrob Chemother 1993;32:133-9.
69. Gokhale PC, Kshirsagar NA, Khan MU, Pandya SK, Merchant RH, Mehta KP, Colaco MP. Treatment of candiduria with liposomal amphotericin-B (L-AmpB-LRC) in children. J Antimicrob Chemother 1993;33:889-91.
70. Gondal JA, Swartz RP, Rahman A. Therapeutic evaluation of free and liposome-encapsulated amphotericin B in the treatment of systemic candidiasis in mice. Antimicrob Agents Chemother 1989;33:1544-8.
71. Grant SM, Clissold SP. Fluconazole. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in superficial and systemic mycoses. Drugs 1990;39:877-916.
72. Graybill JR, Craven PC, Taylor RL, Williams DM, Magee WE. Treatment of murine cryptococcosis with liposome-associated amphotericin B. J Infect Dis 1982;145:748-52.
73. Gregoriadis G. Overview of liposomes. J Antimicrob Chemother 1991;28(Suppl.B): 39-48.
74. Gregoriadis G. The carrier potential of liposomes in biology and medicine. N Engl J Med 1976;295:704-10.
75. Guo LSS, Fielding RM, Lasic DD, Hamilton RL, Mufson D. Novel antifungal drug delivery: stable amphotericin B-cholesteryl sulfate discs. Intern J Pharm 1991;75:45-54.
76. Guo LS, Fielding RM, Mufson D. Pharmacokinetic study of a novel amphotericin B colloidal dispersion with improved therapeutic index. Ann NY Acad Sci 1990;618: 586-8.
77. Hanson LH, Stevens DA. Comparison of antifungal activity of amphotericin B deoxycholate suspension with that of amphotericin B cholesteryl sulfate colloidal dispersion. Antimicrob Agents Chemother 1992;36:486-8.
78. Hector RF, Yee E. Evaluation of Bay R 3783 in rodent models of superficial and systemic candidiasis, meningeal cryptococcosis, and pulmonary aspergillosis. Antimicrob Agents Chemother 1990;34:448-54.
79. Heinemann V, Jehn U, Scholz P, Gleixner B, Möhrle W, Wachholz K, Kolb HJ. AmBisome®: Pharmacokinetics and clinical results. Bone Marrow Transplant 1993; 12(Suppl.4):S147-8.
80. Heymans C, Van der Auwera P, Sculier JP, Coune A, Brassine C, Laduron C, Hollaert C, Meunier F. *In vitro* evaluation of the antifungal activity of amphotericin B entrapped into liposomes during storage for one year. J Antimicrob Chemother 1991; 28(Suppl.B):17-25.
81. Hoeprich PD. Clinical use of amphotericin B and derivatives: Lore, mystique, and fact. Clin Infect Dis 1992;14(Suppl. 1):S114-9.
82. Hopfer RL, Mills K, Mehta R, Lopez-Berestein G, Fainstein V, Juliano RL. *In vitro* antifungal activities of amphotericin B and liposome-encapsulated amphotericin B. Antimicrob Agents Chemother 1984;25:387-9.
83. Hospenthal DR, Rogers AL, Mills GL. Development of amphotericin B liposomes bearing antibodies specific to *Candida albicans*. Mycopathologia 1988;101:37-45.

84. Hospenthal D, Gretzinger K, Rogers A. Treatment of a murine model of systemic candidiasis with liposomal amphotericin B bearing antibody to *Candida albicans*. J Med Microbiol 1989;30:193-7.
85. Hospenthal DR, Rogers AL, Beneke ES. Effect of attachment of anticandidal antibody to the surface of liposomes encapsulating amphotericin B in the treatment of murine candidiasis. Antimicrob Agents Chemother 1989;33:16-8.
86. Hostetler JH, Clemons KV, Hanson LH, Stevens DA. Efficacy and safety of amphotericin B colloidal dispersion compared with those of amphotericin B deoxycholate suspension for treatment of dissemiated murine cryptococcosis. Antimicrob Agents Chemother 1992;36:2656-60.
87. Hudson J, Scott GL, Warnock DW. Treatment of hepatosplenic candidosis with liposomal amphotericin B in patient with acute leukemia. Lancet 1991;338:1534-5.
88. Humphreys H, Oliver DA, Winter R, Warnock DW. Liposomal amphotericin B and continuous venous-venous haemofiltration. J Antimicrob Chemother 1994;33:1070-1.
89. Janknegt R, De Marie S, Bakker Woudenberg IAJM, Crommelin DJA. Liposomal and lipid formulations of amphotericin B. Clinical Pharmacokinetics. Clin Pharmacokinet 1992;23:279-91.
90. Janoff AS, Boni LT, Popescu MC, Minchey SR, Cullis PR, Madden TD, Taraschi T, Gruner SM, Shyamsunder E, Tate MW, Mendelsohn R, Bonner D. Unusual lipid structures selectively reduce the toxicity of amphotericin B. Proc Natl Acad Sci USA 1988;85:6122-6.
91. Janoff AS. Liposomes and lipid structures as carriers of amphotericin B. Eur J Clin Microbiol Infect Dis 1990;9:146-51.
92. Joly V, Dromer F, Barge J, Yeni P. Seta N, Molas G, Carbon C. Incorporation of amphotericin B (AMB) into liposomes alters AMB-induced acute nephrotoxicity in rabbits. J Pharmacol Exp Ther 1989;251:311-6.
93. Joly V, Saint-Julien L, Carbon C, Yeni P. Interactions of free and liposomal amphotericin B with renal proximal tubular cells in primary culture. J Pharmacol Exp Ther 1990;255:17-22.
94. Joly V, Bolard J, Saint-Julien L, Carbon C, Yeni P. Influence of phospholipid / amphotericin B ratio and phospholipid type on *in vitro* renal cell toxicities and fungicidal activities of lipid-associated amphotericin B formulations. Antimicrob Agents Chemother 1992;36:262-6.
95. Joly V, Farinotti R, Saint-Julien L, Chéron, Carbon C, Yeni P. *In vitro* renal toxicity and *in vivo* therapeutic efficacy in experimental murine cryptococcosis of amphotericin B (Fungizone) associated with Intralipid. Antimicrob Agents Chemother 1994;38:177-83.
96. Juliano RL, Lopez-Berestein G, Hopfer R, Mehta R, Mehta K, Mills K. Selective toxicity and enhanced therapeutic index of liposomal polyene antibiotics in systemic fungal infections. Ann NY Acad Sci 1985;446:390-402.
97. Juliano RL, Grant CWM, Barber KR, Kalp MA. Mechanism of the selective toxicity of amphotericin B incorporated into liposomes. Mol Pharmacol 1987;31:1-11.
98. Jullien S, Contrepois A, Sligh JE, Domart Y, Yeni P, Brajtburg J, Medoff G, Bolard J. Study of the effects of liposomal amphotericin B on *Candida albicans, Cryptococcus neoformans*, and erythrocytes by using small unilamellar vesicles prepared from saturated phospholipids. Antimicrob Agents Chemother 1989;33:345-9.
99. Jullien S, Brajtburg J, Bolard J. Affinity of amphotericin B for phosphatidylcholine vesicles as a determinant of the *in vitro* cellular toxicity of liposomal preparations. Biochim Biophys Acta 1990;1021:39-45.

100. Kan V, Bennett J, Amantea M, Smolskis MC, McManus E, Grasela DM, Sherman JW. Comparative safety, tolerance, and pharmacokinetics of amphotericin B lipid complex and amphotericin B desoxycholate in healthy male volunteers. J Infect Dis 1991;164:418-21.
101. Karyotakis NC, Anaissie EJ. Efficacy of escalating doses of liposomal amphotericin B (AmBisome) against hematogenous *Candida lusitaniae* and *Candida krusei* infection in neutropenic mice. Antimicrob Agents Chemother 1994;38:2660-2.
102. Katz NM, Pierce PF, Anzeck RA, Visner MS, Canter HG, Foegh ML, Pearle DL, Tracy C, Rahman A. Liposomal amphotericin B for treatment of pulmonary aspergillosis in a heart transplant patient. J Heart Transplant 1990;9:14-7.
103. Kirsch R, Goldstein R, Tarloff D, Paris D, Hook J, Hanna N, Bugelski P, Poste. An emulsion formulation of amphotericin B improves the therapeutic index when treating systemic murine candidiasis. J Infect Dis 1988;158:1065-70.
104. Klibanov AL, Maruyama K, Torchillin VP, Huang L. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett 1990;268:235-7.
105. Klibanov AL, Huang L. Long circulating liposomes: development and perspectives. J Liposome Res 1992;2:321-34.
106. Krause HJ, Juliano RL. Interactions of liposome-incorporated amphotericin B with kidney epithelial cell cultures. Mol Pharmacol 1988;34:286-97.
107. Kullberg BJ, Van 't Wout JW, Poell RJM, Van Furth R. Combined effect of fluconazole and recombinant human interleukin-1 on systemic candidiasis in neutropenic mice. Antimicrob Agents Chemother 1992;36:1225-9.
108. Lackner H, Schwinger W, Urban C, Müller W, Ritschl E, Reiterer F, Kuttnig-Haim M, Urlesberger B, Hauer C. Liposomal amphotericin B (AmBisome) for treatment of disseminated fungal infections in two infants of very low birth weight. Pediatrics 1992;89:1259-61.
109. Laing RBS, Milne LJR, Leen CLS, Malcolm GP, Steers AJW. Anaphylactic reactions to liposomal amphotericin. Lancet 1994;344:682.
110. Lasic D. Liposomes. Am Sci 1992;80:20-31.
111. Le Conte P, Joly V, Saint-Julien L, Gillardin JM, Carbon C, Yeni P. Tissue distribution and antifungal effect of liposomal itraconazole in experimental cryptococcosis and pulmonary aspergillosis. Am Rev Respir Dis 1992;145:424-9.
112. Le Conte P, Faintreny A, Potel G, Legallou F, Bugnon D, Baron D. *In vitro* and *in vivo* antifungal activity of liposomal and free intraconazole against *Candida albicans*. Curr Ther Res Clin Exp 1994:55:938-43.
113. Lee JW, Amantea MA, Francis PA, Navarro EE, Bacher J, Pizzo PA, Walsh TJ. Pharmacokinetics and safety of a unilamellar liposomal formulation of amphotericin B (AmBisome) in rabbits. Antimicrob Agents Chemother 1994;38:713-8.
114. Levine SJ, Walsh TJ, Martinez A, Eichacker PQ, Lopez-Berestein G, Natanson C. Cardiopulmonary toxicity after liposomal amphotericin B infusion. Ann Intern Med 1991;114:664-6.
115. Levitz SM. Overview of host defences in fungal infections. Clin Infect Dis 1992; 14(Suppl.1):S37-42.
116. Levy MY, Polacheck I, Barenholz Y, Benita S. Efficacy evaluation of a novel submicron amphotericin B emulsion in murine candidiasis. J Med Vet Mycol 1993; 31:207-18.
117. Longuet P, Joly V, Amirault P, Seta N, Carbon C, Yeni P. Limited protection by small unilamellar liposomes against the renal tubular toxicity induced by repeated amphotercin B infusions in rats. Antimicrob Agents Chemother 1991;35:1303-8.

118. Lopez-Berestein G, Mehta R, Hopfer RL, Mills, Kasi L, Mehta K, Fainstein V, Luna M, Hersch EM, Juliano RL. Treatment and prophylaxis of disseminated infection due to *Candida albicans* in mice with liposome-encapsulated amphotericin B. J Infect Dis 1983;147:939-45.
119. Lopez-Berestein G, Mehta R, Hopfer RL, Mehta K, Hersh EM, Juliano RL. Effects of sterols on the therapeutic efficacy of liposomal amphotericin B in murine candidiasis. Cancer Drug Deliv 1983;1:37-42.
120. Lopez-Berestein G, Rosenblum MG, Mehta R. Altered tissue distribution of amphotericin B by liposomal encapsulation: comparison of normal mice to mice infected with *Candida albicans*. Cancer Drug Deliv 1984;1:199-205.
121. Lopez-Berestein G, Hopfer RL, Mehta R, Mehta K, Hersh EM, Juliano RL. Prophylaxis of *Candida albicans* infection in neutropenic mice with liposome-encapsulated amphotericin B. Antimicrob Agents Chemother 1984;25:366-7.
122. Lopez-Berestein G, Hopfer RL, Mehta R, Mehta K, Hersch EM, Juliano RL. Liposome-encapsulated amphotericin B for treatment of disseminated candidiasis in neutropenic mice. J Infect Dis 1984;150:278-83.
123. Lopez-Berestein G, McQueen T, Mehta K. Protective effect of liposomal-amphotericin B against *C.albicans* infection in mice. Cancer Drug Deliv 1985;2:183-9.
124. Lopez-Berestein G, Fainstein V, Hopfer RL, Mehta K, Sullivan MP, Keating M, Rosenblum MG, Mehta R, Luna M, Hersh EM, Reuben J, Juliano RL, Bodey GP. Liposomal amphotericin B for treatment of systemic fungal infections in patients with cancer: a preliminary study. J Infect Dis 1985;151:704-10.
125. Lopez-Berestein G, Bodey GP, Frankel LS, Mehta K. Treatment of hepatosplenic cadidiasis with liposomal amphotericin B. J Clin Oncol 1987;5:310-7.
126. Lopez-Berestein G. Liposomes as carriers of antifungal drugs. Ann NY Acad Sci 1988; 544:590-7.
127. Lopez-Berestein G, Bodey GP, Fainstain V, Keating M, Frankel LS, Zeluff B, Gentry L, Mehta K. Treatment of systemic fungal infections with liposomal amphotericin B. Arch Intern Med 1989;149:2533-6.
128. Lyman CA, Walsh TJ. Systemically administered antifungal agents- A review of their clinical pharmacology and therapeutic applications. Drugs 1992;44:9-35.
129. Macedo MCMA, Dulley FL, Ostronoff M, Machado CM, Zambon E, Matsumoto C, Dorlhiac-Llaccer P, Chamone DF. Effectiveness of amphotericin B in lipid emulsion for treating fungal septicemia in granulocytopenic patients. Clin Infect Dis 1994;19: 366-7.
130. Mehta R, Lopez-Berestein G, Hopfer R, Mills K, Juliano RL. Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells. Biochim Biophys Acta 1984; 770:230-4.
131. Mehta RT, Mehta K, Lopez-Berestein G, Juliano RL. Effect of liposomal amphotericin B on murine macrophages and lymphocytes. Infect Immun 1985;47:429-33.
132. Mehta RT, Lopez-Berestein G, Hopfer RL, Mehta K, White RA, Juliano RL. Prophylaxis of murine candidiasis via application of liposome-encapsulated amphotericin B and a muramyl dipeptide analog, alone or on combination. Antimicrob Agents Chemother 1985;28:511-3.
133. Meunier F, Sculier JP, Coune A, Brassinne C, Heymans C, Laduron C, Collette N, Hollaert C, Bron D, Klastersky J. Amphotericin B encapsulated in liposomes administered to cancer patients. Ann NY Acad Sci 1988;544:598-610.
134. Meunier F, Prentice HG, Ringdén O. Liposomal amphotericin B (AmBisome): safety data from a phase II/III clinical trial. J Antimicrob Chemother 1991;28(Suppl.B):83-91.

135. Meunier F, Aoun M, Bitar N. Candidemia on immunocompromised patients. Clin Infect Dis 1992;14(Suppl.2):S120-5.
136. Mills W, Chopra R, Linch DC, Goldstone AH. Liposomal amphotericin B in he treatment of fungal infections in neutropenic patients: a single-centre experience of 133 episodes in 116 patients. Br J Haematol 1994;86:754-60.
137. Miyazaki HM, Kohno S, Miyazaki Y, Mitsutaki K, Tomono K, Kaku M, Koga H, Hara K. Efficacy of intravenous intraconazole against experimental pulmonary aspergillosis. Antimicrob Agents Chemother 1993;37:2762-5.
138. Miyazaki T, Kohno S, Kaku M, Koga H, Yamaguchi K. Liposome-encapsulated amphotericin B in the treatment of experimental murine candidiasis. Tohoku J Exp Med 1990;161:273-81.
139. Miyazaki T, Kohno S, Kaku M, Koga H, Yamaguchi K. Polysaccharide-coated liposomal amphotericin B for the treatment of murine pulmonary candidiasis. Tohoku J Exp Med 1992;168:483-90.
140. Moreau P, Milpied N, Fayette N, Ramee JF, Harousseau JL. Reduced renal toxicity and improved clinical tolerance of amphotericin B mixed with Intralipid compared with conventional amphotericin B in neutropenic patients. J Antimicrob Chemother 1992;30:535-41.
141. Morrison CJ, Stevens DA. Comparative effects of cilofungin and amphotericin B on experimental murine candidiaisis. Antimicrob Agents Chemother 1990;34:746-50.
142. O'Connell B, Crotty G, Cafferkey MT, Stephens R, McDonald GSA, McCann SR. Successful treatment of focal hepatic candidiasis with liposomal amphotericin B. Irish J Med Sci 1992;161:664-5.
143. Olsen SJ, Swerdel MR, Blue B, Clark JM, Bonner DP. Tissue distribution of amphotericin B lipid complex in laboratory animals. J Pharm Pharmacol 1991;43: 831-5.
144. Padula A, Chambers HF. Evaluation of cilofungin (LY 121019) for treatment of experimental *Candida albicans* endocarditis in rabbits. Antimicrob Agents Chemother 1989;33:1822-3.
145. Pahls S, Schaffner A. Comparison of the activity of free and liposomal amphotericin B *in vitro* and in a model of systemic and localized murine candidiasis. J Infect Dis 1994;169:1057-61.
146. Papahadjopoulos D, Allen T, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C, Martin FJ. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci USA 1991;88:11460-4.
147. Pallister CJ, Johnson, EM, Warnock PJ, Reeves DF. *In vitro* effects of liposome-encapsulated amhotericin B (AmBisome) and amphotericin B-deoxycholate (Fungizone) on the phagocytic and candidacidal function of human polymorphonuclear leucocytes. J Antimicrob Chemother 1992;30:313-20.
148. Patterson TF, Miniter P, Dijkstra J, Szoka FC, Ryan JL, Andriole VT. Treatment of experimental invasive aspergillosis with novel amphotericin B/cholesterol sulfate complexes. J Infect Dis 1989;159:717-24.
149. Patterson TF, George D, Ingersoll R, Miniter P, Andriole VT. Efficacy of SCH 39304 in treament of experimental invasive aspergillosis. Antimicrob Agents Chemother 1991;35:1985-8.
150. Patterson TF, George D, Miniter P, Andriole VT. Saperconazole therapy in a rabbit model of invasive aspergillosis. Antimicrob Agents Chemother 1992;36:2681-5.
151. Patterson TF, Fothergill AW, Rinaldi MG. Efficacy of itraconazole solution in a rabbit model of invasive aspergillosis. Antimicrob Agents Chemother 1993;37:2307-10.

152. Perfect JR, Savani DV, Durack DT. Comparison of itraconazole and fluconazole in treatment of cryptococcal meningitis and candida pyelonephritis in rabbits. Antimicrob Agents Chemother 1986;29:579-83.
153. Perfect JR, Wright KA, Hobbs MM, Durack DT. Treatment of experimental cryptococcal meningitis and disseminated candidiasis with SCH 39304. Antimicrob Agents Chemother 1989;33:1735-40.
154. Perfect JR, Wright KA. Amphotericin B lipid complex in the treatment of experimental cryptococcal meningitis and disseminated candidosis. J Antimicrob Chemother 1994;33:73-81.
155. Perkins WR, Minchey SR, Boni LT, Swenson CE, Popescu MC, Pasternack RF, Janoff AS. Amphotericin B-phospholipid interactions resposible for reduced mammalian cell toxicity. Biochim Biophys Acta 1992;1107:271-82.
156. Pfaller MA. Epidemiology and control of fungal infections. Clin Infect Dis 1994; 19(Suppl.1):S8-13.
157. Pisarik L, Joly V, Jullien S, Carbon C, Yeni P. Reduction of amphotericin B acute toxicity after intravenous administration of empty liposomes in mice. J Infect Dis 1990;161:1042-4.
158. Proffitt RT, Satorius A, Chiang SM, Sullivan L, Adler-Moore JP. Pharmacology and toxicology of a liposomal formulation of amphotericin B (AmBisome) in rodents. J Antimicrob Chemother 1991;28(Suppl.B):49-61.
159. Proffitt RT, Adler-Moore JP, Fujii G, Satorius A, Lee MJA, Baily A. Stability and mode of action of AmBisome® (liposomal amphotericin B). J Control Release 1994;28:342-3.
160. Ralph ED, Khazindar AM, Barber KR, Grant CWM. Comparative *in vitro* effects of liposomal amphotericin B, amphotericin B-deoxycholate, and free amphotericin B against fungal strains determined by using MIC and minimal lethal concentration susceptibility studies and time-kill curves. Antimicrob Agents Chemother 1991;35: 188-91.
161. Ralph ED, Barber KR, Grant CWM. Liposomal amphotericin B: and effective, nontoxic preparation for the treatment of urinary tract infections caused by *Candida albicans*. Am J Nephrol 1991;11:118-22.
162. Ralph ED, Barber KR, Grant CWM. Clinical experience with multilamellar liposomal amphotercicin B in patients with proven and suspected fungal infections. Scand J Infect Dis 1993;25:487-96.
163. Richards EM, Apperley JF, Marcus RE. Successful use of liposome-encapsulated amphotericin to treat invasive aspergillosis following failure of conventional amphotericin. Clin Lab Haemat 1992;14:127-30.
164. Richardson K, Brammer KW, Marriot MS, Troke PF. Activity of UK-49,858, a bistriazole derivative against experimental infections with *Candida albicans* and *Trichophyton mentagrophytes*. Antimicrob Agents Chemother 1985;27:832-5.
165. Ringdén O, Meunier F, Tollemar J, Ricci P, Tura S, Kuse E, Viviani MA, Gorin NC, Klastersky J, Fenaux P, Prenice HG, Ksionski G. Efficacy of amphotericin B encapsulated in liposomes (AmBisome) in the treatment of invasive fungal infections in immunocompromised patients. J Antimicrob Chemother 1991;28(Suppl.B):73-82.
166. Ringdén O, Tollemar J, Tydén G. Liposomal amphotericin B. Lancet 1992;339:374.
167. Ringdén O, Tollemar J. Liposomal amphotericin B (AmBisome®) treatment of invasive fungal infections in immunocompromised children. Mycoses 1993;36:187-92.
168. Ringdén O. Clinical use of AmBisome® with special emphasis on experience in children. Bone Marrow Transplant 1993;12(Suppl.4):S149-50.

169. Ringdén O, Andström E, Remberger M, Svahn BM, Tollemar J. Allergic reactions and other rare side effects of liposomal amphotericin B. Lancet 1994;344(8930):1156-7.
170. Rogers TE, Galgiani JN. Activity of fluconazole (UK 49,858) and ketoconazole against *Candida albicans in vitro* and *in vivo*. Antimicrob Agents Chemother 1986;30:418-22.
171. Roilides E, Pizzo PA. Perspectives on the use of cytokines in the management of infectious complications of cancer. Clin Infect Dis 1993;17(Suppl. 2):S385-9.
172. Sanders SW, Buchi KN, Goddard MS, Lang JK, Tolman KG. Single-dose pharmacokinetics and tolerance of a cholesterol sulphate complex of amphotericin B administered to healthy volunteers. Antimicrob Agents Chemother 1991;35:1029-34.
173. Schindler JJ, Warren RP, SD Allen, Jackson MK. Immunological effects of amphotericin B and liposomal amphotericin B on splenocytes from immune-normal and immune-compromised mice. Antimicrob Agents Chemother 1993;37:2716-21.
174. Schürmann D, De Matos Marques B, Grunewald T, Pohle HD, Hahn H, Ruf B. Safety and efficacy of liposomal amphotericin B in treating AIDS-associated disseminated cryptococcosis. J Infect Dis 1991;164:620-2.
175. Sculier JP, Coune A, Meunier F, Brassine C, Laduron C, Hollaert C, Collette N, Heymans C, Klastersky J. Pilot study of amphotericin entrapped in sonicated liposomes in cancer patients with fungal infections. Exp J Cancer Clin Oncol 1988; 24:527-38.
176. Sculier JP, Bron D, Coune A, Meunier F. Successful treatment with liposomal amphotericin B in two patients with persisting fungemia. Eur J Clin Microbiol Infect Dis 1989;8:903-7.
177. Sharland M, Hay RJ, Davies EG. Liposomal amphotericin B in hepatic candidosis. Arch Dis Child 1994;70:546-7.
178. Shaunak S, Cohen J. Lack of toxicity of long-term, high total dose liposomal amphotericin B. J Antimicrob Chemother 1994;34:451-2.
179. Shirkoda A, Lopez-Berestein G, Holbert JM, Luna MA. Hepatosplenic fungal infection: CT and pathologic evaluation after treatment with liposomal amphotericin B. Radiology 1986;159:349-53.
180. Smith KR, Lank KM, Cobbs CG, Cloud GA, Dismukes WE. Comparison of cilofungin and amphtericin B for therapy of murine candidiasis. Antimicrob Agents Chemother 1990;34:1619-21.
181. Sorensen KN, Allen SD, Nejdl MJ, Proffitt RT. Aerosolization of liposomal (AmBisome®) and non-liposomal (Fungizone®) amphotericin B as treatment for pulmonary fungal infections. J Control Release 1994;28:346-7.
182. Souza LC, Maranhao RC, Schreier S, Campa A. *In vitro* and *in vivo* studies of the decrease of amphotericin B toxicity upon association with a triglyceride-rich emulsion. J Antimicrob Chemother 1993;32:123-32.
183. Sternberg S. The emerging fungal threat. Science 1994;266:1632-4.
184. Storm G, Oussoren C, Peeters PAM, Barenholz Y. Tolerability of liposomes *in vivo*. In: Gregoriadis G (Ed.) Liposome technology, volume III. 2nd edition. Boca Raton: CRC Press, 1993:345-383.
185. Sullivan GW, Carper HT, Mandell GL. Lipid complexing decreases amphotericin B inflammatory activation of human neutrophils compared with that of a desoxycholate-suspended preparation of amphotericin B (Fungizone). Antimicrob Agents Chemother 1992;36:39-45.
186. Swerdloff JN, Filler SG, Edwards JE. Severe candidal infections in neutropenic patients. Clin Infect Dis 1993;17(Suppl. 2):S457-67.

187. Szoka FC, Milholland D, Barza M. Effect of lipid composition and liposome size on toxicity and *in vitro* fungicidal activity of liposome-intercalated amphotericin B. Antimicrob Agents Chemother 1987;31:421-9.
188. Tanio T, Ichise K, Nakajima T, Okuda T. *In vivo* efficacy of SM-8668 (Sch 39304), a new oral triazole antifungal agent. Antimicrob Agents Chemother 1990;34:980-4.
189. Taylor RL, Williams DM, Craven PC, Graybill JR, Drutz DJ, Magee WE. Amphotericin B in liposomes: A novel therapy for histoplasmosis. Am Rev Respir Dis 1982;125:610-1.
190. Tollemar J, Ringdén O, Tydén G. Liposomal amphotericin B (AmBisome®) treatment in solid organ and bone marrow transplant recipients. Efficacy and safety evaluation. Clin Transplant 1990;4:167-75.
191. Tollemar J, Andersson S, Ringdén O, Tydén G. A retrospective clinical comparison between antifungal treatment with liposomal amphotercin B in transplant recipients. Mycoses 1992;35:215-20.
192. Tollemar J, Ringdén O, Andersson S, Sundberg B, Ljungman P, Tydén G. Randomized double-blind study of liposomal amphotericin B (AmBisome) prophylaxis of invasive fungal infections in bone marrow transplant recipients. Bone Marrow Transplant 1993;12:577-82.
193. Tollemar J, Hockerstedt K, Ericzon BG, Sundberg B, Ringdén O. Fungal prophylaxis with AmBisome in liver and bone marrow transplant recipients: Results of two randomized double-blind studies. Transplant Proc 1994;26:1833.
194. Tremblay C, Barza M, Fiore C, Szoka F. Efficacy of liposome-intercalated amphotericin B in the treatment of systemic candidiasis in mice. Antimicrob Agents Chemother 1984;26:170-3.
195. Troke PF, Andrews RJ, Brammer KW, Marriott MS, Richardson K. Efficacy of UK-49,858 (fluconazole) against *Candida albicans* experimental infections in mice. Antimicrob Agents Chemother 1985;28:815-8.
196. Van Cutsem J. Oral, topical and parenteral antifungal treatment with itraconazole in normal and immunocompromised animals. Mycoses 1989;32(Suppl. 1):S14-34.
197. Van Cutsem J, Van Gerven F, Janssen PAJ. Oral and parenteral therapy with saperconazole (R66905) of invasive aspergillosis in normal and immunocompromised animals. Antimicrob Agents Chemother 1989;33:2063-8.
198. Van 't Wout JW, Mattie H, van Furth R. The efficacy of amphotericin B, fluconazole and itraconazole against systemic candidiasis in normal and neutropenic mice. Antimicrob Agents Chemother 1989;33:147-51.
199. Walsh TJ, Aoki S, Mechinaud F, Bacher J, Lee JW, Rubin M, Pizzo PA. Effects of preventive, early, and late antifungal chemotherapy with fluconazole in different granulocytopenic models of experimental disseminated candidiasis in granulocytopenic hosts. J Infect Dis 1990;16:755-60.
200. Walsh TJ, Lee JW, Aoki S, Mechinaud F, Bacher J, Lecciones J, Thomas V, Rubin M, Pizzo PA. Experimental basis for use of fluconazole for preventive or early treatment of disseminated candidiasis in granulocytopenic hosts. Rev Infect Dis 1990; 12(Suppl.3):S307-17
201. Walsh TJ, Lee JW, Lecciones J, Kelley P, Peter J, Bacher J, Pizzo PA. SCH-39304 in prevention and treatment of disseminated candidiasis in persitently granulocytopenic rabbits. Antimicrob Agents Chemother 1990;34:1560-4.
202. Walsh TJ. Invasive pulmonary aspergillosis in patients with neoplastic diseases. Semin Respir Infect 1990;5:111-2.
203. Walsh TJ, Lee JW, Roilides E, Francis P, Bacher J, Lyman CA, Pizzo PA. Experimental antifungal chemotherapy in granulocytopenic animal models of disseminated

candidiasis: approaches to understanding investigational antifungal compounds for patients with neoplastic diseases. Clin Infect Dis 1992;14(Suppl. 1):S139-47.
204. Walsh TJ. Management of immunocompromised patients with evidence of an invasive mycosis. Hematol Oncol Clin N Am 1993;7:1003-26.
205. Wasan KM, Vadiei K, Lopez-Berestein G, Luke DR. Pharmacokinetics, tissue distribution, and toxicity of free and liposomal amphotericin B in diabetic rats. J Infect Dis 1990;161:562-6.
206. Wasan KM, Rosenblum MG, Cheung L, Lopez-Berestein G. Influence of lipoproteins on renal cytotoxicity and antifungal activity of amphotericin B. Antimicrob Agents Chemother 1994;38:223-7.
207. Wasan KM, Morton RE, Rosenblum MG, Lopez-Berestein G. Decreased toxicity of liposomal amphotericin B due to association of amphotericin B with high-density lipoproteins. J Pharmac Sci 1994;83:1006-10.
208. Wasan KM, Grossie VB, Lopez-Berestein G. Concentrations in serum and distribution in tissue of free and liposomal amphotericin B in rats during continuous Intralipid infusion. Antimicrob Agents Chemother 1994;38:2224-6.
209. Washington C, Lance M, Davis SS. Toxicity of amphotericin B emulsion formulations. J Antimicrob Chemother 1993;31:806-8.
210. Weber RS, Lopez-Berestein G. Treatment of invasive *Aspergillus* sinusitis with liposomal amphotericin B. Larygoscope 1987;97:937-41.
211. White A, Goetz MB. Azole-resistant *Candida albicans*: report of two cases of resistance to fluconazole and review. Clin Infect Dis 1994;19:687-92.
212. Wingard JR. Infections due to resistant *Candida* species in patients with cancer who are receiving chemotherapy. Clin Infect Dis 1994;19 (Suppl. 1):S49-53.
213. Witt, MD, Bayer AS. Comparison of fluconazole and amphotercin B for prevention and treatment of experimental *Candida* endocarditis. Antimicrob Agents Chemother 1991;35:2481-5.
214. Witt MD, Imhoff T, Li C, Bayer AS. Comparison of fluconazole and amphotericin B for treatment of experimental *Candida* endocarditis caused by non-*C.albicans* strains. Antimicrob Agents Chemother 1993;37:2030-2.
215. Woodle MC, Matthay KK, Newman MS, Hidayat JE, Coolins LR, Redemann C, Martin FJ, Papahadjopoulos D. Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. Biochim Biophys Acta 1992;1105: 193-200.
216. Woodle MC, Lasic DD. 1992. Sterically stabilized liposomes. Biochim Biophys Acta 1992;1113:171-99.
217. Yamada H, Tsuda T, Watanabe T, Ohashi M, Murakami K, Mochizuki H. *In vitro* and *in vivo* antifungal activities of D0870, a new triazole agent. Antimicrob Agents Chemother 1993;37:2412-7.
218. Zoubek A, Emminger W, Emminger-Schmidmeier W, Peters C, Pracher E, Grois N, Gadner H. Conventional versus liposomal amphotericin B in immunosuppressed children. Pediatr Hematol Oncol 1992;9:187-93.

Chapter 2

Effects of amphotericin B-desoxycholate (Fungizone®) versus fluconazole on the extracellular and intracellular growth of *Candida albicans*

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ABSTRACT

The effects of amphotericin B-desoxycholate (AMB-DOC) and fluconazole (FLU) on the extracellular and intracellular growth of *Candida albicans* were studied. With respect to the extracellular growth of *C.albicans*, antifungal activity was measured in terms of MICs and minimal fungicidal concentrations as well as by determination of the concentration that effectively killed (>99.9%) *C.albicans* in the absence or presence (AMB-DOC only) of serum. AMB-DOC was highly active in terms of killing, even at an increased inoculum size. In the presence of serum, AMB-DOC activity was substantially reduced. For FLU, activity was restricted to inhibition of fungal growth, even after the inoculum size was reduced. With respect to the intracellular growth of *C.albicans*, antifungal activity was measured by using monolayers of murine peritoneal macrophages infected with *C.albicans* and was measured in terms of inhibition of germ tube formation as well as effective killing (>99%) of *C.albicans*. AMB-DOC was highly active against *C.albicans*. At an increased ratio of infection, AMB-DOC activity was slightly reduced. FLU had no antifungal activity. Neither a reduction in the ratio of infection nor exposure of *C.albicans* to FLU prior to macrophage ingestion resulted in activity against intracellular *C.albicans* by FLU. Previous exposure of *C.albicans* to AMB-DOC resulted in increased intracellular activity of AMB-DOC. The intracellular antifungal activity of the combination of FLU with AMB-DOC was less than that of AMB-DOC alone. AMB-DOC showed fungicidal activity against *C.albicans* growing both extracellularly and intracellularly, whereas FLU inhibited growth only of extracellular *C.albicans*. A slight antagonistic effect between FLU and AMB-DOC was found with respect to intracellular as well as extracellular *C.albicans*.

INTRODUCTION

Deep-seated candidal infections are an important cause of morbidity and mortality in immunocompromised patients [15,21,22]. The current drug of choice for most systemic mycoses is still amphotericin B-desoxycholate (AMB-DOC), but its use is restricted by a variety of toxic side effects [6,13,15,21,22]. Consequently, there is a need for effective and less toxic drugs for the treatment of patients with these infections. One of these antifungal agents is the triazole fluconazole (FLU) [4,10,14,33,34,41]. *In vivo* experimental studies suggested that FLU is active against disseminated candidiasis [8,9,29,31,32,35,37]. Recently, the antifungal efficacy of FLU was demonstrated in persistently granulocytopenic rabbits when it was used for prevention or early treatment [39,40]. Little is still known, however, on the role of FLU in the treatment of systemic candidiasis in immunocompromised patients. Since the host defense mechanisms in these patients are severely impaired, the intrinsic antifungal activity of the antifungal agent is of great importance for effective treatment. Unfortunately, standardized *in vitro* methods for assessment of the intrinsic activity of antifungal agents are not available. In addition, most of the *in vitro* methods that are used are criticized because of the poor correlation with *in vivo* antifungal activity [5,6,11,12,18,27,28,36].

To gain more insight into the intrinsic antifungal activity of AMB-DOC and FLU, the activities of both agents against extracellular *Candida albicans* in relation to inoculum size were investigated in this study. Known antagonizing factors for the assessment of *in vitro* antifungal activity were excluded by choosing the recommended optimal test conditions for each agent.

The *in vivo* efficacy of an antifungal agent is determined not only by its intrinsic antifungal activity but also by factors that influence the bioavailability of the agent, such as protein binding and intracellular penetrating capacity. Therefore, the effect of serum on the antifungal activity against extracellular *C.albicans* was investigated. Next, the antifungal activities of AMB-DOC and FLU against intracellular *C.albicans* were studied. Activity was determined in relation to the ratio of infection. In addition, the effect of exposure of *C.albicans* to one of the antifungal agents prior to macrophage ingestion as well as the effect of a combination of both antifungal agents on intracellular *C.albicans* were investigated.

MATERIALS AND METHODS

***Candida* strains.** *C.albicans* ATCC 44858 was used in the experimental studies. *C.albicans* ATCC 28516 and *C.kefyr* (formerly *C.pseudotropicalis*, Carshalton strain; Pfizer Code Y0601; kindly provided by Pfizer Central Research, Sandwich, England) were used as reference strains in the MIC and minimal fungicidal concentration (MFC) determinations with AMB-DOC and FLU, respectively. The yeasts were

maintained at -80°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing 10% (v/v) glycerol.

Antifungal agents. AMB-DOC (Fungizone® for intravenous infusion; Bristol Myers-Squibb, Woerden, The Netherlands) was reconstituted with 10 ml of distilled water to give a standard solution of 5 g/L. Further dilutions were made in distilled water. FLU was kindly provided by Pfizer Nederland B.V. as a standard solution of 2 g/L in saline (FLU for intravenous infusion; Pfizer France). Further dilutions were made in phosphate-buffered saline (PBS).

Antifungal activity of AMB-DOC versus that of FLU against extracellular *C.albicans*. (I) MIC and MFC. Stationary-growth-phase cultures were obtained after incubation of the yeasts in Sabouraud maltose broth (Difco Laboratories) for 24 h at 37°C. Yeasts were washed in PBS, counted in a hemacytometer, and adjusted to the desired inoculum in assay medium. For experiments with AMB-DOC, the assay medium was Antibiotic Medium no. 3 (Penassay broth; Difco Laboratories), whereas for FLU the assay medium was high-resolution medium (kindly provided by Pfizer Central Research). At twofold increasing inocula ranging from $1.3x10^7$ to $1x10^8$ CFU/L, the MICs and MFCs of AMB-DOC and FLU at twofold increasing concentrations ranging from 0.025 to 102 mg/L were determined. In order to ensure accurate determination of the 99.9% endpoint, the MFC was determined after a total volume of 1.0 ml was subcultured onto Sabouraud dextrose agar (Oxoid, Basingstoke, England). Before plating, the concentrations of AMB and FLU in these specimens were reduced to an inactive level by washing the samples three times with PBS.

(II) Short-term growth. Logarithmic-growth-phase cultures were prepared by reincubation of a stationary-phase inoculum in assay medium for 5 h under continuous agitation at 37°C. The desired inoculum was obtained after dilution of this *C.albicans* suspension in assay medium at 37°C. The extracellular activity of the drug in terms of effective killing (>99.9%) of *C.albicans* during 6 h of incubation was determined. At eightfold increasing inocula, ranging from $1.3x10^7$ to $8.0x10^8$ CFU/L for AMB-DOC and from $2.0x10^5$ to $1.3x10^7$ CFU/L for FLU, the extracellular activity of AMB-DOC versus that of FLU was assessed at various concentrations ranging from 0.05 to 12.8 mg/L for AMB-DOC and 0.4 to 102 mg/L for FLU. The controls contained only the solvent of the antifungal agent in the appropriate dilution. During incubation, under continuous rotation at 8 rpm at 37°C and protected from light, the numbers of viable organisms were determined at 2-h intervals by making plate counts of 10-fold serial dilutions of the washed specimen on Sabouraud dextrose agar. Additionally, at the concentrations that effected >99.9% killing of *C.albicans*, the effect of serum on the activity of the antifungal agent was measured. At an inoculum of $1.3x10^7$ CFU/L, *C.albicans* was exposed during 6 h of incubation to antifungal agent in assay medium supplemented with various amounts (5, 50, and 90% [v/v]) of pooled human serum (PHS; from a pool of 350 serum samples from healthy volunteer donors). Because the presence of

serum induces the formation of germ tubes by *C.albicans*, the antifungal activity was expressed not only in terms of effective killing (>99.9%) of *C.albicans* but also in terms of inhibition of germ tube formation, which was determined microscopically in a hemacytometer.

Antifungal activity of AMB-DOC versus that of FLU against intracellular *C.albicans*. (I) Activity in relation to ratio of infection. Macrophages were obtained from peritoneal cavities of 10- to 13-week-old specified-pathogen-free BALB/c mice (Iffa Credo, L'Arbresle, France). The macrophages were washed twice in Dulbecco modified Eagle medium (Flow Laboratories, Irvine, Scotland) supplemented with 1% glutamine. Monolayers of peritoneal macrophages were cultured at 37°C on chamber slides (Lab-Tek, Bayer, The Netherlands) under a humidified atmosphere of 7.5% CO_2 in air in culture medium containing Dulbecco modified Eagle medium supplemented with 1% glutamine and 15% fetal bovine serum (FBS; Hy-Clone, Logan, Utah). After the first 2 h of incubation, fresh culture medium was added to the monolayer, and the macrophages were incubated for 24 h. After this incubation period, various inocula of opsonized *C.albicans* were added to the monolayers, resulting in increasing *C.albicans*-to-macrophage ratios ranging from 1:40 to 5:8. *C.albicans* was opsonized by incubation of a washed stationary-growth-phase suspension with 10% normal mouse serum (BALB/c) under continuous rotation at 8 rpm for 15 min at 37°C, and then the yeasts were washed twice in PBS. After a 30-min uptake period, the noningested yeasts were removed by washing the monolayer three times with Dulbecco modified Eagle medium supplemented with 1% glutamine and 5% FBS at 37°C (time zero). The macrophages were reincubated for 24 h in the presence of twofold increasing concentrations of the antifungal agent in culture medium supplemented with 5% FBS; the concentrations ranged from 0.05 to 1.6 mg/L for AMB-DOC and 0.4 to 102 mg/L for FLU. Control monolayers were incubated with the solvent of the antifungal agent in the appropriate dilution. Parameters for intracellular antifungal activity were inhibition of germ tube formation as well as killing of intracellular *C.albicans* (>99% of the number of viable intracellular yeasts at time zero). Inhibition of germ tube formation was determined microscopically. At 6 and 24 h of incubation, after the monolayer was washed three times with PBS, the monolayers were fixed in methanol and stained with May-Grünwald-Giemsa, after removal of the plastic chamber from the slide. At the concentrations that inhibited germ tube formation, intracellular killing was determined by washing the monolayers three times with ice-cold (0°C) PBS; this was followed by disruption of the macrophages by quickly freezing and thawing them in the presence of distilled water containing 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). Suspensions were mixed vigorously. Freezing and thawing dit not affect the viability of *C.albicans*. The number of viable intracellular yeasts was determined by making plate counts of 10-fold serial dilutions of the specimen.

The toxicities of AMB-DOC up to 1.6 mg/L and FLU up to 102 mg/L to the macrophages were assessed by determining the effects of the agents on the phagocytic capacity and the membrane integrity of the macrophages. The effects of the antifungal agents on the phagocytic capacity of the macrophages were determined after 24 h of exposure of uninfected macrophage monolayers to the antifungal agents. Control monolayers were incubated with the solvent of the antifungal agent in the appropriate dilution. After the monolayer was washed three times, opsonized *Escherichia coli* 128K$^-$ was added to the monolayers. After a 30-min uptake period, the uningested bacteria were removed by washing the monolayer and the number of viable intracellular bacteria was determined. The effects of the antifungal agents on the membrane integrity of the macrophages was determined by ^{51}Cr release. Uninfected macrophage monolayers were labeled during 1 h with $Na_2{}^{51}CrO_4$ (Amersham International plc., Buckinghamshire, England). After labeling, the monolayer was washed to remove non-cell-associated ^{51}Cr; this was followed by 6 h of exposure to the antifungal agents. Control monolayers were incubated with the solvent of the antifungal agent in the appropriate dilution. By this method, exposure to antifungal agent for 24 h could not be tested because of the high spontaneous ^{51}Cr release, because of passive labeling, from the control monolayers after 24 h. After incubation, ^{51}Cr release was determined by measuring the radioactivity in the supernatants as well as in the disrupted monolayers in a gamma counter (Minaxy 5530; Packard Instrument Co. Inc., Downers Grove, Ill.).

(II) Effect of previous exposure of *C.albicans* to antifungal agent. Monolayers of peritoneal macrophages were cultured as described above. After 24 h of incubation, opsonized *C.albicans* was added to the monolayers, resulting in a *C.albicans*-to-macrophage ratio of 1:8, together with twofold increasing concentrations of the antifungal agent in culture medium supplemented with 5% FBS; antifungal agent concentrations ranged from 0.05 to 1.6 mg/L for AMB-DOC and 0.4 to 102 mg/L for FLU. After a 30-min uptake period, the monolayer was washed to remove uningested yeasts and antifungal agent and was reincubated for 24 h in the presence of antifungal agent. Control monolayers were reincubated with the solvent of the antifungal activity in the appropriate dilution. Intracellular antifungal activity was determined at 6 and 24 h of incubation, as described above.

(III) Antifungal activity of a combination of AMB-DOC and FLU against intracellular *C.albicans*. Monolayers of peritoneal macrophages were cultured as described above. After 24 h of incubation, opsonized *C.albicans* was added to the monolayers, resulting in a *C.albicans*-to-macrophage ratio of 1:8. After a 30-min uptake period, the uningested yeasts were removed by washing the monolayer. The macrophages were reincubated for 24 h in the presence of combinations of the antifungal agents in culture medium supplemented with 5% FBS; antifungal agent concentrations ranged from 0.1 to 1.6 mg/L for AMB-DOC and 0.4 to 102 mg/L for FLU. Control monolayers were reincubated with the solvent of the agent in the

appropriate dilution. Intracellular antifungal activity was determined at 6 and 24 h of incubation, as described above. The effect of combinations of AMB-DOC and FLU on the extracellular growth of *C.albicans* during 24 h of incubation was tested, after the addition of fourfold increasing FLU concentrations ranging from 0.4 to 102 mg/L to AMB-DOC at 0.2 mg/L by the method described above.

RESULTS

Antifungal activity of AMB-DOC versus that of FLU against extracellular *C.albicans* in relation to inoculum size. (I) MIC and MFC. At a *C.albicans* inoculum of $1.3x10^7$ CFU/L, the MIC and MFC of AMB-DOC were 0.1 and 3.2 mg/L, respectively, and did not change at an inoculum size increased to $1.0x10^8$. The MIC and FLU was 0.8 mg/L and could be assessed only by using the lowest inoculum ($1.3x10^7$ CFU/L). FLU did not show fungicidal activity when it was tested up to 102 mg/L.

(II) Short-term growth. The survival of *C.albicans* at various concentrations of AMB or FLU during 6 h of incubation at 37°C is shown in Figure 1.

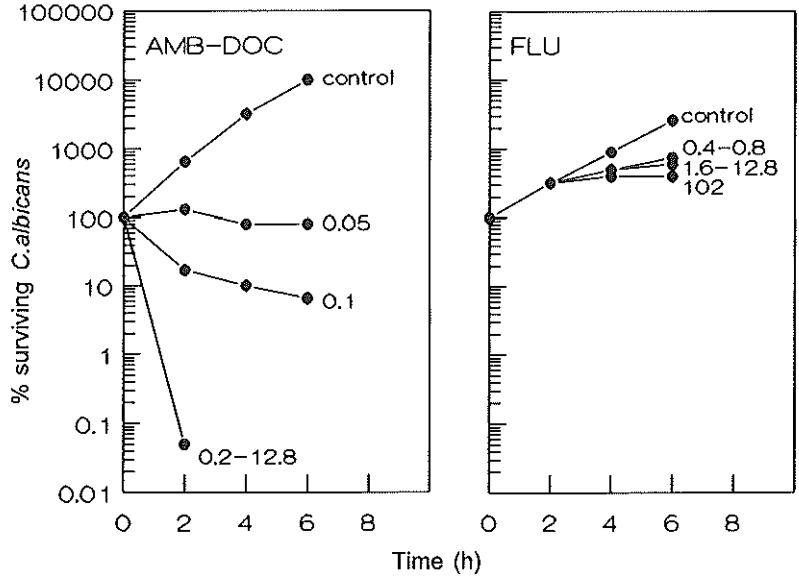

Figure 1. *Antifungal activity of AMB-DOC and FLU at the indicated concentrations (mg/L) against extracellular C.albicans during short-term growth at an inoculum size of $1.3x10^7$ CFU/L in the logarithmic phase of growth. The control contained only the solvent of the antifungal agent at the appropriate dilution. Each symbol represents the mean of three experiments.*

Because the MIC of FLU could be assessed only at $1.3x10^7$ CFU/L, the antifungal effect on the short-term growth of *C.albicans* was initially determined at this inoculum size. With AMB-DOC, killing of *C.albicans* (>99.9%) was obtained at 0.2 mg/L. Killing was not affected by an increase in the inoculum by 8- or 64-fold, respectively (data not shown). FLU tested up to a concentration of 102 mg/L did not result in the killing of *C.albicans* but only inhibited fungal growth. A reduction in the inoculum size by 8- or 64-fold, respectively, did not result in the killing of *C.albicans* (data not shown).

The effect of serum on the antifungal activity of AMB-DOC against extracellular *C.albicans* is presented in Table 1. The minimal AMB-DOC concentration required for the inhibition of germ tube formation as well as for effective killing (>99.9%) of *C.albicans* in the presence of serum are given. FLU was not tested, since it is known that protein binding is low for FLU. In the presence of 5% PHS, inhibition of germ tube formation as well as effective killing were observed at an AMB-DOC concentration of 0.2 mg/L. An increase in the amount of serum did result in an increase in the concentration of AMB required to obtain the effects. Eventually, compared with the effect of 5% PHS, the presence of 90% PHS resulted in a 4-fold increase in the concentrations required to inhibit germ tube formation, whereas a 16-fold increase in concentration was needed to obtain effective killing.

Table 1. *Effect of serum on the antifungal activity of AMB-DOC against extracellular C.albicans during 6 h of incubation.*[a]

Medium		Minimal AMB concn (mg/L) required for:	
		Inhibition of germ tube formation	Effective killing (>99.9%)
Serum-free			0.2
PHS	5%	0.2	0.2
	50%	0.4	1.6
	90%	0.8	3.2

[a]*C.albicans* was used at an inoculum size of $1.3x10^7$ CFU/L
[b]Percentages of PHS are by volume

Antifungal activity of AMB-DOC versus that of FLU against intracellular *C.albicans*. (I) Activity in relation to ratio of infection. The minimal concentrations of AMB-DOC and FLU required for the inhibition of germ tube formation as well as for effective killing (>99.9%) of *C.albicans* at 6 and 24 h of incubation at various ratios of infection are given in Table 2. In the absence of antifungal agent, *C.albicans* grows intracellularly by the formation of germ tubes, and within 24 h mycelium is formed. At a *C.albicans*-to-macrophage ratio of 1:8, inhibition of germ

tube formation and killing were observed within the first 6 h of incubation at AMB concentrations of 0.2 and 0.4 mg/L, respectively. After prolonged incubation (24 h), this activity was not changed. At a fivefold increased ratio of infection, twofold increased AMB concentrations were required to obtain both effects. FLU tested up to 102 mg/L was not effective intracellularly during 6 h of incubation. After prolonged incubation (24 h), germ tubes and hyphae were still observed when FLU was tested up to 102 mg/L. However, the formation of mycelium was inhibited. A fivefold reduction in the ratio of infection also did not result in intracellular activity of FLU. These data were derived from three separate experiments, in which each concentration was tested in triplicate.

Cellular toxicities of the antifungal agents measured in terms of inhibition of the phagocytic capacity of the macrophages or a decrease in membrane integrity were not observed with AMB-DOC or FLU when they were tested at concentrations up to 1.6 and 102 mg/L, respectively (data not shown).

Table 2. *Antifungal activity of AMB-DOC versus that of FLU after 6 and 24 h of incubation against intracellular C.albicans in relation to ratio of infection.*[a]

Antifungal agent and *C.albicans* : macrophage ratio	Minimal concn (mg/L) required for:			
	Inhibition of germ tube formation		Effective killing (>99%)	
	6 h	24 h	6 h	24 h
AMB-DOC				
1:8	0.2	0.2	0.4	0.4
5:8	0.4	0.4	0.8	0.8
FLU				
1:8	>102	>102	ND	ND
1:40	>102	>102	ND	ND

[a]Antifungal agent was present in the incubation medium after *C.albicans* ingestion
ND = not done

(II) Effect of previous exposure of *C.albicans* to antifungal agent. The minimal concentrations of AMB-DOC and FLU required for the inhibition of germ tube formation as well as for effective killing (>99%) of *C.albicans* and *C.albicans*-to-macrophage ratio of 1:8 after 6 and 24 h of incubation are given in Table 3. Exposure of *C.albicans* for 30 min to one of the antifungal agents at the concentrations used during macrophage ingestion did not reduce the number of viable intracellular yeasts after 30 min of ingestion. With AMB-DOC, inhibition of germ tube formation and killing were observed within 6 h of incubation at

concentrations of 0.1 and 0.2 mg/L, respectively. FLU tested up to 102 mg/L was not effective intracellularly. These data were derived from three separate experiments, in which each concentration was tested in triplicate.

Table 3. *Antifungal activity of AMB-DOC versus that of FLU after 6 and 24 h of incubation against intracellular C.albicans: effect of previous exposure to antifungal agent.*

Antifungal agent and *C.albicans* : macrophage ratio	Minimal concn (mg/L) required for:			
	Inhibition of germ tube formation		Effective killing (>99%)	
	6 h	24 h	6 h	24 h
AMB-DOC				
1:8[a]	0.2	0.2	0.4	0.4
1:8[b]	0.1	0.1	0.2	0.2
FLU				
1:8[a]	>102	>102	ND	ND
1:8[b]	>102	>102	ND	ND

[a]Antifungal agent was present in the incubation medium after *C.albicans* ingestion
[b]Antifungal agent was present in the incubation medium during and after *C.albicans* ingestion
ND = not done

(II) Antifungal activity of a combination of AMB-DOC and FLU against intracellular *C.albicans*. The antifungal activity of combinations of AMB-DOC and FLU against intracellular *C.albicans* at a *C.albicans*-to-macrophage ratio of 1:8 after 24 h of incubation in terms of inhibition of germ tube formation as well as effective killing (>99%) are shown in Tables 4 and 5, respectively. In the absence of FLU, inhibition of germ tube formation and effective killing were observed at AMB-DOC concentrations of 0.2 and 0.4 mg/L, respectively. The addition of FLU up to 102 mg/L to AMB-DOC did not result in a reduction of the AMB-DOC concentrations required for inhibition of germ tube formation and effective killing. In contrast, at combinations of AMB-DOC at 0.2 mg/L plus FLU at 1.6 mg/L and of AMB-DOC at 0.4 mg/L plus FLU at 1.6 mg/L, inhibition of germ tube formation and effective killing, respectively, were no longer observed. In the presence of FLU at concentrations from 1.6 mg/L, an increase in the AMB-DOC concentration up to 0.8 mg/L was required to obtain both effects. After 6 h of incubation, this antagonistic effect of FLU was not observed (data not shown). These data were derived from three separate experiments, in which each concentration was tested in triplicate.

Regarding the effect of combinations of AMB-DOC with FLU on the extracellular growth of *C.albicans*, we did observe a slight antagonistic effect of FLU during 24 h of incubation after the addition of fourfold increasing FLU concentrations, ranging from 0.4 to 102 mg/L, to AMB-DOC at 0.2 mg/L (data not shown).

Table 4. *Antifungal activity of combinations of AMB-DOC and FLU at various concentrations against intracellular C.albicans[a] after 24 h of incubation: inhibition of germ tube formation.*

AMB concn (mg/L)	Antifungal activity with FLU concn (mg/L) of:									
	102	51.2	25.6	12.8	6.4	3.2	1.6	0.8	0.4	0
1.6	+		+		+		+	+	+	+
0.8	+		+		+		+	+	+	+
0.4	—		—		—		—	+	+	+
0.2	—		—		—		—	+	+	+
0.1	—		—		—		—	—	—	—
0	—	—	—	—	—	—	—	—	—	—

[a]The *C.albicans*-to-macrophage ratio was 1:8
+, inhibition of germ tube formation; —, no inhibition of germ tube formation

Table 5. *Antifungal activity of combinations of AMB-DOC and FLU at various concentrations against intracellular C.albicans[a] after 24 h of incubation: effective killing (>99%).*

AMB concn (mg/L)	Antifungal activity with FLU concn (mg/L) of:									
	102	51.2	25.6	12.8	6.4	3.2	1.6	0.8	0.4	0
1.6	+		+		+		+	+	+	+
0.8	+		+		+		+	+	+	+
0.4	—		—		—		—	+	+	+
0.2	—		—		—		—	—	—	—
0.1	—		—		—		—	—	—	—
0	—	—	—	—	—	—	—	—	—	—

[a]The *C.albicans*-to-macrophage ratio was 1:8
+, effective killing; —, no effective killing

DISCUSSION

The observations of the activity of AMB-DOC against extracellular *C.albicans* after an incubation period of 24 h showed that, in the absence of serum, AMB-DOC is highly active in terms of growth inhibition and killing of *C.albicans*. This antifungal activity was maintained when the inoculum was increased. The presence of serum greatly influenced the activity of AMB-DOC against extracellular *C.albicans*. This is in agreement with observations of others [23], who found a twofold increase in the MIC and MFC of AMB-DOC when *C.albicans* was exposed to AMB-DOC in the presence of 25% PHS. This reduction of antifungal activity in the presence of serum may be explained by the high protein binding of AMB-DOC, resulting in reduced bioavailability.

FLU was not active in terms of killing of extracellular *C.albicans*. Only an inhibitory effect by FLU was observed at a low inoculum. This is in agreement with the observations of other investigators [16,28,32,37,38]. Since protein binding is reported to be low for FLU [1,14,17], the effect of serum on its antifungal activity was not tested.

The antifungal effect against intracellular *C.albicans* was studied by using monolayers of murine peritoneal macrophages infected with *C.albicans*. The experimental design varied with respect to different ratios of infection, exposure of *C.albicans* to one of the antifungal agents prior to macrophage ingestion, and exposure to the antifungal agents alone or in combination. It is known that resident peritoneal macrophages are unable to kill *C.albicans* during incubation *in vitro* and are even unable to prevent intracellular germ tube formation [2,3,7,24,38]. As a consequence, the antifungal effect on intracellular *C.albicans* could be investigated without interference from the direct fungistatic or fungicidal effects of the macrophages themselves. The intracellular efficacies of the antifungal agents were determined in terms of inhibition of germ tube formation as well as effective killing of *C.albicans* intracellularly. With AMB-DOC, inhibition of the development of germ tubes was found at concentrations lower than those required for killing of intracellular *C.albicans*, which has also been reported by others [25,26,38]. After an increase in the ratio of infection, slightly increased AMB concentrations were required to obtain both effects. The cellular toxicity of AMB-DOC in terms of a decrease in membrane integrity (^{51}Cr release) or a reduction in the phagocytic capacity of the macrophages (*E.coli* phagocytosis) was not observed when macrophages were exposed to AMB-DOC for 6 and 24 h, respectively. Other investigators [20] reported, however, that AMB-DOC is highly suppressive to macrophage differentiation, as measured by transglutaminase induction, and to effector functions, as measured by superoxide anion release after 16 h of incubation. Immunostimulatory effects of AMB-DOC after *in vitro* exposure of peritoneal macrophages have also been reported. Increased phagocytosis of polystyrene beads in macrophages after *in vitro* exposure to AMB-DOC was

observed [19]. In our study, however, an increase in the phagocytosis of *E.coli* was not found. The discrepancies in these observations cannot readily be explained.

Exposure of *C.albicans* to AMB-DOC prior to macrophage ingestion slightly influenced the antifungal activity of AMB-DOC in terms of a decrease in the AMB concentrations required for inhibition of germ tube formation as well as for the effective killing of intracellular *C.albicans*.

The antifungal activity of AMB-DOC was observed within 6 h. Prolonged incubation (24 h) did not change this effect, provided that AMB-DOC was present in the incubation medium. It seemed that the continuous presence of AMB-DOC extracellularly was necessary to maintain intracellular antifungal activity.

FLU was not effective intracellularly when macrophages were exposed to FLU for 6 h, not even at a reduced *C.albicans*-to-macrophage ratio, nor after exposure of *C.albicans* to FLU prior to macrophage ingestion. Although the formation of mycelium was inhibited after prolonged incubation (24 h), germ tubes and hyphae were still observed with FLU, and therefore, FLU was not effective intracellularly. The results of the activity of FLU against intracellular *C.albicans* are in agreement with the results of activity against extracellular *C.albicans*, in which no complete growth inhibition was observed. It has been reported by others [42] that FLU, despite its observed good intracellular penetrating capacity, did not have a significant effect on the killing of intracellular *C.albicans* or on the formation of germ tubes within 4 h of incubation of *C.albicans*-infected macrophage monolayers derived from human blood monocytes, which is in agreement with the results of our study. Those investigators [42] also mentioned the prevention of the formation of mycelium by FLU after 24 h of incubation and reported a significant growth inhibition of *C.albicans*, as measured by CFU counts of viable intracellular *C.albicans*. CFU counts of intracellular *C.albicans* can only be determined accurately when germ tube formation is completely inhibited. In the presence of germ tubes, the CFU count may be an overestimation of viable *C.albicans* because of fragmentation of the germ tubes during disruption of the monolayer, giving rise to an increase in CFU. On the other hand, the CFU count can be an underestimation of viable *C.albicans*, since viable *C.albicans* may be washed away after destruction of the macrophages by germ tubes. Other investigators [38] described that FLU had only a minimal effect on the number of viable intracellular *C.albicans* in murine macrophage monolayers, but that it significantly inhibited germ tube formation within 4 h of incubation. The number of yeasts that produced germ tubes as well as the length of the germ tubes appeared to be significantly reduced, as measured microscopically. The intracellular activities of antifungal agents can only be defined as complete inhibition of germ tube formation, at the least.

To investigate whether the AMB concentrations required for intracellular activity could be reduced further, combinations of AMB-DOC and FLU were tested. However, the potentiation of intracellular activity was not observed after

coincubation of AMB-DOC with FLU. In contrast, antagonism between FLU and AMB-DOC was found with respect to intracellular *C.albicans*. This antagonism was observed only after 24 h of incubation but was not observed after 6 h of incubation. With respect to extracellular *C.albicans*, a slight antagonistic effect between FLU and AMB-DOC measured during 24 h of incubation was observed, as was also recently described by others [30]. They speculated that competition for binding sites on *C.albicans* could be responsible for this antagonism. Intracellular activity could also be reduced by competition during intracellular penetration. However, these explanations for the observed antagonism are speculative, and further study is needed for elucidation.

In summary, it was observed that although the activity of AMB-DOC against extracellular *C.albicans* was high, the antifungal activity was influenced by factors relevant to its efficacy in the treatment of infection. The antifungal activity of AMB-DOC was substantially reduced by the presence of serum; however, it was only slightly hindered by the intracellular localization of the yeasts. At an increased ratio of infection of intracellular *C.albicans*, the antifungal activity of AMB-DOC was slightly reduced. For FLU, antifungal activity against extracellular *C.albicans* was restricted to inhibition of fungal growth, despite the use of optimal test conditions, such as the recommended assay medium and the use of low *C.albicans* inocula, whereas FLU was not effective against intracellular *C.albicans*. Therefore, it is questionable whether FLU effectively contributes to the eradication of *C.albicans* when host defense mechanisms are severely impaired. A slight antagonistic effect between FLU and AMB-DOC was found with respect to intracellular as well as extracellular *C.albicans*.

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REFERENCES

1. Brammer KW. Pharmacokinetics and tissue penetration of fluconazole in humans. Rev Infect Dis 1990;12(Suppl.3):S318-26.
2. Brummer E, Stevens DA. Candidacidal mechanisms of peritoneal macrophages activated with lymphokines or γ-interferon. J Med Microbiol 1989;28:173-81.
3. Cummings NP, Pabst MJ, Johnston RB. Activation of macrophages for enhanced release of superoxide anion and greater killing of *Candida albicans* by injection of muramyl dipeptide. J Exp Med 1980;152:1659-69.
4. Dismukes WE. Azole antifungal drugs: old and new. Ann Intern Med 1988;109:177.
5. Drutz DJ. *In vitro* antifungal susceptibility testing and measurement of levels of antifungal agents in body fluids. Rev Infect Dis 1987;9:392-7.
6. Espinel-Ingroff A, Shadomy S. *In vitro* and *in vivo* evaluation of antifungal agents. Eur J Clin Microbiol Infect Dis 1989;8:352-61.

7. Evron R. *In vitro* phagocytosis of *Candida albicans* by peritoneal mouse macrophages. Infect Immun 1980;28:963-71.
8. Fisher MA, Lee PG, Tarry WF. Fluconazole (UK-49,858) treatment of candidiasis in normal and diabetic rats. Antimicrob Agents Chemother 1989;33:1042-5.
9. Fisher MA, Shen SH, Haddad J, Tarry WF. Comparison of *in vivo* activity of fluconazole with that of amphotericin B against *Candida tropicalis, Candida glabrata,* and *Candida krusei.* Antimicrob Agents Chemother 1989;33:1443-6.
10. Fromtling RA. Overview of medically important antifungal azole derivatives. Clin Microbiol Rev 1988;1:187.
11. Galgiani JN. Antifungal susceptibility tests. Antimicrob Agents Chemother 1987;31: 1867-70.
12. Galgiani JN. Susceptibility of *Candida albicans* and other yeasts to fluconazole: relation between *in vitro* and *in vivo* studies. Rev Infect Dis 1990;12(Suppl.3): S272-5.
13. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-29.
14. Grant SM, Clissold SP. Fluconazole. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in superficial and systemic mycoses. Drugs 1990;39:877-916.
15. Horn RB, Wong B, Kiehn TE, *et al.* Fungemia in a cancer hospital: changing frequency, earlier onset, and results of therapy. Rev Infect Dis 1985;76:646-55.
16. Hughes CE, Beggs WH. Action of fluconazole (UK-49,858) in relation to other systemic antifungal azoles. J Antimicrob Chemother 1987;19:171-4.
17. Humphrey MJ, Jevons S, Tarbit MH. Pharmacokinetic evaluation of U.K.-49,858, a metabolically stable triazole antifungal in animals and humans. Antimicrob Agents Chemother 1985;28:648-53.
18. Kobayashi GS, Spitzer ED. Testing of organisms for susceptibility to triazoles: is it justified? Eur J Clin Microbiol Infect Dis 1989;8:387-9.
19. Lin HS, Medoff G, Kobayashi GS. Effects of amphotericin B on macrophages and their precursor cells. Antimicrob Agents Chemother 1977;11:154-60.
20. Mehta RT, Mehta K, Lopez-Berestein G, Juliano RL. Effect of liposomal amphotericin B on murine macrophages and lymphocytes. Infect Immun 1985;47:429-33.
21. Meunier F. Fungal infections in the compromised host. In: Rubin RH, Young LS (Eds.) Clinical approach to infection in the compromised host. Plenum Publishing Corporation, New York. 1988:193-220.
22. Meunier F. Candidiasis. Eur J Clin Microbiol Infect Dis 1989;8:438-47.
23. Meunier F, Lambert C, Van der Auwera P. *In vitro* activity of SCH39304 in comparison with amphotericin B and fluconazole. J Antimicrob Chemother 1990;25:227-36.
24. Morrison RP, Cutler JE. *In vitro* studies of the interactions of murine phagocytic cells with *Candida albicans.* J Reticuloendothel Soc 1981;29:23-34.
25. Nugent KM, Couchot KR. Effects of sublethal concentrations of amphotericin B on *Candida albicans.* J Infect Dis 1986;154:665-9.
26. Nugent KM, Couchot KR, Gray LD. The effect of *Candida* morphology on amphotericin B susceptibility. Antimicrob Agents Chemother 1987;31:335-6.
27. Odds FC, Abbott AB. Relative inhibition factors - a novel approach to the assessment of antifungal antibiotics *in vitro.* J Antimicrob Chemother 1984;13:31-43.
28. Odds FC, Cheesman SL, Abbott AB. Antifungal effects of fluconazole (UK 49858), a new triazole antifungal, *in vitro.* J Antimicrob Chemother 1986;18:473-8.
29. Perfect JR, Savani DV, Durack DT. Comparison of itraconazole and fluconazole in treatment of cryptococcal meningitis and candida pyelonephritis in rabbits. Antimicrob Agents Chemother 1986;29:579-83.

30. Petrou MA, Rogers TR. Interactions *in vitro* between polyenes and imidazoles against yeasts. J Antimicrob Chemother 1991;27:491-506.
31. Richardson K, Brammer KW, Marriot MS, Troke PF. Activity of UK-49,858, a bistriazole derivative against experimental infections with *Candida albicans* and *Trichophyton mentagrophytes*. Antimicrob Agents Chemother 1985;27:832-5.
32. Rogers TE, Galgiani JN. Activity of fluconazole (UK 49,858) and ketoconazole against *Candida albicans in vitro* and *in vivo*. Antimicrob Agents Chemother 1986;30:418-22.
33. Saag MS, Dismukes WE. Azole antifungal agents: Emphasis on new triazoles. Antimicrob Agents Chemother 1988;32:1-8.
34. Stevens DA. The new generation of antifungal drugs. Eur J Clin Microbiol Infect Dis. 1988;7:732-5.
35. Troke PF, Andrews RJ, Brammer KW, Marriot MS, Richardson K. Efficacy of UK-49,858 (fluconazole) against *Candida albicans* experimental infections in mice. Antimicrob Agents Chemother 1985;28:815-8.
36. Troke PF, Andrews RJ, Pye GW, Richardson K. Fluconazole and other azoles: translation of *in vitro* activity to *in vivo* and clinical efficacy. Rev Infect Dis 1990; 12(Suppl.3):S276-80.
37. Van 't Wout JW, Mattie H, Van Furth R. The efficacy of amphotericin B, fluconazole and itraconazole against systemic candidiasis in normal and neutropenic mice. Antimicrob Agents Chemother 1989;33:147-51.
38. Van 't Wout JW, Meynaar I, Linde I, Mattie H, Van Furth R. Effect of amphotericin B, fluconazole and itraconazole on intracellular *C.albicans* and germ tube development in macrophages. J Antimicrob Chemother 1990;25:803-11.
39. Walsh TJ, Aoki S, Mechinaud F, Bacher J, Lee J, Rubin M, Pizzo PA. Effects of preventive, early, and late antifungal chemotherapy with fluconazole in different granulocytopenic models of experimental disseminated candidiasis. J Infect Dis 1990; 161:755-60.
40. Walsh TJ, Lee J, Aoki S, Mechinaud F, Bacher J, Lecciones J, Thomas V, Rubin M, Pizzo PA. Experimental basis for use of fluconazole for preventive or early treatment of disseminated candidiasis in granulocytopenic hosts. Rev Infect Dis 1990;12(Suppl.3): S307-17.
41. Walsh TJ, Pizzo PA. Treatment of systemic fungal infections: recent progress and current problems. Eur J Clin Microbiol 1988;7:460-75.
42. Wildfeuer A, Laufen H, Haferkamp O. Interaction of fluconazole and human phagocytic cells. Arzneim-Forsch/Drug Res 1990;40:1044-7.

Chapter 3

Efficacies of amphotericin B-desoxycholate (Fungizone®), liposomal amphotericin B (AmBisome®) and fluconazole in the treatment of systemic candidiasis in immunocompetent and leukopenic mice

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ABSTRACT

The *in vitro* activities of amphotericin B-desoxycholate (AMB-DOC), liposomal amphotericin B (AmBisome®) and fluconazole (FLU) were determined against a single strain of *Candida albicans*. In addition, the efficacies of these agents in the treatment of systemic candidiasis in both immunocompetent and leukopenic mice were compared. The minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of AMB-DOC and AmBisome® were similar, but on the basis of time-kill studies, the fungicidal activity of AmBisome® was significantly less than that of AMB-DOC.

In immunocompetent mice, the dosage of AMB-DOC was limited by toxicity, resulting in a maximum tolerated dosage (MTD) of 0.4 mg AMB/kg/day during treatment for 5 days; AmBisome® was less toxic, the MTD being 7 mg AMB/kg/day following a treatment period of the same duration. Both AMB-DOC and AmBisome® led to significant reductions in the numbers of *C.albicans* in the kidneys of these mice and prevented relapse of infection after completion of treatment. FLU in dosages of 0.4 and 64 mg FLU/kg/day resulted in initial reductions in the numbers of CFU but failed to prevent relapse.

In leukopenic mice, treatment for 5 days with AMB-DOC in a dosage of 0.3 mg AMB/kg/day resulted in survival of the animals and a significant reduction in the numbers of CFU in the liver, spleen and lungs. However, there was no reduction in the number of CFU in the kidneys and this led to relapse of infection once therapy was terminated. FLU in a dosage of 64 mg FLU/kg/day produced effects which were similar to those of AMB-DOC; prolonged treatment with FLU for 18 days did not improve the efficacy of this agent. Only treatment with high-dosage (7 mg AMB/kg/day) AmBisome® was effective in significantly reducing the numbers of CFU of *C.albicans* in the kidneys and other organs of leukopenic mice, as well as preventing relapse, even in severely infected animals.

INTRODUCTION

Deep-seated fungal infection is an important cause of morbidity and mortality in immunocompromised patients [22,23]. Currently, the treatment of choice for most systemic mycoses is amphotericin B-desoxycholate (AMB-DOC), although the amount of this drug which can be administered is limited by a number of side-effects, some of which may be serious [6,10,23,41]. There is, therefore, a need to identify effective and less toxic alternatives for the treatment of patients with life-threatening fungal infections. One such agent is the triazole, fluconazole (FLU) [6,12,41]. Both experimental studies and data in humans have shown that FLU is effective therapy for disseminated candidiasis [2,7-9,18,26,30,32,36,38,43]. The efficacy of FLU as prophylaxis or early treatment has also been demonstrated in persistently granulocytopenic rabbits [39,40], but there is a paucity of evidence to support the efficacy of this drug as treatment for immunocompromised hosts with systemic candidiasis.

Another promising approach in the treatment of invasive fungal infections is amphotericin B (AMB) which has been incorporated into liposomes or other lipid carriers [25,42]. In both experimental [1,5,11,14-16,20,21,27,35]and human studies [4,17,19,34,24,28,31,33] a reduction in the incidence of side-effects and an improvement in the therapeutic index have been reported for various lipid-associated formulations of AMB. One such preparation is AmBisome® which has been shown to be effective in the treatment of immunocompetent mice with candidiasis [1,11]. However, its efficacy in leukopenic mice with severe disseminated candidiasis has not yet been determined. In the present study, we have compared the efficacies of FLU, liposomal amphotericin B (AmBisome®) and AMB-DOC as therapy for both immunocompetent and leukopenic mice with invasive candidiasis.

MATERIALS AND METHODS

Candida strains. *Candida albicans* ATCC 44858 was used in all of the experiments and was stored at -80°C in Todd-Hewitt broth (Difco Laboratories, Detroit, MI, USA) containing 10% (v/v) glycerol.

Antifungal agents. AMB-DOC (Fungizone®; Bristol Myers-Squibb, Woerden, The Netherlands) was reconstituted with distilled water to obtain a stock solution of 5 g/L. AmBisome®, AMB encapsulated in small unilamellar vesicles consisting of hydrogenated soybean phosphatidylcholine, cholesterol and distearoylphosphatidyl-glycerol in a molar ratio of 2:1:0.8, was kindly provided by Vestar Inc. (San Dimas, CA, USA) as a lyophilized preparation. The powder was reconstituted according to the manufacturer's instructions with distilled water at 65°C to give a liposomal suspension containing 4 g AMB/L and 35 g lipid/L. FLU was provided by Pfizer Nederland B.V. as a stock solution containing 2g/L.

Animals. Specified pathogen-free, 10- to 13-week-old, female BALB/c mice were obtained from Iffa Credo (L'Arbresle, France).

Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs). MICs and MFCs were determined by a broth dilution method as described previously [37]. Two-fold serial dilutions of the antifungal agents in concentrations ranging from 0.025-102 mg/L were prepared in Antibiotic Medium No. 3 (Difco Laboratories). Stationary growth phase cultures of the *Candida albicans* strain were obtained after incubation in Sabouraud maltose broth (Difco Laboratories) for 24 h at 37°C. The organisms were washed in phosphate-buffered saline (PBS), counted in an hemocytometer and adjusted to the desired inoculum in Antibiotic Mediuim No. 3; the suspension contained $1.3x10^7$ CFU/L. The MIC was defined as the lowest concentration of each antifungal agent which inhibited visible growth after 24h of incubation at 37°C. Samples from clear tubes were washed in PBS and inoculated on to solid medium. The MFC was defined as the lowest concentration which killed > 99.9% of the original inoculum after overnight incubation at 37°C.

***In vitro* activities of AMB-DOC and AmBisome® during short-term exposure.** The fungicidal activities of AMB-DOC and AmBisome® were determined by a killing curve method as described previously [37]. Briefly, two-fold serial dilutions of each agent in concentrations ranging from 0.05-25.6 mg/L were prepared in Antibiotic Medium No. 3. A logarithmic growth phase culture of *C.albicans* was diluted and added to each tube to give an initial suspension of $1.3x10^7$ CFU/L. The tubes were incubated for 6 h at 37°C. Samples were withdrawn at 2-h intervals and the numbers of viable organisms were determined.

Determination of maximum tolerated dosage (MTD). The MTDs of AMB-DOC and AmBisome® were determined in uninfected mice and expressed in terms of death of the animals during the course of treatment or a significant change in renal and/or hepatic function in the survivors. Twenty mice (two groups of ten) received the targeted dosage of either AMB-DOC (ranging from 0.1 to 1.5 mg AMB/kg/day) or AmBisome® (ranging from 1 to 32 mg AMB/kg/day) by the iv route for 5 consecutive days. Dosages of AMB-DOC of 0.1-1.0 mg AMB/kg/day were reached by increments of 0.1 mg AMB/kg/day and a dosage of 1.5 mg AMB/kg/day was reached by a single increment of 0.5 mg AMB/kg/day; dosages of AmBisome® were reached by increments of ≥ 1 mg AMB/kg/day. Time until death was monitored from administration of the antifungal agent. Abnormalities of renal function in surviving mice were detected by measuring the serum urea and creatinine concentrations; abnormalities of hepatic function were detected by measuring the serum aspartate aminotransferase and alanine aminotransferase concentrations. All biochemical parameters were determined by established methods (Merck Diagnostica, Darmstadt, Germany) from samples of blood obtained from the surviving mice in one cohort which were killed 24 h after termination of treatment and from those in a second cohort killed 14 days after termination of treatment. The

MTD was defined as the maximum dosage which did not lead to death during treatment or to a statistically significant increase in the concentrations of the biochemical parameters used to monitor renal and hepatic functions compared with the concentrations of these indices in untreated mice (two groups of ten which were killed at the same time intervals as the treated mice).

Efficacies of AMB-DOC, AmBisome® and FLU in infected immunocompetent mice. Mice were infected by the iv inoculation of $5x10^4$ CFU of *C.albicans* into the tail vein. AMB-DOC and AmBisome® were each administered once daily by the iv route to three groups of ten mice. The dosages of the two agents corresponded to their MTDs which had been determined previously in immunocompetent mice infected with *C.albicans* according to the method and criterion (i.e. death) described above for uninfected mice; for comparison, AmBisome® was also administered to separate cohorts in a dosage which was equivalent to the MTD of AMB-DOC. FLU, in a range of dosages up to 64 mg FLU/kg/day, was administered twice daily by the ip route to three groups of ten mice. Additional cohorts of ten mice received one of the following placebos: desoxycholate (DOC; Sigma Chemical Co., St Louis, MO, USA) administered iv once daily, liposomes without AMB (placebo liposomes, PLACEBO-LIP) administered iv once daily, or PBS administered ip twice daily. Treatment with the antifungal agents and placebos was started 48 h after infection was initiated, and continued for 5 consecutive days. Just after the start of treatment and at 24 h and 14 days after treatment had been completed, surviving animals in separate groups of ten mice receiving each of the regimens were killed. The kidneys were removed and homogenized in 20 mL of PBS for 30 sec at 10,000 rpm in VirTis homogenizer (The VirTis Co. Inc., Gardiner, NY, USA). The antifungal agents were eliminated from the homogenates by washing three times with PBS. Serial ten-fold dilutions of the homogenates were prepared in PBS and 0.2 mL samples of each dilution, as well as 2 mL samples of the undiluted homogenates, were spread on to Sabouraud dextrose agar (SDA, Oxoid, Basingstoke, UK); large (20 cm) diameter plates were used for the 2 mL samples. The remainder of each homogenate, together with an equal volume of double-concentrated SDA, were poured into plastic plates. Following incubation at 37°C for 48 h, the numbers of *C.albicans* CFU in the kidneys were calculated and expressed as the geometric means. The following criteria were used to assess the efficacy of treatment: a statistically significant reduction in the numbers of CFU in the kidneys 24 h after termination of treatment compared with those at the start of treatment; a statistically significant reduction in the numbers of CFU in the kidneys of antifungal-treated mice compared with those in the kidneys of mice receiving the placebos; and prevention of a significant increase in the numbers of CFU in the kidneys 14 days after termination of treatment compared with the numbers 24 h after termination of treatment (equating to relapse of infection).

In order to determine if treatment prevented hyphal growth, separate cohorts of mice (each consisting of ten animals) were killed at 24 h or 14 days after

termination of treatment. The kidneys were removed and fixed in 10% buffered formalin. Following sectioning and staining by the periodic acid-Schiff method, the tissues were examined microscopically.

Efficacies of AMB-DOC, AmBisome® and FLU in infected leukopenic mice. Leukopenia was induced by the ip administration of cyclophosphamide (Sigma Chemical Co.); a dosage of 100 mg/kg was administered 4 days before initiation of infection followed by additional doses of 75 mg/kg on the day of inoculation and at 3-day intervals thereafter whilst the mice were still alive. This resulted in persistent granulocytopenia ($<0.1 \times 10^9$/L) from the onset of infection. The mice were infected by the iv inoculation of 10^4 CFU of the *C.albicans* strain into the tail vein. AMB-DOC and AmBisome® were each administered iv once daily to groups of ten mice in dosages which corresponded to their MTDs (determined previously in infected leukopenic mice according to the method and criterion i.e. death during treatment described above for uninfected mice); for comparison, AmBisome® was also given to separate cohorts in a dosage which was equivalent to the MTD of AMB-DOC. FLU, in a range of dosages up to 64 mg FLU/kg/day, was administered twice daily by the ip route. The placebos were the same as those used in the experiment with immunocompetent mice. The efficacy of treatment in relation to severity of infection was investigated by commencing therapy with AMB-DOC, AmBisome®, FLU or one of the three placebos at 6 h, 16 h or 24 h after inoculation and continuing for 5 consecutive days. In a separate experiment, the efficacies of prolonged treatment with FLU and as maintenance therapy following initial treatment with AmBisome® were evaluated. The treatment regimens were commenced 6 h after initiation of infection and consisted of the administration of FLU for 18 days, AmBisome® for 5 days or AmBisome® for 5 days followed by FLU for a further 13 days. Just after the start of treatment and at 24h and 14 days after completing treatment, the surviving animals in separate groups which initially comprised ten mice were killed. The kidneys, liver, spleen and lungs were removed and processed for the determination of viable counts and histological examination as described above for the immunocompetent animals. The efficacies of the various treatment regimens were also assessed according to the criteria described above for immunocompetent mice and, additionally, in terms of percentage survival.

AMB concentrations in the blood and tissues of leukopenic mice following treatment with AMB-DOC and AmBisome®. In a separate experiment with different cohorts of leukopenic mice infected with *C.albicans*, treatment with AMB-DOC or AmBisome® in dosages which corresponded to their MTDs was initiated 6 h after inoculation and continued for 5 days as described previously. At 24 h and 14 days after completion of treatment, the mice were killed, samples of blood were collected in heparinized tubes and the kidneys, liver, spleen and lungs were removed, weighed, homogenized in distilled water and prepared for analysis by high-performance liquid chromatography (HPLC). The blood and tissue homogenates from five mice were pooled. AMB standards were prepared by adding

AMB-DOC in concentrations ranging from 0.1-5 mg/L to blood or tissue homogenate obtained from untreated mice, followed by incubation at 37°C for 90 min to allow binding of the antifungal agent to blood and tissue components. Both samples and standards were stored at -80°C until just before extraction. The AMB was extracted from the blood and homogenized tissues with ethanol in a mixture of sample and ethanol in a 4:6 (v/v) ratio. The extracts were centrifuged for 5 min at 13,000xg and the concentrations of AMB in the supernatants were determined by HPLC; a C_{18} column (Chromspher, 100x3 mm; Perking-Elmer, Norwalk, CT, USA) with a Guard precolumn (Chromguard, 10x3 mm; Chrompack, Middelburg, The Netherlands) was used for this purpose. The mobile phase, which consisted of a 0.1 M sodium acetate solution (pH 7.2) containing 0.2% (v/v) triethylamine and 60% (v/v) acetonitrile, was run at a flow rate of 0.3 mL/min. AMB was detected at 382 nm (LC 95 detector; Perking-Elmer). The sensitivity of the assay was 0.1 mg/L.

Statistical analysis. Results were expressed as the geometric means ± S.D. Differences between the various treatment groups and times at which the mice were killed, in terms of the numbers of CFU, were analyzed by the Mann-Whitney test.

RESULTS

***In vitro* susceptibilities.** The MICs of AMB-DOC and AmBisome® were 0.1 and 0.05 mg/L, respectively; the corresponding MFCs were 3.2 and 6.4 mg/L. The MIC of FLU was 0.8 mg/L; fungicidal activity could not be demonstrated with FLU at concentrations up to 102 mg/L.

***In vitro* activities during time-kill studies.** The survival of *C.albicans* in the presence of varying concentrations of AMB-DOC and AmBisome® during 6 h of incubation at 37°C is shown in Figure 1. In the presence of 0.2 mg/L, AMB-DOC killed > 99.9% of the initial inoculum within 2 h. With AmBisome®, on the other hand, a concentration of 25.6 mg/L was required to produce the same fungicidal activity and this was only achieved after 6 h of incubation.

MTDs of AMB-DOC and AmBisome®. The MTDs of AMB-DOC and AmBisome® in terms of death or significant abnormalities in the renal and/or hepatic functions of survivors 24 h after termination of treatment are presented in Table 1; similar result were observed 14 days after termination of treatment (data not shown). For AMB-DOC, the MTD which resulted in death was 0.8 mg AMB/kg/day. In contrast, neither death nor a significant increase in the parameters used to detect abnormalities in renal function was observed with dosages of AmBisome® of up to 32 mg AMB/kg/day. However, the MTD of AmBisome® in terms of significant abnormalities in hepatic function was 7 mg AMB/kg/day; from a dosage of 8 mg AMB/kg/day, the concentrations of both hepatic enzymes were increased by up to four-fold.

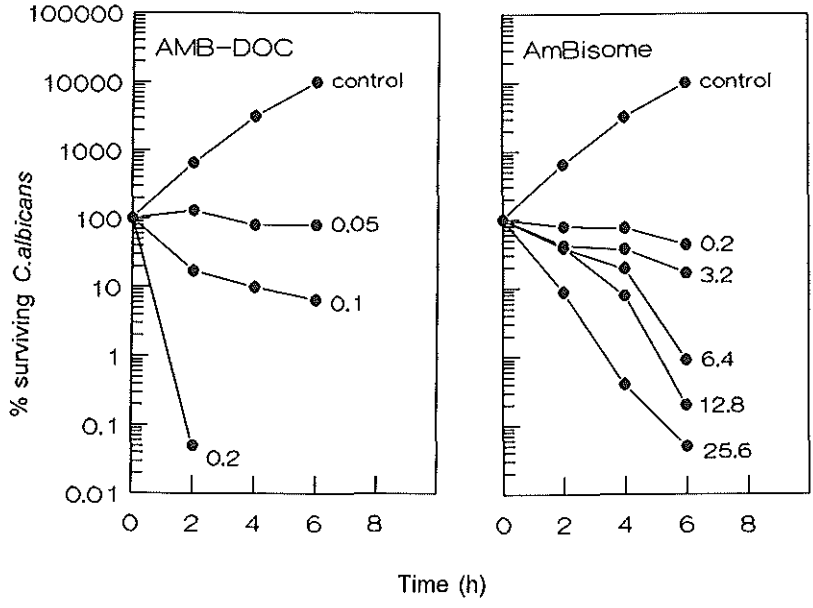

Figure 1. *In vitro fungicidal activities of AMB-DOC and AmBisome® in varying concentrations (mg/L) against C.albicans. Each symbol represents the mean of three experiments.*

Table 1. *Maximum tolerated dosages (MTDs)[a] of AMB-DOC and AmBisome® in uninfected mice.*

Criterion		MTD (mg AMB/kg/day)	
		AMB-DOC	AmBisome®
Death		0.8	>32
Renal function	BUN[b]	>0.8	>32
	CREAT[c]	>0.8	>32
Hepatic function	ASAT[d]	>0.8	7
	ALAT[e]	>0.8	7

[a]Expressed in terms of death during treatment or significant increase in the parameters used to monitor renal and hepatic functions 24 h after termination of treatment
[b]Blood urea nitrogen; [c]Serum creatinine
[d]Aspartate aminotransferase, [e]Alanine aminotransferase

Efficacies of AMB-DOC, AmBisome® and FLU in infected immunocompetent mice. In immunocompetent mice infected with *C.albicans*, infection is confined to the kidney (Van Etten *et al.*, unpublished data). The MTD of AMB-DOC for these animals in terms of death during treatment was 0.4 mg AMB/kg/day; for AmBisome®, dosages of up to 7 mg AMB/kg/day did not lead to death. The effects of the various treatments on the growth of *C.albicans* in the kidneys are shown in Table 2. During the study period, both an increase in the numbers of CFU and hyphal growth were observed following administration of the placebos. Therapy with AMB-DOC in a dosage of 0.4 mg AMB/kg/day (corresponding to the MTD) resulted in a significantly lower number of CFU in the kidneys 24 h after termination of treatment than did the placebos ($P \leq 0.001$) and compared with the number of CFU at the start of treatment ($P \leq 0.001$); AMB-DOC also prevented the number of CFU from increasing significantly at 14 days after termination of treatment i.e. relapse of infection. AmBisome®, in the same dosage (0.4 mg AMB/kg/day), was equally effective in respect of these outcome measures. Increasing of the dosage of AmBisome® to 7 mg AMB/kg/day (the MTD) resulted in an even lower number of CFU 24 h after termination of treatment than AmBisome® at 0.4 mg AMB/kg/day ($P \leq 0.01$); the number of CFU was reduced still further 14 days after termination of treatment ($P \leq 0.001$). FLU in a dosage of 0.4 mg FLU/kg/day produced a significant reduction in the number of CFU 24 h after completion of treatment compared with the number of CFU at the start of treatment ($P \leq 0.025$), but the number was not significantly lower than that observed following administration of a placebo. Although there were significantly fewer CFU in the kidneys at the 14-day post-treatment assessment with FLU than with the placebos ($P \leq 0.01$), relapse of infection, in terms of both an increase in the number of CFU compared with the number 24 h after termination of treatment and hyphal growth, was not prevented. Nor did increasing the dosage of FLU to 64 mg FLU/kg/day improve the efficacy of this agent.

Efficacies of AMB-DOC, AmBisome® and FLU in infected leukopenic mice. In leukopenic mice infected with *C.albicans*, infection is disseminated to the kidneys, liver, spleen and lungs, and untreated mice die between 24 h and 7 days after inoculation (Van Etten *et al.*, unpublished data). The MTD of AMB-DOC in terms of death during treatment was 0.3 mg AMB/kg/day; for AmBisome®, dosages of up to 7 mg AMB/kg/day did not cause death. The effects of treatment on the survival of leukopenic mice and growth of candida in the kidneys of survivors are shown in Tables 3 and 4, respectively. In common with immunocompetent mice, treatment with a placebo was associated with both an increase in the number of CFU and hyphal growth in the kidneys. Furthermore death of the animals was observed. Since almost all placebo-treated mice died, the numbers of CFU in the organs of the animals receiving a placebo were not compared with those in the organs of the animals receiving one of the antifungal agents. AMB-DOC, in a dosage equivalent to the MTD of this agent in leukopenic mice (0.3 mg AMB/kg/day), resulted in

Table 2. *Effect of treatment on growth of C.albicans in the kidneys of immunocompetent mice.*

treatment[a]	dosage (mg/kg/day)	start of treatment	24 h after termination of treatment		14 days after termination of treatment		
		Log_{10} CFU/kidney[b]	hyphal growth	Log_{10} CFU/kidney[b] in surviving mice	hyphal growth	Log_{10} CFU/kidney[b] in surviving mice	no. of mice with sterile kidneys/no. of surviving mice
untreated		4.58 ± 0.29					
AMB-DOC	0.4		—	2.44 ± 0.32[c]	—	2.15 ± 0.88	0/10
AmBisome®	0.4		—	3.06 ± 0.62[c]	—	2.81 ± 1.18	0/10
AmBisome®	7		—	2.20 ± 0.87[c]	—	0.40 ± 0.25	1/10
FLU	0.4		—	3.57 ± 1.10[c]	+	4.90 ± 1.32[d]	0/10
FLU	64		—	2.97 ± 0.58[c]	+	4.41 ± 1.39[d]	0/10
PLACEBO-DOC	-		+	4.19 ± 0.62	+	6.08 ± 0.85[d]	0/10
PLACEBO-LIP	-		+	4.78 ± 0.79	+	6.67 ± 0.80[d]	0/10
PLACEBO-PBS	-		+	4.40 ± 0.76	+	6.56 ± 0.65[d]	0/10

[a]Started 48 h after inoculation and continued for 5 days
[b]Geometric mean ± S.D.
[c]$P \leq 0.025$ compared with number of CFU at start of treatment
[f]$P \leq 0.025$ compared with number of CFU 24 h after termination of treatment
— = no hyphal growth; + = hyphal growth

100% survival, as well as significant reductions in the numbers of CFU in the liver, spleen and lungs ($P \leq 0.001$) (data not shown). However, at this dosage, growth of *Candida* in the kidneys was only inhibited during treatment i.e. the number of CFU 24 h after termination of treatment was not significantly different from the number at the start of treatment. Consequently, at the 14-day post-treatment assessment there was relapse of infection, manifested by hyphal growth and a significant increase in the number of CFU compared with the number at the 24 h post-treatment assessment. AmBisome®, at the equivalent dosage of 0.3 mg AMB/kg/day, was completely ineffective and did not even inhibit growth in the kidneys; at 24 h following completion of treatment, the number of CFU was greater than that at the start of treatment. At a dosage of 7 mg AMB/kg/day (the MTD), however, the number of CFU 24 h after treatment was discontinued was significantly reduced compared with the number at the start of treatment ($P \leq 0.001$). In addition, relapse at the 14-days post-treatment assessment was prevented and the number of CFU was further reduced compared with that at 24 h ($P \leq 0.001$). In a dosage of 0.3 mg FLU/kg/day, FLU was not effective. But at the higher dosage of 64 mg FLU/kg/day, its activity was similar to that of AMB-DOC, except that 24h after termination of treatment, the number of CFU in the kidneys was significantly lower than that at the start of treatment ($P \leq 0.001$); despite this initial reduction, relapse of infection was not prevented.

Table 3. *Effects of treatment on survival of leukopenic mice infected with C.albicans.*

treatment[a]	dosage (mg/kg/day)	survival (%)	
		24 h after termination of treatment	14 days after termination of treatment
AMB-DOC	0.3	100	100
AmBisome®	0.3	90	50
AmBisome®	7	100	100
FLU	0.3	60	0
FLU	64	100	100
PLACEBO-DOC	-	80	0
PLACEBO-LIP	-	80	10
PLACEBO-PBS	-	40	0

[a]Started 6 h after inoculation and continued for 5 days

The effects of prolonged therapy (18 days) with FLU in a dosage of 64 mg FLU/kg/day and maintenance therapy (13 days) at the same dosage following an

Table 4. *Effects of treatment on growth of C.albicans in the kidneys of leukopenic mice.*

treatment[a]	dosage (mg/kg/day)	start of treatment	24 h after termination of treatment		14 days after termination of treatment		
		Log_{10} CFU/kidney[b]	hyphal growth	Log_{10} CFU/kidney[b] in surviving mice	hyphal growth	Log_{10} CFU/kidney[b] in surviving mice	no. of mice with sterile kidneys/no. of surviving mice
untreated		2.65 ± 0.22					
AMB-DOC	0.3		—	2.81 ± 0.49	+	5.28 ± 1.14[d]	0/10
AmBisome®	0.3		ND	4.20 ± 0.67	ND	3.96 ± 1.86	0/5
AmBisome®	7		—	1.21 ± 0.30[c]	—	0.39 ± 0.52	6/10
FLU	0.3		ND	5.83 ± 0.61	ND	ND	ND
FLU	64		—	1.99 ± 0.47[c]	+	5.20 ± 1.28[d]	0/10
PLACEBO-DOC	-		ND	5.67 ± 1.10	ND	ND	ND
PLACEBO-LIP	-		ND	5.60 ± 0.93	ND	5.56	0/1
PLACEBO-PBS	-		ND	6.70 ± 0.48	ND	ND	ND

[a]Started 6 h after inoculation and continued for 5 days
[b]Geometric mean ± S.D.
[c]$P \leq 0.025$ compared with number of CFU at start of treatment
[d]$P \leq 0.025$ compared with number of CFU 24 h after termination of treatment
— = no hyphal growth; + = hyphal growth
ND = Not done

initial 5 days of treatment with AmBisome® (7 mg AMB/kg/day) are shown in Table 5. Extending the duration of FLU treatment did not result in a significantly lower number of CFU in the kidneys 19 days after starting treatment compared with the number of CFU after only 5 days of treatment. Similarly, the effects of treatment with AmBisome® followed by FLU were not significantly different in terms of the number of CFU in the kidneys compared with the effects of treatment with AmBisome® alone.

Table 5. *Effects of prolonged or maintenance treatment with FLU on growth of C.albicans in the kidneys of leukopenic mice.*

treatment[a]	dosage (mg/kg/day)	Log_{10} CFU/kidney[b] in surviving mice[c]	no. of mice with sterile kidneys/no. of surviving mice[c]
FLU[d]	64	2.28 ± 0.36	0/10
AmBisome®[e]	7	0.39 ± 0.52	6/10
AmBisome®/FLU[f]	7/64	0.25 ± 0.41	7/10

[a]Started 6 h after inoculation
[b]Geometric mean ± S.D.
[c]Effects of treatment were assessed 19 days after starting
[d]Administered for 18 days
[e]Administered for 5 days
[f]AmBisome® for 5 days followed by FLU for 13 days

The results of the experiment to assess the effects of treatment in relation to the severity of infection are presented in Table 6. Infection of varying degrees of severity was produces by increasing the delay between inoculation and the initiation of therapy. This was reflected in the number of CFU in the kidneys at the start of treatment (2.65 ± 0.22 log_{10} when treatment was commenced 6 h after inoculation compared with 3.97 ± 0.38 log_{10} when treatment was delayed until 24 h after inoculation). The greater severity of infection resulting from 16- or 24 h delays in starting treatment was also reflected in the lower MTDs of AmB-DOC (both 0.1 mg AMB/kg/day) compared with the MTD (0.3 mg AMB/kg/day) when the delay was only 6 h. The ultimate effect of withholding treatment until 16 h after inoculation was that the efficacy of AMB-DOC was reduced. On the other hand, despite a 16 h delay, AmBisome®, in a dosage of 7 mg AMB/kg/day, retained its efficacy and still produced 100% survival and a significant reduction in the number of CFU in the kidneys. The effects of prolonging the delay to 16 h before administering FLU at a dosage of 64 mg FLU/kg/day were similar to those of AMB-DOC. Even when the initiation of treatment with the higher dosage (7 mg AMB/kg/

Table 6. *Effects of treatment in relation to severity of infection, assessed in terms of survival and growth of C.albicans in the kidneys of leukopenic mice.*

start of treatment (after inoculation)	treatment[a]	dosage (mg/kg/day)	survival[c] (%)	hyphal growth[c]	Log_{10} CFU/kidney[b] in surviving mice[c]	no. of mice with sterile kidneys/no. of surviving mice[c]
6 h	AMB-DOC	0.3	100	+	5.28 ± 1.14	0/10
	AmBisome®	7	100	—	0.39 ± 0.52	6/10
	FLU	64	100	+	5.20 ± 1.28	0/10
16 h	AMB-DOC	0.1	40	ND	6.20 ± 0.97	0/4
	AmBisome®	7	100	—	1.90 ± 0.66	0/10
	FLU	64	70	ND	6.17 ± 0.61	0/7
24 h	AMB-DOC	0.1	ND	ND	ND	ND
	AmBisome®	7	90	ND	1.29 ± 0.82	0/9
	FLU	64	ND	ND	ND	ND

[a]Continued for 5 days
[b]Geometric mean ± S.D.
[c]Determined 14 days after termination of treatment
— = no hyphal growth; + = hyphal growth
ND = Not done

Table 7. *AMB concentrations in blood and tissues after treatment of infected leukopenic mice with AMB-DOC or AmBisome®.*

treatment[a]	dosage (mg/kg/day)	AMB concentration[b] 24h after termination of treatment					AMB concentration[b] 14 days after termination of treatment				
		blood mg/L	liver µg/g	spleen µg/g	kidney µg/g	lung µg/g	blood mg/L	liver µg/g	spleen µg/g	kidney µg/g	lung µg/g
AMB-DOC	0.3	0.2	<	3.3	<	0.6	<	<	2.3	<	<
AmBisome®	7	1.7	356	700	14.5	5.9	<	170	243	7.8	<

[a]Started 6 h after inoculation and continued for 5 days
[b]Blood samples and tissue homogenates from five mice were pooled
< = not detectable

day) of AmBisome® was delayed for 24 h, it remained partially effective, with 90% of animals surviving.

Concentrations of AMB in the blood and tissues of leukopenic mice treated with AMB-DOC and AmBisome®. The concentrations of AMB in the blood and tissues of leukopenic mice following treatment with AMB-DOC and AmBisome® in dosages which corresponded to their respective MTDs are shown in Table 7. At 24 h after completing treatment with AMB-DOC (0.3 mg AMB/kg/day), only low concentrations of AMB were detected in the blood, spleen and lungs; 14 days after completion of treatment, AMB was detectable only in the spleen. In contrast, the administration of AmBisome® at 7 mg AMB/kg/day led to the detection of much higher concentrations of AMB in the blood, liver, spleen, kidneys and lungs 24 h after completing treatment. AMB was still detectable in the liver, spleen and kidneys 14 days after completing treatment.

DISCUSSION

It has previously been demonstrated *in vitro* [37] that AMB-DOC, following short-term exposure, is fungicidal against *C.albicans*, whilst FLU only inhibits growth. Other investigators have reported that the activity of AmBisome® against *C.albicans*, based on the MICs and MFCs was similar to that of free AMB [3]. The present study has confirmed that the activities of AMB-DOC and AmBisome®, in terms of the MICs and MFCs, are similar, but with short-term exposure (time-kill studies), the activity of AmBisome® is significantly less than that of AMB-DOC. Moreover, the *in vitro* activity of another liposomal formulation of AMB, prepared from dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol, has also been shown with time-kill studies to be less than that of either AMB-DOC or free AMB but to have comparable MICs and MFCs [13,29]. In the present study, the effective killing of *C.albicans* by AmBisome® within a 6-h period of incubation was observed only at high concentrations, suggesting that the cidal activity of this agent during long-term exposure might be attributed to the release of AMB from the liposomes into the medium. It is not clear whether the antifungal activity of AmBisome® is the result of a direct interaction between AmBisome® and *C.albicans* or the release of AMB from liposomes during prolonged incubation or both.

Treatment with AMB in the form of AMB-DOC was limited to toxicity. When AMB was incorporated into liposomes (AmBisome®), much higher dosages of this agent were tolerated, although, from dosages of 8 mg AMB/kg/day, disturbances of hepatic function were detected. However, as the increase in hepatic enzymes in plasma was at most fourfold, according to criteria from hospital practice, the disturbances of hepatic function were only mild. It is paradoxical that the mice were killed by lower dosages of AMB-DOC than were required to produce abnormalities of renal and hepatic function. It may be that the death of animals observed with AMB-DOC was related to the way in which the drug was administered. As AMB-

DOC cannot be administered by slow infusion, it is given as a bolus, and this may produce acute adverse reactions, including death. If these reactions could be suppressed, it is possible that the dosages of AMB-DOC required to produce abnormalities of renal or hepatic function would then be lower than those causing death.

In the immunocompetent mice, treatment was started when infection was well established in the kidneys. With this model, both AMB-DOC and AmBisome® were shown to be effective in reducing the numbers of CFU in the kidneys and in preventing relapse after treatment had been stopped. Other investigators have used a lethal model of *Candida* infection to show that high dosages of AmBisome® are able to increase the median survival time and to bring about significant reductions in the numbers of CFU in the kidneys of infected immunocompetent mice [1,11]. In common with others [8,9,38], we have shown that even high dosages of FLU only reduce the numbers of CFU in the kidneys during treatment.

Treatment was inititiated in leukopenic mice at various intervals after inoculation in order to investigate the influence of the severity of infection on the efficacy of therapy. When treatment with AMB-DOC was commenced shortly after inoculation, the animals survived but relapse of infection was not prevented. The administration of high dosages of FLU led to reductions in the numbers of CFU in the kidneys soon after termination of treatment; this has also been reported by other investigators [39,40] who found low numbers of CFU in the kidneys of granulocytopenic rabbits when treatment was started shortly after initiation of infection. However, FLU failed to prevent eventual relapse. Nor did prolonged treatment with FLU improve the efficacy of this agent in terms of reducing further the number of CFU in the kidneys. The use of AmBisome® enabled higher dosages of AMB to be administered and treatment with this drug led to the isolation of a low number of organisms from the kidney, even at two weeks after termination of therapy. Moreover, only with AmBisome® was it possible to sterilize the tissues of some of the animals. Maintenance therapy with FLU following initial treatment with AmBisome® did not enhance the efficacy of the latter drug. In severe infections resulting from a prolonged delay in initiating treatment, the dosage of AMB-DOC was limited by its toxicity and this led to a reduction in efficacy. Treatment with high dosages of FLU was also relatively ineffective under these circumstances. The efficacy of AmBisome®, however, was preserved in the presence of severe infection; at 14 days after completion of treatment, the number of CFU in the kidney remained low.

Following treatment with AmBisome® in dosages corresponding to its MTD, high concentrations of AMB were detected in the tissues, an observation which is in agreement with the results of other studies in which multiple doses of AmBisome® were administered [1,27]. No toxic effects were observed with these high AMB concentrations and 14 days after treatment had been discontinued, the drug was still detectable in the liver, spleen and kidneys. Other investigators have also reported

the presence of AMB in the kidneys 8 days after a 5-day course of AmBisome® [1]. It has not been determined whether these high AMB concentrations represent the liposome formulation or the free drug.

The initial reduction in the number of CFU in the kidneys of immunocompetent mice shortly after completing treatment with FLU, which has no fungicidal activity *in vitro*, can be accounted for by the contribution of host defence mechanisms to the eradication of the *Candida*. In leukopenic animals, however, the role of host defence factors is markedly diminished. None the less, killing of candida following the administration of FLU to immunocompromised animals was noted in the present study and has also been reported by others [39,40] there is no obvious explanation for this observation.

The efficacies of AMB-DOC and FLU in immunocompetent and leukopenic mice demonstrated here have also been described by others [38] who reported that AMB-DOC was superior to FLU in the treatment of immunocompetent mice but that both agents were equally effective in leukopenic mice.

In summary, treatment with AMB-DOC was effective in immunocompetent mice but could not prevent relapse of infection in leukopenic mice once therapy had been stopped. With AmBisome®, much higher dosages of AMB were tolerated, thereby leading to effective treatment in both immunocompetent and severely infected leukopenic mice. Treatment with high dosages of FLU failed to prevent relapse in both groups of mice. The results of the present study should provide the impetus for clinical trials to evaluate the efficacy of AmBisome® in the treatment of invasive candidiasis in neutropenic patients.

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REFERENCES

1. Adler-Moore JP, Chiang SM, Satorius A, Guerra D, McAndrews B, McManus EJ, Proffitt RT. Treatment of murine candidosis and cryptococcosis with a unilamellar liposomal amphotericin B formulation (AmBisome). J Antimicrob Chemother 1991;28(Suppl.B):63-71.
2. Anaissie E, Bodey GP, Kantarjian H, David C, Barnett K, Bow E, *et al.* Fluconazole therapy for chronic disseminated candidiasis in patients with leukemia and prior amphotericin B therapy. Am J Med 1991;91:142-50.
3. Anaissie E, Paetznick V, Proffitt R, Adler-Moore J, Bodey GP. Comparison of the *in vitro* antifungal activity of free and liposome-encapsulated amphotericin B. Eur J Clin Microbiol Infect Dis 1991;10:665-8.

4. Björkholm M, Kållberg N, Grimfors G, Eklund LH, Eksborg S, Juneskans OT, Udén M. Successful treatment of hepatosplenic candidiasis with a liposomal amphotericin B preparation. J Intern Med 1991;2:173-7.
5. Clark MJ, Whitney RR, Olsen SJ, George RJ, Swerdel MR, Kunselman R, Bonner DP. Amphotericin B lipid complex therapy of experimental fungal infections in mice. Antimicrob Agents Chemother 1991;35:615-21.
6. Espinel-Ingroff A, Shadomy S. *In vitro* and *in vivo* evaluation of antifungal agents. Eur J Clin Microbiol Infect Dis 1989;8:352-61.
7. Filler SG, Crislip MA, Mayer CL, Edwards, JE (1991), Comparison of fluconazole and Amphotericin B for treatment of disseminated candidiasis and endolphthalmitis in rabbits. Antimicrob Agents Chemother 1991:35:288-92.
8. Fisher MA, Lee PG, Tarry WF. Fluconazole (UK-49,858) treatment of candidiasis in normal and diabetic rats. Antimicrob Agents Chemother 1989;33:1042-5.
9. Fisher MA, Shen SH, Haddad J, Tarry WF. Comparison of *in vivo* activity of Fluconazole with that of Amphotericin B against *Candida tropicalis, Candida glabrata*, and *Candida krusei*. Antimicrob Agents Chemother 1989;33:1443-6.
10. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-29.
11. Gondal JA, Swartz RP, Rahman A. Therapeutic evaluation of free and liposome-encapsulated amphotericin B in the treatment of systemic candidiasis in mice. Antimicrob Agents Chemother 1989;33:1544-8.
12. Hay RJ. Antifungal therapy and the new azole compounds. J Antimicrob Chemother 1991;28(Suppl.A):35-46.
13. Hopfer RL, Mills K, Mehta R, Lopez-Berestein G, Fainstein V, Juliano RL. *In vitro* antifungal activities of amphotericin B and liposome-encapsulated amphotericin B. Antimicrob Agents Chemother 1984;25:387-9.
14. Hospenthal D, Gretzinger K, Rogers A. Treatment of a murine model of systemic candidiasis with liposomal amphotericin B bearing antibody to *Candida albicans*. J Med Microbiol 1989;30:193-7.
15. Hospenthal DR, Rogers AL, Beneke ES. Effect of attachment of anticandidal antibody to the surface of liposomes encapsulating amphotericin B in the treatment of murine candidiasis. Antimicrob Agents Chemother 1989;33:16-8.
16. Janoff AS, Boni LT, Popescu MC, Minchey SR, Cullis PR, Madden TD, Taraschi T, Gruner SM, Shyamsunder E, Tate MW, Mendelsohn R, Bonner D. Unusual lipid structures selectively reduce the toxicity of amphotericin B. Proc Natl Acad Sci USA 1988;85:6122-6.
17. Kan V, Bennett J, Amantea M, Smolskis MC, McManus E, Grasela DM, Sherman JW. Comparative safety, tolerance, and pharmacokinetics of amphotericin B lipid complex and amphotericin B desoxycholate in healthy male volunteers. J Infect Dis 1991;164: 418-21.
18. Kauffman CA, Bradley SF, Ross SC, Weber DR. Hepatosplenic candidiasis: succesful treatment with fluconazole. Am J Med 1991;91:137-41
19. Lopez-Berestein G, Bodey GP, Fainstain V, Keating M, Frankel LS, Zeluff B, Gentry L, Mehta K. Treatment of systemic fungal infections with liposomal amphotericin B. Arch Intern Med 1989;149:2533-6.
20. Lopez-Berestein G, Hopfer RL, Mehta R, Mehta K, Hersch EM, Juliano RL. Liposome-encapsulated amphotericin B for treatment of disseminated candidiasis in neutropenic mice. J Infect Dis 1984;150:278-83.
21. Lopez-Berestein G, Mehta R, Hopfer RL, Mills, Kasi L, Mehta K, Fainstein V, Luna M, Hersch EM, Juliano RL. Treatment and prophylaxis of disseminated infection due to

Candida albicans in mice with liposome-encapsulated amphotericin B. J Infect Dis 1983;147:939-45.
22. Meunier, F. Fungal infections in the compromised host. In: Rubin RH, Young LS (Eds.) Clinical approach to infection in the compromised host. Plenum Publishing Corporation, New York. 1988:193-220.
23. Meunier F. Candidiasis. Eur J Clin Microbiol Infect Dis 1989;8:438-47.
24. Meunier F, Prentice HG, Ringdén O. Liposomal amphotericin B (AmBisome): safety data from a phase II/III clinical trial. J Antimicrob Chemother 1991;28(Suppl.B):83-91.
25. Patterson TF, Andriole VT. The role of liposomal amphotericin B in the treatment of systemic fungal infections. Eur J Cancer Clin Oncol 1989;25(Suppl.2):S63-8.
26. Perfect JR, Savani DV, Durack DT. Comparison of itraconazole and fluconazole in treatment of cryptococcal meningitis and candida pyelonephritis in rabbits. Antimicrob Agents Chemother 1986;29:579-83.
27. Proffitt RT, Satorius A, Chiang SM, Sullivan L, Adler-Moore JP. Pharmacology and toxicology of a liposomal formulation of amphotericin B (AmBisome) in rodents. J Antimicrob Chemother 1991;28(Suppl.B):49-61.
28. Ralph ED, Barber KR, Grant CWM. Liposomal amphotericin B: and effective, nontoxic preparation for the treatment of urinary tract infections caused by *Candida albicans*. Am J Nephrol 1991;11:118-22.
29. Ralph ED, Khazindar AM, Barber KR, Grant CWM. Comparative *in vitro* effects of liposomal amphotericin B, amphotericin B-deoxycholate, and free amphotericin B against fungal strains determined by using MIC and minimal lethal concentration susceptibility studies and time-kill curves. Antimicrob Agents Chemother 1991;35:188-91.
30. Richardson K, Brammer KW, Marriot MS, Troke PF. Activity of UK-49,858, a bistriazole derivative against experimental infections with *Candida albicans* and *Trichophyton mentagrophytes*. Antimicrob Agents Chemother 1985;27:832-5.
31. Ringdén O, Meunier F, Tollemar J, Ricci P, Tura S, Kuse E, Viviani MA, Gorin NC, Klastersky J, Fenaux P, Prenice HG, Ksionski G. Efficacy of amphotericin B encapsulated in liposomes (AmBisome) in the treatment of invasive fungal infections in immunocompromised patients. J Antimicrob Chemother 1991;28(Suppl.B):73-82.
32. Rogers TE, Galgiani JN. Activity of fluconazole (UK 49,858) and ketoconazole against *Candida albicans in vitro* and *in vivo*. Antimicrob Agents Chemother 1986;30:418-22.
33. Schürmann D, De Matos Marques B, Grunewald T, Pohle HD, Hahn H, Ruf B. Safety and efficacy of liposomal amphotericin B in treating AIDS-associated disseminated cryptococcosis. J Infect Dis 1991;164:620-2.
34. Sculier JP, Coune A, Meunier F, Brassine C, Laduron C, Hollaert C, Collette N, Heymans C, Klastersky J. Pilot study of amphotericin entrapped in sonicated liposomes in cancer patients with fungal infections. Exp J Cancer Clin Oncol 1988;24:527-38.
35. Tremblay C, Barza M, Fiore C, Szoka F. Efficacy of liposome-intercalated amphotericin B in the treatment of systemic candidiasis in mice. Antimicrob Agents Chemother 1984; 26:170-3.
36. Troke PF, Andrews RJ, Brammer KW, Marriott MS, Richardson K. Efficacy of UK-49,858 (fluconazole) against *Candida albicans* experimental infections in mice. Antimicrob Agents Chemother 1985;28:815-8.
37. Van Etten EWM, Van de Rhee NE, Van Kampen KM, Bakker-Woudenberg IAJM. Effects of amphotericin B and fluconazole on the extracellular and intracellular growth of *Candida albicans*. Antimicrob Agents Chemother 1991;35:2275-81.

38. Van 't Wout JW, Mattie H, van Furth R. The efficacy of amphotericin B, fluconazole and itraconazole against systemic candidiasis in normal and neutropenic mice. Antimicrob Agents Chemother 1989;33:147-51.
39. Walsh TJ, Aoki S, Mechinaud F, Bacher J, Lee JW, Rubin M, Pizzo PA. Effects of preventive, early, and late antifungal chemotherapy with fluconazole in different granulocytopenic models of experimental disseminated candidiasis in granulocytopenic hosts. J Infect Dis 1990;16:755-60.
40. Walsh TJ, Lee JW, Aoki S, Mechinaud F, Bacher J, Lecciones J, Thomas V, Rubin M, Pizzo PA. Experimental basis for use of fluconazole for preventive or early treatment of disseminated candidiasis in granulocytopenic hosts. Rev Infect Dis 1990;12(Suppl.3): S307-17.
41. Walsh TJ, Pizzo PA. Treatment of systemic fungal infections: recent progress and current problems. Eur J Clin Microbiol 1988;7:460-75.
42. Wiebe VJ, DeGregorio MW. Liposome-encapsulated amphotericin B: a promising new treatment for disseminated fungal infections. Rev Infect Dis 1988;10:1097-101.
43. Witt, MD, Bayer AS. Comparison of fluconazole and amphotericin B for prevention and treatment of experimental *Candida* endocarditis. Antimicrob Agents Chemother 1991; 35:2481-5.

Chapter 4

Biodistribution of liposomal amphotericin B (AmBisome®) versus amphotericin B-desoxycholate (Fungizone®) in immunocompetent uninfected mice as well as in leukopenic mice infected with *Candida albicans*

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Biodistribution of liposomal amphotericin B (AmBisome) and amphotericin B-desoxycholate (Fungizone) in uninfected immunocompetent mice and leucopenic mice infected with *Candida albicans*

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ABSTRACT

The biodistribution of liposomal amphotericin B (AmBisome®) and amphotericin B-desoxycholate (AMB-DOC) were compared after a single injection of drug in uninfected immunocompetent mice and in leukopenic mice 6 h after inoculation with *Candida albicans*. AMB-DOC was administered at the maximum tolerated dose (MTD) of 0.3 mg AMB/kg whereas AmBisome® was given at either 0.3 mg AMB/kg or the MTD of 7 mg AMB/kg. Amphotericin B (AMB) concentrations in the blood, liver, spleen, lungs and kidneys were determined by HPLC analysis at various intervals during the 48 h after administration. The biodistribution of both preparations of AMB followed similar patterns in both uninfected immunocompetent mice as well as those that were leukopenic and infected with *C.albicans*. Administration of AmBisome® resulted in increased concentrations of drug in the blood, liver and spleen but decreased concentrations in the kidney and lung. Hepatosplenic uptake of AmBisome® was highly dose dependent with 7 mg AMB/kg resulting in a relatively prolonged blood circulation. Blood and tissues retained high AMB concentrations after administration of AmBisome® at the MTD. By using radiolabeled AmBisome®, it was found that the high AMB concentrations in blood represented liposome-associated AMB and that during circulation in blood slow release of AMB occurred.

INTRODUCTION

Parenteral administration of amphotericin B (AMB) remains the treatment of choice for most invasive fungal infections but its clinical utility is limited by the toxic side effects resulting from treatment with the desoxycholate preparation [11,17]. Recently, AMB has been incorporated into liposomes or other lipid-carriers leading to a reduction in toxicity thereby improving the therapeutic index [6,12,17,21]. One of these formulations, AmBisome®, has been shown to result in improved efficacy in the treatment of various experimental fungal infections in both immunocompetent and imunocompromised animals [2,4,5,9,13,23] although little is known about the way in which AmBisome® exerts its antifungal activity *in vivo*. There are some data on the biodistribution of AmBisome® in uninfected immunocompetent mice [2,13], rats [20], and rabbits [9] but there are no published data for infected leukopenic animals. Moreover, the published data do not allow a distinction to be made between liposome-encapsulated AMB and non-encapsulated AMB because only data on the total concentrations of AMB in serum and tissues are available. We therefore undertook a study to compare the biodistribution of AmBisome® with that of amphotericin B-desoxycholate (AMB-DOC) in leukopenic mice infected with *Candida albicans* and in uninfected immunocompetent mice by radiolabeling AmBisome® with a radioactive marker that represents intact liposomes.

MATERIALS AND METHODS

***Candida* strain.** *Candida albicans* ATCC 44858 was used in all of the experiments and was stored at -80°C in Todd-Hewitt broth (Difco laboratories, Detroit, MI, USA) containing 10% (v/v) glycerol.

Animals. Specified pathogen-free, 14- to 20-weeks-old female BALB/c mice were obtained from Iffa Credo (L'Arbresle, France).

Amphotericin B preparations. Amphotericin B-desoxycholate (AMB-DOC; Fungizone®, Bristol Myers-Squibb, Woerden, The Netherlands) was reconstituted with distilled water to obtain a stock solution of 5 g AMB/L. Liposomal AMB, AmBisome®, AMB encapsulated in small unilamellar vesicles consisting of hydrogenated soybean phosphatidylcholine, cholesterol and distearoylphosphatidylglycerol in a molar ratio of 2:1:0.8, was kindly provided by Vestar Inc. (San Dimas, CA, USA) as a lyophilized preparation. The powder was reconstituted in distilled water at 65°C according to the manufacturer's instructions to give a liposomal suspension containing 4 g AmB/L and 35 g lipid/L.

Induction of leukopenia and infection with *C.albicans*. Leukopenia was induced by injecting 100 mg/kg cyclophosphamide (Sigma Chemical Co., St.Louis, MO) ip 4 days before inoculation with *C.albicans*. A further dose of 75 mg/kg was given on the day of infection. Infection was induced by inoculating 10^4 CFU of *C.albicans*

into the tail vein. This treatment resulted in persistent granulocytopenia of $<0.1 \times 10^9$/L granulocytes from the time of inoculation up to the end of the study.

Radiolabeling of AmBisome®. Lyophilized AmBisome® powder was reconstituted with distilled water containing 5 mM deferoxamine mesylate (DF; Ciba Geigy, Basel, Switzerland). AmBisome® was radiolabeled with ^{67}Ga-DF as described by Woodle [24] using ^{67}Ga-citrate from Nordian (Montreal, Canada) which formed a ^{67}Ga-DF complex in the aqueous interior of the liposomes. This complex is appropriate for tracing of intact liposomes *in vivo* because there is minimal translocation of radioactive label to plasma proteins and renal clearance is rapid once the label is released from the liposomes [10]. Liposomes were separated from free DF and radiolabel by gel filtration on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden) eluted with a buffer solution containing 10 mM sodium-succinate and 5% (w/v) glucose (pH 5.5) and concentrated using 300 kDa Microsep filters (Filtron, Breda, The Netherlands). The concentration of AMB was determined spectrofotometrically at 405 nm, after destruction of the liposomes in DMSO-methanol (1:1, v/v).

Determining the biodistribution of AMB following administration of both preparations. The biodistribution of AMB was determined in groups of mice after iv administration of AMB-DOC or AmBisome® at 0.3 mg AMB/kg, which was the maximum tolerated dosage (MTD) of AMB-DOC after five consecutive days of treatment of infection due to *C.albicans* in leukopenic mice [23]. AmBisome® was also given at the MTD of 7 mg AMB/kg, which had been determined previously [23]. Each preparation of the drug was administered to leukopenic mice 6 h after inoculation with *C.albicans*.

Determining the concentrations of AMB. AMB concentrations were determined in two separate sets of experiments in the blood, liver, spleen, lungs and kidneys of five mice at each of the various time points during the first 48 h after administration. Blood was collected in heparinized tubes immediately before the mice were killed. After removal, the spleen, kidneys, lungs, and liver were weighed and homogenized separately in distilled water. The blood samples and tissue homogenates from each group of five mice were pooled and stored at -80°C until required for assay using high-performance liquid chromatography (HPLC) analysis [23]. ^{111}In-labeled syngeneic red blood cells were employed to determine the total blood volume and the correction factors for the blood in each organ [14].

Blood levels of AMB and ^{67}Ga-DF following administration of radiolabeled AmBisome®. Radiolabeled AmBisome® was administered to uninfected mice as a single iv dose of 7 mg AMB/kg in two separate experiments. The concentrations of ^{67}Ga-DF were determined in heparinized blood of each of the five mice at each of various time points during the first 24 h after administration. Counts of ^{67}Ga-DF were determined in 200 μL samples of blood as well as in the liposomes contained in the original dose using a gamma counter (Minaxy 5530, Packard Instruments,

Downers Grove, USA). Blood samples from each of five mice were then pooled and stored at -80°C until required for HPLC analysis for AMB concentrations.

RESULTS

Biodistribution of AMB following administration of AmB-DOC or AmBisome® at an equivalent dosage. In both infected leukopenic mice and uninfected immunocompetent mice, liposomal encapsulation of AMB resulted in increased concentrations of AMB in the blood, liver, and spleen but lower concentrations in the kidney and lungs (Figure 1). Five minutes after injection, 41% of the dose of AmBisome® was recovered from the blood of uninfected mice and 4% of the original dose was retrieved 4 h after administration whereas 61% AMB was recovered from liver and 10% from spleen 1 h after administration and there was no decrease in drug recovery during the first 12 h after injection. In contrast, 13% of AMB-DOC was recovered in blood 5 min after injection and 4% was retrieved 2 h after administration whereas 26% of the drug was recovered from the liver and only 3% was recovered from the spleen after 15 min. At 12 h after administration 16% was retrieved from the liver. Up to 6% of AMB was detected in the kidneys 15 min after administration of AMB-DOC and up to 5% in the lungs 4 h after injection whereas none of the AmBisome® was retrieved in these organs. The biodistribution of AMB in both infected and uninfected mice followed a similar pattern.

Biodistribution of AMB following administration of AmBisome® in relation to dosage. The biodistribution of AmBisome® in both infected and uninfected mice was similar and hepatosplenic uptake of AmBisome® was highly dosage dependent (Figure 2). Five minutes after injecting 0.3 mg AMB/kg, 54% of the drug was recovered from the liver and spleen of uninfected mice and increased to 81% within 12 h after administration whereas 41% of the drug was recovered from blood 5 min after injection and less than 3% 12 h later. In contrast, the hepatosplenic uptake was less than 3% 5 min after injection of 7 mg AMB/kg whereas 99% of the dose was retrieved in blood. Twelve hours after administration, uptake by the liver and spleen had increased to 64% while the amount recovered from blood decreased to 15%.

Blood and tissue levels of AMB following administration of AMB-DOC or AmBisome® both at their MTDs. Within 48 h after administration of AmBisome®, blood concentrations of AMB in uninfected mice decreased from 104 mg/L to 0.2 mg/L (Table 1) Peak concentrations occurred in the liver 12 h after injection, in the kidneys and lungs 12 h later and in the spleen 48 h after administering the drug, and there was no decrease in drug recovery during the first 24 h after injection. Blood concentrations of AMB decreased from 0.6 to 0.2 mg/L within 2 h after injecting AMB-DOC. Peak concentrations occurred in the liver, and spleen 5 min

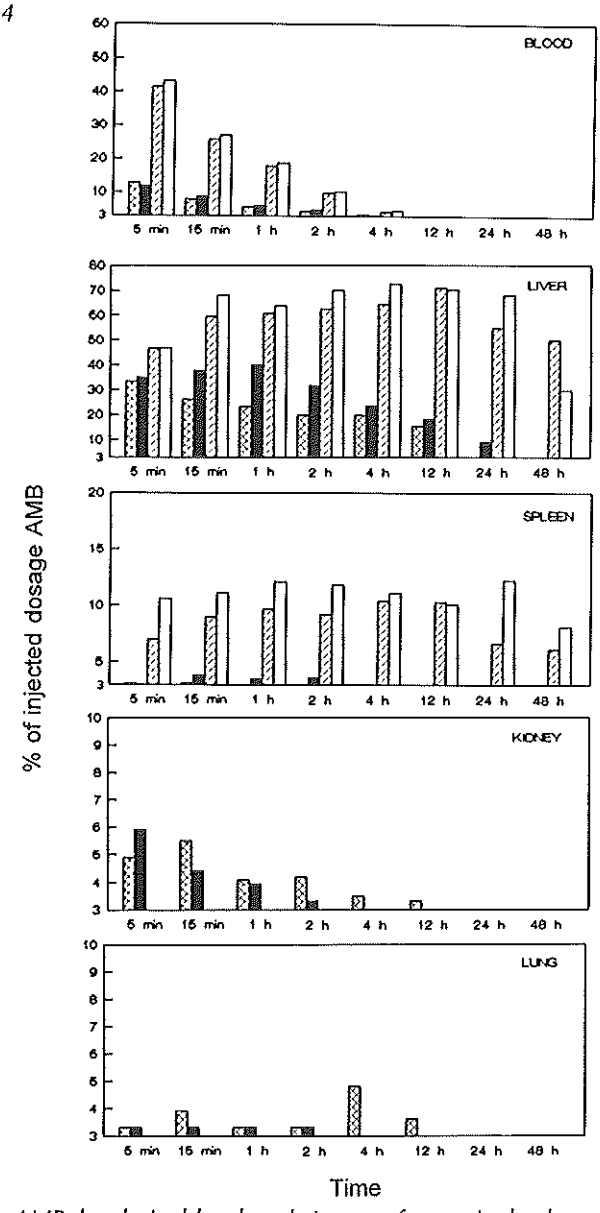

Figure 1. *AMB levels in blood and tissues after a single dose iv of AMB-DOC or AmBisome® at an equivalent dosage of 0.3 mg AMB/kg in uninfected immunocompetent mice as well as in leukopenic mice infected with C.albicans.*
▨ *AMB-DOC in uninfected mice;* ▩ *AMB-DOC in infected leukopenic mice;* ▨ *AmBisome® in uninfected mice;* □ *AmBisome® in infected leukopenic mice. Each symbol represents the mean of two experiments. Blood and tissue samples from five mice were pooled.*

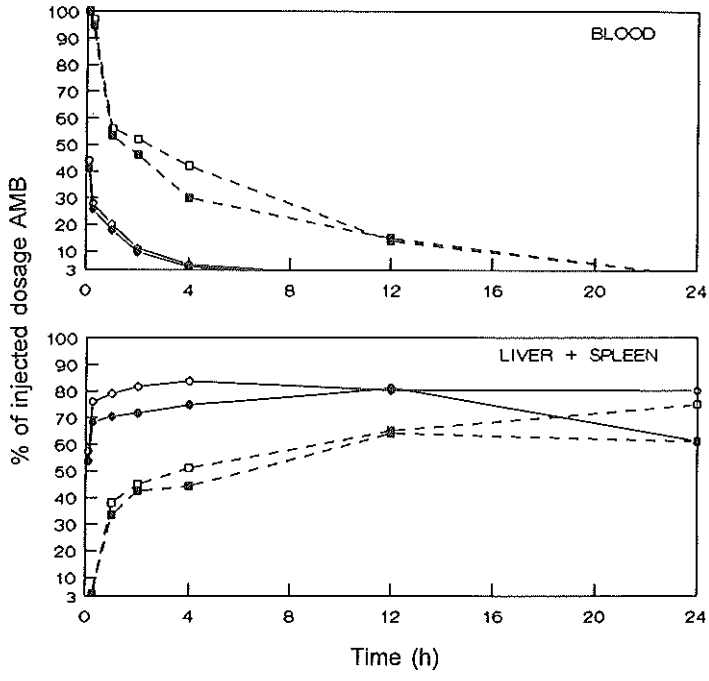

Figure 2. *Blood levels and hepatosplenic uptake of AMB after a single dose iv of AmBisome® in relation to dosage in uninfected immunocompetent mice as well as in leukopenic mice infected with C.albicans.* ● *AmBisome® at 0.3 mg AMB/kg in uninfected mice;* ○ *AmBisome® at 0.3 mg AMB/kg in infected leukopenic mice;* ■ *AmBisome® at 7 mg AMB/kg in uninfected mice;* □ *AmBisome® at 7 mg AMB/kg in infected leukopenic mice. Each symbol represents the mean of two experiments. Blood and tissue samples from five mice were pooled.*

after injection and 10 min later in the kidney and lung. Concentrations of drug gradually decreased in liver, spleen and kidney during the first 12 h after administration. The biodistribution of AMB in both infected (Table 2) and uninfected mice followed a similar pattern.

Blood levels of AMB and ^{67}Ga-DF following administration of radiolabeled AmBisome®. Following injection of 7 mg AMB/kg radiolabeled AmBisome®, a relatively prolonged circulation of intact liposomes indicated by the detection of ^{67}Ga-DF was observed, while the concentration of AMB declined more rapidly (Figure 3). Incubating AmBisome® in 90% (v/v) serum at 37°C *in vitro* for 6 h did not lead to any detectable loss of drug from the liposomes (data not shown).

Table 1. *AMB concentrations in blood and tissues after a single dose iv of AMB-DOC or AmBisome® in uninfected immunocompetent mice.*

Time after injection	AMB concn[a] (mg/L)		AMB concn[a] (μg/g)							
	BLOOD		LIVER		SPLEEN		KIDNEY		LUNG	
	A-DOC[b]	L-A[b]	A-DOC[b]	L-A[b]	A-DOC[b]	L-A[b]	A-DOC[b]	L-A[b]	A-DOC[b]	L-A[b]
5 min	0.6	104.4	1.8	5.7	2.0	29.4	0.9	3.0	0.8	<
15 min	0.3	95.4	1.4	19.4	2.0	37.0	1.2	3.0	1.3	<
1 h	0.2	53.8	1.2	38.7	<	51.1	0.9	4.3	0.8	<
2 h	0.2	46.5	1.1	49.1	<	64.2	0.9	5.2	0.8	<
4 h	0.1	30.3	1.1	50.4	<	77.3	0.6	7.3	1.3	<
12 h	<	15.1	0.8	76.1	<	69.6	0.6	3.9	0.8	8.3
24 h	<	0.7	<	70.9	<	88.1	<	9.1	<	15.6
48 h	<	0.2	<	62.7	<	106.6	<	6.1	<	15.0

[a]Blood samples and tissue homogenates from five mice were pooled
[b]A dosage of 0.3 mg AMB/kg AmB-DOC (A-DOC) or 7 mg AMB/kg AmBisome® (L-A) was administered iv
< = not detectable

Table 2. *AMB concentrations in blood and tissues after a single dose iv of AMB-DOC or AmBisome® in infected leukopenic mice.*

Time after injection	AMB concn[a] (mg/L)		AMB concn[a] (μg/g)							
	BLOOD		LIVER		SPLEEN		KIDNEY		LUNG	
	A-DOC[b]	L-A[b]	A-DOC[b]	L-A[b]	A-DOC[b]	L-A[b]	A-DOC[b]	L-A[b]	A-DOC[b]	L-A[b]
5 min	0.5	102.0	1.9	17.7	<	28.0	1.3	6.3	1.1	<
15 min	0.4	96.7	2.1	25.9	4.9	38.0	1.0	4.0	1.1	<
1 h	0.3	76.8	2.2	48.6	3.3	92.0	1.0	1.3	1.1	<
2 h	0.2	53.9	1.8	57.9	3.3	90.0	0.7	1.3	1.1	<
4 h	0.2	39.7	1.4	64.6	<	122.0	<	2.7	<	<
12 h	<	13.1	1.0	85.2	<	118.0	<	3.0	<	1.4
24 h	<	1.5	0.8	97.8	<	140.0	<	4.0	<	7.1
48 h	<	0.3	<	82.3	<	134.0	<	4.0	<	6.2

[a]Blood and tissue samples from five mice were pooled
[b]A dosage of 0.3 mg AMB/kg AmB-DOC (A-DOC) or 7 mg AMB/kg AmBisome® (L-A) was administered iv at 6 h after *C.albicans* inoculation
< = not detectable

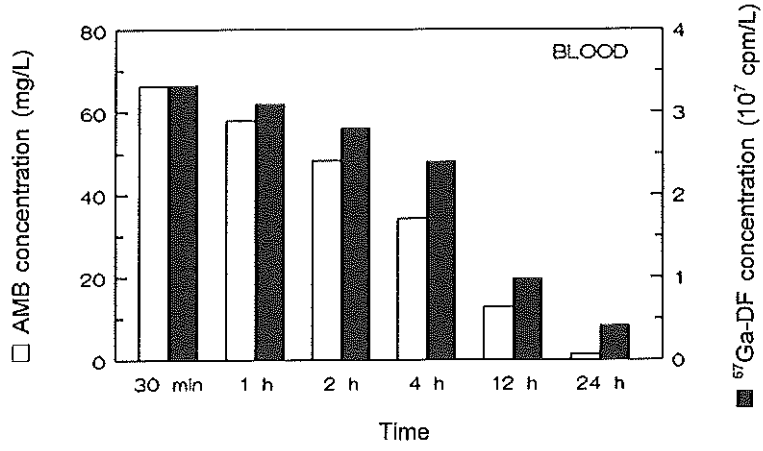

Figure 3. *Blood levels of AMB (□) as well as ^{67}Ga-DF (■) after a single dose iv of radiolabeled AmBisome® at a dosage of 7 mg AMB/kg in uninfected immunocompetent mice. Each symbol represents the mean of two experiments.*

DISCUSSION

The blood, liver and spleen concentrations of AMB were consistently higher after administration of AmBisome® than were those of the desoxycholate preparation. The biodistribution of both preparations of AMB exhibited a similar pattern in uninfected immunocompetent mice as well as in leukopenic mice infected with *C.albicans* which suggests that infection does not influence the disposition of the drug. Lopez-Berestein *et al.* [16] demonstrated the same phenomenon when comparing a different liposomal preparation of AMB (dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol in a 7:3 molar ratio) with AMB-DOC in immunocompetent mice. The immune status of the mice also had little influence on the biodistribution of the drug. The higher AMB concentrations found in the tissues of leukopenic mice, especially in the spleen, was probably due to the decrease in organ weight as a result either of infection or the aplasia induced by cyclophosphamide to inhibit hematopoiesis.

Hepatosplenic uptake of AmBisome® was highly dose dependent and the higher dose of AmBisome® produced a relatively long AMB circulation time as also shown by others [9,13,20]. When AmBisome® was given at the MTD, blood and tissue retained relatively high drug concentrations without evidence of toxicity as also has been noted by others [13,20].

When an equivalent dose of the liposomal preparation and AMB-DOC was given to immunocompetent uninfected mice, encapsulation of AMB resulted in increased

drug concentrations in blood, liver and spleen as has also been reported by Gondal *et al.* [13] and Francis *et al.* [9]. Gondal *et al.* [13] reported increased AMB levels in the liver and spleen and found equal drug concentrations in the kidneys and slightly elevated concentrations in the lungs during the first hour after administration. In contrast, concentrations in the kidneys and lungs were decreased in our study which we believe explains the lower efficacy of AmBisome® to an equivalent dosage of the desoxycholate preparation when treating systemic candidiasis in neutropenic mice [23]. The discrepancies might be the result of residual blood in the organs which will inflate the concentration unless a correction factor, such as the one we used, is employed.

Different patterns of biodistribution in animals were observed for the other industrially produced AMB lipid preparations, AmB-Lipid-Complex (ABLC) and AmB-Colloidal-Dispersion (ABCD, Amphocil®). Tissue concentrations of AMB in the liver and spleen were higher after administering ABLC than were those of AMB-DOC. Lung concentrations were similar to those after AMB-DOC whereas those in the kidney were slightly higher after the desoxycholate preparation [3,18]. Due to its large size (1.6-11 μm) ABLC is rapidly entrapped by the mononuclear phagocyte system resulting in blood levels that are considerably lower than those achieved with AMB-DOC. Animals receiving ABCD showed reduced peak concentrations of AMB in plasma and decreased concentrations in the spleen, kidney and lung but increased concentrations in the liver compared to the concentrations after treatment with AMB-DOC [7,8].

It is still thought that AMB is pharmacologically inactive when bound to liposomes. It was recently shown by Adler-Moore [1] and Proffitt *et al.* [19] that AMB remains tightly associated with the liposomal strucuture of AmBisome® *in vitro* and *in vivo* and that AmBisome® can interact directly with both phagocytized and non phagocytized fungal cells, resulting in death of the fungi. The extent to which release of AMB is important to the antifungal activity of AmBisome® is at present under investigation. However, a distiction should be made between liposome-encapsulated AMB and non-encapsulated AMB in blood and tissues in order to gain more insight into the antifungal activity of AmBisome® *in vivo* [6,15]. None of the assays originally used to measure AMB concentrations allowed this distinction to be made until Sculier *et al.* [22] developed a method for estimating the ratio of non-encapsulated and encapsulated liposomal AMB concentrations in serum by determining the total AMB concentrations by HPLC and the concentration of liposomes by the amount of inorganic phosphorus. Accurate assay of blood concentrations of liposomal AMB is further complicated if liposomal drug sediments together with erythrocytes during centrifugation to prepare plasma or serum. To obviate this, we measured AMB concentrations in whole blood and also investigated whether AMB concentrations in blood represented liposome-associated AMB by measuring the residence time of both AMB as well as intact liposomes in blood.

The data from this study suggest that the largest proportion of the total AMB concentration in blood represented liposome-encapsulated AMB. We tested various methods for separating AMB bound to lipoproteins and other plasma components from liposome-encapsulated AMB. Gel filtration on Sephacryl S500 or S1000, Sepharose 4B, Biogel A.15 columns were inappropriate for this purpose as was filtration/centrifugation through 300kDa Microsep filters. In the present study we opted to investigate whether AMB concentrations in blood represented liposome-associated AMB by labeling AmBisome® with the radioactive marker ^{67}Ga-DF which is a representative for intact liposomes. A relatively prolonged residence time of intact liposomes was shown after administration of AmBisome® at 7 mg AMB/kg. AMB levels in blood were equal or lower than the levels of intact liposomes suggesting that total AMB levels represent liposome-associated AMB, with slow release of AMB from the liposomes during circulation. As no AMB was released from AmBisome® in 90 % (v/v) serum at 37°C (unpublished data), the AMB released in whole blood was thought to be due, at least partly, to the interaction of AmBisome® with blood cells.

In summary, the biodistribution of neither preparation of AMB in mice was influenced by immunocompetence nor by infection with *C.albicans*. Encapsulation of AMB in AmBisome® yielded increased concentrations of drug in the blood, liver and spleen whereas levels in kidney and lung were decreased. Hepatosplenic uptake of AmBisome® was highly dosage dependent resulting in a relatively prolonged blood circulation time at the higher doses of 7 mg AMB/kg. After administration of AmBisome® at the MTD, blood and tissues retained high AMB concentrations which represented liposome-encapsulated AMB and the drug was released slowly during circulation in the blood. The extent to which the release of AMB is important for effective antifungal activity is presently under investigation.

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REFERENCES

1. Adler-Moore J. *In vivo* and *in vitro* evidence for reduced toxicity and mode of action of AmBisome®. Bone Marrow Transplant 1993;12(Suppl.4):S146.
2. Adler-Moore JP, Chiang SM, Satorius A, Guerra D, McAndrews B, McManus EJ, Proffitt RT. Treatment of murine candidosis and cryptococcosis with a unilamellar liposomal amphotericin B formulation (AmBisome). J Antimicrob Chemother 1991;28(Suppl.B): 63-71.

3. Clark MJ, Whitney RR, Olsen SJ, George RJ, Swerdel MR, Kunselman R, Bonner DP. Amphotericin B lipid complex therapy of experimental fungal infections in mice. Antimicrob Agents Chemother 1991;35:615-21.
4. Clemons KV, Stevens DA. Comparison of a liposomal amphotericin B formulation (AmBisome) and deoxycholate amphotericin B (Fungizone) for the treatment of murine paracoccidioidomycosis. J Med Vet Mycol 1993;31:387-94.
5. Clemons KV, Stevens DA. Therapeutic efficacy of a liposomal formulation of amphotericin B (AmBisome) against murine blastomycosis. J Antimicrob Chemother 1993;32:465-72.
6. De Marie S, Janknegt R, Bakker-Woudenberg IAJM. Clinical use of liposomal and lipid-complexed amphotericin B. J Antimicrob Chemother 1994;33:907-16.
7. Fielding RM, Singer AW, Wang LH, Babbar S, Guo LSS. Relationship of pharmacokinetics and drug distribution in tissue to increased safety of amphotericin B colloidal dispersion in dogs. Antimicrob Agents Chemother 1992;36:299-307.
8. Fielding RM, Smith PC, Wang LH, Porter J, Guo LSS. Comparative pharmacokinetics of amphotericin B after administration of a novel colloidal delivery system, ABCD, and a conventional formulation to rats. Antimicrob Agents Chemother 1991;35:1208-13.
9. Francis P, Lee JW, Hoffman A, Peter J, Francesconi A, Bacher J, Shelhamer J, Pizzo A, Walsh TJ. Efficacy of unilamellar liposomal amphotericin B in treatment of pulmonary aspergillosis in persistently granulocytopenic rabbits: the potential role of bronchoalveolar D-mannitol and serum galactomannan as marker of infection. J Infect Dis 1994;169:356-68.
10. Gabizon A, Huberty J, Straubinger RM, Price DC, Papahadjopoulos D. An improved method for in vivo tracing and imaging of liposomes using a gallium 67-deferoxamine complex. J Liposome Res 1988-89;1:123-35.
11. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-29.
12. Gates C, Pinney RJ. Amphotericin B and its delivery by liposomal and lipid formulations. J Clin Pharm Ther 1993;18:147-53.
13. Gondal JA, Swartz RP, Rahman A. Therapeutic evaluation of free and liposome-encapsulated amphotericin B in the treatment of systemic candidiasis in mice. Antimicrob Agents Chemother 1989;33:1544-8.
14. Heaton WA, Davis HH, Welch MJ, Mathias CJ, Joist JH, Sherman LA, Siegel BA. Indium-111: a new radionulclide label for studying human platelet kinetics. Br J Haematol 1979;42:613-22.
15. Janknegt R, De Marie S, Bakker Woudenberg IAJM, Crommelin DJA. Liposomal and lipid formulations of amphotericin B. Clinical Pharmacokinetics. Clin Pharmacokinet 1992;23:279-91.
16. Lopez-Berestein G, Rosenblum MG, Mehta R. Altered tissue distribution of amphotericin B by liposomal encapsulation: comparison of normal mice to mice infected with *Candida albicans*. Cancer Drug Deliv 1984;1:199-205.
17. Lyman CA, Walsh TJ. Systemically administered antifungal agents- A review of their clinical pharmacology and therapeutic applications. Drugs 1992;44:9-35.
18. Olsen SJ, Swerdel MR, Blue B, Clark JM, Bonner DP. Tissue distribution of amphotericin B lipid complex in laboratory animals. J Pharm Pharmacol 1991;43:831-5.
19. Proffitt RT, Adler-Moore JP, Fujii G, Satorius A, Lee MJA, Baily A. Stability and mode of action of AmBisome® (liposomal amphotericin B). J Control Release 1994;28:342-3.

20. Proffitt RT, Satorius A, Chiang SM, Sullivan L, Adler-Moore JP. Pharmacology and toxicology of a liposomal formulation of amphotericin B (AmBisome) in rodents. J Antimicrob Chemother 1991;28(Suppl.B):49-61.
21. Schmitt HJ. New methods of delivery of amphotericin B. Clin Infect Dis 1993; 17(Suppl.2):S501-6.
22. Sculier JP, Coune A, Meunier F, Brassine C, Laduron C, Hollaert C, Collette N, Heymans C, Klastersky J. Pilot study of amphotericin entrapped in sonicated liposomes in cancer patients with fungal infections. Exp J Cancer Clin Oncol 1988;24:527-38.
23. Van Etten EWM, Van den Heuvel-de Groot C, Bakker-Woudenberg IAJM. Efficacies of amphotericin B-desoxycholate (Fungizone), liposomal amphotericin B (AmBisome) and fluconazole in the treatment of systemic candidosis in immunocompetent and leucopenic mice. J Antimicrob Chemother 1993;32:723-39.
24. Woodle MC. ^{67}Gallium-labeled liposomes with prolonged circulation: Preparation and potential as nuclear imaging agents. Nucl Med Biol 1993;20:149-55.

Chapter 5

Interactions of AmBisome® with extracellular and intracellular *Candida albicans*

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ABSTRACT

The *in vitro* activities of liposomal amphotericin B (AmBisome®) and amphotericin B-desoxycholate (AMB-DOC) against extracellular *C.albicans* during 6 h of incubation in the presence of human serum were determined. With AMB-DOC inhibition of germ tube formation and effective killing were observed at AMB-concentrations of 0.8 mg/L and 3.2 mg/L, respectively. With AmBisome® for both parameters tested, 32-fold increased AMB concentrations were needed. Preincubation of AmBisome® in human serum during 6 h did not influence the rate of killing of *C.albicans*.

Antifungal activity against intracellular *C.albicans* was assessed at 4 h and 24 h after incubation of *C.albicans*-infected monolayers of peritoneal macrophages with antifungal agent. In the absence of antifungal agent *C.albicans* grows intracellularly by formation of germ tubes, and within 24 h mycelium is formed. Antifungal activity was evaluated in terms of both stabilization of the state of *C.albicans* infection, as well as eradication of *C.albicans* from infected macrophages. For AmBisome® only stabilization was observed at a concentration of 102 mg/L after 24 h of incubation. For AMB-DOC stabilization and eradication were observed only after 24 h of incubation at 0.8 mg/L and 1.6 mg/L, respectively. After previous exposure of macrophages to AmBisome® during 6 h before infection, increased antifungal activity of AmBisome® was observed: stabilization was observed at 4 h of incubation at 102 mg/L; at 24 h of incubation stabilization and eradication were observed at 25.6 mg/L and 102 mg/L, respectively. Prolongation of the exposure time before *C.albicans* infection from 6 h up to 24 h resulted in a further increase in antifungal activity of AmBisome®. Localization studies of AmBisome® and *C.albicans* in macrophages were performed using fluorescent-labeled *C.albicans* and fluorescent-labeled AmBisome®. It was observed that the presence of AmBisome® within a macrophage does not influence uptake of *C.albicans* by the same macrophage, or the other way around.

INTRODUCTION

Systemic candidiasis is seen with increasing frequency in the immuno-compromised patient. Although restricted by a variety of toxic side effects, parenteral amphotericin B (AMB) is still the drug of choice for most invasive fungal infections [6,12]. AMB is administered as amphotericin B-desoxycholate (AMB-DOC or Fungizone®). It has been shown that incorporation of AMB in liposomes or other lipid-carriers results in a reduction of toxic effects, and thereby in improvement of therapeutic index [5,7]. For one of the industrially prepared AMB-lipid preparations, AmBisome®, little is still known about the way in which this formulation exerts its antifungal activity *in vivo*. It is still thought by many investigators that AMB is biologically inactive when associated with liposomes. Still, it has been shown in two short reports [2,18] that AMB remains tightly associated with the liposomal structure of AmBisome® *in vitro* and *in vivo* and that AmBisome® can act on both phagocytized and non-phagocytized fungal cells, resulting in death of the fungi. In a study from our laboratory [24] it was previously shown that during circulation of AmBisome® in blood slow release of AMB occurred. As it is still not clear to what extent release of AMB is of importance for antifungal activity, in the present study the interactions of AmBisome® versus AMB-DOC with *Candida albicans* in the presence of serum were investigated.

Another issue on which only very limited data are available [16], is the antifungal activity of AmBisome® against intracellularly growing *C.albicans*. *C.albicans* can be considered as a facultative intracellular pathogen, as it is able to survive within unactivated macrophages and to grow out of these cells by forming germ tubes [22,25]. AMB-DOC is higly active against intracellular *C.albicans*, as was previously shown [13,22,25]. Therefore, it is questionable whether encapsulation of AMB in AmBisome® will be an advantage with respect to antifungal activity against intracellular *C.albicans*. Numerous studies have demonstrated that liposomes are phagocytized effectively by macrophages both *in vitro* and *in vivo* [20]. It is expected that high concentrations of AMB can be obtained intracellularly after exposure of macrophages to AmBisome®. It was also demonstrated in studies investigating liposomal ampicillin [3], that the liposomal lipid composition greatly influenced the rate of intracellular liposomal degradation and hence the rate at which liposome encapsulated agent was released intracellularly and became available to exert its antibacterial effect. As AmBisome® is a solid liposome type, containing saturated phospholipids with high transition temperatures (above 37°C), slow intracellular degradation and thereby delayed intracellular antifungal activity might be expected.

Furthermore, as the mononuclear phagocyte system (MPS) plays an important role in the host defence against *C.albicans* [10,11], it is important to know whether the uptake and degradation of AmBisome® by uninfected macrophages has effects on subsequent *C.albicans* infection.

In the present study several aspects on the interactions of AmBisome® with intracellular *C.albicans* were investigated: antifungal activity of AmBisome® against intracellular *C.albicans*, the effect of previous exposure of uninfected macrophages to AmBisome® on *C.albicans* ingestion and intracellular antifungal activity, and the localization of both *C.albicans* and AmBisome® in macrophages.

MATERIALS AND METHODS

Materials. Antibiotic Medium no. 3 (Penassay broth) and Todd-Hewitt broth were from Difco Laboratories (Detroit, MI). Sabouraud dextrose agar was from Oxoid (Basingstoke, England). Amphotericin B-desoxycholate (AMB-DOC or Fungizone® for intravenous infusion) was kindly provided by Bristol Myers-Squibb, Woerden, The Netherlands. AMB-DOC was reconstituted with distilled water to obtain a stock solution of 5 g/L. AmBisome®, AMB encapsulated in small unilamellar vesicles consisting of hydrogenated soybean phosphatidylcholine, cholesterol and distearoylphosphatidylglycerol in a molar ratio of 2:1:0.8, was kindly provided by Vestar Inc. (San Dimas, CA) as a lyophilized preparation. The powder was reconstituted according to the manufacturer's instructions with distilled water at 65°C to give a liposomal suspension containing 4 g AMB/L and 35 g lipid/L. Fluorescein isothiocyanate (FITC) was obtained from Sigma (St. Louis, MO). Pooled human serum (PHS) was obtained from a pool of 350 serum samples from healthy volunteer donors. Dulbecco modified Eagle medium (DMEM) and Trypan Blue (0.4% v/v in saline) were from Flow Laboratories, Irvine, Scotland. Lab Tek® chamber slides were from Nunc Inc.(Naperville, IL). Fetal bovine serum (FBS) was from Hy-Clone (Logan, Utah). Fluorescent marker 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate D282 (DiI) was kindly provided by Eric Claassen from the Dept. of Immunology and Medical Microbiology, TNO Medical Biological Laboratory, Rijswijk, The Netherlands. Glycerol was from Merck (Darmstadt, Germany). Glutamine was from Flow (Amstelstad, The Netherlands).

Candida strain. *Candida albicans* ATCC 44858 was used in all experiments and was stored at -80°C in Todd-Hewitt broth containing 10% (v/v) glycerol. Of AMB-DOC and AmBisome® the MIC values were 0.1 mg/L and 0.05 mg/L, respectively, and MFC values were 3.2 mg/L and 6.4 mg/L, respectively, as described previously [23].

Fluorescent labeling of *C.albicans*. A stationary-growth-phase suspension of *C.albicans* was washed three times with a buffer solution containing 0.1 M $NaHCO_3/Na_2CO_3$ (pH 9.5). Then the pellet of *C.albicans* was resuspended and incubated with 1 g FITC/L buffer solution for 72 h at 4°C, after which the nonbinding FITC molecules were removed by washing three times with buffer solution.

Opsonization of *C.albicans*. *C.albicans* was opsonized by incubation of a washed stationary-growth-phase suspension with 10% normal mouse serum

(BALB/c) under continuous rotation at 8 rpm for 15 min at 37°C, after which the yeasts were washed twice in PBS.

Fluorescent labeling of AmBisome®. AmBisome® was labeled with the fluorescent marker DiI. As shown by Claassen [4] this label is appropriate for determining the localization of liposomes *in vitro* and *in vivo*. Furthermore, this label can be used for labelling of preformed liposomes [4], such as AmBisome®. DiI was dissolved in 100% ethanol (2.5 mg/ml) and the stock solution was kept at 4°C. DiI was added to AmBisome® at an amount of 4-5 mg/g liposomal lipid. To obtain homogeneously labeled material, the addition of DiI was always performed under rigorous vortexing. Non-incorporated DiI was removed by filtration of the labeled AmBisome® suspension through 0.2 μm membranes.

Antifungal activity of AmBisome® versus AMB-DOC in the presence of serum against extracellular *C.albicans* during short-term growth. The *in vitro* activities of AmBisome® and AMB-DOC against *C.albicans* at an inoculum of $1.3x10^7$ CFU/L during 6 h of incubation in the presence of serum were determined as decribed previously [22]. Briefly, a logarithmic-growth-phase culture of *C.albicans* was prepared. *C.albicans* was exposed during 6 h of incubation under continuous rotation to twofold increasing concentrations of each agent in Antibiotic Medium no. 3 supplemented with 90% (v/v) PHS ranging from 0.05-102 mg/L. During incubation the numbers of viable *C.albicans* were determined at 2 h intervals by making plate counts of ten-fold serial dilutions of the washed specimen on Sabouraud dextrose agar. Because the presence of serum induces the formation of germ tubes by *C.albicans*, the antifungal activity was expressed not only in terms of effective killing (>99.9%) of *C.albicans*, but also in terms of inhibition of germ tube formation, which was determined microscopically in a hemacytometer.

Effect of previous incubation of AmBisome® or AMB-DOC in serum on antifungal activity against extracellular *C.albicans* during short-term growth. The *in vitro* activities of AmBisome® at 102 mg/L and AMB-DOC at 3.2 mg/L against *C.albicans* at an inoculum of $1.3x10^7$ CFU/L during 6 h of incubation in the presence of 90% (v/v) PHS were also determined after previous incubation of AmBisome® and AMB-DOC during 6 h in PHS in the absence of *C.albicans*.

Antifungal activity of AmBisome® versus AMB-DOC against intracellular *C.albicans*. Macrophages were obtained from peritoneal cavities of 12- to 14-weeks-old specified pathogen-free female BALB/c mice (Iffa Credo, L'arbresle, France). The macrophages were washed twice in DMEM supplemented with 1% glutamine. Monolayers of peritoneal macrophages were cultured at 37°C on chamber slides under a humidified atmosphere of 7.5% CO_2 in air in culture medium containing DMEM supplemented with 1% glutamine and 15% FBS. After the first 2 h of incubation, fresh culture medium was added to the monolayer, and the macrophages were incubated for 24 h. After this incubation period, an inoculum of $2x10^5$ CFU of opsonized *C.albicans* were added to the monolayer, resulting in a *C.albicans*-to-macrophage ratio of 5:8. After a 30-min uptake period, the

noningested yeasts were removed by washing the monolayer three times with DMEM supplemented with 1% glutamine and 5% FBS at 37°C (time zero). The macrophages were reincubated for 24 h in the presence of twofold increasing concentrations AmBisome® (3.2 up to 102 mg/L, equivalent to 28 up to 896 mg lipid/L) or AMB-DOC (0.1 up to 1.6 mg/L) in culture medium supplemented with 1% glutamine and 5% FBS. Control monolayers were incubated with the solvent of the antifungal agent in the appropriate dilution. The proportion of viable macrophages following incubation for 24 h with the antifungal agents was assessed by means of a trypan blue exclusion method (0.2% v/v). At time zero and at 4 h and 24 h of incubation, the monolayers were washed three times with PBS, fixed in methanol, stained with May-Grünwald-Giemsa, and examined under a microscope. Three experiments were performed in which in each individual experiment for each incubation condition a total number of 150 macrophages were scanned. The treatment status pertaining to individual slides was not known to the observer. The results were expressed as percentages of total macrophages in which the macrophages were divided into four categories: (1) uninfected macrophages, (2) macrophages infected with *C.albicans* blastospores, (3) macrophages infected with both *C.albicans* blastospores as well as germ-tubes or hyphae, and (4) macrophages infected with *C.albicans* germ-tubes, hyphae or even mycelium.

Using the method of microscopic examination, no discrimination was made between *C.albicans* attached to the macrophages or *C.albicans* interiorized by the macrophages. As endocytosis does not occur at 4°C [26], attachment of *C.albicans* to the macrophages was studied by incubation of the macrophages and opsonized *C.albicans* at a *C.albicans*-to-macrophage ratio of 5:8 during 30 min at 4°C instead of 37°C, followed by washing of the monolayer.

Effect of previous exposure of macrophages to AmBisome® on antifungal activity against intracellular *C.albicans*. Monolayers of peritoneal macrophages were cultured as described above. After 24 h of incubation, macrophages were reincubated for 6 h or 24 h in the presence of twofold increasing concentrations AmBisome® (3.2 up to 102 mg/L) in culture medium supplemented with 1% glutamine and 5% FBS. Control monolayers were incubated with the solvent of the antifungal agent in the appropriate dilution. After this incubation period, the monolayer was washed three times with culture medium at 37°C. Opsonized *C.albicans* was added to the monolayer, and ingested as described above. Noningested yeasts were removed by washing the monolayer three times with culture medium at 37°C (time zero). The macrophages were reincubated for 24 h in the presence of AmBisome® just as before *C.albicans* ingestion. Intracellular antifungal activity was determined at time zero and at 4 h and 24 h of incubation, as described above.

Localization of AmBisome® in macrophages infected with *C.albicans*. Monolayers of peritoneal macrophages were cultured as described above. After 24 h of incubation, FITC-labeled *C.albicans* was added to the monolayer, and ingested

as described above. Noningested yeasts were removed by washing with culture medium at 37°C. Macrophages were reincubated for 4 h in the presence of twofold increasing concentrations of DiI-labeled AmBisome® (3.2 up to 102 mg/L) in culture medium, as described above. After this incubation period, the monolayer was washed three times with ice-cold PBS and observed directly by fluorescence microscopy after mounting with PBS with 10% (v/v) glycerol.

Localization of *C.albicans* in macrophages previously exposed to AmBisome®. Monolayers of peritoneal macrophages were cultured as described above. After 24 h of incubation, macrophages were reincubated for 4 h in the presence of twofold increasing concentrations of DiI-labeled AmBisome® (3.2 up to 102 mg/L) in culture medium, as described above. After this incubation period, the monolayer was washed three times with culture medium at 37°C. FITC-labeled *C.albicans* was added to the monolayer, and ingested as described above. Noningested yeasts were removed by washing with ice-cold PBS and the monolayer was observed directly by fluorescence microscopy after mounting with PBS with 10% (v/v) glycerol.

RESULTS

Antifungal activity of AmBisome® versus AMB-DOC in the presence of serum against extracellular *C.albicans* during short-term growth. The *in vitro* activities of AmBisome® and AMB-DOC against *C.albicans* at an inoculum of $1.3x10^7$ CFU/L during 6 h of incubation in the presence of PHS are presented in Table 1. With AMB-DOC inhibition of germ tube formation and effective killing were observed at AMB-concentrations of 0.8 mg/L and 3.2 mg/L, respectively. With AmBisome® for both parameters tested, 32-fold increased AMB concentrations were needed.

Table 1. *Antifungal activity of AmBisome® versus AMB-DOC in the presence of 90% (v/v) PHS against C.albicans (inoculum $1.3x10^7$ CFU/L) during 6 h incubation.*

	Minimal AMB concn (mg/L) required for:	
	Inhibition of germ tube formation	Effective killing (>99.9%)
AMB-DOC	0.8	3.2
AmBisome®	25.6	102

Effect of previous incubation of AmBisome® or AMB-DOC in serum on antifungal activity against extracellular *C.albicans* during short-term growth. Antifungal activities of AMB-DOC at 3.2 mg/L or AmBisome® at 102 mg/L against *C.albicans* at an inoculum $1.3x10^7$ CFU/L during 6 h of incubation in PHS, without preincubation or after preincubation during 6 h in PHS is shown in Figure 1. For

both AMB-DOC as well as for AmBisome®, preincubation in PHS did not influence the rate of killing of *C.albicans*.

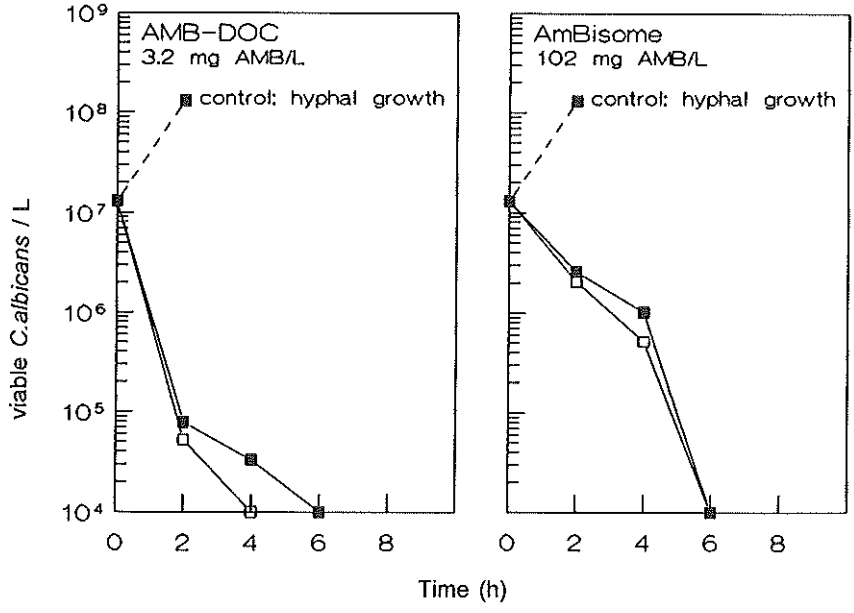

Figure 1. *Effect of preincubation of AMB-DOC and AmBisome® in PHS on antifungal activity against C.albicans. Antifungal activity of AMB-DOC at 3.2 mg/L or AmBisome® at 102 mg/L against C.albicans (inoculum 1.3x10⁷ CFU/L) during 6 h of incubation in 90 % (v/v) PHS, without preincubation (■) or after preincubation during 6 h in PHS (□). Each symbol represents the mean of three experiments.*

Antifungal activity of AmBisome® versus AMB-DOC against intracellular *C.albicans*. Intracellular growth of *C.albicans* in monolayers of peritoneal macrophages in the absence of antifungal agent is shown in Figure 2. Immediately after the 30-min *C.albicans* ingestion period (time zero) 65% of total macrophages were infected with *C.albicans* blastospores. Attachment of *C.albicans* to the macrophages after 30 min of incubation at 4°C was shown to be very low: *C.albicans* association was observed for only 3% of macrophages (data not shown). After 4 h of incubation all intracellular *C.albicans* had formed germ-tubes or hyphae. After 24 h of incubation massive hyphal or mycelial growth of *C.albicans* was observed within a heavily disrupted monolayer. Antifungal activities of AmBisome® or AMB-DOC after 4 h and 24 h of incubation against intracellular *C.albicans* is shown in Figure 3. Cellular toxicity of AmBisome® or AMB-DOC at the concentrations used was not observed as measured by trypan blue exclusion.

Furthermore, total numbers of macrophages in the monolayers were not decreased following antifungal treatment.

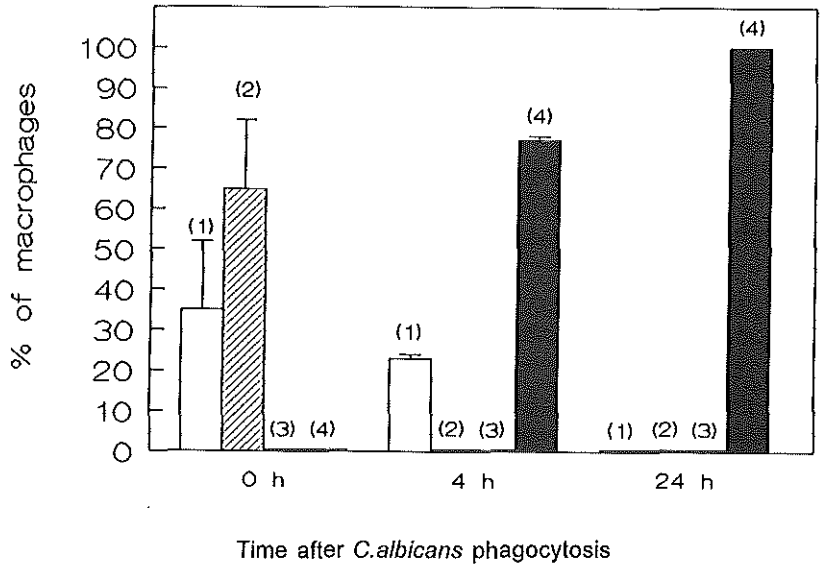

Figure 2. *State of C.albicans infection in macrophage monolayers. (1) uninfected macrophages □; (2) macrophages with intracellular C.albicans blastospores ▨; (3) macrophages with intracellular C.albicans blastospores and germtubes or hyphae or mycelium ▩; (4) macrophages with intracellular C.albicans germtubes or hyphae or mycelium ■.*

Antifungal activity was evaluated in terms of both stabilization of the state of *C.albicans* infection, as well as eradication of *C.albicans* from infected macrophages. As about 65% of total macrophages were infected with *C.albicans* blastospores immediately after the *C.albicans* ingestion period, stabilization of this state of *C.albicans* infection was considered when percentage of *C.albicans*-infected macrophages did not exceed 65%, with percentage of macrophages infected with *C.albicans* was considered when percentage of *C.albicans*-infected macrophages was reduced from 65% to less than 10%. For AmBisome® eradication of *C.albicans* was not observed up to a concentration of 102 mg/L within 4 h or even 24 h of incubation of the monolayer infected with *C.albicans*. Stabilization was observed

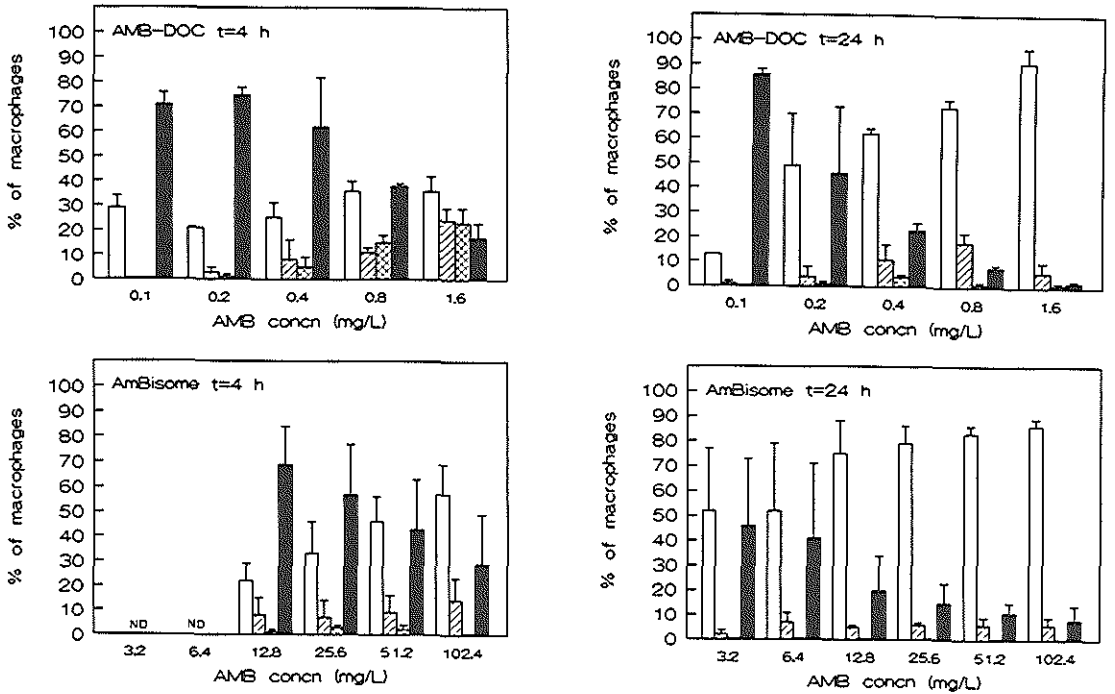

Figure 3. *State of C.albicans infection in macrophage monolayers during incubation with AmBisome® versus AMB-DOC. Uninfected macrophages □; macrophages with intracellular C.albicans blastospores ▨; macrophages with intracellular C.albicans blastospores and germtubes or hyphae or mycelium ▩; macrophages with intracellular C.albicans germtubes or hyphae or mycelium ■.*
ND = not determined

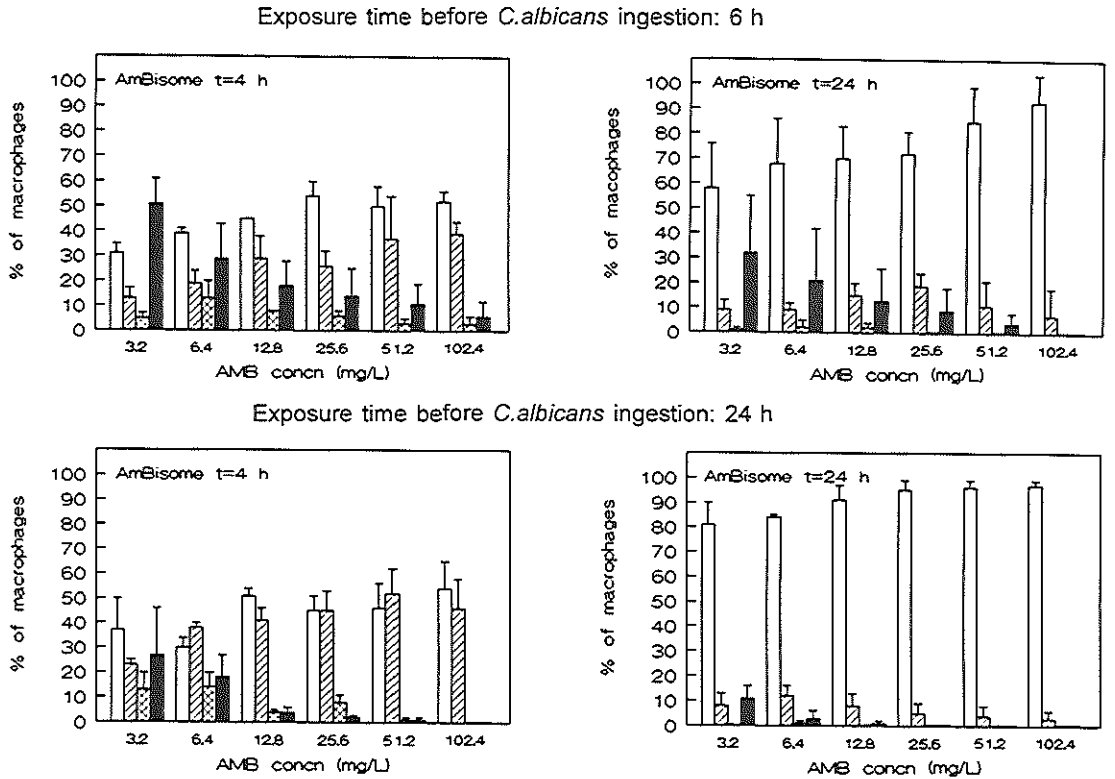

Figure 4. *State of C.albicans infection in macrophage monolayers previously exposed to AmBisome®, during incubation with AmBisome®. Uninfected macrophages □; macrophages with intracellular C.albicans blastospores ▨; macrophages with intracellular C.albicans blastospores and germtubes or hyphae or mycelium ▩; macrophages with intracellular C.albicans germtubes or hyphae or mycelium ■.*

only after 24 h of incubation at 102 mg/L. For AMB-DOC stabilization and eradication of *C.albicans* were observed only after 24 h of incubation at 0.8 mg/L and 1.6 mg/L, respectively.

Effect of previous exposure of macrophages to AmBisome® on antifungal activity against intracellular *C.albicans*. The effect of exposure to AmBisome® of uninfected macrophages during 6 h or 24 h before *C.albicans* infection on antifungal activity of AmBisome® after 4 h and 24 h of incubation against intracellular *C.albicans* is shown in Figure 4. Previous exposure of the macrophages to AmBisome® up to a concentration of 102 mg/L during 6 h or 24 h did not have any effect on the total numbers of macrophages in the monolayers or on the numbers of macrophages infected with *C.albicans* at time zero (data not shown). Increased antifungal activity was observed after previous exposure to AmBisome® during 6 h: stabilization was observed after 4 h of incubation at 102 mg/L; after 24 h of incubation stabilization and eradication of *C.albicans* were observed at 25.6 mg/L and 102 mg/L, respectively. Prolongation of the exposure time before *C.albicans* infection from 6 h up to 24 h increased antifungal activity even further. After 4 h of incubation stabilization was observed at 12.8 mg/L; after 24 h of incubation stabilization and eradication of *C.albicans* were observed at 6.4 mg/L and 12.8 mg/L, respectively.

Localization of AmBisome® in macrophages infected with *C.albicans*. Macrophages infected with FITC-labeled *C.albicans* were thereafter incubated for 4 h in the presence of various concentrations of DiI-labeled AmBisome® ranging from 3.2 up to 102 mg/L. As significant autofluorescence was observed with air-dried and fixed slides, it should be stressed that the slides were observed directly after incubation and subsequent rinsing, without drying and fixation. Another reason for leaving out fixation of the slides with organic solvent was to prevent possible dissolution and thereby removal of the liposomal phospholipid bilayers together with fluorescent label, subsequent to the fixation procedure.

It was observed that AmBisome® was localized in all macrophages infected with *C.albicans* as well as in part of the uninfected macrophages, with increased intensity of fluorescence at increased AmBisome® concentrations.

Localization of *C.albicans* in macrophages previously exposed to AmBisome®. Uninfected macrophages were incubated for 4 h in the presence of various concentrations of DiI-labeled AmBisome® ranging from 3.2 up to 102 mg/L, followed by infection with FITC-labeled *C.albicans*. It was observed that all of the macrophages in which *C.albicans* was localized already contained AmBisome®, with increased intensity of fluorescence at increased AmBisome® concentrations.

DISCUSSION

For two different liposomal formulations of AMB, AmBisome® and AMB in dimyristoylphosphatidylcholine / dimyristoylphosphatidylglycerol (DMPC / DMPG

7:3, 5 mol% AMB), the *in vitro* antifungal activities against *C.albicans* in the absence of serum have been investigated previously [1,8,15,19,23]. It was shown for both liposomal formulations that the *in vitro* activity of liposomal AMB was less than that of AMB-DOC after short term exposure of *C.albicans* [19,23], whereas after long-term exposure (MIC, MFC) liposomal AMB was either less active than AMB-DOC [15,19] or equally active as AMB-DOC [1,8,19,23]. It is still thought by many investigators that AMB is biologically inactive when associated to liposomes. AMB should be released from the liposomes to exert antifungal activity. The antifungal activity of liposomal AMB during short term incubation is low as a consequence of marginal release of AMB in medium without serum. After long term incubation release of AMB may be substantial, resulting in antifungal activity similar to that of AMB-DOC. However, for AmBisome® it was reported [18] that AMB remains tightly associated with the liposomal structure for up to 24 h *in vitro*, either in the absence or in the presence of 50% (v/v) human plasma, as well as *in vivo* during circulation after intravenous administration of AmBisome® in mice. The investigators also reported a direct interaction between AmBisome® and fungal cells. For a lipid formulation of AMB at 50 mol% in DMPC/DMPG (7:3), it was reported that AMB can be released from the lipid-complex by fungal lipases [17]. Whether fungal lipases play a role in the release of AMB from AmBisome® in the close vicinity of the fungus, and whether this is necessary for antifungal activity has to be investigated. We have previously shown for AmBisome® [24] that slow release of AMB occurred during circulation in blood. Until now it remained unclear to what extent release of AMB is of importance for antifungal activity. In the present study the interactions of AmBisome® with *C.albicans* in the presence of serum were examined. During short-term incubation in the presence of 90 % (v/v) human serum still 32-fold increased AMB concentrations are required for effective killing of *C.albicans* as compared to AMB-DOC. This indicates that even in 90% (v/v) serum AMB remains tightly associated to the liposome in AmBisome®, as effective killing is expected at only 3% release of AMB from AmBisome®. Furthermore, it was investigated for AmBisome® whether killing of *C.albicans* was due to serum-induced AMB release. As killing of *C.albicans* with AMB-DOC was much faster than with AmBisome®, release of AMB from AmBisome® during preincubation in serum would result in an increased killing rate of *C.albicans*. We found that the rate of killing of *C.albicans* by AmBisome® was not influenced by preincubation of AmBisome® in serum, indicating that serum-induced AMB release is not necessary for antifungal activity against extracellular *C.albicans*.

With respect to the interactions of AmBisome® versus AMB-DOC with intracellular *C.albicans*, in the present study the parameters for intracellular antifungal activity used, were different from those used in a previous study from our laboratory [22]. In that previous study [22] the parameters for intracellular antifungal activity were inhibition of germ tube formation and effective killing (>99%) of intracellular *C.albicans*. Effective killing was measured by CFU counts of viable

intracellular *C.albicans*. As discussed previously [22], CFU counts of intracellular *C.albicans* can only be determined accurately in case only *C.albicans* blastospores are present and germ tube formation is completely inhibited. In the present study, it became clear for two reasons that use of these parameters could lead to misinterpretation of the results. First, under conditions in which the total number of *C.albicans*-infected macrophages was reduced substantially, intracellular *C.albicans* germ tubes or hyphae were still observed. Secondly, intracellular *C.albicans* germ tubes or hyphae were sometimes eradicated from infected macrophages between 4 h and 24 h of incubation, indicating that *C.albicans* germ tubes or hyphae are still susceptible for the antifungal activity of AMB. It is also reported by other investigators [14] that *C.albicans* germ tubes or hyphae are susceptible for AMB, even more susceptible than *C.albicans* blastospores. We decided that for determining antifungal activity against intracellular *C.albicans* it is more relevant to investigate what is actually changing in the state of infection of macrophages, instead of just focussing on aspects of the intracellular *C.albicans* (germ tube formation and viability) alone. Therefore, microscopic observation of the infected monolayers was used in the present study.

The data clearly show that, in parallel with the antifungal activities against extracellular *C.albicans*, the intracellular antifungal activity of AMB-DOC is much higher than that of AmBisome®. Of course, it should also be noted that blood and tissue levels of AMB that can be achieved *in vivo* with AmBisome® are much higher than with AMB-DOC. We have previously reported [24] that after administration of a single dose of AMB-DOC at the MTD of 0.3 mg AMB/kg, a maximum concentration of 0.6 mg/L in blood, and tissue concentrations up to 2.2 μg/g in liver and 4.9 μg/g in spleen were achieved. With AmBisome®, at the MTD of 7 mg AMB/kg, concentrations up to 104 mg/L in blood, 98 μg/g in liver and 140 μg/g in spleen were obtained. In human patients [9] peak levels of 1.5-2 mg/L in serum were observed with AMB-DOC at 1 mg AMB/kg , whereas with AmBisome® at 2.5 mg AMB/kg plasma concentrations were measured up to 20.3 mg/L. It is clear that although for intracellular antifungal activity much higher dosages of AmBisome® than AMB-DOC are required, much higher extracellular concentrations can be achieved with AmBisome® than with AMB-DOC. Still, extrapolation of the data derived from the *in vitro* experiments in the present study to the clinically relevant situation *in vivo* remains speculative.

Although numerous studies have demonstrated that liposomes are phagocytosed effectively by macrophages both *in vitro* and *in vivo* [20], an explanation for the low intracellular activity of AmBisome® might be low uptake of AmBisome® by macrophages infected with *C.albicans*. However, from our localization experiments with flourescent-labeled AmBisome® it is clear that AmBisome® is avidly taken up by macrophages, both by uninfected macrophages as well as by macrophages infected with *C.albicans*. Another more reasonable explanation for the low intracellular activity is slow intracellular degradation of AmBisome®. AmBisome®

is a solid liposome type, composed of saturated phospholipids with high transition temperatures and cholesterol, which is expected to be slowly degraded intracellularly. For ampicillin encapsulated in two types of liposomes, differing in the rigidity of the lipid bilayer, it was clearly demonstrated [3], that the liposomal lipid composition greatly influenced the rate of intracellular liposomal degradation. Relatively slow intracellular degradation was demonstrated for the solid liposome type. This relatively slow degradation resulted in a delayed intracellular release of the encapsulated ampicillin, as reflected in absent or delayed intracellular killing of intracellular *Listeria monocytogenes*.

As macrophages seem to be occupied with the intracellular degradation of AmBisome® for a substantial period of time, an important question is whether uptake and degradation of AmBisome® in uninfected macrophages has unfavourable effects on subsequent *C.albicans* infection. Other investigators [16] reported that after previous exposure of human polymorphonuclear neutrophils (PMNs) to AmBisome® at a concentration of 20 mg/L subsequent uptake of *C.albicans* was not influenced, whereas intracellular killing of *C.albicans* during subsequent incubation for 60 min with AmBisome® was increased. The rapid effect of AmBisome® reported in this study may result from a rapid degradation of AmBisome® in human PMNs. Another explanation is related to the fact that the PMNs used in this study had already candidacidal activity of their own. This candidacidal activity is possibly easily enhanced after only minimal damage of intracellular *C.albicans* by AmBisome®. In the present study it was shown that exposure to AmBisome® of uninfected macrophages does not influence the state of *C.albicans* infection in macrophage monolayers. From our localization experiments it is clear that the presence of AmBisome® within a macrophage does not influence uptake of *C.albicans* in the same macrophage. It is clearly shown that previous exposure of uninfected macrophages to AmBisome® is advantageous for antifungal activity of AmBisome® against intracellular *C.albicans*. These results indicate once more that for intracellular degradation of AmBisome® time is needed.

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REFERENCES

1. Anaissie E, Paetznick V, Proffitt R, Adler-Moore J, Bodey GP. Comparison of the *in vitro* antifungal activity of free and liposome-encapsulated amphotericin B. Eur J Clin Microbiol Infect Dis 1991;10:665-8.
2. Adler-Moore J. *In vivo* and *in vitro* evidence for reduced toxicity and mode of action of AmBisome®. Bone Marrow Transplant 1993;12(Suppl.4):S146.

3. Bakker-Woudenberg IAJM, Lokerse AF, Roerdink, FH. Effect of lipid composition on activity of liposome-entrapped ampicillin against intracellular *Listeria monocytogenes*. Antimicrob Agents Chemother 1988;32:1560-4.
4. Claassen E. Post-formation fluorescent labelling of liposomal membranes. *In vivo* detection, localisation and kinetics. J Immunol Methods 1992:147:231-40.
5. De Marie S, Janknegt R, Bakker-Woudenberg IAJM. Clinical use of liposomal and lipid-complexed amphotericin B. J Antimicrob Chemother 1994;33:907-16.
6. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-29.
7. Gates C. Pinney RJ. Amphotericin B and its delivery by liposomal and lipid formulations. J Clin Pharm Ther 1993;18:147-53.
8. Hopfer RL, Mills K, Mehta R, Lopez-Berestein G, Fainstein V, Juliano RL. *In vitro* antifungal activities of amphotericin B and liposome-encapsulated amphotericin B. Antimicrob Agents Chemother 1984;25:387-9.
9. Janknegt R, De Marie S, Bakker Woudenberg IAJM, Crommelin DJA. Liposomal and lipid formulations of amphotericin B. Clinical Pharmacokinetics. Clin Pharmacokinet 1992;23:279-91.
10. Jensen J, Warner T, Balish E. The role of phagocytic cells in resistence to disseminated candidiasis in granulocytopenic mice. J Infect Dis 1994;170:900-5.
11. Levitz SM. Overview of host defences in fungal infections. Clin Infect Dis 1992; 14(Suppl.1):S37-42.
12. Lyman CA, Walsh TJ. Systemically administered antifungal agents- A review of their clinical pharmacology and therapeutic applications. Drugs 1992;44:9-35.
13. Martin E, Stüben A, Görz A, Weller U, Bhakdi S. Novel aspect of amphotericin B action: accumulation in human monocytes potentiates killing of phagocytosed *Candida albicans*. Antimicrob Agents Chemother 1994;38:13-22.
14. Nugent KM, Couchot KR, Gray LD. The effect of *Candida* morphology on amphotericin B susceptibility. Antimicrob Agents Chemother 1987;31:335-6.
15. Pahls S, Schaffner A. Comparison of the activity of free and liposomal amphotericin B *in vitro* and in a model of systemic and localized murine candidiasis. J Infect Dis 1994; 169:1057-61.
16. Pallister CJ, Johnson EM, Warnock DW, Elliot PJ, Reeves DF. *In vitro* effects of liposome-encapsulated amphotericin B (AmBisome) and amphotericin B-deoxycholate (Fungizone) on the phagocytic and candidacidal function of human polymohonuclear leucocytes. J Antimicrob Chemother 1992;30:313-20.
17. Perkins WR, Minchey SR, Boni LT, Swenson CE, Popescu MC, Pasternack RF, Janoff AS. Amphotericin B-phospholipid interactions resposible for reduced mammalian cell toxicity. Biochim Biophys Acta 1992;1107:271-82.
18. Proffitt RT, Adler-Moore JP, Fujii G, Satorius A, Lee MJA, Baily A. Stability and mode of action of AmBisome® (liposomal amphotericin B). J Control Release 1994;28:342-3.
19. Ralph ED, Khazindar AM, Barber KR, Grant CWM. Comparative *in vitro* effects of liposomal amphotericin B, amphotericin B-deoxycholate, and free amphotericin B against fungal strains determined by using MIC and minimal lethal concentration susceptibility studies and time-kill curves. Antimicrob Agents Chemother 1991;35:188-91.
20. Scherphof GL, Dijkstra J, Spanjer HH, Derksen JTP, Roerdink FH. Uptake an intracellular processing of targeted and non-targeted liposomes by rat Kupffer cells *in vivo* and *in vitro*. Ann NY Acad Sciences 1985;446:368-84.
21. Schmitt HJ. New methods of delivery of amphotericin B. Clin Infect Dis 1993; 17(Suppl.2):S501-6.

22. Van Etten EWM, Van de Rhee NE, Van Kampen KM, Bakker-Woudenberg IAJM. Effects of amphotericin B and fluconazole on the extracellular and intracellular growth of *Candida albicans*. Antimicrob Agents Chemother 1991;35:2275-81.
23. Van Etten EWM, Van den Heuvel-de Groot C, Bakker-Woudenberg IAJM. Efficacies of amphotericin B-desoxycholate (Fungizone), liposomal amphotericin B (AmBisome) and fluconazole in the treatment of systemic candidosis in immunocompetent and leucopenic mice. J Antimicrob Chemother 1993;32:723-39.
24. Van Etten EWM, Otte-Lambillion M, Van Vianen W, Ten Kate MT, Bakker-Woudenberg IAJM. Biodistribution of liposomal amphotericin B (AmBisome) and amphotericin B-desoxycholate (Fungizone) in uninfected immunocompetent mice and leucopenic mice infected with *Candida albicans*. J Antimicrob Chemother 1995;35:509-19
25. Van 't Wout JW, Meynaar I, Linde I, Mattie H, Van Furth R. Effect of amphotericin B, fluconazole and itraconazole on intracellular *C.albicans* and germ tube development in macrophages. J Antimicrob Chemother 1990;25:803-11.
26. Willingham MC, Pastan IH. Receptor mediated endocytosis: general considerations and morphological approaches. In: Curatrecasas P, Roth TF (Eds.) Receptor mediated Endocytosis: Receptors and recognition-Series B. Vol. 15. Chapmann & Hall, London. 1983:1-17.

Chapter 6

Sterically stabilized pegylated amphotericin B-liposomes: toxicity and biodistribution in mice

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Sterically stabilized amphotericin B-liposomes: toxicity and biodistribution in mice

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ABSTRACT

In this study it was investigated whether long-circulating amphotericin B (AMB) containing liposomes could be prepared by incorporation of polyethyleneglycol (1900) derivatized distearoylphosphatidylethanolamine (PEG-DSPE), and whether the incorporation of PEG-DSPE affected toxicity and biodistribution of the preparation in mice. Toxicity of two formulations of liposomes containing both PEG-DSPE and AMB (PEG-AMB-LIP, types 1 and 2) was compared with that of AMB-liposomes without PEG-DSPE (AMB-LIP) as well as that of AMB-desoxycholate (AMB-DOC). The maximum tolerated dosage (MTD) of AMB-DOC, expressed in terms of death during treatment for 5 consecutive days or significant increases in the parameters used to monitor renal and hepatic functions, was 0.8 mg AMB/kg/day. AMB-LIP were the least toxic, the MTD being 11 mg AMB/kg/day. The formulation with AMB complexed to DSPG (PEG-AMB-LIP type 1) was as toxic as AMB-DOC. This PEG-AMB-LIP formulation was omitted from further studies on biodistribution. With AMB complexed to PEG-DSPE (PEG-AMB-LIP type 2) toxicity was substantially reduced, resulting in a MTD of 9 mg AMB/kg/day. Biodistribution of radiolabeled PEG-AMB-LIP type 2 was compared with that of AMB-LIP. Blood residence time of PEG-AMB-LIP type 2 was prolonged as compared to AMB-LIP; For PEG-AMB-LIP type 2 at 24 h after administration 30% of the injected dosage of AMB in intact liposomes was circulating versus 6% for AMB-LIP.

INTRODUCTION

The therapy of choice for most invasive fungal infections remains amphotericin B (AMB), administered as amphotericin-desoxycholate (AMB-DOC or Fungizone®). However, its clinical use is limited by toxic side effects [17]. A promising approach in the treatment of deep systemic fungal infections is AMB incorporated in liposomes or other lipid-carriers [11,17,19]. With respect to the industrially produced preparations AmBisome®, ABLC (AMB Lipid Complex) and Amphocil® it is evident that these have quite different structural and pharmacokinetic characteristics [13]. The relatively large structures of ABLC as well as the small discoidal particles of Amphocil® are rapidly taken up by the mononuclear phagocyte system (MPS), whereas small liposomes as in AmBisome® remain in the circulation for relatively prolonged periods. Until now it is not known whether blood residence time is an important variable influencing the therapeutic index of AMB-liposomes.

The ability to achieve a significantly longer blood residence time of liposomes creates new opportunities for improving the delivery of antimicrobial agents to infected tissues [6,7]. Recently, many reports have shown that hydrophilic phosphatidylethanolamine derivatives of monomethoxy polyethyleneglycols (PEG-PE) attached to the liposomes enhance blood circulation time without the limitations of lipid dose or rigid composition [1-4,7-9,14-16,18,20,22,23]. Such long-circulating liposomes have been named sterically stabilized liposomes.

As the effects of inclusion of PEG-DSPE on the therapeutic index of AMB-liposomes are not yet reported, we have investigated whether long-circulating AMB containing liposomes can be prepared by incorporation of PEG-DSPE, and whether the incorporation of PEG-DSPE affects the toxicity and biodistribution of the preparation.

Successful liposomal encapsulation of AMB requires that AmB is complexed e.g. to DSPG, as in the current AmBisome® formulation. We have demonstrated that complexation of AMB to PEG-DSPE can also be achieved (manuscript in preparation). In this study the preparation of two different formulations of liposomes containing both PEG-DSPE and AMB is described. PEG-AMB-LIP type 1, in which PEG-DSPE was added to AMB-liposomes with AMB complexed to DSPG, as well as PEG-AMB-LIP type 2, in which AMB was complexed to PEG-DSPE and DSPG was omitted from the preparation, were prepared. These pegylated formulations were compared with non-pegylated AMB-liposomes (referred to as AMB-LIP) regarding toxicity and biodistribution.

MATERIALS AND METHODS

Materials. AMB and AMB-DOC (Fungizone® for intravenous infusion) were kindly provided by Bristol Myers-Squibb, The Netherlands. AMB-DOC was

reconstituted according to the manufacturer's instructions with distilled water to give a standard solution of 5 g AMB/L. Lipid powder consisting of HSPC:Chol: DSPG:AMB in a molar ratio of 2:1:0.8:0.4 in which AMB is complexed to DSPG was kindly provided by Vestar Inc. (San Dimas, CA). Hydrogenated soybean phosphatidylcholine (HSPC), cholesterol (Chol), monomethoxy polyethyleneglycol (1900) succinimidyl succinate (activated PEG), distearoylphosphatidylethanolamine (DSPE), were all kindly provided by Vestar Inc. Dimethylsulphoxid (DMSO) was from Janssen Chimica (Tilburg, The Netherlands). Deferoxamine mesylate (DF) was from Ciba-Geigy (Basel, Switzerland). ^{67}Ga-citrate was from Nordian (Montreal, Canada), ^{111}In-chloride from Frosst (Quebec, Canada). Hydroxyquinoline sulfate (oxine) was from Sigma (St. Louis, MO). PEG(1900) derivative of DSPE (PEG-DSPE) was synthesized as described by Blume and Cevc [8].

Liposome preparation. Liposome preparations consisted of HSPC:Chol:DSPG: AMB in a molar ratio of 2:1:0.8:0.4 (AMB-LIP), HSPC:Chol:DSPG in a molar ratio of 2:1:0.8 (PLACEBO-LIP), PEG-DSPE:HSPC:Chol:DSPG:AMB in a molar ratio of 0.29:2:1:0.8:0.4 (PEG-AMB-LIP type 1), PEG-DSPE:HSPC:Chol:AMB in a molar ratio of 0.21:1.79:1:0.32 (PEG-AMB-LIP type 2), and PEG-DSPE:HSPC:Chol in a molar ratio of 0.21:1.79:1 (PEG-PLACEBO-LIP type 2). AMB is very poorly soluble in chloroform-methanol (1:1, v/v), and therefore as a first step in the preparation of AMB containing liposomes AMB was complexed to either DSPG (AMB-LIP and PEG-AMB-LIP type 1) or to PEG-DSPE (PEG-AMB-LIP type 2). For AMB-LIP, PLACEBO-LIP, PEG-AMB-LIP type 1, and PEG-PLACEBO-LIP type 2 a chloroform-methanol (1:1, v/v) solution of lipid powder or lipid mixture was evaporated to dryness in a round-bottom flask at 65 °C. For PEG-AMB-LIP type 2, AMB was complexed to PEG-DSPE by adding small volumes (20-50 μl) of 1N HCl to a suspension of AMB and PEG-DSPE in 2 mL chloroform-methanol (1:1, v/v), followed by heating at 65 °C and Vortex mixing until the solution cleared. Small volumes (10-15 μl) of 1N NaOH were added, followed by addition of HSPC and Chol. When precipitation of AMB was observed, again small volumes of 1N HCl were added until the solution cleared. This lipid mixture was evaporated to dryness in a round-bottom flask at 65 °C. The lipid film was hydrated by Vortex mixing in a buffer solution containing 10 mM sodium-succinate and 10% (w/v) sucrose (pH 5.5) at 65 °C. For biodistribution experiments 5 mM DF was added to this buffer. Liposomes were sonicated resulting in an average particle size of 100 nm (range 95-105 nm), as measured by dynamic light scattering (Malvern 4700 system, Malvern, U.K.). Biodistribution of intact liposomes was performed using liposomes radio-labeled with ^{67}Ga-DF as described by Woodle [24]. The labeling resulted in formation of a ^{67}Ga-DF complex in the aqueous interior of liposomes. As shown by Gabizon et al. [10], this complex is appropriate for in vivo tracing of intact liposomes because of the advantages of minimal translocation of radioactive label to plasma proteins and the rapid renal clearance rate when the label is released from the liposomes extracellularly. Liposomes were separated from non-entrapped

AMB, DF, or radiolabel by gel filtration on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden), and concentrated by using 300 kDa Microsep filters (Filtron, Breda, The Netherlands). Phospholipid concentration was determined by a phosphate assay [5]. AMB concentration was determined spectrophotometrically at 405 nm, after destruction of the liposomes in DMSO-methanol (1:1, v/v).

Determination of Maximum Tolerated Dosage (MTD). Toxicity of AMB-DOC, AMB-LIP, PEG-AMB-LIP type 1, and PEG-AMB-LIP type 2 was measured in uninfected specified pathogen free female BALB/c mice (14 to 20-week-old, 22-27 g Iffa Credo, L'arbresle, France). Mice (6 per group) were treated iv for five consecutive days with each of the preparations once daily (q24h) with AMB dosages ranging from 0.1-25 mg AMB/kg/day. Acute mortality was assessed directly following injection of the preparation. Blood urea nitrogen (BUN) and serum creatinine (CREAT), as parameters for renal toxicity, and aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), as parameters for liver toxicity, were determined by established methods in serum samples of mice sacrificed at 24h after termination of treatment. The MTD was defined as the maximum dosage that did not result in death during treatment or more than threefold increase in the indices for renal and liver function, as compared to untreated mice.

Biodistribution studies. Biodistribution of AMB-LIP, PLACEBO-LIP, PEG-AMB-LIP type 2 and PEG-PLACEBO-LIP type 2 was investigated. Radiolabeled liposomes were administered iv at 85 μmol lipid/kg (equivalent to 9 mg AMB/kg) as a single dose in uninfected mice. In two separate experiments of three mice for each different time interval after injection, 200 μl blood was collected from the mice in heparinized tubes. Mice were sacrificed, and whole organs (liver, spleen, lungs, and kidneys) were excised. Blood and organs, as well as the injected dosage of liposomes were counted for ^{67}Ga-DF in a gamma counter (Minaxy 5530, Packard Instruments, Downers Grove, U.S.A.). For the AMB containing preparations blood or organs from three mice were pooled and organs were homogenized in distilled water. Blood and tissue samples were stored at -80 °C until just before HPLC analysis, as decribed before [21]. In a separate experiment using ^{111}In-labeled red blood cells [12] blood volume was determined to be 7.5% of body weight and blood correction factors were determined for each organ.

RESULTS AND DISCUSSION

The MTDs in uninfected mice of AMB-DOC, AMB-LIP, PEG-AMB-LIP type 1, and PEG-AMB-LIP type 2 are presented in Table 1. The MTD of AMB-DOC in terms of death during treatment was 0.8 mg AMB/kg/day. At this dosage renal or liver toxicity was not seen. With AMB-LIP (a formulation with a lipid composition as in AmBisome®) the MTD with no death during treatment was 19 mg AMB/kg/day. At this dosage renal toxicity was not yet observed, whereas the MTD with respect to liver toxicity was 11 mg AMB/kg/day. Incorporation of PEG-DSPE resulting in PEG-

AMB-LIP type 1 led to a preparation that was as toxic as AMB-DOC. For PEG-AMB-LIP type 2 death during treatment was shown at dosages higher than 13 mg AMB/kg/day. At this dosage renal toxicity was not seen. For liver toxicity the MTD was 9 mg AMB/kg/day.

Table 1. *Maximum Tolerated Dosage (MTD) of AMB-DOC, AMB-LIP, PEG-AMB-LIP type 1, and PEG-AMB-LIP type 2 in uninfected mice*[a].

parameter of toxicity	MTD[b] (mg AMB/kg/day)			
	AMB-DOC	AMB-LIP	PEG-AMB-LIP type 1	PEG-AMB-LIP type 2
death during treatment	0.8	19	0.8	13
impaired renal function	>0.8	>19	>0.8	>13
impaired liver function	>0.8	11	>0.8	9

[a]Mice were treated iv for five consecutive days once daily (q24h) with AMB-DOC or with sonicated liposomes with an average diameter of 100 nm: AMB-LIP (HSPC/Chol/DSPG/AMB), PEG-AMB-LIP type 1 (PEG-DSPE/HSPC/Chol/DSPG/AMB), or PEG-AMB-LIP type 2 (PEG-DSPE/HSPC/Chol/AMB). AMB dosages ranged from 0.1-1.0 mg AMB/ kg/day in steps of 0.1 mg AMB/kg/day, and above 1.0 mg AMB/kg/day in steps of 2.0 mg AMB/kg/day. Two experiments of three mice each (n=6) for each dosage were performed.
[b]Toxicity was determined in terms of death during treatment, or more than threefold increase in the indices for renal function (BUN and CREAT) and liver function (ASAT, ALAT) as compared to placebo-treated mice, determined at 24h after termination of treatment

The high toxicity of the PEG-AMB-LIP type 1 preparation is probably due to a very toxic subpopulation of small particles containing AMB. A heterogeneity in particle size was observed after ultracentrifugation of the PEG-AMB-LIP type 1 preparation at 260,000xg. It was shown by dynamic light scattering measurements that particles of approximately 100 nm containing AMB were sedimented at this speed, whereas small particles of approximately 40 nm containing AMB remained in the supernatant. There was a marked difference with respect to toxicity in terms of acute death in mice between the particles of 40 nm and 100 nm. Acute death was observed after iv administration of the 40 nm AMB-particles at a dosage of 0.6 mg AMB/kg, whereas no toxicity was observed with the 100 nm AMB-particles at a dosage of 6 mg AMB/kg. As a first step in the preparation of PEG-AMB-LIP type 1, AMB is complexed to DSPG. This lipophilic AMB-DSPG complex is inserted in the phospholipid bilayer during spray-drying and thus helps to retain AMB in the liposome. PEG-DSPE is added to this formulation in which AMB is already complexed to DSPG. It is conceivable that competition between PEG-DSPE and

DSPG for complexation of AMB occurred, resulting in the formation of highly toxic AMB-containing lipid particles of 40 nm beside less toxic 100 nm AMB-liposomes. In PEG-AMB-LIP type 2 in which DSPG was omitted from the preparation, AMB was complexed to PEG-DSPE. PEG-AMB-LIP type 2 did not show the heterogeneity in particle size, and this was also not observed for AMB-LIP. As the PEG-AMB-LIP type 1 preparation was very toxic, these liposomes were omitted from further studies on biodistribution that were performed with AMB-LIP and PEG-AMB-LIP type 2.

Biodistribution studies were performed with ^{67}Ga-DF labeled liposomes. ^{67}Ga-DF was used as marker for intact liposomes. In studies with AMB-containing liposomes both ^{67}Ga-DF levels as well as AMB concentrations were determined. For both AMB-LIP as well as PEG-AMB-LIP type 2, AMB levels as percentage of injected dosage in blood and liver+spleen were equivalent to levels of intact liposomes (Figures 1 and 2), suggesting that AMB levels primarily represent liposome-associated AMB. For both AMB-LIP and PEG-AMB-LIP type 2 levels in kidney and lung never exceeded 3% and 1% of injected liposome dose, respectively (data not shown). Comparison of the blood residence of AMB-LIP versus PLACEBO-LIP shows that the presence of AMB in the liposome preparation induced a dramatic drop in blood level of intact liposomes within five minutes after administration (Figure 1). As this initial drop is seen in both the ^{67}Ga-DF marker as well as in AMB concentration (Figure 1) it is unlikely that this initial decrease is due to leakage of ^{67}Ga-DF or AMB. Although the initial hepatosplenic uptake of AMB-LIP within five minutes after administration is high (29% of injected dosage), this can still not account for the low blood level (47% of injected dosage) at this time point. As the exact localization of the distributed liposomes in other organs, such as bone marrow, brain, muscle, and skin at 5 min after administration was not determined, the rapid elimination of AMB-LIP is unexplained. After this initial drop in blood level of liposomal AMB a relatively prolonged blood residence time was observed with 6% of injected dosage circulating at 24h after administration. In line with expectations, it was shown that inclusion of PEG-DSPE into PEG-AMB-LIP type 2 resulted in a prolonged blood residence time of liposomal AMB as compared to AMB-LIP. Unfortunately, due to problems with repect to aggregation of liposomes during preparation, it was not possible to compare PEG-AMB-LIP type 2 with its appropriate control, i.e. non-pegylated AMB-liposomes with omission of not only PEG-DSPE but also DSPG. Successful liposomal encapsulation of AMB requires that AMB is complexed to either DSPG, as in AMB-LIP, or to PEG-DSPE, as in PEG-AMB-LIP type 2. An effect of AMB on blood residence time of the pegylated liposomes was not observed with PEG-AMB-LIP type 2 (Figure 2). Both PEG-PLACEBO-LIP type 2 as well as PEG-AMB-LIP type 2 showed a long blood residence time with 20-30% of the injected dosage of liposomes still circulating at 24h after administration. These results are in good agreement with those reported elsewhere for PEG-liposomes with similar particle size [1,2,7,22]. However, in the

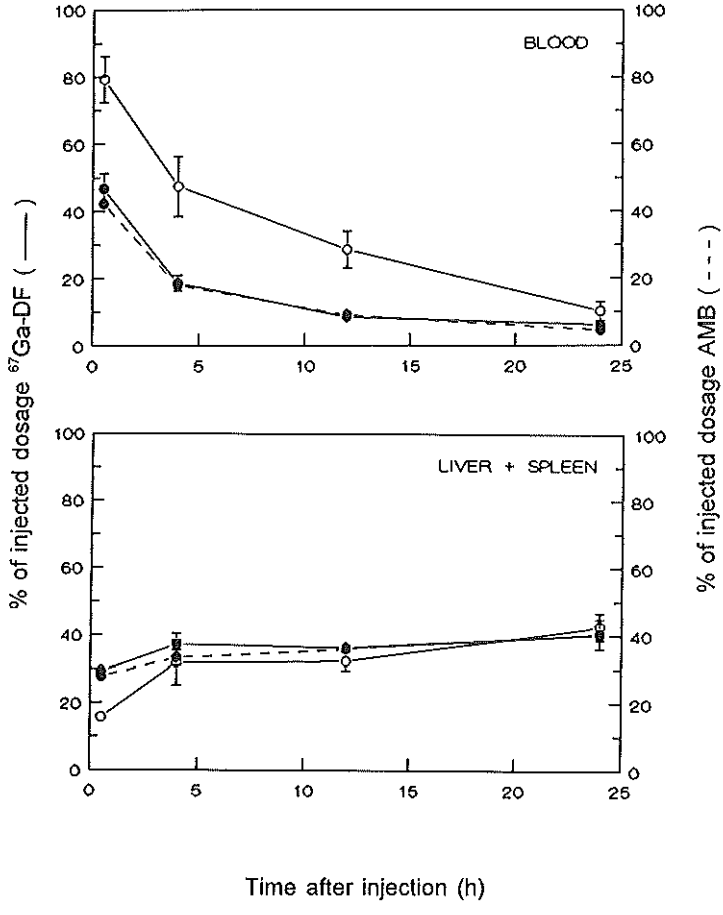

Figure 1. *Biodistribution of ^{67}Ga-DF labeled liposomes at various intervals after iv injection. Sonicated liposomes with an average diameter of 100 nm were injected intravenously in mice at a dosage of 85 µmol lipid/kg.*
(○) PLACEBO-LIP: HSPC/Chol/DSPG; (●) AMB-LIP: HSPC/Chol/DSPG/AMB. Data are expressed as percentage of injected dosage cpm ^{67}Ga-DF (solid line) as mean ± S.D. for two separate experiments of three mice each (n = 6), or as percentage of injected dosage AMB (broken line) as mean ± range for two separate experiments with in each experiment blood or tissue from three mice pooled. Where no error-bars are seen the range is smaller than the data points.

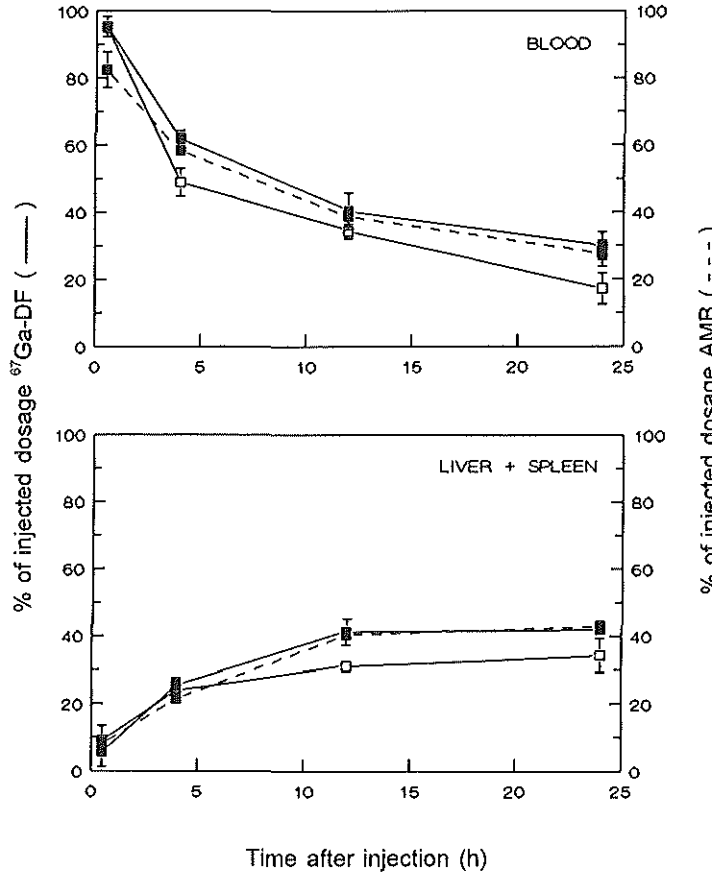

Figure 2. *Biodistribution of ^{67}Ga-DF labeled liposomes at various intervals after iv injection. Sonicated liposomes with an average diameter of 100 nm were injected intravenously in mice at a dosage of 85 μmol lipid/kg.*
(□) PEG-PLACEBO-LIP type 2: PEG-DSPE/HSPC/Chol; (■) PEG-AMB-LIP type 2: PEG-DSPE/HSPC/Chol/AMB. Data are expressed as percentage of injected dosage cpm ^{67}Ga-DF (solid line) as mean ± S.D. for two separate experiments of three mice each (n=6), or as percentage of injected dosage AMB (broken line) as mean ± range for two separate experiments with in each experiment blood or tissue from three mice pooled. Where no error-bars are seen the range is smaller than the data points.

present study a relatively high hepatosplenic uptake of the liposomes of 34-43% was observed at 24h after aministration, as compared to the other studies [1,2,7,22] in which the hepatosplenic uptake ranged from 7-28% at 24h after iv administration. This might be related to differences in size distribution of the liposome dispersion, as different methods for liposome sizing were used (sonication in the present study versus extrusion in the other studies). It is possible that sonication, as compared to the extrusion method, yields a preparation with relatively increased numbers of liposomes smaller than 100 nm. Extravascular localization of these small liposomes after passage through the fenestrated vascular endothelial lining of the liver sinusoids may occurr.

In conclusion, incorporation of PEG-DSPE in AMB-LIP with AMB complexed to DSPG (PEG-AMB-LIP type 1) results in an extremely toxic preparation. When AMB is complexed to PEG-DSPE and DSPG is omitted from the preparation (PEG-AMB-LIP type 2) toxicity is substantially reduced. In comparison with AMB-LIP this PEG-AMB-LIP type 2 preparation shows a slightly increased toxicity. Blood residence time of liposomal AMB is prolonged with PEG-AMB-LIP type 2 as compared to AMB-LIP. To what extent the increased toxicity as well as prolonged blood residence time of liposomal AMB with PEG-AMB-LIP type 2 are determining factors with respect to improved efficacy as compared to AMB-LIP in the treatment of fungal infections is now under investigation.

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REFERENCES

1. Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivaties of poly(ethylene glycol) show prolonged circulation half lives *in vivo*. Biochim Biophys Acta 1991;1066:29-36.
2. Allen TM, Hansen C. Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochim Biophys Acta 1991;1068:133-41.
3. Allen TM. Stealth liposomes: five years on. J Liposome Res 1992;2:289-305.
4. Allen TM, Hansen CB, Guo LSS. Subcutaneous administration of liposomes - a comparison with the intravenous and intraperitoneal routes of injection. Biochim Biophys Acta 1993;1150:9-16.
5. Ames B.N., Dubin D.T. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J Biol Chem 1960;235:769-75.

6. Bakker-Woudenberg IAJM, Ten Kate MT, Stearne-Cullen LET, Woodle MC. Efficacy of gentamycin or ceftazidime entrapped in liposomes with prolonged blood circulation and enhanced localization in *Klebsiella pneumoniae* infected lung tissue. J Infect Dis 1995;171:938-47.
7. Bakker-Woudenberg IAJM, Lokerse AF, Ten Kate MT, Mouton JW, Woodle MC, Storm G. Liposomes with prolonged blood circulation and selective localization in *Klebsiella pneumoniae*-infected lung tissue. J Infect Dis 1993;168:164-71.
8. Blume G, Cevc G. Liposomes for the sustained drug release *in vivo*. Biochim Biophys Acta 1990;1029:91-7.
9. Blume G, Cevc G, Crommelin DJA, Bakker-Woudenberg IAJM, Kluft C, Storm G. Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. Biochim Biophys Acta 1993;1149:180-4.
10. Gabizon A, Huberty J, Straubinger RM, Price DC, Papahadjopoulos D. An improved method for in vivo tracing and imaging of liposomes using a gallium 67-deferoxamine complex. J Liposome Res 1988-89;1:123-35.
11. Gates C. Pinney RJ. Amphotericin B and its delivery by liposomal and lipid formulations. J Clin Pharm Ther 1993;18:147-53.
12. Heaton WA, Davis HH, Welch MJ, Mathias CJ, Joist JH, Sherman LA, Siegel BA. Indium-111: a new radionuclide label for studying human platelet kinetics. Br J Haematol 1979;42:613-22 .
13. Janknegt R, De Marie S, Bakker Woudenberg IAJM, Crommelin DJA. Liposomal and lipid formulations of amphotericin B. Clinical Pharmacokinetics. Clin Pharmacokinet 1992;23:279-91.
14. Klibanov AL, Maruyama K, Torchillin VP, Huang L. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett 1990;268:235-7.
15. Klibanov AL, Huang L. Long circulating liposomes: development and perspectives. J Liposome Res 1992;2:321-34.
16. Lasic DD, Martin FJ, Gabizon A, Huang SK, Papahadjopoulos D. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. Biochim Biophys Acta 1991;1070:187-92.
17. Lyman CA, Walsh TJ. Systemically administered antifungal agents- A review of their clinical pharmacology and therapeutic applications. Drugs 1992;44:9-35.
18. Papahadjopoulos D, Allen T, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C, Martin FJ. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci USA 1991;88:11460-4.
19. Patterson TF, Andrioli VT. The role of liposomal Amphotericin B in the treatment of systemic fungal infections. Eur J Cancer Clin Oncol 1989;25(Suppl 2):S63-8.
20. Senior J, Delgado C, Fisher D, Tilcock C, Gregoriadis D. Influence of surface hydrophilicity of liposomes on their interaction with plasma proteins and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. Biochim Biophys Acta 1991;1062:77-82.
21. Van Etten EWM, Van den Heuvel-De Groot C, Bakker-Woudenberg IAJM. Efficacies of amphotericin B-desoxycholate (Fungizone), liposomal amphotericin B (AmBisome) and fluconazole in the treatment of systemic candidosis in immunocompetent and leucopenic mice. J Antimicrob Chemotherapy 1993;32:723-39.
22. Woodle MC, Matthay KK, Newman MS, Hidayat JE, Coolins LR, Redemann C, Martin FJ, Papahadjopoulos D. Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. Biochim Biophys Acta 1992;1105:193-200.

23. Woodle MC, Lasic DD. 1992. Sterically stabilized liposomes. Biochim Biophys Acta 1992;1113:171-99.
24. Woodle MC. ^{67}Gallium-labeled liposomes with prolonged circulation: Preparation and potential as nuclear imaging agents. Nucl Med Biol 1993;20:149-55.

Chapter 7

Pegylated long-circulating amphotericin B-liposomes versus AmBisome®: *in vitro* antifungal activity, toxicity, and efficacy in the treatment of systemic candidiasis in leukopenic mice

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Amphotericin B liposomes with prolonged circulation in blood: *in vitro* antifungal activity, toxicity, and efficacy in systemic candidiasis in leukopenic mice

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ABSTRACT

Pegylated AMB-liposomes (PEG-AMB-LIP) were compared with laboratory prepared non-pegylated AMB-liposomes (AMB-LIP), a formulation with a lipid composition as in AmBisome®, as well as with industrially prepared AmBisome®, regarding *in vitro* antifungal activity, toxicity, blood residence time, and therapeutic efficacy. Killing of *C.albicans* (>99.9%) during short-term (6 h) incubation was observed at 0.2 mg/L for amphotericin B desoxycholate (AMB-DOC), 0.4 mg/L for PEG-AMB-LIP, 0.8 mg/L for AMB-LIP and 12.8 mg/L for AmBisome®. The maximum tolerated dosages (MTDs) of PEG-AMB-LIP, AMB-LIP, and AmBisome® were 15 mg AMB/kg, 19 mg AMB/kg and >31 mg AMB/kg, respectively. In contrast to AMB-LIP, blood residence time of PEG-AMB-LIP was prolonged and dosage independent. In a model of systemic candidiasis in leukopenic mice at a dosage of 5 mg AMB/kg, PEG-AMB-LIP was completely effective, AMB-LIP was partially effective, whereas AmBisome® was not effective. AMB-LIP at 11 mg AMB/kg was partially effective. AmBisome® at 29 mg AMB/kg was completely effective. In conclusion, therapeutic efficacy of AMB-liposomes can be improved by preparing AMB-liposomes in which a substantial reduction of toxicity is achieved, while antifungal activity is retained. In addition, therapeutic efficacy is favored by a prolonged residence time of AMB-liposomes in blood.

INTRODUCTION

There has been a steady increase in the incidence of invasive fungal infections in immunocompromised patients. Presently, these infections are among the most important causes of morbidity and mortality among patients with cancer and among other severely immunocompromised hosts. As the overall prognosis for patients with invasive fungal infections remains poor, there is a critical need to improve methods for treating these infections.

Parenteral administration of amphotericin B (AMB) remains the therapy of choice for most invasive fungal infections. AMB is administered as amphotericin B-desoxycholate (AMB-DOC, Fungizone®), but its clinical use is limited by toxic side effects [15,23]. A promising approach in the treatment of deep systemic fungal infections is AMB incorporated in liposomes or other lipid-carriers [12,16,27]. With respect to the industrially produced preparations AmBisome®, ABLC (AMB Lipid Complex) and Amphocil® it is evident that these have quite different structural and pharmacokinetic characteristics [20,27]. The relatively large structures of ABLC as well as the small discoidal particles of Amphocil® are rapidly taken up by the mononuclear phagocyte system (MPS), whereas small liposomes as in AmBisome® remain in the circulation for relatively prolonged periods. Until now it is not known whether long blood residence time is of importance for improved efficacy of AMB-liposomes.

The ability to achieve a significantly longer blood residence time of liposomes opens new ways to achieving improved delivery of antimicrobial agents to infected tissues including infections in non-MPS tissues [8]. Recently, many reports have shown that hydrophilic phosphatidylethanolamine derivatives of monomethoxy polyethyleneglycols (PEG-PE) attached to the liposomes can effectively prolong their blood circulation time without the limitations of lipid dose, small particle size, or rigid lipid composition [2-5,8,9,21,22,25,31,32]. Such liposomes have been named sterically stabilized liposomes. The distinctive properties of sterically stabilized liposomes makes them excellent candidates for many therapeutic applications.

In our laboratory it was recently shown [30] that long-circulating AMB containing liposomes can be prepared by incorporation of PEG-DSPE. In the present study the effects of incorporation of PEG-DSPE in AMB-liposomes on *in vitro* antifungal activity, toxicity, and efficacy in systemic candidiasis in leukopenic mice is reported. The pegylated AMB-liposome formulation was compared with laboratory prepared non-pegylated AMB-liposomes, a formulation with a lipid composition as in AmBisome®, as well as with industrially prepared AmBisome®.

MATERIALS AND METHODS

Materials. Antibiotic Medium No.3 was from Difco Laboratories (Detroit, MI). Sabouraud dextrose agar was from Oxoid (Basingstoke, England). Amphotericin B

and amphotericin B-desoxycholate (AMB-DOC, Fungizone® for iv infusion) were kindly provided by Bristol Myers-Squibb, The Netherlands. AMB-DOC was reconstituted with distilled water to give a standard solution of 5 g/L. AmBisome®, consisting of HSPC:Chol:DSPG:AMB in a molar ratio of 2:1:0.8:0.4, and lipid powder, consisting of HSPC:Chol:DSPG:AMB in a molar ratio of 2:1:0.8:0.4 in which AMB is complexed to DSPG were both kindly provided by Vestar, Inc. (San Dimas, CA). Hydrogenated soybean phosphatidylcholine (HSPC), cholesterol (Chol), monomethoxy polyethyleneglycol (1900) succinimidyl succinate (activated PEG), distearoylphosphatidylethanolamine (DSPE), were all kindly provided by Vestar Inc. Dimethylsulphoxid (DMSO) was from Janssen Chimica (Tilburg, The Netherlands). Deferoxamine mesylate (DF) was from Ciba-Geigy (Basel, Switzerland). ^{67}Ga-citrate was from Nordian (Montreal, Canada), ^{111}In-chloride from Frosst (Quebec, Canada). Hydroxyquinoline sulfate (oxine), and cyclophosphamide were from Sigma (St. Louis, MO). PEG(1900) derivative of DSPE (PEG-DSPE) was synthesized as described by Blume and Cevc [9].

***Candida* strain.** *Candida albicans* ATCC 44858 was used in all of the experiments and was stored at -80°C in Todd-Hewitt broth (Difco laboratories, Detroit, MI, USA) containing 10% (v/v) glycerol.

Animals. Specified pathogen-free, 12- to 20-weeks-old female BALB/c mice were obtained from Iffa Credo (L'Arbresle, France).

Liposome preparation. Liposome preparations consisted of PEG-DSPE:HSPC: Chol:AMB in a molar ratio of 0.21:1.79:1:0.32 (PEG-AMB-LIP), PEG-DSPE:HSPC: Chol in a molar ratio of 0.21:1.79:1 (PEG-PLACEBO-LIP), HSPC:Chol:DSPG:AMB in a molar ratio of 2:1:0.8:0.4 (AMB-LIP), and HSPC:Chol:DSPG in a molar ratio of 2:1:0.8 (PLACEBO-LIP). AMB is very poorly soluble in chloroform-methanol (1:1, v/v), and therefore as a first step in the preparation of AMB containing liposomes AMB was complexed to either PEG-DSPE (PEG-AMB-LIP) or to DSPG (AMB-LIP). For PEG-AMB-LIP, AMB was complexed to PEG-DSPE by adding small volumes (20-50 μl) of 1N HCl to a suspension of AMB and PEG-DSPE in 2 mL chloroform-methanol (1:1, v/v), followed by heating at 65 °C and Vortex mixing untill the solution cleared. Small volumes (10-15 μl) of 1N NaOH were added, followed by addition of HSPC and Chol. When precipitation of AMB was observed, again small volumes of 1 N HCl were added untill the solution cleared. This lipid mixture was evaporated to dryness in a round-bottom flask at 65 °C. For PEG-PLACEBO-LIP, AMB-LIP, and PLACEBO-LIP a chloroform-methanol (1:1, v/v) solution of lipid mixture or lipid powder was evaporated to dryness in a round-bottom flask at 65°C. The lipid film was hydrated by Vortex mixing in a buffer solution containing 10 mM sodium-succinate and 10% (w/v) sucrose (pH 5.5) at 65 °C. For biodistribution experiments 5 mM DF was added to this buffer. Liposomes were sonicated resulting in an average particle size of 100 nm (range 95-105 nm), as measured by dynamic light scattering (Malvern 4700 system, Malvern, U.K.). Biodistribution of intact liposomes was performed using liposomes radiolabeled with ^{67}Ga-DF as described

by Woodle [33]. The labeling resulted in formation of a ^{67}Ga-DF complex in the aqueous interior of liposomes. As shown by Gabizon et al. [14], this complex is appropriate for *in vivo* tracing of intact liposomes because of the advantages of minimal translocation of radioactive label to plasma proteins and the rapid renal clearance rate when the label is released from the liposomes. Liposomes were separated from non-entrapped AMB, DF, or radiolabel by gel filtration on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden), and concentrated by using 300 kDa Microsep filters (Filtron, Breda, The Netherlands). Phospholipid concentration was determined by a phosphate assay [6]. AMB concentration was determined spectrofotometrically at 405 nm, after destruction of the liposomes in DMSO-methanol (1:1, v/v).

AmBisome®, consisting of HSPC:Chol:DSPG:AMB in a molar ratio of 2:1:0.8:0.4 was provided as a lyophilized preparation. The powder was reconstituted according to the manufacturer's instructions with distilled water at 65 °C to give a liposomal suspension containing 4 g AMB/L and 35 g lipid/L, with an average liposome particle size of 90 nm (range 88-92 nm).

***In vitro* antifungal activities of AMB-DOC, PEG-AMB-LIP, AMB-LIP, and AmBisome®.** The *in vitro* activities of AMB-DOC, PEG-AMB-LIP, AMB-LIP, and AmBisome® in terms of effective killing (>99.9%) of *C.albicans* at an inoculum of $1.3x10^7$ CFU/L during 6 h of incubation were determined as described previously [28]. Briefly, a logarithmic-growth-phase culture of *C.albicans* was prepared. *C.albicans* was exposed during 6 h of incubation to twofold increasing concentrations of each agent in Antibiotic Medium No.3, ranging from 0.05-25.6 mg/L. During incubation the numbers of viable *C.albicans* were determined at 2 h intervals by making plate counts of ten-fold serial dilutions of the washed specimen on Sabouraud dextrose agar.

Determination of Maximum Tolerated Dosage (MTD). Toxicity of PEG-AMB-LIP, AMB-LIP and AmBisome® was measured in uninfected mice. Mice (6 per group) were treated iv with a single dose. AMB dosages ranged from 1 to 31 mg AMB/kg in steps of 2 mg AMB/kg. Acute mortality was assessed directly following injection of the preparation. Blood urea nitrogen (BUN) and serum creatinine (CREAT), as parameters for renal toxicity, and aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), as parameters for liver toxicity, were determined by established methods in serum samples of mice sacrificed at 48h after termination of treatment. The MTD was defined as the maximum dosage that did not result in death or more than threefold increase in the indices for renal and liver function, as compared to untreated mice.

Blood residence time of PEG-AMB-LIP versus AMB-LIP in uninfected mice at various dosages. ^{67}Ga-DF labeled liposomes were administered iv at 0.5 mg AMB/kg or 5 mg AMB/kg (equivalent to 4.7 μmol liposomal lipid/kg or 47 μmol lipsomal lipid/kg, respectively) as a single dose in uninfected mice. In two separate experiments levels of ^{67}Ga-DF as well as AMB in blood were determined at various

time points during 24 h after administration, using three mice for each time point in each experiment. Blood was collected in heparinized tubes. Blood samples (200 μl), as well as the dosage of injected liposomes were counted for ^{67}Ga-DF in a gamma counter (Minaxy 5530, Packard Instruments, Downers Grove, USA). Additionally, at 5 mg AMB/kg, AMB concentrations were determined in these blood samples. For determination of AMB in blood, samples from three mice were pooled and stored at -80 °C until just before high-performance-liquid-chromatography (HPLC) analysis, as described before [28]. In a separate experiment using ^{111}In-labeled syngeneic red blood cells [18] total blood volume in the mice was determined.

Efficacies of PEG-AMB-LIP, AMB-LIP, and AmBisome® in leukopenic mice infected with *C.albicans*. Leukopenia was induced by ip administration of cyclophosphamide at 100 mg/kg 4 days before *C.albicans* inoculation followed by an additional dose of 75 mg/kg on the day of inoculation, and at 3-day intervals thereafter. This treatment resulted in persistent granulocytopenia ($<0.1 \times 10^9$/L) from the time of *C.albicans* inoculation up to termination of the study. Leukopenic mice were infected by inoculation of 3×10^4 CFU *C.albicans* into the tail vein. PEG-AMB-LIP, AMB-LIP, AmBisome® and placebo's were each administered iv as a single dose at 20 h after *C.albicans* inoculation at dosages corresponding to their MTDs. AMB-LIP and AmBisome® were also administered at a dosage which was equivalent to the MTD of PEG-AMB-LIP. Just before treatment and at 48 h as well as 6 days after treatment, the surviving mice were sacrificed. The kidneys, liver, spleen and lungs were removed and processed for the determination of viable counts, as described previously [28]. The following criteria were used to assess the efficacy of treatment: survival of mice up to 6 days after treatment; a statistically significant reduction in the numbers of CFU *C.albicans* in the kidneys at 48 h after treatment as compared to the numbers at the time of treatment, as well as prevention of a significant increase in the numbers of CFU *C.albicans* in the kidneys at 6 days after treatment as compared to the numbers at 48 h after treatment (equating to relapse of infection).

Statistical analysis. Results were expressed as the geometric means $\pm$ S.D. Differences in *C.albicans* CFU between the various treatment groups were analyzed by the Mann-Whitney test.

RESULTS

***In vitro* antifungal activities of AMB-DOC, PEG-AMB-LIP, AMB-LIP, and AmBisome®.** For AMB-DOC, PEG-AMB-LIP, AMB-LIP, and AmBisome® the minimal AMB concentrations required for killing >99.9% of the initial *C.albicans* inoculum within 6 h of incubation were determined. With AMB-DOC, PEG-AMB-LIP and AMB-LIP effective killing was obtained at 0.2 mg/L, 0.4 mg/L and 0.8 mg/L,

respectively. With AmBisome®, on the other hand, a concentration of 12.8 mg/L was required to produce the same fungicidal activity.

MTDs of PEG-AMB-LIP, AMB-LIP and AmBisome® in uninfected mice. The MTDs of PEG-AMB-LIP, AMB-LIP, an AmBisome® are presented in Table 1. With PEG-AMB-LIP the MTD with no death after treatment was 15 mg AMB/kg. At this dosage renal or liver toxicity were not yet observed. For AMB-LIP (a formulation with a lipid composition as in AmBisome®) death during treatment was shown at dosages higher than 19 mg AMB/kg. At this dosage no renal or liver toxicity was seen. With AmBisome® no toxicity was observed in terms of death, or impairments in renal or liver function up to a dosage of 31 mg AMB/kg.

Table 1. *Maximum Tolerated Dosage (MTD) of PEG-AMB-LIP, AMB-LIP and AmBisome® in uninfected mice*[a].

	MTD[b] (mg AMB/kg)		
parameter of toxicity	PEG-AMB-LIP	AMB-LIP	AmBisome®
death after treatment	15	19	>31
impaired renal function	>15	>19	>31
impaired liver function	>15	>19	>31

[a]Mice were treated iv with a single dose. AMB dosages ranged from 1 to 31 mg AMB/kg in steps of 2 mg AMB/kg

[b]Toxicity was determined in terms of death after treatment, or more than threefold increase in the indices for renal function (blood urea nitrogen and serum creatinine) and liver function (aspartate amino transferase and alanine amino transferase) as compared to placebo-treated mice, determined at 48h after termination of treatment

Blood residence time of PEG-AMB-LIP versus AMB-LIP in uninfected mice at various dosages. Blood levels of ^{67}Ga-DF following administration of radiolabeled PEG-AMB-LIP and AMB-LIP at dosages of 0.5 mg AMB/kg (4.7 μmol lipid/kg) and 5 mg AMB/kg (47 μmol lipid/kg) are shown in Figure 1. ^{67}Ga-DF was used as marker for intact liposomes. With PEG-AMB-LIP independent of dosage a prolonged blood residence time of intact liposomes was observed, with 20% of the injected dosage of liposomes still circulating at 24h after administration. For AMB-LIP it was shown that levels of intact liposomes dropped within five minutes after administration to 26% and 35% of the injected dosage of liposomes at the dosages of 0.5 mg AMB/kg and 5 mg AMB/kg, respectively. After this initial drop only at the highest dosage of 5 mg AMB/kg a prolonged blood residence time was observed, with 6% of injected dosage circulating at 12 h after administration. At the lower

dosage of 0.5 mg AMB/kg blood levels of liposomes declined to 6% of injected dosage within 4 h after administration.

Blood levels of both ^{67}Ga-DF and AMB following administration of radiolabeled PEG-AMB-LIP and AMB-LIP at 5 mg AMB/kg were determined. For both types of liposomes AMB levels declined more rapidly than levels of intact liposomes during circulation (data not shown).

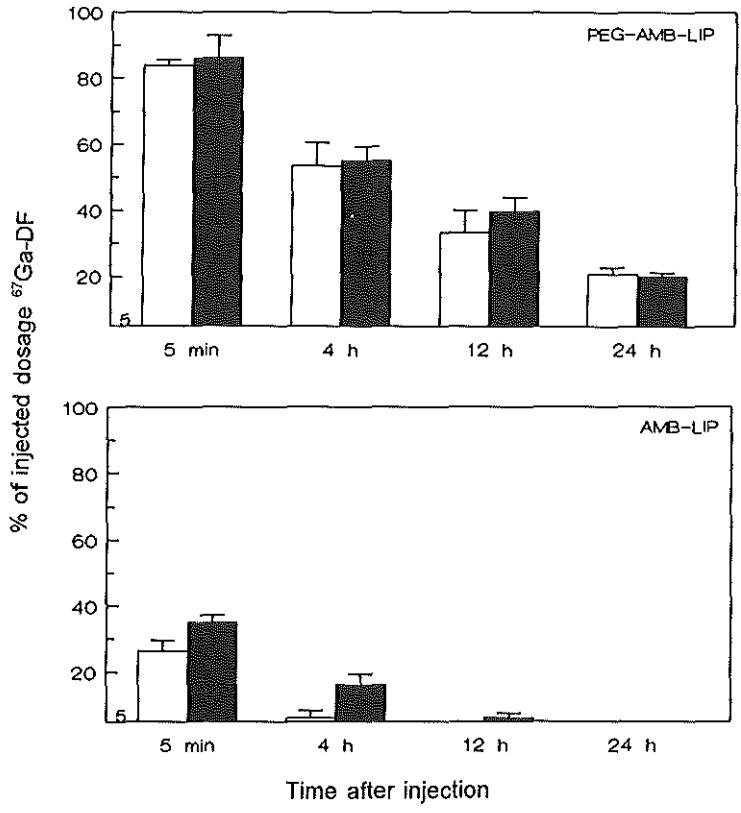

Figure 1. *Blood levels of ^{67}Ga-DF after administration of radiolabeled PEG-AMB-LIP and AMB-LIP in uninfected mice at various dosages. A single dose was administered iv at* □ *0.5 mg AMB/kg (4.7 μmol liposomal lipid/kg), or* ■ *5 mg AMB/kg (47 μmol liposomal lipid/kg). Data are expressed as percentage of injected dosage ^{67}Ga-DF as mean ± S.D. for two separate experiments of three mice each (n=6). Where no error-bars are seen the range is smaller than the data points.*

Efficacies of PEG-AMB-LIP, AMB-LIP, and AmBisome® in leukopenic mice infected with *C.albicans*. In leukopenic mice infected with *C.albicans* the infection is disseminated to the kidney, liver, spleen and lung, and untreated mice die between 24 h and eight days after *C.albicans* inoculation. The MTDs in these infected mice of PEG-AMB-LIP, AMB-LIP, and AmBisome® in terms of acute death were 5 mg AMB/kg, 11 mg AMB/kg, and 29 mg AMB/kg, respectively. The effect of treatment on survival of the animals and growth of *C.albicans* in the kidney is presented in Table 2. Increase of *C.albicans* CFU and death of the animals was observed after placebo treatment. As the placebo-treated animals died, numbers of *C.albicans* in the kidney of these mice could not be compared to those of AMB-treated mice. Treatment with PEG-AMB-LIP at the MTD (5 mg AMB/kg) was completely effective: 100% survival of mice was observed, as well as a significant reduction of *C.albicans* in liver ($P \leq 0.01$), spleen ($P \leq 0.001$), and lung ($P \leq 0.025$) (data not shown). In the kidney numbers of *C.albicans* were significantly reduced at 48 h after treatment ($P \leq 0.001$), relapse of infection was prevented; numbers of *C.albicans* were even further significantly reduced at 6 days after treatment ($P \leq 0.01$). Treatment with AMB-LIP at an equivalent dosage (5 mg AMB/kg) was partially effective: survival of all animals was observed, as well as a significant reduction of *C.albicans* in liver ($P \leq 0.025$), spleen ($P \leq 0.001$), and lung ($P \leq 0.001$) (data not shown); however, in the kidney growth of *C.albicans* was only inhibited, and numbers of *C.albicans* were not significantly reduced. Although increase of the dosage of AMB-LIP up to the MTD (11 mg AMB/kg) resulted in a slight improvement of efficacy compared to 5 mg AMB/kg, it did not result in complete efficacy of treatment as seen with PEG-AMB-LIP at 5 mg AMB/kg; at 11 mg AMB/kg numbers of *C.albicans* in the kidney were significantly reduced as compared to the time of treatment only at 6 days after treatment. AmBisome® at a dosage of 5 mg AMB/kg was not effective, as animals died after treatment. AmBisome® at the MTD (29 mg AMB/kg) was as effective as PEG-AMB-LIP at 5 mg AMB/kg.

DISCUSSION

With respect to the *in vitro* antifungal activity of the AMB-liposome formulations against *C.albicans*, it is derived from the present study that after encapsulation of AMB in PEG-AMB-LIP or in AMB-LIP the high antifungal activity as is seen with AMB-DOC is almost fully retained, whereas entrapment of AMB in AmBisome® is accompanied with substantial loss of antifungal activity. Although it was previously shown that AMB-DOC and AmBisome® were equally active after long-term exposure (MIC, MFC) [7, 28] the activity of AmBisome® during short term exposure was significantly less than that of AMB-DOC [28]. Recently, it is reported [24] that even after long-term exposure (24 h) AmBisome® was four to eight times less active than AMB-DOC against *C.albicans*.

Table 2. *Effect of treatment on survival of mice and growth of C.albicans in the kidney in leukopenic mice*[a].

treatment[b]	dosage (mg/kg)	start of treatment[b]	48 h after treatment[c]		6 days after treatment[c]		
		Log_{10} CFU/kidney[d]	survival %	Log_{10} CFU/kidney[d] in surviving mice	survival %	Log_{10} CFU/kidney[d] in surviving mice	no. mice with sterile kidney/no. surviving mice
untreated		4.16 ± 0.23					
PEG-AMB-LIP	5		100	3.05 ± 0.76[e]	100	1.99 ± 0.42[f]	0/10
AMB-LIP	5		100	4.48 ± 0.83	100	4.04 ± 0.91	0/10
AMB-LIP	11		100	4.27 ± 0.20	100	2.54 ± 0.50[f]	0/10
AmBisome®	5		70	5.26 ± 0.57	40	3.92 ± 0.51	0/4
AmBisome®	29		100	2.60 ± 0.32[e]	100	1.01 ± 1.72[f]	2/10
PEG-PLACEBO-LIP	-		40	6.38 ± 0.22	30	6.12 ± 0.39	0/3
PLACEBO-LIP	-		50	6.56 ± 0.21	10	7.39	0/1

[a]Leukopenic mice were inoculated iv at zero time with $3x10^4$ CFU of *C.albicans*
[b]PEG-AMB-LIP, AMB-LIP, AmBisome®, PEG-PLACEBO-LIP, and PLACEBO-LIP were administered iv 20 h after inoculation
[c]Effect of treatment was determined at 48 h (n = 10) as well as 6 days (n = 10) after treatment
[d]Each value represents the geometric mean ± SD
[e]$P \leq 0.01$ compared with the number of CFU at 20 h after inoculation (time of treatment)
[f]$P \leq 0.01$ compared with the number of CFU at 48 h after treatment

Comparison of the MTD of the industrially prepared AmBisome® in normal mice (>31 mg AMB/kg) and the MTD of a laboratory prepared AMB-liposome formulation (AMB-LIP) having the same lipid composition (19 mg AMB/kg) revealed that the method of preparation greatly influences the toxicity of the AMB-liposome formulation. As the MTD of AMB-DOC in terms of acute death after a single dose treament is 0.8 mg AMB/kg (unpublished data), encapsulation of AMB in AMB-LIP still substantially reduced the toxicity of AMB. To the same extent this also applied to PEG-AMB-LIP (MTD of 15 mg AMB/kg).

The prolonged residence time of PEG-AMB-LIP as compared to AMB-LIP was previously demonstrated at a dosage of 9 mg AMB/kg (corresponding to 85 μmol lipid/kg) [30]. A valuable asset of the PEG-PE-containing liposomes is that they show dosage-independent pharmacokinetics [2,3,25,31]. Taking together the data from our present and our previous [30] study, it is concluded that for PEG-AMB-LIP prolonged blood residence time is not depending on lipid dosage for a dosage range of 4.7-85 μmol lipid/kg. These results are in good agreement with those reported elsewhere for PEG-PE-liposomes with similar particle size [2,3,25,31], demonstrating dosage independent blood circulation times for dosage ranges of 0.5-500 μmol/kg in mice [3,25,31], and 3-100 μmol/kg [2,31] in rats. On the contrary, as reported previously by Allen *et al.* [3], blood residence times of small liposomes of approximately 100 nm in diameter, with a rigid liposomal bilayer and without surface modifications are dependent on the lipid dose given. In the present study it is shown that AMB-LIP blood residence time is depending on lipid dosage, which is in accordance with these previous observations [3]. For AmBisome® dosage dependent blood circulation of AMB was already reported previously [13,17,26,29].

The extent to which a prolonged residence time of liposomal AMB is of importance for improved therapeutic efficacy was studied in a model of systemic candidiasis in leukopenic mice after a single dose treatment. Efficacy of PEG-AMB-LIP, AMB-LIP, and AmBisome® were compared at an equivalent dosage of 5 mg AMB/kg (being the MTD of PEG-AMB-LIP in leukopenic infected mice). At this dosage treatment with PEG-AMB-LIP was completely effective, treatment with AMB-LIP was only partially effective. We conclude that therapeutic efficacy in this model of deep systemic candidiasis is favored by a prolonged residence time of AMB-liposomes in the blood compartment. Whether this is due to sustained release of AMB during blood circulation or increased localization of AMB liposomes at the sites of infection, as was described by others for localized site of infection [8] or solid tumor [25,19], is not yet clear. AmBisome® at 5 mg AMB/kg was not effective at all. Additionally, AMB-LIP and AmBisome® were both studied at their own MTD. Complete efficacy of treatment could not be achieved with AMB-LIP, not even after increase of the dosage up to 11 mg AMB/kg (MTD). AmBisome® at 29 mg AMB/kg (MTD) was as effective as treatment with PEG-AMB-LIP at the almost six-fold lower dosage of 5 mg AMB/kg. Until now, efficacy of AmBisome® is compared with that of AMB-DOC in several models of fungal infections [1,10,11,13,17,28]. From these

studies it can be concluded that, depending on the model of fungal infection, immune status of the host, and parameter for efficacy used, the antifungal activity of AmBisome® is either somewhat less or equal to that of AMB-DOC at equivalent dosages. However, treatment with AMB-DOC is restricted by acute toxicity. Using AmBisome®, much higher dosages are tolerated resulting in improved antifugal efficacy, even in severe infection in immunocompromised animals. Only recently [24], the interpretation of the data from these experimental studies on AmBisome® has been critically discussed. The authors rightly note that a difference in toxicity, and thereby in therapeutic index, between AmBisome® and AMB-DOC has always been claimed on the basis of acute toxicity of rapidly injected AMB-DOC in small laboratory animals. As in patients AMB-DOC is not administered as an iv bolus, but by slow infusion, the authors studied therapeutic efficacy of both AMB-DOC and AmBisome® in localized and systemic murine candidiasis, with AMB-DOC administered at high dosages in multiple fractions. The observation that AmBisome® was less active than high dosages of AMB-DOC indicate that by entrapment of AMB in liposomes therapeutic efficacy might be reduced. From the present study it is also clear that in AmBisome® reduction of toxicity is concomitant with reduction in antifungal activity, and thereby in antifungal efficacy *in vivo*. At an equivalent dosage, AMB-LIP, a formulation in which somewhat less reduction in toxicity goes together with retainment of antifungal activity, shows better antifungal efficacy *in vivo*.

By combining the data on toxicity, antifungal activity, blood residence time and efficacy of treatment, two important conclusions can be drawn: First, from a comparison between laboratory prepared AMB-LIP with industrially prepared AmBisome®, it is clear that the method of preparation of an AMB-liposome formulation greatly influences the toxicity, antifungal activity, and thereby the antifungal efficacy *in vivo*. It should be stressed that maximal reduction of AMB toxicity results in concomitant loss of antifungal activity and efficacy. Therefore for optimization of the preparation of AMB-liposomes it is important to focus on both reduction in AMB toxicity, while retaining antifugal activity. In both AMB-LIP and PEG-AMB-LIP this is achieved. The second conclusion derived by comparing PEG-AMB-LIP and AMB-LIP, is that therapeutic efficacy is favored by a prolonged residence time of AMB-liposomes in the blood compartment.

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REFERENCES

1. Adler-Moore JP, Chiang SM, Satorius A, Guerra D, McAndrews B, McManus EJ, Proffitt RT. Treatment of murine candidosis and cryptococcosis with a unilamellar liposomal Amphotericin B formulation. J Antimicrob Chemother 1991;28(Suppl.B):63-71.
2. Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivaties of poly(ethylene glycol) show prolonged circulation half lives *in vivo*. Biochim Biophys Acta 1991;1066:29-36.
3. Allen TM, Hansen C. Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochim Biophys Acta 1991;1068:133-41.
4. Allen TM. Stealth liposomes: five years on. J Liposome Res 1992;2:289-305.
5. Allen TM. The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. Adv. Drug Deliv. Rev. 1994;13: 285-309.
6. Ames BN, Dubin DT. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J Biol Chem 1960;235:769-75.
7. Anaissie E, Paetznick V, Proffitt R, Adler-Moore J, Bodey GP. Comparison of the *in vitro* antifungal activity of free and liposome-encapsulated Amphotericin B. Eur J Clin Microbiol Infect Dis 1991;10:665-8.
8. Bakker-Woudenberg IAJM, Lokerse AF, Ten Kate MT, Mouton JW, Woodle MC, Storm G. Liposomes with prolonged blood circulation and selective localization in *Klebsiella pneumoniae*-infected lung tissue. J Infect Dis 1993;168:164-71.
9. Blume G, Cevc G. Liposomes for the sustained drug release *in vivo*. Biochim Biophys Acta 1990;1029:91-7.
10. Clemons KV, Stevens DA. Comparison of a liposomal amphotericin B formulation (AmBisome) and deoxycholate amphotericin B (Fungizone) for the treatment of murine paracoccidioidomycosis. J Med Vet Mycol 1993;31:387-94.
11. Clemons KV, Stevens DA. Therapeutic efficacy of a liposomal formulation of amphotericin B (AmBisome) against murine blastomycosis. J Antimicrob Chemother 1993;32:465-72.
12. De Marie S, Janknegt R, Bakker-Woudenberg IAJM. Clinical use of liposomal and lipid-complexed amphotericin B. J Antimicrob Chemother 1994;33:907-16.
13. Francis P, Lee JW, Hoffman A, Peter J, Francesconi A, Bacher J, Shelhamer J, Pizzo PA, Walsh TJ. Efficacy of unilamellar liposomal amphotericin B in treatment of pulmonary aspergillosis in persistently granulocytopenic rabbits: The potential role of bronchoalveolar D-mannitol and serum galactomannan as markers of infection. J Infect Dis 1994;169:356-68.
14. Gabizon A, Huberty J, Straubinger RM, Price DC, Papahadjopoulos D. An improved method for *in vivo* tracing and imaging of liposomes using a gallium 67-deferoxamine complex. J Liposome Res 1988-89;1:123-35.
15. Gallis HA, Drew RH, Pickard WW. Amphotercin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-29.
16. Gates C, Pinney RJ. Amphotericin B and its delivery by liposomal and lipid formulations. J Clin Pharm Ther 1993;18:147-53.
17. Gondal JA, Swartz RP, Rahman A. Therapeutic evaluation of free and liposome-encapsulated amphotericin B in the treatment of systemic candidiasis in mice. Antimicrob Agents Chemother 1989;33:1544-8.

18. Heaton WA, Davis HH, Welch MJ, Mathias CJ, Joist JH, Sherman LA, Siegel BA. Indium-111: a new radionulclide label for studying human platelet kinetics. Br J Haematol 1979;42:613-22.
19. Huang SK, Lee KD, Hong K, Friend DS, Papahadjopoulos D. Microscopic localizationofsterically stabilized liposomes in colon carcinoma-bearing mice. Cancer Res 1992;52:5135-43.
20. Janknegt R, De Marie S, Bakker-Woudenberg IAJM, Crommelin DJA. Liposomal and lipid formulations of amphotericin. Clinical pharmacokinetics. Clin Pharmacokinet 1992;23:279-91.
21. Klibanov AL, Maruyama K, Torchillin VP, Huang L. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett 1990;268:235-7.
22. Klibanov AL, Huang L. Long circulating liposomes: development and perspectives. J Liposome Res 1992;2:321-34.
23. Lyman CA, Walsh TJ. Systemically administered antifungal agents - a review of their clinical pharmacology and therapeutic applications. Drugs 1992;44:9-35.
24. Pahls S, Schaffner A. Comparison of the activity of free and liposomal amphotericin B *in vitro* and in a model of localized murine candidiasis. J Infect Dis 1994;169:1057-61.
25. Papahadjopoulos D, Allen T, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee K-D, Woodle MC, Lasic DD, Redemann C, Martin FJ. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci USA 1991;88:11460-4.
26. Proffitt RT, Satorius A, Chiang SM, Sullivan L, Adler-Moore JP. Pharmacology and toxicology of a liposomal formulation of Amphotericin B (AmBisome) in rodents. J Antimicrob Chemother 1991;28(Suppl.B):49-61.
27. Schmitt HJ. New methods of delivery of amphotericin B. Clin Infect Dis 1993; 17(Suppl.2):S501-6.
28. Van Etten EWM, Van den Heuvel-de Groot C, Bakker-Woudenberg IAJM. Efficacies of amphotericin B-desoxycholate (Fungizone), liposomal amphotericin B (AmBisome) and fluconazole in the treatment of systemic candidosis in immunocompetent and leucopenic mice. J Antimicrob Chemother 1993;32:723-39.
29. Van Etten EWM, Otte-Lambillion M, Van Vianen W, Ten Kate MT, Bakker-Woudenberg IAJM. Biodistribution of liposomal amphotericin B (AmBisome) versus amphotericin B-desoxycholate (Fungizone) in immunocompetent uninfected mice as well as in leucopenic mice infected with *Candida albicans*. J Antimicrob Chemother 1995;35: 509-19.
30. Van Etten EWM, Van Vianen W, Tijhuis R, Storm G, Bakker-Woudenberg IAJM. Sterically stabilized amphotericin B-liposomes: toxicity and biodistribution in mice. J Control Release 1995;in press.
31. Woodle MC, Matthay KK, Newman MS, Hidayat JE, Coolins LR, Redemann C., Martin FJ, Papahadjopoulos D. Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. Biochim Biophys Acta 1992;1105:193-200.
32. Woodle MC, Lasic DD. Sterically stabilized liposomes. Biochim Biophys Acta 1992; 1113:171-99.
33. Woodle MC. ^{67}Gallium-labeled liposomes with prolonged circulation: Preparation and potential as nuclear imaging agents. Nucl Med Biol 1993;20:149-55.

Chapter 8

Efficacy of pegylated long-circulating amphotericin B-liposomes versus AmBisome® in the treatment of systemic candidiasis in leukopenic mice in relation to the severity of infection

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ABSTRACT

The therapeutic efficacy of pegylated long-circulating amphotericin B-liposomes (PEG-AMB-LIP) was compared with that of commercially prepared AmBisome® in a model of systemic candidiasis in leukopenic mice in relation to the severity of infection. AmBisome® administered as a single dose (5 mg AMB/kg) 6 h after *Candida albicans* inoculation was only partially effective: all mice survived up to 7 days after *C.albicans* inoculation. However, the number of *C.albicans* CFU in the kidney had neither increased nor decreased as compared to the start of treatment. PEG-AMB-LIP at the same dosage was much more effective: all mice survived, and the number of *C.albicans* CFU in the kidney was significantly reduced 3 to 7 days after inoculation. If treatment was delayed to 20 h after inoculation, AmBisome® was not effective anymore (60% mortality). In contrast, PEG-AMB-LIP retained its efficacy. When multidose AmBisome® treatment was started 6 h after inoculation, 5 daily doses were needed to significantly reduce *C.albicans* CFU. When treatment was delayed to 16 h after inoculation, even 5 daily doses of AmBisome® were not effective anymore, and some of animals died. Further delay in treatment to 20 h after inoculation resulted in increase in mortality to 70%. In sharp contrast, a single dose of PEG-AMB-LIP was efficacious (zero mortality) even if treatment was delayed to 20 h after inoculation. Since the efficacy of PEG-AMB-LIP could not be matched by AmBisome®, even when sustained blood levels of AmBisome® were assessed by a multidose regimen, it is concluded that prolonged blood residence time of AMB-liposomes is not the only factor contributing to superior antifungal efficacy of PEG-AMB-LIP observed *in vivo*. The high intrinsic antifungal activity of PEG-AMB-LIP may be of great clinical significance in the treatment of severe invasive fungal infection.

INTRODUCTION

The ability to achieve a significantly longer blood residence time of liposomes opens new ways for improved delivery of antimicrobial agents to infected tissue sites outside those that are included in the mononuclear phagocyte system (MPS) [5]. Many reports have shown that hydrophilic phosphatidylethanolamine derivatives of monomethoxy polyethyleneglycols (PEG-PE) attached to the liposomes can effectively prolong their blood circulation time without the limitations of a high lipid dose, small particle size, or rigid lipid bilayer [1,3,7,12,13]. Such pegylated liposomes have been named sterically stabilized liposomes. The distinctive properties of sterically stabilized liposomes makes them excellent candidates for many therapeutic applications [4,5,8].

We recently showed [10] that long-circulating amphotericin B (AMB) containing liposomes can be prepared by incorporation of PEG-DSPE. With the pegylated AMB-liposome formulation developed at our laboratory (PEG-AMB-LIP) [10], prolonged blood residence time, as well as substantial reduction of AMB toxicity without loss of antifungal activity were achieved. Furthermore, from a study on the effects of incorporation of PEG-DSPE in AMB-liposomes on *in vitro* antifungal activity, toxicity, and efficacy in systemic candidiasis in leukopenic mice [11] two important conclusions were drawn: First, from a comparison between laboratory prepared AMB-liposomes with commercially prepared AmBisome®, it became clear that the method of preparation of an AMB-liposome formulation greatly influences the toxicity, as well as antifungal activity, and thereby the antifungal efficacy *in vivo*. For AmBisome® it was found that reduction of toxicity of AMB is associated with a reduction of its antifungal activity. Therefore, for optimization of the formulation of AMB-liposomes it is important to focus on reduction of AMB toxicity, without reducing its antifungal activity. The second conclusion, derived by comparing PEG-AMB-LIP and laboratory prepared AMB-liposomes, was that therapeutic efficacy is favoured by a prolonged residence time of AMB-liposomes in the blood compartment. In the present study, the significance of the characteristics of prolonged blood residence time and high intrinsic activity of PEG-AMB-LIP in the treatment of systemic candidiasis were studied by comparing the antifungal efficacy of single dose PEG-AMB-LIP treatment to single- or multidose AmBisome® treatment in relation to the severity of infection.

MATERIALS AND METHODS

Materials. Sabouraud dextrose agar was from Oxoid (Basingstoke, England). Amphotericin B was kindly provided by Bristol Myers-Squibb, The Netherlands. AmBisome®, consisting of HSPC:Chol:DSPG:AMB in a molar ratio of 2:1:0.8:0.4, was kindly provided by Vestar, Inc. (San Dimas, CA). Hydrogenated soybean

phosphatidylcholine (HSPC), cholesterol (Chol), monomethoxy polyethyleneglycol (1900) succinimidyl succinate (activated PEG), distearoylphosphatidylethanolamine (DSPE), were all kindly provided by Vestar, Inc. Dimethylsulphoxid (DMSO) was from Janssen Chimica (Tilburg, The Netherlands). Cyclophosphamide was from Sigma (St. Louis, MO). PEG(1900) derivative of DSPE (PEG-DSPE) was synthesized as described by Blume and Cevc [6].

***Candida* strain.** *Candida albicans* ATCC 44858 was used in all of the experiments and was stored at -80°C in Todd-Hewitt broth (Difco laboratories, Detroit, MI, USA) containing 10% (v/v) glycerol.

Animals. Specified pathogen-free, 12- to 20-weeks-old female BALB/c mice were obtained from Iffa Credo (L'Arbresle, France).

Liposome preparation. PEG-DSPE:HSPC:Chol:AMB in a molar ratio of 0.21:1.79:1:0.32 (PEG-AMB-LIP) was prepared as described previously [10]. Briefly, AMB was complexed to PEG-DSPE in chloroform-methanol (1:1, v/v) at 65°C, followed by addition of HSPC and Chol. This lipid mixture was evaporated to dryness, and subsequently hydrated by Vortex mixing in a buffer solution containing 10 mM sodiumsuccinate and 10% (w/v) sucrose (pH 5.5) at 65°C. Phospholipid concentration was determined by a phosphate assay [2]. AMB concentration was determined spectrophotometrically at 405 nm, after destruction of the liposomes in DMSO-methanol (1:1, v/v).

AmBisome®, consisting of HSPC:Chol:DSPG:AMB in a molar ratio of 2:1:0.8:0.4 was provided as a lyophilized preparation. The powder was reconstituted according to the manufacturer's instructions with distilled water at 65 °C to give a liposomal suspension containing 4 g AMB/L and 35 g lipid/L, with an average liposome particle size of 90 nm (range 88-92 nm).

Efficacies of PEG-AMB-LIP and AmBisome® in leukopenic mice infected with *C.albicans*. Leukopenia was induced by ip administration of cyclo-phosphamide at 100 mg/kg 4 days before *C.albicans* inoculation followed by an additional dose of 75 mg/kg on the day of inoculation, and at 3-day intervals thereafter. This treatment resulted in persistent granulocytopenia ($<0.1 \times 10^9$/L) from the time of *C.albicans* inoculation up to termination of the study. Leukopenic mice were infected by inoculation of 3×10^4 *C.albicans* CFU into the tail vein. Various treatment regimens were studied:

Single dose PEG-AMB-LIP versus single dose AmBisome®, in relation to severity of infection. PEG-AMB-LIP and AmBisome® were each administered iv as a single dose of 5 mg AMB/kg at 6 h, or 20 h after *C.albicans* inoculation. This dosage is the maximum tolerated dosage (MTD) of PEG-AMB-LIP in leukopenic mice administered as a single dose at 20 h after inoculation with *C.albicans*, as determined previously [11].

Single- versus multidose AmBisome®. AmBisome® was administered iv at 5 mg AMB/kg as a single dose, or daily for three, or five consecutive days at 5 mg AMB/kg/day. Treatment was started 6 h after *C.albicans* inoculation.

Single dose PEG-AMB-LIP versus multidose AmBisome®, in relation to severity of infection. PEG-AMB-LIP was administered iv as a single dose, whereas AmBisome® was administered iv daily for five consecutive days. Treatment was started 6 h, 16 h, or 20 h after *C.albicans* inoculation.

Just prior to the start treatment and at three days as well as seven days after *C.albicans* inoculation, surviving mice were sacrificed. The kidneys were removed and processed for the determination of viable *C.albicans*, as described previously [9]. The following criteria were used to assess the efficacy of treatment: survival of mice up to seven days after *C.albicans* inoculation; a statistically significant reduction of the numbers of *C.albicans* CFU in the kidneys at three days as well as seven days after *C.albicans* inoculation as compared to the CFU at the start of antifungal treatment.

Statistical analysis. Results were expressed as the geometric means ± S.D. Differences in *C.albicans* CFU between the various treatment groups were analyzed by the Mann-Whitney test.

RESULTS AND DISCUSSION

In leukopenic mice infected with *C.albicans* the infection is disseminated to the kidney, liver, spleen and lung, and untreated as well as placebo-treated mice die between 24 h and eight days after *C.albicans* inoculation [11]. As it was previously shown [9,11] that both PEG-AMB-LIP as well as AmBisome® treatment resulted in significantly reduced numbers of *C.albicans* CFU in liver, spleen and lung, in the present study only the numbers of *C.albicans* CFU in the kidney were determined.

By increasing the delay between *C.albicans* inoculation and the start of treatment, the efficacy of treatment in relation to the severity of infection could be investigated. This was reflected in the number of *C.albicans* CFU in the kidney at the start of treatment (Tables 1 and 3). The effect of single dose PEG-AMB-LIP versus AmBisome®, in relation to severity of infection, is presented in Table 1. AmBisome® administered 6 h after *C.albicans* inoculation was only partially effective: all mice survived up to 7 days; however, the number of *C.albicans* CFU in the kidney had neither increased nor decreased as compared to the start of treatment. PEG-AMB-LIP at the same dosage was much more effective as all mice survived, and the number of *C.albicans* CFU decreased significantly up to seven days after inoculation. If treatment was delayed to 20 h after inoculation, AmBisome® was not effective anymore, as 60% of animals died; in contrast PEG-AMB-LIP retained its efficacy (zero mortality).

To investigate whether prolongation of the blood residence of AmBisome®, obtained by repeated administration, would result in improved efficacy, the effect of single- versus multidose treatment of AmBisome® was investigated (Table 2). AmBisome® treatment started 6 h after *C.albicans* inoculation, and administered daily for three or for five consecutive days, did not result in a significant reduction

Table 1. *Effect of single dose PEG-AMB-LIP versus single dose AmBisome® on survival of mice and growth of C.albicans in the kidney in leukopenic mice[a], in relation to severity of infection.*

start of treatment	treatment[b]	number of doses	start of treatment	day 3 after inoculation[c]		day 7 after inoculation[c]		
			Log_{10} CFU/kidney[d]	survival %	Log_{10} CFU/kidney[d] in surviving mice	survival %	Log_{10} CFU/kidney[d] in surviving mice	no. mice with sterile kidney/no. surviving mice
6 h	untreated		3.20 ± 0.16					
	PEG-AMB-LIP	1		100	1.04 ± 0.39[e]	100	1.16 ± 0.70[e]	0/10
	AmBisome®	1		100	3.28 ± 0.20	100	3.47 ± 0.41	0/10
20 h	untreated		4.16 ± 0.23					
	PEG-AMB-LIP	1		100	3.05 ± 0.76[e]	100	1.99 ± 0.42[e]	0/10
	AmBisome®	1		70	5.26 ± 0.57	40	3.92 ± 0.51	0/4

[a]Leukopenic mice were inoculated iv at zero time with $3x10^4$ *C.albicans* CFU; untreated as well as placebo-treated mice died between 24 h and eight days after *C.albicans* inoculation
[b]PEG-AMB-LIP or AmBisome® was administered iv as a single dose at 5 mg AMB/kg
[c]Effect of treatment was determined at day 3 (n = 10) as well as day 7 (n = 10) after inoculation.
[d]Each value represents the geometric mean ± SD
[e]$P \leq 0.01$ compared with number of CFU at the start of treatment

Table 2. *Effect of single- versus multidose AmBisome® on survival and growth of C.albicans in the kidney in leukopenic mice*[a].

start of treatment	treatment[b]	number of doses	start of treatment	day 3 after inoculation[c]		day 7 after inoculation[c]		
			Log_{10} CFU/kidney[d]	survival %	Log_{10} CFU/kidney[d] in surviving mice	survival %	Log_{10} CFU/kidney[d] in surviving mice	no. mice with sterile kidney / no. surviving mice
6 h	untreated		3.20 ± 0.16					
	AmBisome®	1		100	3.28 ± 0.20	100	3.47 ± 0.41	0/10
	AmBisome®	3		100	3.08 ± 0.26	100	3.46 ± 0.57	0/10
	AmBisome®	5		100	2.90 ± 0.44	100	2.63 ± 0.46[e]	0/10

[a]Leukopenic mice were inoculated iv at zero time with $3x10^4$ *C.albicans* CFU; untreated as well as placebo-treated mice died between 24 h and eight days after *C.albicans* inoculation

[b]AmBisome® was administered iv as a single dose, or daily for three, or five consecutive days at 5 mg AMB/kg/day. Treatment was started at 6 h after inoculation

[c]Effect of treatment was determined at day 3 (n = 10) as well as day 7 (n = 10) after inoculation

[d]Each value represents the geometric mean ± SD

[e]$P \leq 0.01$ compared with number of CFU at the start of treatment

Table 3. *Effect of single dose PEG-AMB-LIP versus multidose AmBisome® on survival of mice and growth of C.albicans in the kidney in leukopenic mice[a], in relation to severity of infection.*

start of treatment	treatment[b]	number of doses	start of treatment	day 3 after inoculation[c]		day 7 after inoculation[c]		
			Log_{10} CFU/kidney[d]	survival %	Log_{10} CFU/kidney[d] in surviving mice	survival %	Log_{10} CFU/kidney[d] in surviving mice	no. mice with sterile kidney / no. surviving mice
6 h	untreated		3.20 ± 0.16					
	PEG-AMB-LIP	1		100	1.04 ± 0.39[e]	100	1.16 ± 0.70[e]	0/10
	AmBisome®	5		100	2.90 ± 0.44	100	2.63 ± 0.46[e]	0/10
16 h	untreated		4.01 ± 0.22					
	PEG-AMB-LIP	1		100	2.29 ± 0.42[e]	100	1.35 ± 0.51[e]	0/10
	AmBisome®	5		90	4.79 ± 0.40	90	3.43 ± 0.38	0/9
20 h	untreated		4.16 ± 0.23					
	PEG-AMB-LIP	1		100	3.05 ± 0.76[e]	100	1.99 ± 0.42[e]	0/10
	AmBisome®	5		60	5.39 ± 0.45	30	4.57 ± 1.11	0/3

[a]Leukopenic mice were inoculated iv at zero time with $3x10^4$ *C.albicans* CFU; untreated as well as placebo-treated mice died between 24 h and eight days after *C.albicans* inoculation

[b]PEG-AMB-LIP was administered iv as a single dose at 5 mg AMB/kg. AmBisome® was administered iv daily for five consecutive days at 5 mg AMB/kg/day

[c]Effect of treatment was determined at day 3 (n = 10) as well as day 7 (n = 10) after inoculation

[d]Each value represents the geometric mean ± SD

[e]$P \leq 0.01$ compared with number of CFU at the start of treatment

of *C.albicans* CFU at three days after inoculation. Only after five-day treatment the number of *C.albicans* CFU was significantly lowered shortly after treatment (as determined seven days after inoculation). Although antifungal efficacy was improved after the five-day treatment with AmBisome®, the response to this therapy was, thus, relatively slow.

In this model of systemic candidiasis, in which untreated animals start to die 24h after *C.albicans* inoculation, it was questioned what would be the efficacy of five-day treatment with AmBisome® if the start of treatment was delayed. The effect of single dose PEG-AMB-LIP versus multidose AmBisome® for five consecutive days, in such delayed treatment protocol, is presented in Table 3. As was already shown in the previous experiments both PEG-AMB-LIP as well as AmBisome® administered 6 h after *C.albicans* inoculation were both effective. When treatment was delayed to 16 h after inoculation, AmBisome® was not effective anymore, as a small percentage of animals died. Further delay of treatment to 20 h after inoculation resulted in a further increase in mortality. In contrast, a single dose of PEG-AMB-LIP retained its efficacy even if it was only given 20 h after inoculation.

It is, thus, clear that single dose treatment with PEG-AMB-LIP is superior to five-day treatment with AmBisome® in this model of systemic candidiasis in the leukopenic host. We have previously shown [11], by comparing PEG-AMB-LIP and laboratory prepared AMB-liposomes, that therapeutic efficacy is favoured by a prolonged residence time of AMB-liposomes in the blood compartment. The present study shows that the high efficacy as observed for PEG-AMB-LIP could not be matched by AmBisome® even if, by a multidose regimen, its presence in blood was prolonged. It is concluded that prolonged blood residence time of AMB-liposomes is not the only factor contributing to the antifungal efficacy of such formulations *in vivo*. Our previous studies [11] showed that in AmBisome® reduction of toxicity of AMB is associated with a reduction of its antifungal activity, *in vitro* as well as *in vivo*. In PEG-AMB-LIP a substantial reduction of AMB toxicity was achieved, without such a loss in antifungal activity. The present study clearly demonstrates the high intrinsic activity of PEG-AMB-LIP: Only treatment with PEG-AMB-LIP and not with AmBisome® resulted in decreasing numbers of *C.albicans* CFU in the kidney within short period of time after *C.albicans* inoculation. The high intrinsic antifungal activity of PEG-AMB-LIP may be of great clinical significance in the treatment of severe invasive fungal infection, especially in the immunocompromised host.

REFERENCES

1. Allen TM. The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. Adv Drug Deliv Rev 1994;13:285-309.
2. Ames B.N., Dubin D.T. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J Biol Chem 1960;235:769-75.

3. Bakker-Woudenberg IAJM, Lokerse AF, Ten Kate MT, Mouton JW, Woodle MC, Storm G. Liposomes with prolonged blood circulation and selective localization in *Klebsiella pneumoniae*-infected lung tissue. J Infect Dis 1993;168:164-71.
4. Bakker-Woudenberg IAJM, Ten Kate MT, Stearne-Cullen LET, Woodle MC. Efficacy of gentamycin or ceftazidime entrapped in liposomes with prolonged blood circulation and enhanced localization in *Klebsiella pneumoniae* infected lung tissue. J Infect Dis 1995;171:938-47.
5. Bergers JJ, Ten Hagen TLM, Van Etten EWM, Bakker-Woudenberg IAJM. Liposomes as delivery systems in the prevention and treatment of infectious diseases. Pharmacy World & Science 1995;17:1-11.
6. Blume G, Cevc G. Liposomes for the sustained drug release *in vivo*. Biochim Biophys Acta 1990;1029:91-7.
7. Klibanov AL, Huang L. Long circulating liposomes: development and perspectives. J. Liposome Res. 1992;2:321-34.
8. Papahadjopoulos D, Allen T, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C, Martin FJ. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci USA 1991;88:11460-4.
9. Van Etten EWM, Van den Heuvel-De Groot C, Bakker-Woudenberg IAJM. Efficacies of amphotericin B-desoxycholate (Fungizone), liposomal amphotericin B (AmBisome) and fluconazole in the treatment of systemic candidosis in immunocompetent and leucopenic mice. J Antimicrob Chemotherapy 1993;32:723-39.
10. Van Etten EWM, Van Vianen W, Tijhuis R, Storm G, Bakker-Woudenberg IAJM. Sterically stabilized amphotericin B-liposomes: toxicity and biodistribution in mice. J. Control. Release. 1995;in press.
11. Van Etten EWM, Ten Kate MT, Stearne LET, Bakker-Woudenberg IAJM. Amphotericin B liposomes with prolonged circulation in blood: *in vitro* antifungal activity, toxicity, and efficacy in systemic candidiasis in leukopenic mice. Antimicrob Agents Chemother 1995;39:1954-8.
12. Woodle MC, Lasic DD. Sterically stabilized liposomes. Biochim Biophys Acta 1992;1113:171-99.
13. Woodle MC, Matthay KK, Newman MS, Hidayat JE, Coolins LR, Redemann C, Martin FJ, Papahadjopoulos D. Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. Biochim Biophys Acta 1992;1105:193-200.

Chapter 9

First evidence of efficacy of pegylated long-circulating amphotericin B-liposomes in the treatment of pulmonary aspergillosis in leukopenic rats

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ABSTRACT

In the present study, first evidence of the antifungal efficacy of pegylated long-circulating amphotericin B-liposomes (PEG-AMB-LIP) in a model of pulmonary aspergillosis in persistently leukopenic rats is presented. Treatment started 30 h after *Aspergillus fumigatus* inoculation with a single dose of 10 mg AMB/kg PEG-AMB-LIP was evaluated in terms of survival of rats as well as in numbers of viable *Aspergillus fumigatus* in post-mortem cultures of left and right lung, and liver. At the start of treatment the *Aspergillus fumigatus* conidia had germinated to hyphae. Survival was significantly prolonged by PEG-AMB-LIP treatment. Post-mortem cultures in treated rats were not different from those in untreated rats. These data are encouraging and warrant further exploration of the therapeutic efficacy of PEG-AMB-LIP in invasive pulmonary aspergillosis.

INTRODUCTION

As in granulocytopenic patients the mortality from pulmonary aspergillosis remains high despite aggressive antifungal therapy, there is an urgent need for therapeutic advances [10]. Although experimental studies on the efficacy of various antifungal agents and amphotericin B (AMB)-lipid formulations have been performed [1,2,3,5,9,10,11,12,13,14], in only two models of pulmonary aspergillosis, one in rabbits [3,11] and another in rats [13], the respiratory route of infection as well as profound and persistent granulocytopenia were part of the experimental design. In these two models of invasive pulmonary aspergillosis, improved survival of animals after multidose treatment with Amphocil® [3] or AmBisome® [11,13] has been reported. It is evident that Amphocil® and AmBisome® have quite different structural and pharmacokinetic characteristics [3,11]. The small discoidal particles of Amphocil® are rapidly taken up by the mononuclear phagocyte system, whereas small liposomes as in AmBisome® remain in the circulation for relatively prolonged periods. Until now it is not known whether prolonged residence of AMB-liposomes in the blood compartment is relevant for the efficacy of such formulations in invasive pulmonary aspergillosis. A pegylated long-circulating amphotericin B (AMB)-liposome formulation (PEG-AMB-LIP) was developed in our laboratory [16]. PEG-AMB-LIP has recently been shown to greatly improve the antifungal efficacy of AMB in severe invasive candidiasis after only single dose treatment [17]. With respect to the treatment of pulmonary infection, it has been shown in our laboratory that pegylated liposomes selectively localize at the site of *Klebsiella pneumoniae*-infected lung tissue [7], resulting in improved efficacy of gentamicin or ceftazidime entrapped in such long-circulating liposomes [8]. In the present study it was investigated whether a single-dose of long-circulating PEG-AMB-LIP was effective in the treatment of invasive pulmonary aspergillosis in persistently leukopenic rats.

MATERIALS AND METHODS

Materials. Sabouraud dextrose agar (SDA) was from Oxoid (Basingstoke, England). Amphotericin B was kindly provided by Bristol Myers-Squibb, The Netherlands. Hydrogenated soybean phosphatidylcholine (HSPC), cholesterol (Chol), and polyethylene glycol (1900) derivatized distearoylphosphatidylethanolamine (PEG-DSPE), were all kindly provided by LTI, Inc. (Menlo Park, CA). Dimethylsulphoxid (DMSO) was from Janssen Chimica (Tilburg, The Netherlands). Cyclophosphamide was from Sigma (St. Louis, MO).

***Aspergillus* strain.** A clinical isolate of *Aspergillus fumigatus* from an immunocompromised patient with invasive pulmonary aspergillosis was used. This strain was stored under oil on SDA. For inoculation a suspension of conidia of *A.fumigatus* in sterile saline was prepared.

Animals. Specified pathogen-free, 18- to 25-weeks-old (weight 185-225 g) female R strain albino rats were obtained from Harlan CPB, Rijswijk, The Netherlands.

Immunosuppression and supportive care. Leukopenia was induced by ip administration of cyclophosphamide at 90 mg/kg 5 days before *A.fumigatus* inoculation followed by an additional dose of 60 mg/kg on the day before inoculation, and at 4-day intervals thereafter. This treatment resulted in persistent granulocytopenia ($<0.5x10^9$/L) from the time of *A.fumigatus* inoculation up to termination of the study. To prevent bacterial superinfection strict hygienic care was applied, and rats received ciprofloxacin (660 mg/L) and polymyxin E (100 mg/L) in their drinking water during the whole experiment. From the day before inoculation daily amoxicillin (40 mg/kg/dose), administered intramusculary, was added to this regime for the remainder of the experiment. Shortly before and after inoculation gentamicin (40 mg/kg/dose) was administered intramusculary.

Liposome preparation. PEG-DSPE:HSPC:Chol:AMB in a molar ratio of 0.21:1.79:1:0.32 (PEG-AMB-LIP) was prepared as described previously [16]. Briefly, AMB was complexed to PEG-DSPE in chloroform-methanol (1:1, v/v) at 65°C, followed by addition of HSPC and Chol. This lipid mixture was evaporated to dryness, and subsequently hydrated by Vortex mixing in a buffer solution containing 10 mM sodium-succinate and 10% (w/v) sucrose (pH 5.5) at 65°C. Phospholipid concentration was determined by a phosphate assay [4]. AMB concentration was determined spectrophotometrically at 405 nm, after destruction of the liposomes in DMSO-methanol (1:1, v/v).

Experimental lung infection. Experimental pulmonary aspergillosis was obtained according to the method used in the experimental pneumonia model (left lung) with *Klebsiella pneumoniae* as described by Bakker-Woudenberg [6]. Briefly, rats were anaesthetized after which the left main bronchus was intubated and the left lung was inoculated with 0.02 ml of conidial suspension.

Efficacy of PEG-AMB-LIP in leukopenic rats infected with *A.fumigatus*. Leukopenic rats were inoculated with 10^5 conidia *A.fumigatus*. PEG-AMB-LIP (10 mg AMB/kg) was administered iv as a single dose at 30 h after *A.fumigatus* inoculation. Survival (days after inoculation) was recorded for each rat. Post-mortem the left and right lung, and the liver were removed and processed for the determination of viable *A.fumigatus*, as described previously for *C.albicans* [15]. The following criteria were used to assess the efficacy of treatment: survival of rats up to twelve days after *A.fumigatus* inoculation and reduction in the numbers of *A.fumigatus* CFU in the various organs at time of death as compared to the numbers found in rats left untreated.

Statistical analysis. Statistical evaluation of differences in the survival curves between PEG-AMB-LIP-treated and untreated groups of rats was performed by the log rank test. This test examines the decrease in survival with time as well as the final percentage of survival. Results of post-mortem cultures were expressed as the

geometric means ± S.D. Differences in *A.fumigatus* CFU between the two groups were analyzed by the Mann-Whitney test.

RESULTS AND DISCUSSION

In this model of pulmonary aspergillosis untreated rats died between 5 to 8 days after inoculation. Post-mortem cultures in these untreated rats (Table 1) revealed that at the time of death the infection had disseminated to the right lung in all rats and to the liver in the majority of the rats (11 out of 15). Whether mortality is directly related to the dissemination of the infection remained unclear. The effect of treatment started 30 h after inoculation with a single dose of PEG-AMB-LIP (10 mg AMB/kg) on survival of the rats is presented in Figure 1. At the start of treatment the conidia had already germinated to hyphae. Treatment resulted in significantly ($P \leq 0.001$) prolonged survival. However, post-mortem cultures in the treated rats (Table 1) were not different from those in untreated rats.

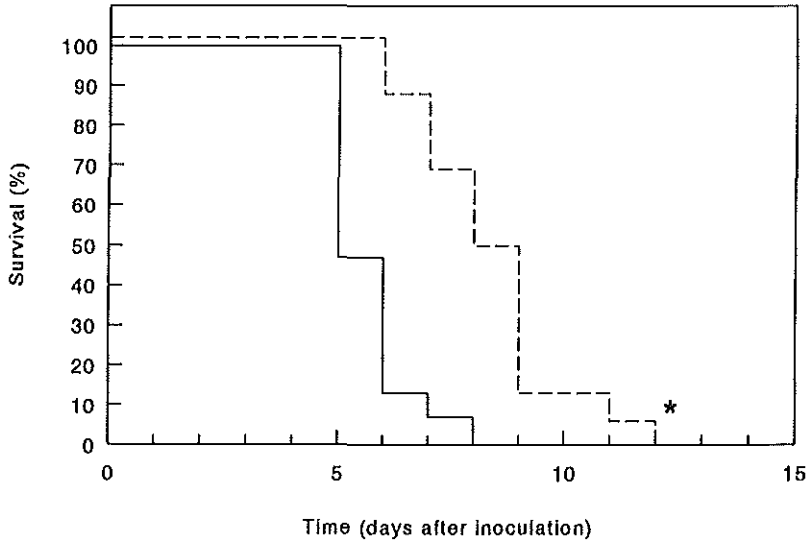

Figure 1. *Effect of single dose administration of PEG-AMB-LIP on survival of leukopenic rats with pulmonary aspergillosis (Kaplan-Meier plot). Leukopenic rats were inoculated in the left lobe of the lung with 10^5 conidia A.fumigatus at zero time. Rats were treated with PEG-AMB-LIP (- - - -) adminstered at 30 h after inoculation, or left untreated(——). *$P \leq 0.001$ versus untreated rats.*

Table 1. *Effect of single dose PEG-AMB-LIP on lung infection with A.fumigatus and dissemination to other organs in leukopenic rats*[a].

start of treatment	treatment[b]	dosage (mg/kg)	Log_{10} CFU/organ[c] in deceased rats[d]			no. culture positive organs[d] / total no. rats		
			left lung	right lung	liver	left lung	right lung	liver
	untreated		3.4 ± 0.4	1.8 ± 0.9	1.4 ± 1.0	16/16	15/15	11/15
30 h	PEG-AMB-LIP	10	3.1 ± 0.8	1.4 ± 0.9	0.7 ± 1.0	15/15	14/16	6/16

[a]Leukopenic rats were inoculated in the left lung at zero time with 10^5 conidia *A.fumigatus*
[b]PEG-AMB-LIP was administered iv as a single dose
[c]Each value represents the geometric mean ± SD
[d]Effect of treatment was determined by post-mortem cultures

With respect to other AMB-lipid formulations only limited data on efficacy in animal models of invasive aspergillosis are available [1,3,9,11,12,13,14]. The clinical relevance of some of the animal models is questionable, as *A.fumigatus* was mostly inoculated iv instead of via the respiratory route [1,9,12,14]. In addition, some of the studies were in animals that were not persistently granulocytopenic [1,9,12]. In immunocompetent animals or in animals treated with corticosteroids for suppresion of macrophage antifungal actvity, high doses of various AMB-lipid formulations proved to be efficacious in terms of survival of animals and/or clearance of *A.fumigatus* from infected organs. A recent study reported on the effect of prophylaxis of pulmonary aspergillosis with aerosolized AmBisome® [2], which was found to be highly protective against the development of pulmonary aspergillosis after intranasal challenge with *A.fumigatus* conidia.

In the two clinically relevant models of pulmonary aspergillosis in granulocytopenic animals, one in rabbits [3,11] and another in rats [13] improved survival of animals after multidose treatment with Amphocil® [3] or AmBisome® [11,13] has been reported. The data from the present study indicate increased survival of rats after only a single dose of long-circulating PEG-AMB-LIP, and are encouraging for further exploring the therapeutic efficacy of PEG-AMB-LIP in invasive pulmonary aspergillosis.

REFERENCES

1. Ahmad I, Sarkar AK, Bachhawat BK. Design of liposomes to improve delivery of amphotericin B in the treatment of aspergillosis. Mol Cell Biochem 1989;91:85-90.
2. Allen SD, Sorensen KN, Nejdl MJ, Proffitt RT. Efficacy of aerosolized liposomal amphotericin B (AmBisome®) as a prophylactic treatment in an immune compromised murine model of pulmonary aspergillosis. J Control Rel 1994;28:348-9.
3. Allende MC, Lee JW, Francis P, Garrett K, Dollenberg H, Berenguer J, Lyman CA, Pizzo PA, Walsh TJ. Dose-dependent antifungal activity and nephrotoxicity of amphotericin B colloidal dispersion in experimental pulmonary aspergillosis. Antimicrob Agents Chemother 1994;38:518-22.
4. Ames B.N., Dubin D.T. The role of polyamines in the neutralization of bacteriophage deoxyribonulceic acid. J Biol Chem 1960;235:769-75.
5. Andriole VT, Miniter P, George D, Kordick D, Patterson TF. Animal models: Usefuless for studies of fungal pathogenesis and drug efficacy in aspergillosis. Clin Infect Dis 1992;14(Suppl. 1):S134-8.
6. Bakker-Woudenberg IAJM, Van den Berg JC, Michel MF. Therapeutic activities of cefazolin, cefotaxime, and ceftazidime against experimentally induced *Klebsiella pneumoniae* pneumonia in rats. Antimicrob Agents Chemother 1982;22:1042-50.
7. Bakker-Woudenberg IAJM, Lokerse AF, Ten Kate MT, Mouton JW, Woodle MC, Storm G. Liposomes with prolonged blood circulation and selective localization in *Klebsiella pneumoniae*-infected lung tissue. J Infect Dis 1993;168:164-71.
8. Bakker-Woudenberg IAJM, Ten Kate MT, Stearne-Cullen LET, Woodle MC. Efficacy of gentamycin or ceftazidime entrapped in liposomes with prolonged blood circulation

and enhanced localization in *Klebsiella pneumoniae* infected lung tissue. J Infect Dis 1995;171:938-47.
9. Clark MJ, Whitney RR, Olsen SJ, George RJ, Swerdel MR, Kunselman R, Bonner DP. Amphotericin B lipid complex therapy of experimental fungal infections in mice. Antimicrob Agents Chemother 1991;35:615-21.
10. Denning DW, Stevens DA. Antifungal and surgical treatment of invasive aspergillosis: review of 2121 published cases. Rev Infect Dis 1990;12:1147-1201.
11. Francis P, Lee JW, Hoffman A, Peter J, Francesconi A, Bacher J, Shelhamer J, Pizzo PA, Walsh TJ. Efficacy of unilamellar liposomal amphotericin B in treatment of pulmonary aspergillosis in persistently granulocytopenic rabbits: The potential role of bronchoalveolar D-mannitol and serum galactomannan as markers of infection. J Infect Dis 1994;169:356-68.
12. Gokhale PC, Kshirsagar NA, Pandya SK. Preclinical and pharmaceutical testing of liposomal amphotericin B. Indian J Med Res [B] 1993;98:75-8.
13. Leenders ACAP, Ten Kate MT, Bakker-Woudenberg IAJM, De Marie S. AmBisome® reduces dissemination of infection in a rat model for one-sided pulmonary aspergillosis. [abstract] Eur Congress Clin Microbiol Infect Dis, Vienna, 1995.
14. Patterson TF, Miniter P, Dijkstra J, Szoka FC, Ryan JL, Andriole VT. Treatment of experimental invasive aspergillosis with novel amphotericin B/cholesterol sulfate complexes. J Infect Dis 1989;159:717-24.
15. Van Etten EWM, Van den Heuvel-De Groot C, Bakker-Woudenberg IAJM. Efficacies of amphotericin B-desoxycholate (Fungizone), liposomal amphotericin B (AmBisome) and fluconazole in the treatment of systemic candidosis in immunocompetent and leukopenic mice. J Antimicrob Chemother 1993;32:723-39.
16. Van Etten EWM, Van Vianen W, Tijhuis R, Storm G, Bakker-Woudenberg IAJM. Sterically stabilized amphotericin B-liposomes: toxicity and biodistribution in mice. J Control Release 1995;in press.
17. Van Etten EWM, Ten Kate MT, Stearne LET, Bakker-Woudenberg IAJM. Amphotericin B liposomes with prolonged circulation in blood: In vitro antifungal activity, toxicity, and efficacy in systemic candidiasis in leukopenic mice. Antimicrob Agents Chemother 1995;39:1954-8.

Chapter 10

General Discussion

There has been a steady increase in the incidence of invasive fungal infections. Presently, these infections are among the most important causes of morbidity and mortality among patients with cancer and among other severely immunocompromised hosts [5,39,42]. As the overall prognosis for patients with invasive fungal infections remains poor, there is a critical need to improve the treatment of these infections. Parenteral administration of amphotericin B (AMB) remains the therapy of choice for most invasive fungal infections. AMB is administered as AMB-desoxycholate (AMB-DOC or Fungizone®), but its use is significantly limited by its toxic side effects [17]. New antifungal agents, such as the triazole fluconazole (FLU) and liposomal AMB have been developed for the management of invasive fungal infections [9,13,18,20,21,29,42]. The role of these new agents in the treatment of invasive fungal infections in the leukopenic host is not clearly defined nor well understood.

The **primary aim** of the work presented in this thesis was **to gain further insight into the therapeutic value of existing and newly developed liposomal formulations of AMB in the treatment of invasive fungal infections.**

To this end, different animal models of invasive fungal infections were developed. Clinically relevant issues including persistent leukopenia and dissemination of infection, are addressed. One model was an invasive *Candida albicans* infection with dissemination to the kidney, liver, spleen, and lung in mice which were rendered persistently leukopenic. In addition, a model of invasive candidiasis restricted to the kidney in immunocompetent mice was used. Finally, a model of *Aspergillus fumigatus* one-sided pulmonary infection in persistently leukopenic rats was developed, in which the fungus disseminated to the right lung and to the liver during the course of the infection [28].

The question of the impact of a fungistatic agent, FLU, versus that of the fungicidal agent, AMB-DOC, on treatment of invasive candidiasis was covered in the first two studies (chapters 2 and 3). Although the *in vitro* activity of AMB-DOC against *C.albicans* was high, the antifungal activity was influenced by factors relevant to its efficacy in the treatment of infection: activity of AMB-DOC was substantially reduced by the presence of serum. AMB-DOC activity was only slightly hindered by an intracellular localization of the yeast (in peritoneal macrophages). For FLU, even at high concentrations, antifungal activity against extracellular *C.albicans* was restricted to inhibition of fungal growth. Furthermore, FLU was not effective against intracellular *C.albicans*. Therefore, it was questioned whether FLU could effectively eradicate *C.albicans in vivo*, in particular when host defense mechanisms are severely impaired. In immunocompetent mice FLU was effective as long as treatment was given, whereas in leukopenic mice efficacy was only obtained if treatment was started in the early phase of the disease. In well established *C.albicans* infection in leukopenic mice FLU was definitely not efficacious when treatment was delayed. Other investigators [40,41] came to a similar conclusion regarding FLU efficacy in their models of invasive candidiasis in

persistently granulocytopenic rabbits. It is now generally accepted that treatment with FLU in established invasive candidiasis in granulocytopenic patients is not recommended, although limited studies in such patients have shown some encouraging results [9,20,29]. Another issue that became clear once more in the present study, was that in contrast with the potent antifungal activity of AMB-DOC *in vitro*, the *in vivo* therapeutic efficacy of AMB-DOC is very limited, especially in leukopenic animals, due to the low maximum tolerated dosage (MTD).

The rationale for the use of AmBisome®, one of the industrially prepared AMB-lipid formulations, was to make use of the potent fungicidal effect of AMB while, at the same time, reducing its toxicity by liposomal encapsulation. The antifungal efficacy of AMB was far improved with AmBisome®, being highly effective in multidose treatment regimens. High dosages reduced *C.albicans* in the kidney and other organs as well as prevented relapse of infection, even in severe infection (chapter 3). It should be noted, however, that at equivalent dosages, AmBisome® was less effective than AMB-DOC. On the other hand, much higher dosages were tolerated for AmBisome® in these mice, resulting in improved therapeutic efficacy. Studies of AmBisome® in other models of fungal infection showed either equivalent or reduced antifungal efficacy at equal dosages when compared to AMB-DOC [1, 11,12,14,19,31].

With respect to the biodistribution of AMB after administration of AMB-DOC or AmBisome® (chapter 4), it was shown for both formulations that the condition of the animals (immunocompetent uninfected mice versus leukopenic mice infected with *C.albicans*) did not influence on the biodistribution patterns. Comparing the biodistribution of AMB-DOC and AmBisome® at equivalent dosages revealed that encapsulation of AMB resulted in increased AMB levels in blood. In addition increased AMB concentrations were present in the mononuclear phagocyte system (MPS), particularly the liver and spleen, whereas concentrations in kidney and lung were lower. The low AMB levels in the kidney after AmBisome® treatment may explain the decreased antifungal efficacy in invasive candidiasis when compared with AMB-DOC at equivalent dosages. Uptake of AmBisome® by MPS-tissues was highly dosage dependent and saturable. As a result, at high lipid dosages a prolonged circulation of AmBisome® in blood could be obtained. After administration of AmBisome® at the MTD, blood and tissues retained high AMB concentrations. It should be emphasized that these high concentrations of AMB did not lead to toxicity. Recently, similar results were published with respect to the biodistribution of AmBisome® in rabbits [27].

In studies from other investigators on biodistribution of AmBisome® and other AMB-lipid formulations only data on total AMB concentrations in serum and tissues have been reported [14,19,23,27,34]. No distinction could be made between lipid-associated AMB and non-lipid-associated AMB. Radiolabeling of AmBisome® using a radioactive marker which is a representative of intact liposomes [15], allowed us to determine whether AMB concentrations measured in blood represented liposome

associated AMB or not. The high AMB concentrations in blood represented liposome entrapped AMB. During circulation slow release of AMB occurred (chapter 4).

It is suggested by many investigators that AMB is biologically inactive when associated with liposomes. Even today it remains unclear whether release of AMB is of importance for antifungal activity. *In vitro* AMB remains tightly associated to the liposomes in AmBisome® in the presence of serum, and AMB has not necessarily to be released from AmBisome® in order to express antifungal activity against *C.albicans* (chapter 5). Similar results have recently been reported by others. These authors even showed that AmBisome® can act on both phagocytized and non-phagocytized fungal cells, resulting in death of the fungi [2,35]. For a lipid formulation of AMB in DMPC/DMPG (7:3), it was reported that AMB can be released from the lipid-complex by fungal lipases [33]. Whether fungal lipases play a role in the release of AMB from AmBisome® in the close vicinity of the fungus, and whether this is necessary for antifungal activity is not yet clear.

An important issue on which only very limited data were available, is the antifungal activity of AmBisome® against intracellularly growing *C.albicans*. *C.albicans* can be considered as a facultative intracellular pathogen, as it is able to survive inside unactivated macrophages and to grow out of these cells by forming germ tubes (chapter 2). AMB-DOC is higly active against intracellular *C.albicans* (chapter 2). Therefore, it was questioned whether encapsulation of AMB in AmBisome® would be an advantage with respect to antifungal activity against intracellular *C.albicans* in peritoneal macrophages (chapter 5). In parallel with the antifungal activities against extracellular *C.albicans* (chapter 3), the intracellular antifungal activity of AMB-DOC was much higher than that of AmBisome®. Although numerous studies have demonstrated that liposomes are phagocytized effectively by macrophages both *in vitro* and *in vivo* [37], an explanation for the low intracellular activity of AmBisome® might be low uptake of AmBisome® by macrophages infected with *C.albicans*. However, in the present study localization experiments with fluorescens-labeled AmBisome® clearly showed that AmBisome® is taken up by macrophages, both by uninfected macrophages and macrophages infected with *C.albicans*. A more likely explanation for the relatively low intracellular activity is slow intracellular degradation of AmBisome®. AmBisome® is a solid type liposome, composed of saturated phospholipids with high transition temperatures and cholesterol, which is expected to be slowly degraded intracellularly. In another study using ampicillin entrapped in a solid liposome type, relatively slow intracellular degradation in macrophages was demonstrated [6], resulting in a delayed intracellular release of the entrapped ampicillin, and hence absent or delayed killing of intracellular *Listeria monocytogenes*.

As macrophages seemed to be occupied with the intracellular degradation of AmBisome® for a substantial period of time, an important question was whether uptake and degradation of AmBisome® in uninfected macrophages had effects on

subsequent *C.albicans* ingestion and on intracellular growth of *C.albicans* (chapter 5). The presence of AmBisome® inside uninfected macrophages did not influence uptake of *C.albicans* by the same macrophages. Furthermore, previous exposure of uninfected macrophages to AmBisome® was advantageous for antifungal activity of AmBisome® against intracellular *C.albicans*. It was recently reported that phagocytic cells may even play a role in transport and accumulation of liposomal AMB into inflammatory sites *in vivo* [30]. Whether leukocytes play an important role in transport or degradation of AmBisome® at inflammatory sites should be investigated.

We hypothesize that AmBisome® exerts its antifungal activity in the leukopenic host at sites of infection outside the MPS via two different routes, as illustrated in Figure 1.

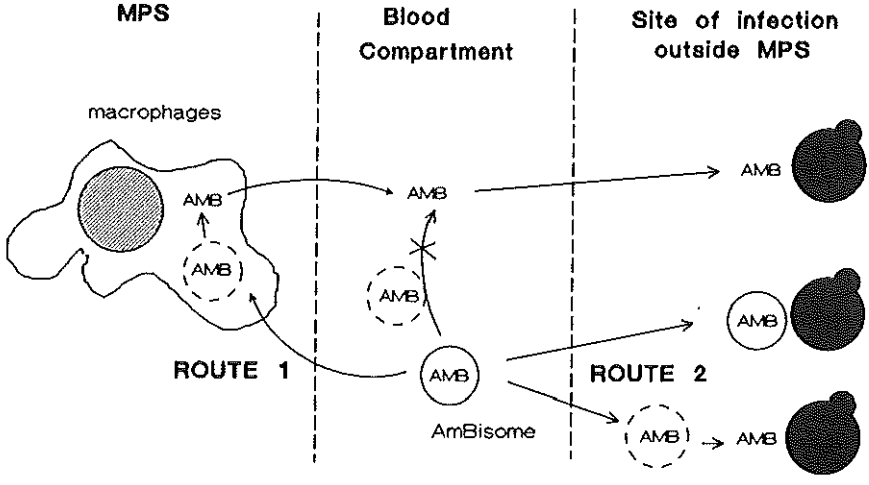

Figure 1. *Schematic representation of the routes via which AmBisome® exerts its antifungal activity.*

After intravenous administration of AmBisome®, intact liposomes circulate in blood with only negligible release of AMB from the liposomes during circulation. One route is that following uptake and processing of AmBisome® by macrophages of the MPS, AMB is released inside the macrophages. In this way the cells of the MPS serve as a 'depot' from which AMB is slowly released without reaching toxic levels in blood. The second route is that intact AmBisome® reaches the site of infection; at the site of infection direct interaction between AmBisome® and the fungal cell may occur or AMB may be released from AmBisome® in the close vicinity of the fungus.

In case direct interaction between AmBisome® and the fungal cells is crucial for the *in vivo* antifungal efficacy, it is important that intact AMB-liposomes localize at the site of infection. Prolonged blood residence probably allows the localization of more intact liposomes at sites of infection outside MPS-tissues. For liposome types such as AmBisome®, that have a small particle size and a rigid bilayer without surface modifications, blood residence time is primarily dependent on the lipid dose administered. Only at high lipid dosages blood circulation times are prolonged due to decrease in hepatosplenic uptake of liposomes, indicative of saturation of the MPS (chapter 4). Advances in liposome technology, especially the incorporation of hydrophilic phosphatidylethanolamine derivatives of monomethoxy polyethyleneglycols (PEG-PE) into liposomes, have resulted in the ability to achieve longer blood residence time of liposomes without the constraints of high lipid dose, small particle size, or rigid nature of the bilayer [4,25,32,43,44]. Such pegylated long-circulating liposomes have been named sterically stabilized liposomes or MPS avoiding liposomes.

Can pegylated long-circulating AMB-liposomes be prepared? Successful liposomal encapsulation of AMB requires that AMB is complexed, e.g. to the negatively charged phospholipid distearoylphosphatidylglycerol (DSPG), as in AmBisome®. We reasoned that the net negative charge of PEG-DSPE could also be used for complexation of AMB. Two different formulations of PEG-DSPE containing liposomes with AMB were prepared (chapter 6). PEG-AMB-LIP type 1, in which AMB was complexed to DSPG, as well as PEG-AMB-LIP type 2, in which AMB was complexed to PEG-DSPE and DSPG was omitted from the preparation. PEG-AMB-LIP type 1 was as toxic as AMB-DOC, and was omitted from further studies on biodistribution. With PEG-AMB-LIP type 2 a relatively non-toxic preparation with a prolonged blood residence time was obtained. Now that a pegylated long-circulating AMB-liposome formulation was available, an urgent question was that of the efficacy of this PEG-AMB-LIP type 2 (further referred to as PEG-AMB-LIP) in the treatment of fungal infections in the leukopenic host.

In our model of invasive candidiasis in leukopenic mice PEG-AMB-LIP was compared with industrially prepared AmBisome® and with AMB-liposomes (AMB-LIP), that had the lipid composition as in AmBisome®, but were prepared at our laboratory (chapter 7). PEG-AMB-LIP and AMB-LIP were almost equal in *in vivo* toxicity and *in vitro* antifungal activity, however they differed in blood residence time. Blood residence time of PEG-AMB-LIP was more prolonged, even at a low lipid dosage. Industrially prepared AmBisome® and laboratory prepared AMB-LIP differed in *in vivo* toxicity and *in vitro* antifungal activity. AmBisome® was less toxic than AMB-LIP, however antifungal activity *in vitro* was substantially reduced as compared to AMB-LIP. In our model of invasive candidiasis in leukopenic mice PEG-AMB-LIP was completely effective after a single dose of its MTD, whereas at this AMB dosage AMB-LIP was partially effective and AmBisome® was not effective.

At its own MTD, which was almost six-fold higher than the MTD of PEG-AMB-LIP, AmBisome® was also completely effective.

Evaluating the data from these three different AMB-liposome formulations on *in vivo* toxicity, *in vitro* antifungal activity, blood residence time and efficacy of treatment *in vivo* allow two important conclusions to be drawn: First, from a comparison between laboratory prepared AMB-LIP and industrially prepared AmBisome®, it became clear that the method of preparation of an AMB-liposome formulation greatly influences the toxicity, *in vitro* antifungal activity, and thereby the antifungal efficacy *in vivo*. A substantial reduction of AMB toxicity is clearly associated with considerable loss of antifungal activity *in vitro* and efficacy *in vivo*. Ideally, for optimization of an AMB-liposome formulation it is important to focus on reduction in AMB toxicity, without reducing its potent antifungal activity. The second conclusion, derived by comparing PEG-AMB-LIP and AMB-LIP, was that therapeutic efficacy is favored by a prolonged residence time of AMB-liposomes in the blood compartment. Whether this was due to sustained release of AMB during blood circulation or to an increased localization of AMB liposomes at the sites of infection, as was described by others for localized infection [7,8] or solid tumor [16,22], has to be further investigated. Apart from the prolonged blood circulation time of PEG-AMB-LIP its high intrinsic antifungal activity may be of great clinical significance in the treatment of severe invasive fungal infection. Only treatment with PEG-AMB-LIP, and not with AmBisome®, even if administered repeatedly, resulted in decreasing numbers of *C.albicans* in the kidney within short period of time after *C.albicans* inoculation (chapter 8). It has been reported recently that, even at high doses, AmBisome® treatment was not effective in an experimental model of hematogenous infection with two non-*albicans Candida* strains, showing moderate susceptibility for AMB [24]. For the treatment of such infections the high intrinsic antifungal activity of PEG-AMB-LIP and the reduced frequency of administration of these pegylated long-circulating AMB-liposomes open new therapeutic options.

The final challenge was to investigate whether the improved antifungal efficacy of PEG-AMB-LIP, as observed in our candidiasis model, could be extrapolated to another very difficult to treat fungal infection: pulmonary invasive aspergillosis in the leukopenic host (chapter 9). In granulocytopenic patients the mortality from pulmonary aspergillosis remains high despite aggressive antifungal therapy; thus, there is an urgent need for therapeutic advances. Our first data from a model of invasive pulmonary aspergillosis in persistently leukopenic rats showed that survival was significantly prolonged after only a single dose of PEG-AMB-LIP. These data are very encouraging and warrant further exploration of the therapeutic advantages of PEG-AMB-LIP in this model of pulmonary aspergillosis in rats. Whereas the present study focused on systemic intravenous administration of AMB-liposomes in the treatment of pulmonary aspergillosis, local administration of aerosolized AmBisome® in the prophylaxis of pulmonary aspergillosis has been recently described as an alternative modality for achieving high concentrations of antifungal

agent at the site of infection [3,38]. Whether the approaches of systemic administration of pegylated long-circulating AMB-liposomes, or local administration of AMB-liposomes, or combinations thereof will be successful in the prophylaxis and/or treatment of an established pulmonary fungal infection in man should be further evaluated.

In addition to antifungal chemotherapy, it is important to remember the critical role played by host defense mechanisms. A functional nonspecific and specific immune system is the best defense against opportunistic fungi. Thus, biological response modifiers, such as interferons, interleukins and colony-stimulating factors may in the future prove to be important adjuncts to antifungal chemotherapy [10,26,36].

Coming back to the aim of the present study, stated on the first page of this chapter, a number of clear answers were obtained. For treatment of established invasive fungal infections in the leukopenic host, it is absolutely necessary to use a fungicidal agent, such as AMB-DOC, even though it is highly toxic. With AmBisome® it is possible to virtually eliminate the toxicity of AMB. However, the reduction of intrinsic antifungal activity associated with the reduction of AMB's toxicity is not well recognized. It should be emphasized that high dosages of AmBisome® are needed for treatment to be effective. However, the present study shows that it is now possible to abolish the toxicity of AMB by liposomal encapsulation without reducing its intrinsic antifungal activity. Pegylated long-circulating AMB-liposomes (PEG-AMB-LIP) meet these requirements. Results observed with PEG-AMB-LIP in two models of difficult to treat fungal infections are very encouraging. The development of pegylated AMB-liposomes opens new ways to improve the treatment of invasive fungal infections in the immunocompromised host. Further development of AMB-liposome-technology, thus, remains highly relevant.

REFERENCES

1. Adler-Moore JP, Chiang SM, Satorius A, Guerra D, McAndrews B, McManus EJ, Proffitt RT. Treatment of murine candidosis and cryptococcosis with a unilamellar liposomal amphotericin B formulation (AmBisome). J Antimicrob Chemother 1991;28(Suppl.B):63-71.
2. Adler-Moore J. *In vivo* and *in vitro* evidence for reduced toxicity and mode of action of AmBisome®. Bone Marrow Transplant 1993;12(Suppl.4):S146.
3. Allen SD, Sorensen KN, Nejdl MJ, Proffitt RT. Efficacy of aerolsolized liposomal amphotericin B (AmBisome®) as a prophylactic treatment in an immune compromised murine model of pulmonary aspergillosis. J Control Release 1994;28:348-9.
4. Allen TM. The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. Adv Drug Deliv Rev 1994;13:285-309.

5. Anaissie E. Opportunistic mycoses in the immunocompromised host: experience at a cancer center and review. Clin Infect Dis 1992;14(Suppl. 1):S43-53.
6. Bakker-Woudenberg IAJM, Lokerse AF, Roerdink, FH. Effect of lipid composition on activity of liposome-entrapped ampicillin against intracellular *Listeria monocytogenes*. Antimicrob Agents Chemother 1988;32:1560-4.
7. Bakker-Woudenberg IAJM, Lokerse AF, Ten Kate MT, Mouton JW, Woodle MC, Storm G. 1993. Liposomes with prolonged blood circulation and selective localization in *Klebsiella pneumoniae*-infected lung tissue. J Infect Dis 1993;168:164-71.
8 Bakker-Woudenberg IAJM, Ten Kate MT, Stearne-Cullen LET, Woodle MC. Efficacy of gentamycin or ceftazidime entrapped in liposomes with prolonged blood circulation and enhanced localization in *Klebsiella pneumoniae* infected lung tissue. J Infect Dis 1995;171:938-47.
9. Bodey GP. Azole antifungal agents. Clin Infect Dis 1992;14(Suppl.1):S161-9.
10. Bodey GP, Anaissie E, Gutterman J, Vadhan-Raj S. Role of granulocyte-macrophage colony-stimulating factor as adjuvant treatment in neutropenic patients with bacterial and fungal infection. Eur J Clin Microbiol Infect Dis 1994;13(Suppl.2):S18-22.
11. Clemons KV, Stevens DA. Comparison of a liposomal amphotericin B formulation (AmBisome) and deoxycholate amphotericin B (Fungizone) for the treatment of murine paracoccidioidomycosis. J Med Vet Mycol 1993;31:387-94.
12. Clemons KV, Stevens DA. Therapeutic efficacy of a liposomal formulation of amphotericin B (AmBisome) against murine blastomycosis. J Antimicrob Chemother 1993;32:465-72.
13. De Marie S, Janknegt R, Bakker-Woudenberg IAJM. Clinical use of liposomal and lipid-complexed amphotericin B. J Antimicrob Chemother 1994;33:907-16.
14. Francis P, Lee JW, Hoffman A, Peter J, Francesconi A, Bacher J, Shelhamer J, Pizzo A, Walsh TJ. Efficacy of unilamellar liposomal amphotericin B in treatment of pulmonary aspergillosis in persistently granulocytopenic rabbits: the potential role of bronchoalveolar D-mannitol and serum galactomannan as marker of infection. J Infect Dis 1994;169:356-68.
15. Gabizon A, Huberty J, Straubinger RM, Price DC, Papahadjopoulos D (1988-89). An improved method for *in vivo* tracing and imaging of liposomes using a gallium 67-deferoxamine complex. J Liposome Res 1988-89;1:123-35.
16. Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, Cohen R, Martin F, Huang A, Barenholz Y. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Res 1994;54:987-92.
17. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-29.
18. Gates C. Pinney RJ. Amphotericin B and its delivery by liposomal and lipid formulations. J Clin Pharm Ther 1993;18:147-53.
19. Gondal JA, Swartz RP, Rahman A. Therapeutic evaluation of free and liposome-encapsulated amphotericin B in the treatment of systemic candidiasis in mice. Antimicrob Agents Chemother 1989;33:1544-8.
20. Grant SM, Clissold SP. Fluconazole. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in superficial and systemic mycoses. Drugs 1990;39:877-916.
21. Graybill JR. Future directions of antifungal chemotherapy. Clin Infect Dis 1992; 14(Suppl.1):S170-81.

22. Huang SK, Lee KD, Hong K, Friend DS, Papahadjopoulos D. Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice. Cancer Res 1992; 52:5135-43.
23. Janknegt R, De Marie S, Bakker Woudenberg IAJM, Crommelin DJA. Liposomal and lipid formulations of amphotericin B. Clinical Pharmacokinetics. Clin Pharmacokinet 1992;23:279-91.
24. Karyotakis NC, Anaissie EJ. Efficacy of escalating doses of liposomal amphotericin B (AmBisome) against hematogenous *Candida lusitaniae* and *Candida krusei* infection in neutropenic mice. Antimicrob Agents Chemother 1994;38:2660-2.
25. Klibanov AL, Huang L. Long circulating liposomes: development and perspectives. J Liposome Res 1992;2:321-334.
26. Kullberg BJ, Van 't Wout JW, Poell RJM, Van Furth R. Combined effect of fluconazole and recombinant human interleukin-1 on systemic candidiasis in neutropenic mice. Antimicrob Agents Chemother 1992;36:1225-9.
27. Lee JW, Amantea MA, Francis PA, Navarro EE, Bacher J, Pizzo PA, Walsh TJ. Pharmacokinetics and safety of a unilamellar liposomal formulation of amphotericin B (AmBisome) in rabbits. Antimicrob Agents Chemother 1994;38:713-8.
28. Leenders ACAP, Ten Kate MT, Bakker-Woudenberg IAJM, De Marie S. Efficacy of liposomal amphotericin B compared to amphotericin-desoxycholate in an animal model of one-sided pulmonary aspergillosis. submitted.
29. Lyman CA, Walsh TJ. Systemically administered antifungal agents- A review of their clinical pharmacology and therapeutic applications. Drugs 1992;44:9-35.
30. Mehta RT, McQueen TJ, Keyhani A, Lopez-Berestein G. Phagocyte transport as mechanism for enhanced therapeutic activity of liposomal amphotericin B. Chemotherapy 1994;40:256-64.
31. Pahls S, Schaffner A. Comparison of the activity of free and liposomal amphotericin B *in vitro* and in a model of systemic and localized murine candidiasis. J Infect Dis 1994;169:1057-61.
32. Papahadjopoulos D, Allen T, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C, Martin FJ. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci USA 1991;88:11460-4.
33. Perkins WR, Minchey SR, Boni LT, Swenson CE, Popescu MC, Pasternack RF, Janoff AS. Amphotericin B-phospholipid interactions resposible for reduced mammalian cell toxicity. Biochim Biophys Acta 1992;1107:271-82.
34. Proffitt RT, Satorius A, Chiang SM, Sullivan L, Adler-Moore JP. Pharmacology and toxicology of a liposomal formulation of amphotericin B (AmBisome) in rodents. J Antimicrob Chemother 1991;28(Suppl.B):49-61.
35. Proffitt RT, Adler-Moore JP, Fujii G, Satorius A, Lee MJA, Baily A. Stability and mode of action of AmBisome® (liposomal amphotericin B). J Control Release 1994;28:342-3.
36. Roilides E, Pizzo PA. Perspectives on the use of cytokines in the management of infectious complications of cancer. Clin Infect Dis 1993;17(Suppl. 2):S385-9.
37. Scherphof GL, Dijkstra J, Spanjer HH, Derksen JTP, Roerdink FH. Uptake an intracellular processing of targeted and non-targeted liposomes by rat Kupffer cells *in vivo* and *in vitro*. Ann NY Acad Sciences 1985;446:368-84.
38. Sorensen KN, Allen SD, Nejdl MJ, Proffitt RT. Aerosolization of liposomal (AmBisome®) and non-liposomal (Fungizone®) amphotericin B as treatment for pulmonary fungal infections. J Control Release 1994;28:346-7.
39. Swerdloff JN, Filler SG, Edwards JE. Severe candidal infections in neutropenic patients. Clin Infect Dis 1993;17(Suppl. 2):S457-67.

40. Walsh TJ, Aoki S, Mechinaud F, Bacher J, Lee JW, Rubin M, Pizzo PA. Effects of preventive, early, and late antifungal chemotherapy with Fluconazole in different granulocytopenic models of experimental disseminated candidiasis. J Infect Dis 1990; 161:755-60.
41. Walsh TJ, Lee J, Aoki S, Mechinaud F, Bacher J, Lecciones J, Thomas V, Rubin M, Pizzo PA. Experimental basis for use of Fluconazole for preventive or early treatment of disseminated candidiasis in granulocytopenic hosts. Rev Infect Dis 1990; 12(Suppl.3):S307-17.
42. Walsh TJ. Management of immunocompromised patients with evidence of an invasive mycosis. Hematol Oncol Clin N Am 1993;7:1003-26.
43. Woodle MC, Matthay KK, Newman MS, Hidayat JE, Coolins LR, Redemann C, Martin FJ, Papahadjopoulos D. Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes. Biochim Biophys Acta 1992;1105:193-200.
44. Woodle MC, Lasic DD. 1992. Sterically stabilized liposomes. Biochim Biophys Acta 1992;1113:171-99.

Summary

As the overall prognosis for patients with invasive fungal infections remains poor, there is a critical need to improve the treatment of these infections. Parenteral administration of amphotericin B (AMB) remains the therapy of choice for most invasive fungal infections. AMB is administered as AMB-desoxycholate (AMB-DOC or Fungizone®), but its use is significantly limited by its toxic side effects. New antifungal agents, such as the triazole fluconazole (FLU) and liposomal AMB have been developed for the management of invasive fungal infections. The role of these new agents in the treatment of invasive fungal infections in the leukopenic host is not clearly defined nor well understood.

The **primary aim** of the work presented in this thesis was **to gain further insight into the therapeutic value of existing and newly developed liposomal formulations of AMB in the treatment of invasive fungal infections.**

First of all, the question of the impact of a fungistatic agent, FLU, versus that of the fungicidal agent, AMB-DOC, on treatment of invasive candidiasis was addressed (chapters 2 and 3). Although the *in vitro* activity of AMB-DOC against *C.albicans* was high, the antifungal activity was influenced by factors relevant to its efficacy in the treatment of infection: activity of AMB-DOC was substantially reduced by the presence of serum. AMB-DOC activity was only slightly hindered by an intracellular localization of the yeast (in peritoneal macrophages). For FLU, even at high concentrations, antifungal activity against extracellular *C.albicans* was restricted to inhibition of fungal growth. Furthermore, FLU was not effective against intracellular *C.albicans*. Therefore, it was questioned whether FLU could effectively eradicate *C.albicans in vivo*, in particular when host defense mechanisms are severely impaired. In immunocompetent mice FLU was effective as long as treatment was given, whereas in leukopenic mice efficacy was only obtained if treatment was started in the early phase of the disease. In well established *C.albicans* infection in leukopenic mice FLU was definitely not efficacious when treatment was delayed. In contrast with the potent antifungal activity of AMB-DOC *in vitro*, the *in vivo* therapeutic efficacy of AMB-DOC in invasive candidiasis was very limited, especially in leukopenic animals, due to the low maximum tolerated dosage (MTD).

The rationale for the use of AmBisome®, one of the industrially prepared AMB-lipid formulations, was to make use of the potent fungicidal effect of AMB while, at the same time, reducing its toxicity by liposomal encapsulation. The antifungal efficacy of AMB was far improved with AmBisome®, being highly effective in multidose treatment regimens. High dosages reduced *C.albicans* in the kidney and other organs as well as prevented relapse of infection, even in severe infection (chapter 3). It should be noted, however, that at equivalent dosages AmBisome® was less effective than AMB-DOC. On the other hand, much higher dosages were tolerated for AmBisome® in these mice, resulting in improved therapeutic efficacy.

With respect to the biodistribution of AMB after administration of AMB-DOC or AmBisome® (chapter 4), it was shown for both formulations that the condition of the animals (immunocompetent uninfected mice versus leukopenic mice infected with

C.albicans) did not influence the biodistribution patterns. Comparing the biodistribution of AMB-DOC and AmBisome® at equivalent dosages revealed that encapsulation of AMB resulted in increased AMB levels in blood. In addition increased AMB concentrations were present in the mononuclear phagocyte system (MPS), particularly the liver and spleen, whereas concentrations in kidney and lung were lower. Uptake of AmBisome® by MPS-tissues was highly dosage dependent and saturable. As a result, at high lipid dosages a prolonged circulation of AmBisome® in blood could be obtained. After administration of AmBisome® at the MTD, blood and tissues retained high AMB concentrations. It should be emphasized that these high concentrations of AMB did not lead to toxicity. By using radioactively labeled AmBisome® it was found that the high AMB concentrations in blood represented liposome entrapped AMB. During circulation slow release of AMB occurred (chapter 4). Even today it remains unclear whether release of AMB is of importance for antifungal activity. *In vitro* AMB remains tightly associated to the liposomes in AmBisome® in the presence of serum, and AMB has not necessarily to be released from AmBisome® in order to express antifungal activity against *C.albicans* (chapter 5).

The antifungal activity of AmBisome® against intracellularly growing *C.albicans* (in peritoneal macrophages) is described in chapter 5. In parallel with the antifungal activities against extracellular *C.albicans* (chapter 3), the intracellular antifungal activity of AMB-DOC was much higher than that of AmBisome®. Localization experiments with fluorescens-labeled AmBisome® clearly showed that AmBisome® is taken up by macrophages, both by uninfected macrophages and macrophages infected with *C.albicans*. The presence of AmBisome® inside uninfected macrophages did not influence uptake of *C.albicans* by the same macrophages. Furthermore, previous exposure of uninfected macrophages to AmBisome® was advantageous for antifungal activity of AmBisome® against intracellular *C.albicans*.

Prolonged residence of liposomes in blood probably allows the localization of intact liposomes at sites of infection outside MPS-tissues. For liposome types such as AmBisome® blood residence time is primarily dependent on the lipid dose administered: only at high lipid dosages blood circulation times are prolonged (chapter 4). Advances in liposome technology, especially the incorporation of hydrophilic phosphatidylethanolamine derivatives of monomethoxy polyethyleneglycols (PEG-PE) into liposomes, have resulted in the ability to achieve longer blood residence time of liposomes without the constraints of high lipid dose, small particle size, or rigid nature of the bilayer. Such pegylated long-circulating liposomes have been named sterically stabilized liposomes or MPS avoiding liposomes.

It was investigated whether pegylated long-circulating AMB-liposomes could be prepared (chapter 6). Successful liposomal encapsulation of AMB requires that AMB is complexed, e.g. to the negatively charged phospholipid distearoylphosphatidylglycerol (DSPG), as in AmBisome®. We reasoned that the net negative charge of PEG-DSPE could also be used for complexation of AMB. Two different formulations of PEG-DSPE containing liposomes with AMB were prepared (chapter 6). PEG-AMB-LIP type 1, in which AMB was complexed to DSPG, as well as PEG-AMB-LIP type 2, in which AMB

was complexed to PEG-DSPE and DSPG was omitted from the preparation. PEG-AMB-LIP type 1 was as toxic as AMB-DOC, and was omitted from further studies on biodistribution. With PEG-AMB-LIP type 2 a relatively non-toxic preparation with a prolonged blood residence time was obtained. The efficacy of this PEG-AMB-LIP type 2 (further referred to as PEG-AMB-LIP) in the treatment of invasive candidiasis in leukopenic mice was investigated (chapter 7). PEG-AMB-LIP was compared with industrially prepared AmBisome® and with AMB-liposomes (AMB-LIP), that had the lipid composition as in AmBisome®, but were prepared in our laboratory. PEG-AMB-LIP and AMB-LIP were almost equal in *in vivo* toxicity and *in vitro* antifungal activity, however they differed in blood residence time. Blood residence time of PEG-AMB-LIP was more prolonged, even at a low lipid dosage. Industrially prepared AmBisome® and laboratory prepared AMB-LIP differed in *in vivo* toxicity and *in vitro* antifungal activity. AmBisome® was less toxic than AMB-LIP, however antifungal activity *in vitro* was substantially reduced as compared to AMB-LIP. In our model of invasive candidiasis in leukopenic mice PEG-AMB-LIP was completely effective at its MTD, whereas at this AMB dosage AMB-LIP was partially effective and AmBisome® was not effective. At its own MTD, which was almost six-fold higher than the MTD of PEG-AMB-LIP, AmBisome® was also completely effective.

Evaluating the data from these three different AMB-liposome formulations on *in vivo* toxicity, *in vitro* antifungal activity, blood residence time and efficacy of treatment *in vivo* allow two important conclusions to be drawn: First, from a comparison between laboratory prepared AMB-LIP and industrially prepared AmBisome®, it became clear that the method of preparation of an AMB-liposome formulation greatly influences the toxicity, *in vitro* antifungal activity, and thereby the antifungal efficacy *in vivo*. A substantial reduction of AMB toxicity is clearly associated with considerable loss of potent antifungal activity *in vitro* and efficacy *in vivo*. Ideally, for optimization of an AMB-liposome formulation it is important to focus on reduction in AMB toxicity, without reducing its antifungal activity. The second conclusion, derived by comparing PEG-AMB-LIP and AMB-LIP, was that therapeutic efficacy is favored by a prolonged residence time of AMB-liposomes in the blood compartment. Apart from the prolonged blood circulation time of PEG-AMB-LIP its high intrinsic antifungal activity may be of great clinical significance in the treatment of severe invasive fungal infection. Only treatment with PEG-AMB-LIP, and not with AmBisome®, even if administered repeatedly, resulted in decreasing numbers of *C.albicans* in the kidney within short period of time after *C.albicans* inoculation (chapter 8).

The final challenge was to investigate whether the improved antifungal efficacy of PEG-AMB-LIP, as observed in our candidiasis model, could be extrapolated to another very difficult to treat fungal infection: pulmonary invasive aspergillosis in the leukopenic host (chapter 9). Our first data from a model of invasive pulmonary aspergillosis in persistently leukopenic rats showed that survival was significantly prolonged after only a single dose of PEG-AMB-LIP. These data are very encouraging and warrant further exploration of the therapeutic advantages of PEG-AMB-LIP in this model of pulmonary aspergillosis in rats.

Summary

Coming back to the aim of the present study, stated on the first page of this summary, a number of clear answers were obtained. For treatment of established invasive fungal infections in the leukopenic host, it is absolutely necessary to use a fungicidal agent, such as AMB-DOC, even though it is highly toxic. With AmBisome® it is possible to virtually eliminate the toxicity of AMB. However, the reduction of intrinsic antifungal activity associated with the reduction of AMB's toxicity is not well recognized. It should be emphasized that high dosages of AmBisome® are needed for treatment to be effective. However, the present study shows that it is now possible to abolish the toxicity of AMB by liposomal encapsulation without reducing its intrinsic antifungal activity. Pegylated long-circulating AMB-liposomes (PEG-AMB-LIP) meet these requirements. Results observed with PEG-AMB-LIP in two models of difficult to treat fungal infections are very encouraging. The development of pegylated AMB-liposomes opens new ways to improve the treatment of invasive fungal infections in the immunocompromised host. Further development of AMB-liposome-technology, thus, remains highly relevant.

Samenvatting

De prognose van patiënten met invasieve mycosen (gist- en schimmel-infecties) is nog altijd heel slecht. Er bestaat er grote behoefte aan verbetering van de behandeling van deze infecties. Het meest gebruikte middel voor de behandeling van invasieve mycosen is amphotericine B (AMB), parenteraal toegediend in de vorm van amphotericine B-desoxycholate (AMB-DOC of Fungizone®). Het gebruik hiervan is echter beperkt vanwege de ernstige bijwerkingen. Ter verbetering van de behandeling van invasieve mycosen zijn nieuwe antifungale middelen, zoals het triazole fluconazole (FLU) en nieuwe formuleringen van AMB, zoals liposomaal AMB, ontwikkeld. De waarde van deze middelen / formuleringen voor de behandeling van invasieve mycosen in de leukopenische gastheer is echter nog onvoldoende duidelijk.

Het **belangrijkste doel** van de studies zoals beschreven in dit proefschrift was **het verwerven van meer inzicht in de therapeutische waarde van zowel bestaande als ook nieuw-ontwikkelde liposomale formuleringen van AMB in de behandeling van invasieve mycosen.**

In de eerste studies (hoofstukken 2 en 3) werd de bruikbaarheid van een fungistatisch middel, FLU, in de behandeling van invasieve candidiasis vergeleken met die van het fungicide middel AMB-DOC. *In vitro* wordt *Candida albicans* snel gedood bij lage concentraties AMB-DOC. Factoren die belangrijk zijn voor de antifungale effectiviteit *in vivo* bleken van invloed op de actviteit van AMB-DOC. In aanwezigheid van serum was de activiteit van AMB-DOC aanzienlijk verminderd. Ten opzichte van intracellulair gelocaliseerde *C.albicans* (in peritoneaal macrofagen) bleek de antifungale activiteit slechts iets verminderd in vergelijking tot de activiteit t.o.v. extracellulair groeiende *C.albicans*. In aanwezigheid van FLU werd *C.albicans* ook bij hoge concentraties niet gedood, maar slechts geremd in de groei. FLU vertoonde geen antifungale activiteit t.o.v. intracellulair groeiende *C.albicans*. Op grond van deze resultaten werd de vraag gesteld of behandeling met FLU effectief zou zijn in geval van invasieve *C.albicans* infecties *in vivo*, met name in de situatie waarin de afweermechanismen van de gastheer ernstig zijn aangetast. In ons proediermodel van invasieve candidiasis in immuuncompetente muizen bleek FLU slechts effectief gedurende de behandelingsperiode. In het model van invasieve candidiasis in leukopenische muizen bleek FLU slechts effectief te zijn indien heel vroeg (empirisch) gestart werd met behandeling; bij een late start van behandeling, op een moment dat de ernst van de *C.albicans* infectie aanzienlijk was toegenomen, was FLU niet effectief meer. In tegenstelling tot de krachtige antifungale activiteit van AMB-DOC *in vitro*, bleek *in vivo* de therapeutische effectiviteit in genoemde modellen beperkt, in verband met de lage maximaal toelaatbare dosis (MTD) van AMB-DOC.

De gedachte om de krachtige fungicide activiteit van AMB te benutten, en tegelijkertijd de toxiciteit van AMB te verminderen door liposomale inkapseling, vormde de basis voor het gebruik van AmBisome®, een van de industrieel bereide AMB-lipid preparaten. De antifungale effectiviteit van AMB *in vivo* was sterk verbeterd als gevolg van inkapseling in de vorm van AmBisome®. Bij invasieve candidiasis in zowel immuuncompetente als leukopenische muizen bleek AmBisome® zeer effectief

(hoofdstuk 3). Herhaalde toediening van hoge doses AmBisome® deed, zelfs in geval van ernstige *C.albicans* infectie, het aantal *C.albicans* in de nier en andere organen sterk afnemen, zodanig dat terugkeer van de infectie kon worden voorkómen. Weliswaar bleek AmBisome® minder effectief te zijn dan AMB-DOC bij een gelijke dosering. Vanwege de lage toxiciteit van AmBisome® kon echter hoog gedoseerd worden, hetgeen alsnog resulteerde in een sterk verbeterde effectiviteit van AMB.

De verspreiding van AMB via het bloed naar verschillende organen (biodistributie) na toediening van AMB-DOC of AmBisome® in muizen werd bestudeerd en beschreven in hoofdstuk 4. Allereerst bleek de conditie van de dieren (immuuncompetente niet-geïnfecteerde muizen versus leukopenische muizen geïnfecteerd met *C.albicans*) niet van invloed op de biodistributie van beide preparaten. Vergelijking van de biodistibutie van AMB-DOC met AmBisome® bij een gelijke dosering laat zien dat bij liposomale inkapseling hogere AMB concentraties in het bloed worden verkregen; ook zijn hogere AMB concentraties aanwezig in het mononucleaire fagocyten systeem (MPS), met name in de lever en milt, terwijl AMB concentraties in de nier en long lager zijn. De opname van AmBisome® in de MPS-organen (lever en milt) is sterk dosis afhankelijk en verzadigbaar. Na toediening van de MTD van AmBisome®, worden gedurende aanzienlijke tijd hoge concentraties AMB in bloed en organen verkregen, welke niet leiden tot toxiciteit. Door gebruik te maken van radioactief gemerkt AmBisome® kon worden vastgesteld dat de hoge AMB concentraties in het bloed voornamelijk liposoom-geassocieerd AMB representeren. Gedurende de circulatie van AmBisome® komt slechts een gering deel van AMB vrij. Tot voor kort bestond onduidelijkheid over het belang van vrijkomen van AMB voor de antifungale activiteit van AmBisome®. In hoofdstuk 5 wordt getoond dat *in vitro* AMB sterk geassocieerd blijft aan de liposomen in AmBisome®, en dat voor antifungale activiteit van AmBisome® t.o.v. *C.albicans* AMB niet hoeft vrij te komen.

De antifungale activiteit van AmBisome® t.o.v. intracellulair gelocaliseerde *C.albicans* (in peritoneaal macrofagen) werd in hoofdstuk 5 beschreven. Naar analogie met de antifungale activiteit van AMB-DOC en AmBisome® t.o.v. extracellulair groeiende *C.albicans* (hoofdstuk 3), was de intracellulaire activiteit van AMB-DOC vele malen groter dan die van AmBisome®. Lokalisatie experimenten met fluoresceïne-gemerkt AmBisome® (hoofdstuk 5) lieten een goede opname van AmBisome® in macrofagen zien, zowel in niet-geïnfecteerde macrofagen als in macrofagen die al *C.albicans* hadden opgenomen. De aanwezigheid van AmBisome® in macrofagen beïnvloedde de opname van *C.albicans* door diezelfde macrofagen niet. Bovendien bleek voorafgaande blootstelling van niet-geïnfecteerde macrofagen aan AmBisome® een positief effect te hebben op de intracellulaire effectiviteit van AmBisome® indien de macrofagen vervolgens werden geïnfecteerd met *C.albicans*.

Een lange circulatietijd van liposomen in het bloed maakt de lokalisatie van intacte liposomen op plaatsen van infectie buiten het MPS mogelijk. Voor liposoomtypen zoals AmBisome® is de circulatietijd in bloed sterk afhankelijk van de lipid-dosis: een hoge lipid-dosis is nodig voor een lange circulatietijd (hoofdstuk 4). Liposoom-technologische ontwikkelingen van de laatste jaren, met name de inbouw van hydrofiele fosfatidyl-

ethanolamine derivaten van monomethoxy polyethyleenglycol (PEG-PE) in liposomen, hebben het mogelijk gemaakt de circulatietijd van liposomen aanzienlijk te verlengen zonder dat hiervoor een hoge lipid dosis, een kleine deeltjesgrootte van het liposoom, of een rigide karakter van de fosfolipide-bilaag nodig zijn. Deze gepegyleerde lang-circulerende liposomen worden sterisch gestabiliseerde of MPS ontwijkende liposomen genoemd. Onderzocht werd of er gepegyleerde lang-circulerende AMB-liposomen kunnen worden bereid (hoofdstuk 6). Voor een goede liposomale inkapseling van AMB is het nodig dat AMB wordt gecomplexeerd b.v. aan het negatief geladen fosfolipid distearoylfosfatidylglycerol (DSPG) dat voorkomt in AmBisome®. De netto negatieve lading van PEG-DSPE zou mogelijk ook gebruikt kunnen worden voor complexering van AMB. Twee verschillende PEG-DSPE bevattende liposoom-preparaten met AMB werden bereid (hoofdstuk 6): PEG-AMB-LIP type 1, waarin AMB werd gecomplexeerd aan DSPG, en PEG-AMB-LIP type 2 waarin AMB werd gecomplexeerd aan PEG-DSPE en het DSPG was weggelaten uit de formulering. PEG-AMB-LIP type 1 bleek even toxisch als AMB-DOC, zodat verdere studies hiermee m.b.t. biodistributie achterwege werden gelaten. Het PEG-AMB-LIP type 2 preparaat was relatief weinig toxisch, en vertoonde een lange cirulatietijd in bloed. Onderzoek naar de antifungale activiteit van PEG-AMB-LIP type 2 (verder PEG-AMB-LIP genoemd) werd verricht in het model van invasieve candidiasis in leukopenische muizen. PEG-AMB-LIP werd vergeleken met industrieel bereid AmBisome® en met AMB-liposomen (AMB-LIP), die de lipid-samenstelling hadden als in AmBisome®, echter in het laboratorium waren bereid (hoofdstuk 7). PEG-AMB-LIP en AMB-LIP vertoonden een grote overeenkomst in toxiciteit (geringe toxiciteit *in vivo*), en in antifungale activiteit t.o.v. *C.albicans in vitro* (vrijwel gelijk aan die van AMB-DOC), maar verschilden van elkaar in circulatietijd in de bloedbaan. De circulatietijd van PEG-AMB-LIP was langer dan die van AMB-LIP, en dit bleef gehandhaafd als de dosis werd verlaagd. Industrieel bereid AmBisome® en in het laboratorium bereid AMB-LIP verschilden van elkaar in *in vivo* toxiciteit en *in vitro* antifungale activiteit. AmBisome® was nog minder toxisch dan AMB-LIP, maar liet tevens een sterk gereduceerde antifungale activiteit *in vitro* zien. In het model van invasieve candidiasis in leukopenische muizen bleek PEG-AMB-LIP zeer effectief na een enkelvoudige toediening van de MTD, terwijl bij een gelijke dosis AMB-LIP slechts partieel effectief was, en AmBisome® niet effectief was. Bij de MTD van AmBisome®, bijna zes maal hoger dan de MTD van PEG-AMB-LIP, was AmBisome® ook zeer effectief.

Evalueren van de gegevens van deze drie verschillende AMB-liposoom preparaten m.b.t de *in vivo* toxiciteit, *in vitro* antifungale activiteit, circulatietijd in bloed, en therapeutische effectiviteit *in vivo* leidt tot twee belangrijke conclusies. Ten eerste, de vergelijking van in het laboratorium bereid AMB-LIP en industrieel bereid AmBisome® laat duidelijk zien dat de bereidingswijze van het liposoom de *in vivo* toxiciteit, de *in vitro* antifungale activiteit, en daarmee de therapeutische effectiviteit *in vivo* in grote mate kan beïnvloeden. Een substantiële vermindering van toxiciteit gaat blijkbaar samen met een aanzienlijk verlies aan *in vitro* antifungale activiteit, en daarmee aan de *in vivo* effectiviteit. Idealiter zou bij een optimaal AMB-liposoom preparaat de toxiciteit van

AMB verminderd moeten worden, zonder dat de krachtige antifungale activiteit van AMB verloren gaat. Ten tweede, uit de vergelijking tussen PEG-AMB-LIP en AMB-LIP kan worden geconcludeerd dat een lange circulatietijd van AMB-liposomen in bloed gunstig is voor de therapeutische effectiviteit. Naast de lange circulatietijd van PEG-AMB-LIP in bloed zou ook de krachtige 'intrinsieke' antifungale activiteit van PEG-AMB-LIP van grote klinische betekenis kunnen zijn bij de behandeling van ernstige invasieve mycosen. In het model van invasieve candidiasis in leukopenische muizen deed behandeling met PEG-AMB-LIP het aantal *C.albicans* in de nier sterk afnemen kort na start van de behandeling, zelfs indien de ernst van de *C.albicans* infectie aanzienlijk was toegenomen. AmBisome®, zelfs herhaald toegediend, bleek niet meer effectief te zijn bij deze zeer ernstige vorm van *C.albicans* infectie (hoofdstuk 8).

De laatste vraag was of de goede antifungale effectiviteit van PEG-AMB-LIP, zoals waargenomen in het candidiasis model, ook gevonden zou worden in een ander model van een bijzonder moeilijk te behandelen schimmelinfectie: pulmonale invasieve aspergillosis in de leukopenische gastheer (hoofdstuk 9). De eerste gegevens van behandeling met een enkelvoudige dosis PEG-AMB-LIP in een model van invasieve pulmonale aspergillosis in leukopenische ratten laten zien dat de overlevingsduur van de behandelde dieren significant langer is dan die van onbehandelde dieren. Deze resultaten zijn zeer bemoedigend en rechtvaardigen verder onderzoek naar de therapeutische mogelijkheden van PEG-AMB-LIP in dit model van pulmonale aspergillosis.

Terugkomend op het doel van deze studie, zoals vermeld op de eerste pagina van deze samenvatting, zijn een aantal duidelijke antwoorden verkregen. Voor de behandeling van bewezen ernstige invasieve mycosen in de leukopenische gastheer, is het absoluut noodzakelijk om een fungicide middel te gebruiken, zoals AMB-DOC, ofschoon dit middel erg toxisch is. In AmBisome® is de toxiciteit van AMB zeer sterk verminderd. De daarmee samenhangende vermindering van 'intrinsieke' antifungale activiteit van AMB is echter een direct gevolg. Dit wordt als zodanig te weinig onderkend. Immers slechts bij hoge doseringen van AmBisome® wordt een effectieve behandeling bereikt. Het in dit proefschrift beschreven onderzoek laat zien dat het wel mogelijk is de toxiciteit van AMB door liposomale inkapseling aanzienlijk te verminderen, zonder dat er verlies van 'intrinsieke' antifungale activiteit optreedt. Gepegyleerde lang-circulerende AMB liposomen voldoen aan bovengenoemde vereisten. De resultaten die werden verkregen met PEG-AMB-LIP in twee modellen van moeilijk te behandelen mycosen in muizen en ratten zijn zeer bemoedigend. De ontwikkeling van gepegyleerde AMB-liposomen opent een nieuwe richting in de verbetering van de behandeling van invasieve mycosen in de immuun-gecompromitteerde gastheer. Aldus blijft de verdere ontwikkeling van liposoom-technologie uiterst relevant voor de behandeling van invasieve mycosen bij de mens.

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Publications

Diederen JHB, Van Etten EWM, Biegstraaten AIM, Terlou M, Vullings HGB, Jansen WF. Flight-induced inhibition of the cerebral median peptidergic neurosecretory system in *Locusta migratoria*. Gen Comp Endocrinol 1988;71:257-64.

Storm G, Van Bloois L, Steerenberg PA, Van Etten E, De Groot G, Crommelin DJA. Liposome encapsulation of Doxorubicin: Pharmaceutical and therapeutic aspects. J Control Release 1989;9:215-29.

Van Etten EWM, Van de Rhee NE, Van Kampen KM, and Bakker-Woudenberg IAJM. Effects of amphotericin B and fluconazole on the extracellular and intracellular growth of *Candida albicans*. Antimicrob Agents Chemother 1991;35:2275-81.

Bakker-Woudenberg IAJM, Thonus IP, Roosendaal R, Van Etten EWM, De Marie S, Melissen PMB. Rationele behandeling van ernstige infecties. In: Bakker-Woudenberg I, Horrevorts A (Eds) Ontwikkelingen in de behandeling van infectieziekten. Dordrecht: ICG Printing, 1991:13-33.

Van Etten EWM, Van den Heuvel C, Bakker-Woudenberg IAJM. Toxicity and efficacy in systemic candidiasis in leukopenic mice of amphotericin B-desoxycholate (Fungizone®) versus liposomal amphotericin B (AmBisome®). J Liposome Res 1993; 3:154-5.

Van Etten EWM, Otte M, Ten Kate MT, Van Heijst BYM, Bakker-Woudenberg IAJM. Biodistribution in mice and *in vitro* activity of amphotericin B-desoxycholate (Fungizone®) versus liposomal amphotercin B (AmBisome®). J Liposome Res 1993; 3:169-70.

Van Etten EWM., Van den Heuvel-de Groot C, and Bakker-Woudenberg IAJM. Efficacies of amphotericin B-desoxycholate (Fungizone), liposomal amphotericin B (AmBisome) and fluconazole in the treatment of systemic candidosis in immunocompetent and leukopenic mice. J Antimicrob Chemother 1993;32:723-39.

Bakker-Woudenberg IAJM, Lokerse AF, Ten Kate MT, Melissen PMB, Van Vianen W, Van Etten EWM. Liposomes as carriers of antimicrobial agents or immunomodulatory in the treatment of infections. Eur J Clin Microbiol Infect Dis 1993; 12(Suppl.1):61-7.

Van Etten EWM, Stearne-Cullen LET, Ten Kate MT, Van Vianen W, Bakker-Woudenberg IAJM. Lang-circulerende amphotericine B-liposomen: toxiciteit, antifungale *in vitro*-activiteit en effectiviteit in een model van systemische candidiasis bij leukopenische muizen. Ned Tijdschr Geneesk 1994;138:2421-2.

Bergers JJ, Ten Hagen TLM, Van Etten EWM, Bakker-Woudenberg IAJM. Liposomes as delivery systems in the prevention and treatment of infectious diseases. Pharmacy World & Science 1995;17:1-11.

Van Deventer AJM, Goessens WHF, Van Belkum A, Van Vliet HJA, Van Etten EWM, Verbrugh HA. Improved detection of *Candida albicans* by PCR in blood of neutropenic mice with systemic candidiasis. J Clin Microbiol 1995;33:625-8.

Van Etten EWM, Otte-Lambillion M, Van Vianen W, Ten Kate MT, and Bakker-Woudenberg IAJM. Biodistribution of liposomal amphotericin B (AmBisome) versus amphotericin B-desoxycholate (Fungizone) in immunocompetent uninfected mice as well as in leukopenic mice infected with *Candida albicans*. J Antimicrob Chemother 1995;35:509-19.

Van Etten EWM, Ten Kate MT, Stearne-Cullen LET, and Bakker-Woudenberg IAJM. Pegylated long-circulating amphotericin B-liposomes versus AmBisome®: toxicity, antifungal activity, and efficacy in systemic candidiasis in leukopenic mice. Antimicrob Agents Chemother 1995;39:1954-8.

Van Etten EWM, Van Vianen W, Tijhuis RHG, Storm G, and Bakker-Woudenberg IAJM. Sterically stabilized amphotericin B-liposomes: toxicity and biodistribution in mice. J Control Release 1995;in press.

Van Etten EWM, Chander H, Snijders S, and Bakker-Woudenberg IAJM. Interactions of AmBisome with extracellular and intracellular *Candida albicans*. J Antimicrob Chemother 1995;in press.

Van Deventer AJM, Van Etten EWM, Van Vliet HJA, Van Etten EWM, Goessens WHF, Verbrugh HA. Prognostic value of PCR in blood of neutropenic mice with systemic candidiasis treated with AmBisome®. Submitted.

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