Inhalatie van amfotericine B formuleringen ter preventie en behandeling van invasieve pulmonale aspergillose

Een experimenteel onderzoek bij ratten

# Inhalation of amphotericin B formulations for prevention and treatment of Invasive Pulmonary Aspergillosis

## An experimental study in rats

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr.ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 19 juni 2002 om 9.45 uur

door Elisabeth Johanna Ruijgrok
geboren te Wassenaar

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Chapter 1

# General introduction and aim

# The emerging fungal threat

Invasive fungal infections have continued to increase in incidence during the last 20 years and are now significant causes of morbidity and mortality. This is particularly true in patients with haematological malignancies undergoing induction or consolidation chemotherapy (especially during the nadir of their granulocytopenia), in immunosuppressed organ transplant recipients, and in patients with acquired immunodeficiency secondary to infection by human immunodeficiency viruses. The increase in number of patients with invasive fungal disease is a result of several factors, including (1) the development of new chemotherapy regimens for solid tumours, lymphoma, myeloma and leukemia; (2) an increase in the number of transplant recipients; (3) increased use of immunosuppressive regimens for autoimmunediseases and (4) the advent of the AIDS pandemic. Autopsy data indicate that more than half of patients who die with malignancies are infected with *Candida* spp., approximately one-third with *Aspergillus* spp., and increasing numbers with *Cryptococcus* spp. or other fungi such as *Fusarium* spp. [5,6,24,54,94,99,105]. Factors that predispose patients to invasive fungal infections are generally based on an impaired defence mechanism. These factors include: prolonged neutropenia (chemotherapy induced); defective T-lymphocyte function (associated with organ transplantation and HIV infection); impaired macrophage function, particularly of pulmonary macrophages (associated with high doses and prolonged administration of corticosteroids; and barrier defects (associated with invasive medical procedures, vascular catheters, parenteral nutrition and haemodialysis and peritoneal dialysis) in compromised patients [4,5,23,29,80,101].

# Invasive Pulmonary Aspergillosis

Invasive aspergillosis is the most severe and life-threatening disease caused by the fungus *Aspergillus*. In most cases, invasive infections with *Aspergillus* spp. present as invasive pulmonary aspergillosis in which *Aspergillus fumigatus* is the main causative agent. This ubiquitous and usually saprophytic fungus grows and sporulates in humid environments on decaying organic matter. On inhalation of its airborne conidia, *A. fumigatus* can cause a wide spectrum of diseases [10,27]. Invasive pulmonary aspergillosis occurs in severely immunocompromised patients who usually develop rapid and often fatal infections. The incidence of invasive pulmonary aspergillosis can differ per centre and also within centres in time. Notorious are the nosocomial outbreaks of aspergillosis as a complication of construction work in or

Table 1. Incidence of aspergillosis according to underlying condition

| Condition | Range (%) |
|---|---|
| Heart-lung or lung transplantation | 19-26[1] |
| Chronic granulomatous disease | 25-40[2] |
| Acute leukemia | 5-24 |
| Allogeneic BMT | 4-9 |
| Autologous BMT without growth factors | 0.5-6 |
| AIDS | 0-12 |
| Liver transplantation | 1.5-10 |
| Heart and kidney transplantation | 0.5-10 |
| Severe combined immunodeficiency | 3.5 |
| Burns | 1-7 |
| Systemic lupus erythematosus | 1 |
| Autologous BMT with growth factors | <1 |

Data are from [27]

[1]Distinguishing colonisation from infection is particularly difficult in these patients.

[2]Lifetime incidence.

near hospital units in which neutropenic cancer and transplant recipients were housed [27]. Approximate incidence figures for different patient groups are shown in Table 1.

The portal entries for *Aspergillus* include the respiratory tract, damaged skin or operation wounds, the cornea and the ear. The majority of patients (80-90%) has pulmonary disease. Approximately 30% of patients initially has no symptoms attributable to invasive pulmonary aspergillosis. As the disease progresses, symptoms appear. Early symptoms are cough and fever. At autopsy, many patients who die with invasive aspergillosis have disseminated disease [10,20,41,60,102,110,114]. Mortality associated with invasive pulmonary aspergillosis is nearly 100% if left untreated [25]. Treatment with appropriate antifungal agents is still correlated with a mortality of 25-40% [27,63]. Survival depends largely on neutrophil recovery, status of malignancy and early diagnosis followed by early treatment of the infection [2,103].
Diagnosis of invasive pulmonary aspergillosis is problematic and infection is often confirmed at autopsy only [11]. Fever unresponsive to broad-spectrum antibiotics is regarded as an early and common clinical sign of an invasive fungal infection. Definitive diagnosis requires both histopathological evidence and cultures of *Aspergillus* in biopsy, respiratory tract secretions or broncho-alveolar lavage fluid. However, the detection of *Aspergillus* in tissue or fluids is insensitive [25]. As early

diagnosis and early start of treatment of invasive aspergillosis are correlated with favourable treatment outcome, the improvement of diagnostic techniques is warranted [2,103]. Improvements in the diagnosis of invasive pulmonary aspergillosis include the use of high-resolution CT [18], polymerase chain reaction (PCR) for the detection of fungal RNA or DNA [31,46] and enzyme-linked immunosorbent assay (ELISA) testing of serum or broncho-alveolar lavage fluid for galactomannan, a component of the fungal cell wall of *Aspergillus* [17,67]. None of the tests has been proven to identify definitively patients with invasive disease, but positive results are generally highly predictive of invasive disease [59].

# Antifungal agents

At present, only two agents are available for the routine clinical treatment of invasive pulmonary aspergillosis: the fungicidal polyene amphotericin B (AMB) and the fungistatic triazole itraconazole. AMB is available as the desoxycholate salt (AMB-DOC), and as three different lipid formulations. In addition, several new drugs for invasive aspergillosis are in the developmental stage.

## Amphotericin B

AMB has been the treatment of choice for most invasive fungal infections since its introduction in the 1950s. AMB is a macrolide polyene antibiotic derived from Streptomyces nodosus. It acts by binding to ergosterol, the principal sterol in the membrane of susceptible fungal cells, causing increased cell membrane permeability, loss of cell components, metabolic disruption and eventually cell death. In addition to its membrane effects, the drug can cause damage to fungal cells possibly by formation of reactive free oxygen radicals after auto-oxidation of AMB bound to membrane components [13,16].

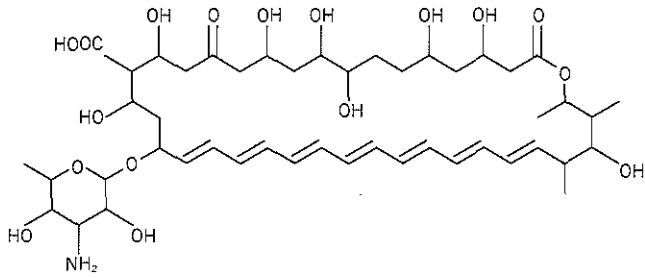


Figure 1. Chemical structure of AMB

AMB has a broad spectrum of activity including *Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans.* AMB is usually administered as the desoxycholate salt (AMB-DOC, Fungizone®). The antifungal activity of AMB is fungicidal and dose-dependent. AMB is poorly absorbed in the gastro-intestinal tract (<5%) [35], and has to be administered intravenously in the treatment of systemic fungal infections. Toxicity associated with intravenous AMB use can be severe and include acute effects during administration (fever, chills), and more delayed effects like thrombophlebitis, nephrotoxicity, hypokalaemia, hypomagnesaemia, anaemia and thrombocytopenia. This toxicity often limits adequate dosing, which reduces the potential clinical efficacy [34,35,45,50,65,66].

### Itraconazole

Itraconazole (Trisporal®, Sporanox®) is the only registered azole agent with activity against *Aspergillus* spp. Itraconazole has fungistatic activity against *Aspergillus* spp., in contrast to the polyenes, which have fungicidal activity. Itraconazole inhibits fungal cytochrome P450 14α-demethylase, which results in a depletion of ergosterol and a loss of membrane integrity and activity [48]. Itraconazole is systemically absorbed after oral administration and has a good tissue distribution in organs that are frequent sites for invasive fungal infections (such as the spleen and lungs), and can therefore be used for prevention and treatment of a variety of invasive fungal infections [15,48]. The recent development of an itraconazole oral solution and an intravenous itraconazole formulation has increased the oral bioavailability and improved the possibilities for the use of this drug [8]. Itraconazole has few side-effects and is generally well-tolerated.

## Lipid based formulations of AMB

Lipid formulations of AMB have been developed to overcome the nephrotoxicity of AMB-DOC [5,42,49]. The reduction of toxicity of lipid formulations of AMB probably results 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 fungal membrane (highest affinity) [56,57]. Three lipid formulations are currently available for clinical use: liposomal AMB (L-AMB), AMB lipid-complex (ABLC), and AMB colloidal dispersion (ABCD) (Table 2).

L-AMB (AmBisome®) consists of small unilamellar liposomes of about 80 nm in diameter, made up of a bilayer membrane of hydrogenated soy phosphatidyl-

Table 2. Formulations of amphotericin B

| Formulation | Brand name | Manufacturer | Carrier | Colloidal type | Size (μm) |
|---|---|---|---|---|---|
| AMB-DOC | Fungizone® | Bristol Myers-Squibb | Desoxycholate | Micelle | 0.035 |
| L-AMB | AmBisome® | Gilead Sciences | HSPC/DSPG/Chol | Liposome | 0.08 |
| ABLC | Abelcet® | The Liposome Company | DMPC/DMPG | Lipid ribbon | 1.6-11 |
| ABCD | Amphocil®, Amphotec® | Alza Corporation | Cholesteryl sulphate | Lipid disk | 0.11-0.12 |

AMB, amphotericin B; HSPC, Hydrogenated Soy Phosphatidylcholine; DSPG, distearoylphosphatidylglycerol; Chol, cholesterol; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol.

choline and distearoylphosphatidylglycerol stabilised with cholesterol and combined with AMB in a 2:0.8:1:0.4 molar ratio.

ABLC (Abelcet®) contains AMB complexed with two phospholipids L-α-dimyristoylphosphatidylcholine (DMPC) and L-α-dimyristoylphosphatidylglycerol (DMPG) in a 7:3 molar ratio with a drug:lipid ratio of 1:1. ABLC is an assembly of ribbon-like structures of a bilayered membrane, measuring 1,600-11,000 nm diameter.

ABCD (Amphocil®, Amphotec®) is a colloidal dispersion composed of disk-like structures of cholesteryl sulphate complexed with AMB in a 1:1 molar ratio. The disk-like structures are approximately 115 nm in diameter.

AMB in lipid complexes or liposomes has antifungal activity comparable with that of AMB-DOC, with respect to MIC/MFC values, but differs in its pharmacological and toxicological properties [5,12]. The pharmacokinetics of the lipid formulations after intravenous administration depend to a large extent on the composition and particle size of the liposomes or lipid carriers. Intravenously administered liposomes and lipid carriers tend to accumulate in the cells of the mononuclear phagocyte system. Animal data show that high concentrations of AMB are obtained in the liver and the spleen after intravenous administration of lipid formulations of AMB as compared to AMB-DOC. Levels in renal tissue after intravenous administration are much lower for the lipid formulations than for AMB-DOC. The serum concentrations are also highly variable between the formulations. The area under the serum concentration time curve (AUC) after a standardised dose is highest for L-AMB and lowest for ABLC [52]. The pharmacokinetic differences between the formulations seem large, however, the clinical relevance of these differences is not yet clear.

Although the nephrotoxicity associated with each of these lipid preparations is lower

than with AMB-DOC, the rates of acute infusion related reactions (which differ among the three lipid formulations) do not differ substantially from those observed with AMB-DOC [5,49,53,92,111] except that they are more frequent with ABCD [109].

## Current treatment strategies for the management of Invasive Pulmonary Aspergillosis

Invasive pulmonary aspergillosis is life-threatening. The poor prognosis in combination with the lack of proper means to make a timely diagnosis necessitates the administration of prophylaxis or empirical treatment in high risk patients without proof of *Aspergillus* infection (usually when the patient has persistent fever of unknown origin that is unresponsive to broad spectrum antibiotics). Both AMB and itraconazole can be employed in the management of *Aspergillus* infection. Table 3 gives a summary of treatment options.

Patients who are at particular risk for infection are eligible for prophylactic strategies. AMB is poorly absorbed after oral administration and can therefore not be employed as an oral prophylactic agent. Intravenous prophylactic use of low dose AMB-DOC (<1mg/kg) is hampered by the severe side-effects and is associated with break-through infections [64,88]. It is not yet clear whether prophylaxis with oral itraconazole can prevent invasive pulmonary aspergillosis, since published studies report conflicting results [28,39,72,74,82,104].

Empirical treatment is usually initiated in immunocompromised patients with persistent fever of unknown origin, that is unresponsive to broad spectrum antibiotics or when pulmonary infiltrates or cavities compatible with *Aspergillus* infection are detected by CT scan. The treatment of choice for empirical treatment remains the parenteral administration of AMB-DOC [7]. However, dose-related toxicity limits the flexible use of this agent. Lipid formulations of AMB show a considerable reduction in especially nephrotoxicity, which allows the use of much higher doses; higher doses lead to an increase in therapeutic index with the potential of higher success rates. In the empirical treatment of systemic fungal infections, the lipid-formulations of AMB and the itraconazole intravenous formulation are at least as effective as AMB-DOC and are less toxic [44,69,71]. There is substantial evidence that L-AMB is at least as effective as AMB-DOC, is associated with fewer break-through systemic fungal infections [44]. There is little information from randomised trials with regard to the clinical use of ABLC and ABCD, but reports from early phase trials and anecdotal studies suggest a lower toxicity as compared with AMB-DOC [106,108].

Table 3. Management options for invasive pulmonary aspergillosis

| Approach | Options | Route | Dosage | References |
|---|---|---|---|---|
| Prophylaxis | HEPA filtration of room | | | Beyer |
| | Itraconazole oral solution | p.o. | 5 mg/kg/day | Prentice, Vreugdenhil, Glasmacher, Delfaverro, Menichetti, Morgenstern |
| | L-AMB | i.v. | 1-2 mg/kg/day | Tollemar, Kelsey |
| Empirical Treatment | AMB-DOC | i.v. | 0.6-1 mg/kg/day for 14 days or until resolution of symptoms | Gallis, Pizzo, EORTC |
| | L-AMB | i.v. | 3-5 mg/kg/day for 14 days or until resolution of symptoms | Walsh |
| | ABLC | i.v. | 5 mg/kg/day for 14 days or until resolution of symptoms | Wingard, Walsh |
| | ABCD | i.v. | 4 mg/kg/day for 14 days or until resolution of symptoms | White |
| | Itraconazole | i.v. | 400 mg/day for 2 days followed by 200 mg/day for 14 days or until resolution of symptoms | Boogaerts |
| Treatment of Confirmed Aspergillosis | Itraconazole oral capsules | p.o. | 400 mg/day for 14 days or more | van't Wout, Stevens, Lebeau |
| | AMB-DOC | i.v. | 0.6-1 mg/kg/day for 14 days or until recovery of granulocytes and resolution of symptoms | Gallis, Lyman, Terrell |
| | L-AMB | i.v. | 3-5 mg/kg/day for 14 days or until recovery of granulocytes and resolution of symptoms | Mills, Ringden, Ellis, Leenders |
| | ABLC | i.v. | 5 mg/kg/day for 14 days or until recovery of granulocytes and resolution of symptoms | Walsh, Anaissie, Clark |
| | ABCD | i.v. | 4 mg/kg/day for 14 days or until recovery of granulocytes and resolution of symptoms | White |
| | Itraconazole | i.v. | 400 mg/day for 2 days followed by 200 mg/day po for 14 days or until recovery of granulocytes and resolution of symptoms | Caillot |

Adapted from [71].

# New approaches in the prevention and treatment of Invasive Pulmonary Aspergillosis

Although potent antifungal agents are available, morbidity and mortality from invasive pulmonary aspergillosis remain unacceptably high. Due to the lack of specific symptoms in the first stages of infection, the diagnosis of invasive pulmonary aspergillosis is often delayed and adequate treatment is started too late in the course of the infection. Therefore, we are nowadays still faced with the severe life-threatening features of invasive pulmonary aspergillosis, which stresses the critical need for optimising management of this infection. New strategies, apart from optimising diagnosis, focus on better pharmacotherapeutic management in developing new antifungal agents or new antifungal strategies for therapy or prophylaxis of invasive pulmonary aspergillosis.

## New antifungal agents

New developments with regard to AMB for aspergillosis are the development of new lipid-carriers of AMB, such as lipid-nanospheres [51,68,79], PEG-ylated liposomes [100] lipid-cochleates [40,91,115], and AMB methoxypoly(ethylene glycol) conjugate (mPEG-AMB) [90]. The polyene nystatin is also developed as a liposomal formulation and is currently under investigation for systemic use [77]. Three new azole derivatives, voriconazole, ravuconazole and posaconazole, are in the process of clinical development [36,76]. These agents appear to be more potent than itraconazole against filamentous fungi, including *Aspergillus*. The new azoles are administered orally; voriconazole will also be available as an intravenous formulation. Clinical studies with voriconazole show a favourable efficacy and safety profile of this agent. [26] Clinical trials of ravuconazole and posaconazole are in progress. New classes of antifungal drugs, the echinocandins and pneumocandins, have been developed. These candins inhibit fungal cell wall synthesis and therefore have been referred to as the 'penicillin for fungi'. Their target in cell wall synthesis, (1,3)-ß-D-glucan synthase, is not present in mammalian cells [78]. By contrast, polyenes and azoles act on a cell membrane common to fungal and mammalian cells, accounting for the toxicity of these agents. Echinocandins are active against *Aspergillus* and *Candida* species; pneumocandins have a similar spectrum but are in addition active against *Pneumocystis*. Neither, however, is active against *Cryptococcus*. The pneumocandins are currently evaluated in phase I, II and III trials for the treatment of oesophageal candidiasis, invasive candidiasis and aspergillosis. The pneumocandin caspofungin has recently entered the market and has the indication treatment of invasive aspergillosis in patients who are refractory

to AMB formulations and/or itraconazole. Chapter 2 of this thesis gives an overview of new agents which are in development for invasive aspergillosis.

An alternative administration mode: Inhalation of AMB

In the last decades, there has been an increasing amount of attention for drug administration via the pulmonary route for either systemic delivery of drugs or for drugs with activity at the pulmonary epithelium. This route of administration is used in the therapy of a number of respiratory disorders, such as asthma, and respiratory infections in patients with cystic fibrosis. The administration of anti-fungal agents via the inhalational route is a new, relatively unexplored strategy for the treatment of invasive pulmonary aspergillosis. The use of inhalation allows easy access to the respiratory tract, directly to the intended sites of action. In general, advantages of inhalation of drugs include: (1) small amounts of drug suffice to prevent or treat symptoms; (2) adverse reactions are usually much less than those after systemic administration; (3) there is a rapid and more predictable onset of action [1,93].

With invasive aspergillosis, the lungs are usually the organs which are affected first. Prior to developing invasive pulmonary aspergillosis, *Aspergillus* spores are inhaled. When local defences fail, like in immunocompromised patients, these spores eventually grow out to be hyphae which penetrate pulmonary tissue with all devastating consequences. By administering antifungal drugs via the pulmonary route, it would be expected that the drug is adequately targeted to the intended site of action. Furthermore, it would be expected that the drug would have minimal systemic toxicity, since there is very little systemic absorption from the pulmonary epithelial cells [70].

In an *in vitro* study on AMB aerosols, the dependency of the aerosol droplet size and aerosol output on the type of nebuliser was demonstrated [86]. A long pulmonary half-life of nebulised AMB experimental liposomes with different surface charges was shown in mice [61]. Administration of nebulised L-AMB was effective in the treatment of pulmonary and systemic *Cryptococcus neoformans* infection in mice [37]. Intensive treatment with nebulised L-AMB was effective in reducing the number of *Candida* organisms in kidneys of mice and prolonging survival time [38]. Prophylaxis or treatment of invasive pulmonary aspergillosis with nebulised (liposomal) AMB was evaluated in a model of aspergillosis in mice and rats with corticosteroid induced immunosuppression [3,87]. In these studies, the nebulised formulations were effective in decreasing the number of *Aspergillus* viable counts in the lungs and in increasing survival of animals.

Inhalational AMB-DOC has also been studied in humans. The toxicity of inhaled AMB-DOC was evaluated in healthy volunteers and in granulocytopenic patients [9,30,43]. The results show that cough, bronchospasms, dyspnea, nausea and an unpleasant after taste are common. Although these side-effects were the cause of discontinuation of treatment in 22% of patients in one study, it was concluded that the safety profile was acceptable.
The prophylactic efficacy of nebulised AMB-DOC was investigated in a number of studies. Among these are several studies in which the value of inhalational AMB was evaluated retrospectively [22,54,84,98]. Although these studies all describe the decrease in the incidence of invasive pulmonary aspergillosis with nebulised AMB, the retrospective design as well as several other factors makes it difficult to draw firm conclusions from these studies.
A prospective randomised trial was performed by Schwartz et al [89]. In this randomised, non-blinded study, inhalational AMB-DOC was compared with placebo in 382 neutropenic patients. The incidence in invasive aspergillosis decreased from 7 to 4%. This difference was not significant, but the overall incidence of invasive pulmonary aspergillosis was lower than expected.
Nebulising lipid formulations might have the advantage of prolonged retention in the lungs [75] and a favourable safety profile due to the pulmonary surfactant like composition of lipid carriers. Up to date, there is only one described case of nebulised liposomal AMB. In this report, nebulised L-AMB was well tolerated [8].

## Aim of the present study

Because current knowledge of inhalational AMB is limited, a rigorous scientific approach is warranted to establish optimal dose, frequency and duration of administration. We therefore decided to study in detail the full potential of nebulised AMB in different formulations, with the aim of answering the following questions:

- What is the pharmacokinetic behaviour of nebulised AMB, which dose would be sufficient and which frequency of dosing?
- Does inhalation of AMB exert any adverse effects?
- What is the value of nebulised lipid formulations of AMB as compared to nebulised AMB-DOC?
- Can the efficacy be demonstrated in an animal model of invasive pulmonary aspergillosis?

The primary aim of the work presented in this thesis was to evaluate the merits of inhalational AMB formulations in the prophylaxis and treatment of invasive pulmonary aspergillosis.

The thesis starts with an overview of Investigational antifungals. Agents that are currently in development for Aspergillus infections are discussed in a review (Chapter 2).

The experimental work addresses the following:

- Characteristics of nebulised AMB
  The aerosol procedure and detailed characteristics of nebulised AMB-DOC and L-AMB, such as aerosol droplet size and aerosol biodistribution are described in Chapter 3.
- Biodistribution of nebulised AMB
  In Chapter 4, the biodistribution of both nebulised AMB-DOC and L-AMB were determined. Parameters such as pulmonary deposition, systemic absorption, retention of AMB were determined in lungs of rats. For this purpose, a model of invasive pulmonary aspergillosis in persistently leukopenic rats was used.
- Toxicity of nebulised AMB
  The toxicity of nebulised AMB formulations to the pulmonary surfactant system, a measure of pulmonary toxicity, was determined *in vitro* (Chapter 5).
- Treatment of experimental invasive pulmonary aspergillosis with nebulised AMB
  In the same rat model that was used for the biodistribution study, the therapeutic efficacy of a single dose of nebulised AMB-DOC and L-AMB was evaluated (Chapter 5). In addition, the therapeutic efficacy of nebulised L-AMB when combined with intravenous therapy, was determined (Chapter 6).
- Prophylaxis of invasive pulmonary aspergillosis with nebulised AMB
  The rat model was used for evaluating the prophylactic efficacy of a single dose of nebulised AMB-DOC and L-AMB (Chapter 7) and ABLC and ABCD (Chapter 8).

# References

1. Adjei, AL, Qiu, Y and Gupta, PK. Bioavailability and pharmacokinetics of inhaled drugs. In *Inhalational aerosols: physical and biological basis of therapy* (Hickey, A.J. Ed.). Marcel Dekker, New York, 1996.
2. Aisner, J, Wiernik, PH, Schimpff, SC. Treatment of invasive aspergillosis: relation of early diagnosis and treatment to response. Ann Intern Med 1977;86:539-43.
3. Allen, SD, Sorensen, KN, Nejdl, MJ, Durrant, C, Proffit, R. Prophylactic efficacy of aerosolised liposomal (AmBisome) and non-liposomal (Fungizone) amphotericin B in murine pulmonary aspergillosis. J Antimicrob Chemother 1994;34:1001-13.
4. Anaissie, EJ. Opportunistic mycoses in the immunocompromised host: experience at a cancer center and review. Clin Infect Dis 1992;14(Suppl. 1):43-53.
5. Andriole, VT. Current and future therapy of invasive fungal infections. In *Current Clinical Topics in Infectious Diseases,* Vol 18 (Remington, J.& Swartz, M., Eds). Blackwell Sciences, Malden, MA 1998:19-36.
6. Andriole, VT. Current and future antifungal therapy: new targets for antifungal agents. The 1998 Garrod Lecture. J Antimicrob Chemother 1999;44:151-62.
7. *Aspergillus*/Aspergillosis website, 2001. http://www.aspergillus.man.ac.uk.
8. De Beule, K, Van Gestel, J. Pharmacology of itraconazole. Drugs 2001;61(Suppl. 1):27-37.
9. Beyer, J, Schwartz, S, Heinemann, V, Siegert, W. Strategies in prevention of invasive pulmonary aspergillosis in immunosuppressed or neutropenic patients. Antimicrob Agents Chemother 1994;37:911-17.
10. Bodey, GP, Vartivarian, S. Aspergillosis. Eur J Clin Microbiol Infect Dis 1989;8:413-37.
11. Bodey, GP, Bueltmann, B, Duguid, W, et al. Fungal infections in cancer patients: an international autopsy survey. Eur J Clin Microbiol Infect Dis 1992;11:99-109.
12. Bodey, GP. Antifungal agents. In Candidosis: Pathogenesis, Diagnosis and Treatment (Bodey, G.P. Ed.), Raven Press, New York, 1993, 371-406.
13. Bolard, J. How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochim Biophys Acta 1986; 864:257-304.
14. Boogaerts, M, Garber, G, Winston, D, et al. Itraconazole compared with amphotericin B as empirical therapy for persistent fever of unknown origin in neutropenic patients. Bone Marrow Transplant 1999; 23 (Suppl. 1): S111.
15. Boogaerts, M, Maertens, J. Clinical experience with itraconazole in systemic infections. Drugs 2001; 61 (Suppl. 1):39-47.
16. Brajtburg, J, Powderly, WG, Kobayashi, GS, Medoff, G. Amphotericin B: Current understanding of its mechanism of action. Antimicrob Agents Chemother 1990;34:2415-6.
17. Bretagne, S, Costa, J-M, Bart-Delabesse, E, Dhédin, N, Rieux, C and Cordonnier, C. Comparison of serum galactomannan antigen detection and competitive polymerase chain reaction for diagnosing invasive aspergillosis. Clin Infect Dis 1998;26:1407-12.
18. Caillot, D, Casanovas, O, Bernard, A, et al. Improved management of invasive pulmonary aspergillosis in neutropenic patients

using early thoracic computed tomographic scan and surgery. J Clin Oncol 1997;15:139-47.

19. Caillot, D, Bassaris, H, Seifert, WF, et al. Efficacy, safety and pharmacokinetics of intravenous followed by oral itraconazole in patients with invasive pulmonary aspergillosis. 39th ICAAC ,1999.
20. Chandrasekar, PH, Weimann, A, Shearer, C. Autopsy-identified infections among bone-marrow transplant recipients: a clinical-pathologic study of 56 patients. Bone Marrow Transplant 1995;16:675-81.
21. Clark, AD, McKendrick, S, Tansey, PJ, et al. A comparative analysis of lipid-complexed and liposomal amphotericin B preparations in hematological oncology. Br J Haematol 1998;103:198-204.
22. De Laurenzi, A, Matteocci, A, Lanti, L, et al. Amphotericin B prophylaxis against invasive fungal infections in neutropenic patients: A single center experience from 1980 to 1995. Infection 1996;24:361-66.
23. Denning, DW. Epidemiology and pathogenesis of systemic fungal infections in the immunocompromised host. J Antimicrob Chemother 1991;28 (Suppl. B):1-6.
24. Denning, DW. Invasive aspergillosis in immunocompromised patients. Curr Opin Infect Dis 1994;7:456-62.
25. Denning, DW, Marinus A, Cohen, J et al. Therapeutic outcome in invasive aspergillosis. Clin Infect Dis 1996;23:608-15.
26. Denning, DW, Del Favero, A, Gluckman, E, et al. The efficacy and tolerability of 109496 (voriconazole) in the treatment of invasive aspergillosis. 13th Congress of the International Society for Human and Animal Mycology, 1997.
27. Denning, DW. Invasive aspergillosis (review). Clin Inf ect Dis 1998;26:781-805.
28. DelFavero, A, Menichetti, F, Micozzi, A, et al. Antifungal prophylaxis in neutropenic patients with hematologic malignancy: a double blind trial to compare itraconazole oral solution with placebo. 37th ICAAC, 1997.
29. Diamond, RD. The growing problem of mycoses in patients infected with the immunodeficiency virus. Rev Inf Dis 1991;13:480-6.
30. Dubois, J, Bartter, T, Gryn J, et al. The physiologic effects of inhaled amphotericin B. Chest 1995;108:750-753.
31. Einsele, H, Hebart, H, Roller, G, et al. Detection and identification of fungal pathogens in blood by using molecular probes. J Clin Microbiol 1997;35:1353-1360.
32. Ellis, M, Spence, D, De Pauw, B, et al. An EORTC international multicentre randomised trial comparing two dosages of liposomal amphotericin B for treatment of invasive aspergillosis. Clin Infect Dis 1998;27:1406-12.
33. EORTC International Antimicrobial Therapy Cooperative Group. Empiric antifungal therapy in febrile granulocytopenic patients. Am J Med 1989;86:668-72.
34. Fanos, V, Cataldi, L. Amphotericin B-induced nephrotoxicity: a review. J Chemother 2000;12:463-70.
35. Gallis, HA, Drew, RH, Pickard, WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-29.
36. George, D, Miniter, P, Andriole, V. Efficacy of UK-109496, a new azole antifungal agent, in an experimental model of invasive aspergillosis. Antimicrob Agents Chemother 1996;40:86-91.
37. 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.

38. Gilbert, BE, Wyde, PR, Lopez-Berestein, G, Wilson, SZ. Aerosolized amphotericin B-liposomes for treatment of systemic Candida infections in mice. Antimicrob Agents Chemother 1994;38:356-59.
39. Glasmacher, A, Molitor, E, Hahn, C, et al. Antifungal prophylaxis with itraconazole in neutropenic patients with acute leukemia. Leukemia 1998;12:1338-43.
40. Graybill, JR, Najvar, LK, Bocanegra, R, Scolpino, A, Mannino, RJ, Zarif, L. Cochleate: a new lipid vehicle for amphotericin B. 39th ICAAC, 1999.
41. Groll, AH, Shah, PM, Mentzel, C, et al. Trends in post-mortem epidemiology of invasive fungal infections at a university hospital. J Infect 1996;33:23-32.
42. Groll, AH, Piscitelli, SC, Walsh, TJ. Clinical pharmacology of systemic antifungal agents: a comprehensive review of agents in clinical use, current investigational compounds, and putative targets for antifungal drug development. Adv Pharmacol 1998;44:343-500.
43. Gryn, J, Goldberg, J, Johnson, E, et al. The toxicity of daily inhaled amphotericin B. Am J Clin Oncol 1993;16:43-46.
44. Hann, IA, Prentice, HG. Lipid-based amphotericin B: a review of the last 10 years of use. Int J Antimicrob Agents 2001;17:161-9.
45. Harrell, CC. Hanf-Kristufek, L. Comparison of nephrotoxicity of amphotericin B products. Clin Infect Dis 2001;32:990-1.
46. Herbart, H, Löffler, J, Meisner, C, et al. Early detection of *Aspergillus* infection after allogeneic stem cell transplantation by polymerase chain reaction screening. J Infect Dis 2000;181:1713-19.
47. Hertenstein, B, Kern, WV, Schmeiser, T, et al. Low incidence of invasive fungal infections after bone marrow transplantation in patients receiving amphotericin B inhalations during neutropenia. Ann. Hematol. 1994;68:21-26.
48. Heykants, J, Van Peer, A, Van de Velde, V, et al. The clinical pharmacokinetics of itraconazole: an overview. Mycoses 1989; 32 (Suppl. 1):67-87.
49. Hiemenz , JW, Walsh, TJ. Lipid formulations of amphotericin B: recent progress and future directions. Clin Infect Dis 1996;22 (Suppl.2):S133-44.
50. Hoeprich, PD. Clinical use of amphotericin B and derivatives: Lore, mystique and fact. Clin Infect Dis 1992;42 (Suppl. 1):S114-9.
51. Hossain, MA, Maesaki, S, Kakeya, H, et al. Efficacy of NS-718, a novel lipid nanosphere-encapsulated amphotericin B, against *Cryptococcus neoformans*. Antimicrob Agents Chemother 1998;42:1722-5.
52. 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.
53. Janknegt, R, Van Etten, EWM, De Marie, S. Lipid formulations of amphotericin B. Curr Opin Infect Dis 1996;9:403-6.
54. Jantunen, E, Ruutu, P, Niskanen, L, et al. Incidence and risk-factors for invasive fungal infections in BMT recipients. Bone Marrow Transplant 1997;19:801-8.
55. Jeffery, GM, Beard MEJ, Ikram, RB, et al.

Intranasal amphotericin B reduces the frequency of invasive aspergillosis in neutropenic patients. Am J Med 1991;90:685-92.

56. Juliano, RL, Lopez-Berestein, G, Hopfer, R, Mehta, R, Mehta, K, Mills, K. Selective toxicity and enhanced therapeutic index of liposome polyene antibiotics in systemic fungal infections. Ann NY Acad Sci 1985;446:390-442.
57. 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.
58. Kelsey, SM, Goldman, JM, McCann, S, et al. Liposomal amphotericin B (AmBisome) in the prophylaxis of fungal infections in neutropenic patients: a randomised, double-blind, placebo-controlled study. Bone Marrow Transplant 1999;23:163-8.
59. Klont, RR, Meis, JFGM, Verweij, PE. Critical assessment of issues in the diagnosis of invasive aspergillosis. Clin Microbiol Infect 2001;7 (Suppl. 6):32-7.
60. Kusne, SJ, Dummer, JS, Singh, N et al. Infections after liver transplantation: an analysis of 101 consecutive cases. Medicine 1988;67:132-43.
61. Lambros, MP, Bourne, DWA, Abbas, SA, Johnson, DL. Disposition of aerosolised liposomal amphotericin B. J Pharm Sci 1997;86:1066-69.
62. Lebeau, B, Pelloux, H, Pinel, C, et al. Itraconazole in the treatment of aspergillosis: a study of 16 cases. Mycoses 1994;37:171-9.
63. Leenders, ACAP, Daenen, S, Jansen, RL. Liposomal amphotericin B compared with amphotericin B deoxycholate in the treatment of documented and suspected neutropenia-associated invasive fungal infections. Br J Haematol 1998;103:205-12.
64. Lortholary, O, Dupont, B. Antifungal prophylaxis during neutropenia and immunodeficiency. Clin Microbiol Rev 1997;10:477-504.
65. Luber, AD, Maa, L, Lam, M, Guglielmo, BJ. Risk factors for amphotericin B induced nephrotoxicity. J Antimicrob Chemother 1999;43:267-271.
66. Lyman, CA, Walsh, TJ. Systemically administered antifungal agents - A review of their clinical pharmacology and therapeutic applications. Drugs 1992;44:9-35.
67. Maertens J, Verhagen, J, Demuynck, H et al. Autopsy-controlled prospective evaluation of serial screening for circulating galactomannan by a sandwich enzyme-linked immunosorbent assay for hematological patients at risk for invasive aspergillosis. J Clin Microbiol 1999;37:3223-9.
68. Maesaki, S, Miyazaki, Y, Higashiyama, Y, Tomono, K, Kohno, S. The efficacy and attenuation of nephrotoxicity of NS-718, a novel lipid-nanosphere-encapsulated amphotericin B against *Cryptococcus neoformans* infection. 39th ICAAC, 1999.
69. De Marie, S. New developments in the diagnosis and management of fungal infections. Haematologica 2000;85:88-93.
70. Marra, F, Partovi, N, Wasan, KM, et al. Amphotericin B disposition after aerosol inhalation in lung transplant recipients. Ann Pharmacother 2002;36:46-51.
71. Meis, JFGM, Verweij, PE. Current management of fungal infections. Drugs 2001;61 (Suppl.1):13-25.
72. Menichetti, F, Favero, AD, Martino P, et al. Itraconazole oral solution as prophylaxis of fungal infections in neutropenic patients with

hematologic malignancies: a randomized, placebo-controlled, double blind, multicenter trial. Clin Infect Dis 1999;28:250-5.

73. Mills, W, Chopra, R, Linch, DC, Goldstone, AH. Liposomal amphotericin B in the treatment of fungal infections in neutropenic patients: a single centre experience of 133 episodes in 116 patients. Br J Hematol 1994;86:754-60.
74. Morgenstern, GR, Prentice, AG, Prentice, HG, et al. A randomized controlled trial of itraconazole versus fluconazole for the prevention of fungal infections in patients with hematologic malignancies. Br J Hematol 1999;105:901-11.
75. Niven, RW, Schreier, H. Nebulization of liposomes. I. Effects of lipid composition. Pharm Res 1990;7:1127-1133.
76. Oakley, K, Morrisey, G, Denning, D. Efficacy of SCH-56592 in a temporarily neutropenic murine model of invasive aspergillosis with an itraconazole susceptible and an itraconazole-resistant isolate of *Aspergillus fumigatus*. Antimicrob Agents Chemother 1997;41:1504-07.
77. Offner, FCJ, Herbrecht, R, Engelhard, D, et al. EORTC-IFCG Phase II study on liposomal nystatin in patients with invasive aspergillus infections, refractory or intolerant to conventional/lipid ampho B. 40th ICAAC, 2000.
78. Onishi, J, Meinz, M, Thompson, J, et al. Discovery of novel antifungal (1,3)-beta-D-glucan synthase. Antimicrob Agents Chemother 1999;43:589-91.
79. Otsubo, T, Maesaki, S, Hossain, MA, et al. In vitro and *in vivo* activities of NS-178, a new lipid nanosphere incorporating amphotericin B, against *Aspergillus fumigatus*. Antimicrob Agents Chemother 1999;43:471-5.
80. Pfaller, MA, Wenzel, R. The impact of changing epidemiology of fungal infections in the 1990s. Eur J Microbiol Infect Dis 1992;11:287-91.
81. Pizzo, PA, Robichaud, KJ, Gill, FA, et al. Empiric antibiotic and antifungal therapy for cancer patients with prolonged fever and granulocytopenia. Am J Med 1982;72:101-11.
82. Prentice, AG, Bradford, GR. Prophylaxis of fungal infections with itraconazole during remission-induction therapy. Mycoses 1989;32 (Suppl.1):96-102.
83. Purcell, IF, Corris, J. Use of nebulised liposomal amphotericin B in the treatment of *Aspergillus fumigatus* empyema. Thorax 1995;50:1321-23.
84. Reichenspurner, H, Gamberg, P, Nitschke, M, et al. Significant reduction in the number of fungal infections after lung-, heart-lung, and heart transplantation using aerosolised amphotericin B prophylaxis. Transplant Proc 1997;29:627-28.
85. Ringden, O, Meunier, F, Tollemar, J, et al. Efficacy of amphotericin B encapsulated in liposomes (AmBisome) in the treatment of invasive fungal infections in immunocompromised patients. J Antimicrob Chemother 1992;28(Suppl B):73-82.
86. Roth, C, Gebhart, J, Just-Nubling, G, von Eisenhart-Rothe, B, Beinhauer-Reeb, I. Characterization of amphotericin B aerosols inhalation treatment of pulmonary aspergillosis. Infection 1996;24:354-60.
87. Schmitt, HJ, Bernard, EM, Hauser, M, Armstrong, D. Aerosol amphotericin B is effective for prophylaxis and therapy in a rat model of invasive pulmonary aspergilosis. Antimicrob Agents Chemother 1988;32:1676-79.

88. Schuler, US, Haag, C. Prophylaxis of fungal infections. Mycoses 1997;40 (Suppl 2):41-4.
89. Schwartz, S, Behre, G, Heinemann, H, et al. Aerosolised amphotericin B inhalations as prophylaxis of invasive Aspergillus infections during prolonged neutropenia: results of a prospective randomised multicenter trial. Blood 1999;11:3654-61.
90. Sedlak, M, Buchta, V, Kubicova, L, Simuringnek, P, Holcapek, M, Kasperova, P. Synthesis and characterisation of a new amphotericin B-methoxypoly(ethylene glycol) conjugate. Bioorg Med Chem Lett 2001;22:2833-35.
91. Segarra, I, Jin, T, Hyra, DA, Mannino, RJ, Zarif, L. Oral administration of amphotericin B with a new AMB-cochleate formulation: tissue distribution after single and multiple oral dose. 39th ICAAC, 1999.
92. Sharkey, PK, Graybill, JR, Johnson, ES, et al. Amphotericin B lipid complex compared with amphotericin B in the treatment of cryptococcal meningitis in patients with AIDS. Clin Infect 1996;22:315-21.
93. Smith, SJ, Bernstein, JA. Therapeutic use of aerosols. In Inhalational aerosols: physical and biological basis of therapy (Hickey, A.J. Ed.). Marcel Dekker, New York, 1996.
94. Sternberg, S. The emerging fungal threat. Science 1994;266:1632-4.
95. Stevens, DA, Lee, JY. Analysis of compassionate use itraconazole therapy for invasive aspergillosis by the NIAID Mycoses Study Group Criteria. Arch Intern Med 1997;157:1857-62.
96. Terrell, CL , Hughes, CE. Antifungal agents used for deep seated mycotic infections. Mayo Clin Proc 1992;67:69-91.
97. Tollemar, J, Ringden, O, Andersson, S, Sundberg, B, Ljungman, P, Tydén, G. Randomised double-blind study of liposomal amohotericin B (AmBisome) prophylaxis of invasive fungal infections in bone marrow transplant recipients. Bone Marrow Transplant 1993;12:577-82.
98. Trigg, ME, Morgan, D, Burns, TL, et al. Successful program to prevent aspergillus infections in children undergoing marrow transplantation: Use of nasal amphotericin. Bone Marrow Transplant 1996;19:43-47.
99. Tumbarello, M, Tacconelli, E, Pagano, L, et al. Comparative analysis of prognostic indicators of aspergillosis in haematologic malignancies and HIV infection. J Infect 1997;34:55-60.
100. Van Etten, EWM, Stearne-Cullen, LET, Verbrugh, HA, Bakker-Woudenberg, IAJM. Efficacy of liposomal amphotericin B with prolonged circulation in blood in the treatment of severe pulmonary aspergillosis in leukopenic rats. Antimicrob Agents Chemother 2000;44:540-45.
101. Vasquez, JA, Sanchez, V, Dmuchowski, C, Demby, LM, Sobel, JD, Zervos, MJ. Nosocomial acquisition of *Candida albicans*: an epidemiologic study. J Infect Dis 1993;168:195-201.
102. Verweij, PE, Donnelly, JP, Kullberg, BJ, Meis, JFGM, De Pauw, BE. Amphotericin B versus amphotericin B plus 5-flucytosine: poor results in the treatment of proven systemic mycoses in neutropenic patients. Infection 1994;22:81-5.
103. Von Eiff, M, Roos, N, Schulten, R, Hesse, M, Zuhlsdorf, M, Van de Loo, J. Pulmonary aspergillosis: early diagnosis improves sur-

vival. Respiration 1995;62:341-47.

104. Vreugdenhil, G, Van Dijke, BJ, Donnelly, JP, et al. Efficacy of itraconazole in the prevention of fungal infections among neutropenic patients with hematologic malignancies and intensive chemotherapy. A double-blind, placebo controlled study. Leuk Lymphoma 1993;11:353-8.

105. Walsh, TJ. Invasive fungal infections: problems and challenges in developing new antifungal compounds. In Emerging Targets in Antibacterial and Antifungal Chemotherapy (Sutcliffe, J. & Georgopapadakou, N.H., Eds). Chapman and Hall, New York 1992:349-73.

106. Walsh, TJ, Hiemenz, JW, Seibel, NL, et al. Amphotericin B lipid complex (ABLC) for invasive fungal infections: Analysis of safety and efficacy in 556 cases. Clin Infect Dis 1998;26:1383-96.

107. Walsh, TJ, Finburg, RW, Arndl, S, et al. Infectious Disease Mycosis Study Group, Liposomal amphotericin B for empirical therapy in patients with persistent fever and neutropenia. New Engl J Med 1999;340:764-71.

108. White, MU, Kusne, S, Wingard, JR, et al. Amphotericin B colloidal dispersion versus amphotericin B as therapy for invasive aspergillosis. Clin Infect Dis 1997;24:635-42.

109. White, MU, Bowden, RA, Sandler, ES, et al. Randomised, double-blind clinical trial of amphotericin B colloidal dispersion (ABCD) versus amphotericin B in the empirical treatment of fever and neutropenia. Clin Infect Dis 1998;27:296-302.

110. Wilkes, MS, Fortin, AH, Felix, JC, Godwin, TA, Thompson, WG. Value of necropsy in acquired immunodeficiency syndrome. Lancet 1988;2:85-8.

111. Wingard, JE. Conventional versus lipid formulations of amphotericin B. In Focus on fungal infections VII (Anaissie, E.J. and Rinaldi, M.G., Chairmen), p. 33. San Antonio, Texas, 1997. Imedex Inc., Alpharetta, GA.

112. Wingard, JR, White, MH, Anaissie, EJ, Rafalli, J, Goodman, J, Arrieta, A, and the L AMPH/ABLC Collaborative Study Group. A randomized, double-blind comparative trial evaluating the safety of liposomal amphotericin B versus amphotericin B lipid complex in the empirical treatment of febrile neutropenia. Clin Infect Dis 2000;31:1155-63.

113. Van't Wout, JW, Novakova, I, Verhagen, CA, et al. The efficacy of itraconazole against systemic fungal infections in neutropenic patients: a randomised comparative study with amphotericin B. J Infect 1991;22:45-52.

114. Young, RC, Bennett, JE, Vogel, CL, Carbone, PP, DeVita, VT. Aspergillosis. The spectrum of the disease in 98 patients. Medicine 1970; 49:147-73.

115. Zarif, L, Jin, T, Scolpino, A, Mannino, RJ. Are cochleates the new lipid-based carrier for oral drug delivery. 39th ICAAC, 1999.

Chapter 2

# Pharmacological agents in development for invasive aspergillosis

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# Abstract

The urgent medical need for new potent antifungal agents in the management of invasive aspergillosis (IA) has resulted in the development of several compounds which may be of value in the future for the treatment or prophylaxis of IA.
In the past years, several novel types of drugs have been discovered and developed, some of which are already in late-stage clinical trials and ready to enter the market. This paper discusses the antifungal agents, classified by their mode of action, that are currently available and the agents which are still in development for treatment or prevention of IA.

# 1. Background

Invasive fungal infections have continued to increase in incidence during the last decade and are now a significant cause of morbidity and mortality [1]. Patients at particular risk for invasive fungal disease include: those who receive chemotherapy, transplant recipients, HIV patients and patients receiving systemic glucocorticoids. The pathogens responsible for fungal infections in these patients are usually *Candida* species or *Aspergillus*. *Aspergillus* is more commonly observed in bone marrow transplant recipients and/or patients with severe and prolonged neutropenia [2,3].Invasive aspergillosis (IA) is the most commonly encountered form of infection caused by *Aspergillus* species. The incidence of IA varies per centre and per underlying illness [4]. IA is associated with a mortality of 25-40% when treated with anti-fungals and nearly 100% if left untreated [5,6]. Diagnosis of IA is problematic and infection is often only confirmed at autopsy [7]. Several factors which complicate the utility of currently used antifungal agents include:

- dose-limiting toxicity
- growing resistance
- multiple drug interactions
- inadequate pharmacokinetic profile
- excessive costs

The poor outcome in patients despite treatment with antifungal agents, emphasises the need for new agents which are superior or supplementary to existing therapies. Ideally, new antifungal agents are potent drugs with a broad spectrum against a fungal target with no mammalian counterpart. The ideal antifungal agent should have favourable pharmacokinetics (oral and intravenous formulation, good tissue distribution), should have no interactions with other drugs and have an acceptable safety profile. There are several compounds in development which might be of value in the future for the treatment or prophylaxis of IA, several novel types of drugs have been discovered and developed, some of which are already in late-stage clinical trials and ready to enter the market. This paper discusses the antifungal agents, classified by their mode of action, that are currently available, and the agents which are still in development for treatment or prevention of invasive aspergillosis.

# 2. Targets of antifungal agents

## 2.1 Fungal cell membrane

The fungal plasma membrane contains sterols and phospholipids as its major lipid components, just like mammalian cell membranes. It functions as a permeability barrier, a conduit for the transportation of small molecules and signals, and as a matrix for proteins. The currently used agents for the treatment of IA interact with ergosterol in the fungal cell membrane. The polyenes amphotericin B (AMB) and nystatin complex with ergosterol in the plasma membrane, causing membrane disruption; this increases membrane permeability which leads to the leakage of the cytoplasmic contents of the cell and subsequently cell death [8]. Polyenes also cause oxidative damage, which may contribute to their fungicidal activity [9]. The triazoles (with itraconazole as the only azole with anti-*Aspergillus* activity) inhibit the activity of the fungal cytochrome P450. Inhibition of cytochrome P450 leads to blocking of lanosterol demethylation to ergosterol, leading to an alteration in the structure and function of the fungal cell membrane [10]. Novel specific antifungal targets in the fungal cell membrane could be [11]:

- phospholipid synthesis
- sphingolipid synthesis
- proton ATPases
- efflux pumps

## 2.2 Fungal cell wall

The fungal cell wall is a structure essential to fungi and lacking in mammalian cells, and is an obvious target for antifungal agents. The three major components are 1,3-ß-glucan, chitin and mannoprotein [12]. Glucan and chitin are responsible for the strength and the shape of the cell wall, while mannoproteins are interstitial components responsible for the cell wall's porosity, antigenicity and adhesion [13]. Glucan consists of glucose polymers, constituting ~ 60% of the cell wall. Glucan synthesis is catalysed by ß-glucan synthase. This enzyme is inhibited non-competitively by the cyclic lipopeptides (echinocandins) and the liposaccharides (papulacandins) [14-17]. Chitin consists of glucosamine homopolymers and is a minor but essential cell wall component. The synthesis of chitin is catalysed by chitin synthases and is competitively inhibited by nikkomycins [15]. Mannoproteins constitute ~ 40% of the fungal cell wall. The benanomycins and the pradimicins interact with the mannoproteins by complexion, resulting in leakage of potassium from the fungal cell membrane [18].

### 2.3 Fungal protein synthesis

Synthesis of proteins unique to fungi represent a novel target for antifungal compounds. The sordarins are a novel class of antifungals that act by inhibiting the protein synthesis elongation cycle [19].

### 2.4 Other targets

Other fungal targets for novel agents include DNA and topoisomerases, intermediate metabolism components (for example, nucleic acids, amino acids, polyamines) and cellular functions such as microtubule formation. Other approaches beyond the interaction of the drug with the microorganism, such as augmentation of host immune response by vaccination or administration of recombinant human cytokines, could have a great impact when combined with antifungal therapy [11].

## 3. Currently used antifungal agents for prophylaxis and treatment of invasive aspergillosis

### 3.1 Polyenes

The polyenes (nystatin and AMB) are fungicidal and have a broad spectrum of antifungal activity. They act by binding to ergosterol located in fungal membranes, this results in the leakage of intracellular constituents and the subsequent death of the cell. The affinity of polyenes to ergosterol in fungal cells is higher than in mammalian cells, therefore the toxicity of polyenes to mammalian cells is less than to fungal cells. Nystatin is not absorbed after oral administration and can not be given parenterally due to high toxicity, therefore it has no place in the treatment of IA. AMB has been the drug of choice for most invasive fungal infections since its introduction in the 1950s. AMB is a macrolide polyene antibiotic derived from *Streptomyces nodosus*. AMB is not absorbed after oral administration. The intravenous formulation consists of the desoxycholate salt of AMB (AMB-DOC, Fungizone®, Bristol-Myers Squibb Co.). Toxicity associated with AMB-DOC (administered intravenously) can be severe and includes acute effects during administration, including:

- fever
- chills
- thrombophlebitis
- nephrotoxicity
- hypokalaemia
- hypomagnesaemia
- anaemia
- thrombocytopenia

This toxicity often limits adequate dosing, which results in suboptimal tissue concentrations reducing potential clinical efficacy [20-23]. Lipid formulations were developed in order to overcome the toxicity of AMB-DOC, and three types of lipid formulations are currently available for clinical use:

- Liposomal AMB (AmBisome®, Gilead Sciences) consists of AMB incorporated into small unilamellar liposomes;
- AMB lipid-complex (ABLC, Abelcet®, The Liposome Company Inc.) contains AMB complexed with two phospholipids. ABLC is an assembly of ribbon-like structures of a bilayer membrane;
- AMB colloidal-dispersion (ABCD, Amphocil®,Amphotec®, Alza Corporation) is a colloidal dispersion composed of disklike structures of AMB complexed with cholesteryl sulfate. Although all three products are not completely free from toxicity, they are generally less toxic than AMB-DOC. However, the lipid formulations are considerably more expensive than AMB-DOC [24-26].

## 3.2 Azoles

The discovery of the triazoles in the late 1980s was a major advancement in the safe and effective treatment of invasive fungal infections. The azole derivatives are synthetic compounds, classified as imidazoles or triazoles depending on whether they have two or three nitrogens in the five-membered azole ring. Depending on the particular compound, azole antifungal agents have fungistatic, broad spectrum activity that includes most yeasts and filamentous fungi. They inhibit C-14 demethylation of lanosterol, thereby causing ergosterol depletion and accumulation of aberrant sterols in the membrane [11]. Of the currently used azoles, only the triazole itraconazole has activity against *Aspergillus* spp. Itraconazole (Trisporal®, Janssen-Cilag, Sporanox®, Gruenenthal GmbH) is systemically absorbed after oral administration and is widely distributed in organs that are frequent sites for IA (such as the spleen and lungs) [27]. The recent development of an itraconazole oral solution (with better bioavailability) and an itraconazole intravenous formulation has increased the options for the use of this drug [28].

# 4. Newly developed antifungal agents with activity against *Aspergillus*

## 4.1 Polyenes

### 4.1.1 Liposomal nystatin

Nystatin is a polyene antifungal agent commonly used in the topical treatment or prophylaxis of several types of superficial mycoses. Liposomal nystatin (Nyotran®, Aronex Pharmaceuticals Inc.), where nystatin is incorporated in a multilamellar liposome, has similar antifungal activity to that of free nystatin and a significantly improved toxicity profile *in vitro* and *in vivo*. *In vitro* experiments show that liposomal nystatin is effective against a large number of fungi, including *Aspergillus* [29]. Liposomal nystatin was well tolerated and improved survival of mice with systemic fungal infections [30,31]. In Phase I and II clinical trials in HIV patients, liposomal nystatin was safe, well tolerated and had an acceptable pharmacokinetic profile. A Phase III trial in 31 patients compared liposomal nystatin to AMB-DOC, with a median duration of therapy of 6 days. There was no significant difference in the incidence of infusion-related toxicity, with a trend towards a higher incidence of grade 3/4 renal dysfunction with AMB-DOC. There was no difference between the two drugs in terms of survival, the secondary end point of the trial. However, Nyotran® was not equivalent to AMB-DOC with respect to the primary efficacy end point as defined in the protocol. In a Phase III trial in 538 neutropenic patients who had previously failed antibacterial therapy, liposomal nystatin (2 mg/kg/day) was compared to AMB-DOC. Both drugs were equivalent in treating fungal infections. The primary endpoint in this study was a composite of survival to 3.5 days post-therapy, reduction of fever for more than 2 days while on therapy, afebrile status at 3.5 days post-therapy, no evidence of progressive or emergent fungal infection and no discontinuation due to drug-related toxicities. In addition, liposomal nystatin showed less renal toxicity than AMB-DOC [32]. In a small Phase II trial, the safety and efficacy of liposomal nystatin (4 mg/kg/day) was evaluated in 24 patients with definite or probable IA. These patients were either intolerant or nonresponsive to liposomal AMB. Liposomal nystatin was well-tolerated in these patients and a mycological response was seen in 5/19 patients who were refractory to liposomal AMB [33].

### 4.1.2 Cochleate AMB

Cochleates are lipid cylinders that can serve as vehicles for the delivery of peptides, DNA or drugs, particularly via the oral route [34]. Orally administered cochleate AMB is rapidly taken up systemically and yields therapeutic concentra-

tions of AMB in key organs [35]. *In vitro*, cochleate AMB had equivalent activity to AMB-DOC against several clinically important fungi, including *Aspergillus* [36]. Cochleate AMB administered in a 20 mg/kg/day dose showed equivalent efficacy to 4 mg/kg/day AMB-DOC in a mouse model of aspergillosis [37].

#### 4.1.3 NS-718

NS-718 is a lipid nanosphere formulation of AMB. Nanospheres are biodegradable polymeric particles in the nanometer size range. Nanospheres serve as drug carriers and can enhance the oral bioavailability of poorly absorbable drugs and tissue uptake after intravenous administration [38]. Intravenous NS-817 was well tolerated and also showed better efficacy than liposomal AMB in a rat model of IA [39]. In rats, NS-817 showed less nephrotoxicity than AMB-DOC [40].

#### 4.1.4 Long-circulating liposomes

A liposomal formulation of AMB was prepared with the incorporation of polyethylene glycol (PEG) in the liposomal membrane. This resulted in liposomes with prolonged circulation and increased accumulation at sites of infection [41]. In contrast to L-AMB, in which liposomal encapsulation effected reduced toxicity as well as reduced antifungal activity of AMB, with the PEGylated AMB liposomal formulation it is possible to reduce AMB toxicity without reducing AMB antifungal activity. The PEGylated AMB liposomal formulation had similar *in vitro* activity to AMB-DOC but had an 18-fold improved single-dose maximum tolerated dose in mice. A single dose of 10 mg/kg of PEGylated AMB intravenous was as effective as a 10-day treatment with 1 mg/kg AMB-DOC intravenously in a model of aspergillosis in persistent neutropenic mice [41]. To further enhance pulmonary drug targeting, PEGylated AMB liposomes were complexed with antibodies against a lung epithelial surface glycoprotein. These immunoliposomes showed enhanced survival and tissue clearance in a murine model of pulmonary aspergillosis [42]. Clinical usefulness of this strategy has not yet been evaluated for treating fungal infection in humans.

#### 4.1.5 Other developments in AMB delivery

By giving AMB via the pulmonary route, it is targeted directly at the site of infection and systemic toxicity is potentially reduced. The therapeutic value and safety of nebulised conventional and liposomal AMB has been shown in a rat model of aspergillosis in severely leukopenic rats [43]. Although this alternative route of administration has been studied in humans, the prophylactic or therapeutic merits still have to be determined [44-46]. Heating AMB is a way of altering the physical properties of the drug. Heated AMB had a maximum tolerated dose which was increased sixfold as compared to AMB-DOC. The heated AMB showed improved survival and lowered fungal tissue burden in a murine candidiasis model [47].

AMB methoxypoly(ethylene glycol) conjugate (mPEG-AMB) was shown to be as effective as AMB-DOC in preliminary *in vitro* screening [48]. AMB hydrosomes are heparin-surfaced nanoparticles designed to target infected sites. These nanoparticles tend to accumulate more in the lungs than AMB-DOC but *in vivo* studies still have to demonstrate potential value in the treatment of IA [49].

#### 4.1.6 SPA-S-843

SPA-S-843 is a derivative of the polyene antibiotic patricin A. The semisynthetic agent SPA-S-843 is water-soluble and chemically stable in an intravenous formulation. The *in vitro* inhibitory and fungicidal activity against *Aspergillus* spp. was equivalent to or even more potent than AMB [50]. Pharmacokinetic evaluation of SPA-S-843 in rats showed that SPA-S-843 has an extensive serum and tissue halflife, longer than AMB [51]. *In vivo* experiments in mice which were intravenously infected with *Aspergillus fumigatus* show a prolonged survival of animals treated with SPA-S-843 as compared to animals treated with AMB [52].

### 4.2 Azoles

Azole compounds have become increasingly important for the treatment of opportunistic fungal infections, as well as superficial and endemic mycoses. Although limited information is available on the new azole antifungals, it seems they all have an excellent *in vitro* profile against a wide range of fungi, including *Aspergillus* [53].

#### 4.2.1 Itraconazole intravenous formulation

The triazole itraconazole exhibits activity against both *Candida* and *Aspergillus* but the absence of an intravenous formulation has restricted its use for the primary treatment of invasive mycoses in neutropenic patients. However, the intravenous cyclodextrin itraconazole formulation may overcome this. Cyclodextrins, given as an intravenous vehicle, serve as solubilisers for the hydrophobic itraconazole but do not alter the pharmacokinetics [54]. Intravenous administration for 2 weeks followed by oral administration of itraconazole for 12 weeks, was shown to yield complete or partial responses in about half of immunocompromised patients with invasive pulmonary aspergillosis [55]. In an open, randomised, controlled, multicentre equivalence trial, 384 neutropenic patients with cancer who had persistent fever unresponsive to antibiotic therapy received either itraconazole (200 mg bid, i.v. for the first 48 h, then 200 mg/day i.v., followed by oral itraconazole solution from day 15) or AMB-DOC (0.7-1 mg/kg). The efficacy of itraconazole was equivalent to AMB-DOC but itraconazole was associated with less toxicity [27]. Since itraconazole is metabolised by the liver and cyclodextrin is excreted renally, the drug should be used with caution in patients with impaired renal function. The adverse effects of itraconazole are:

dose-related nausea, hepatic dysfunction, hypokalaemia, and oedema [56]. Itraconazole is metabolised by the cytochrome P450 system and therefore significant drug interactions are likely.

4.2.2 Voriconazole

Voriconazole is a triazole antifungal agent with an antifungal spectrum similar to itraconazole. Voriconazole can be administered both orally and intravenously. Voriconazole is registered for use in some countries or is currently being evaluated for registration. The Phase III clinical trials have largely been completed for efficacy against a wide range of fungal pathogens. The activity of voriconazole is targeted specifically to include invasive moulds such as *Aspergillus* spp. to which voriconazole has demonstrated fungicidal activity [57-60]. Voriconazole was more effective than itraconazole in different animal models of *Aspergillus* infection [58,61,62]. This new azole inhibits both 14-α-sterol demethylase and 24-methylenedihydrolanosterol demethylation, which might explain its increased activity against some moulds [63]. The pharmacokinetics of voriconazole demonstrate intravenous as well as oral bioavailability, with an excellent absorption of up to 90%. The drug is distributed widely in tissues and the pharmacokinetics of voriconazole are non-linear with extensive hepatic metabolism. The half-life of voriconazole has been reported to be approximately 6 h, which demands twice daily dosing to maintain steady-state plasma concentrations [64]. Cytochrome P450 isoenzymes identified in the metabolism of voriconazole include CYP2C9, CYP2C18 and CYP3A4 [65]. In initial clinical trials, voriconazole was well-tolerated. The most common adverse event in Phase I and II studies was transient and reversible visual disturbance (altered light perception) in 15% of the individuals receiving the drug [65-67]. Other adverse events included [67,68]:

- hepatic disturbances
- skin rash
- nausea
- vomiting
- anorexia

In an open-label Phase II clinical trial, 102 immunocompromised patients with acute IA received voriconazole (6 mg/kg b.i.d. i.v. then 3 mg/kg b.i.d. i.v.) for 6-27 days, followed by 200 mg b.i.d. p.o. for a total duration of 4-24 weeks. Complete resolution was observed in 17% of all patients, 36% showed partial response and 20% showed stable disease [66]. In acute IA with half of the patients refractory to standard therapy, orally administered voriconazole (200 mg b.i.d.) yielded a favourable response in 11/19 patients. In an open, randomised trial in 392 patients with IA, patients received either AMB-DOC (1 mg/kg) or

voriconazole (6 mg/kg b.i.d., then 4 mg/kg b.i.d. followed by 200 mg p.o., b.i.d.). The primary end point was response at week 12. At week 12, complete remission was 53% in patients receiving voriconazole versus 32% in patients receiving AMB [69]. Two case-reports show that due to the extensive penetration in the central nervous system (CNS) fluid, voriconazole has good potential for the treatment of CNS mould infections [68,70]. In a randomised, international, multicentre trial, voriconazole was compared to liposomal AMB for empirical antifungal therapy. 837 patients received either voriconazole (loading dose of 6 mg/kg i.v. every 12 h for two doses and then continued at a dose of 3 mg/kg i.v. or 200 p.o. every 12 h) or liposomal AMB 3 mg/kg. The overall success rates were 26% with voriconazole and 31% with liposomal AMB. Although voriconazole failed to meet the specified criteria for non-inferiority as compared to liposomal AMB, on the basis of the composite end point, it was concluded from this study that voriconazole could be a suitable alternative to AMB preparations for empirical antifungal therapy [71]. Voriconazole promises to be an important new agent in the treatment of IA, with moderate toxicity.

4.2.3 Posaconazole

Posaconazole is a second generation triazole and a structural analogue of itraconazole. The drug has a very low watersolubility and currently only comes in an oral form as a tablet or as a suspension. Overall, the *in vitro* profile of posaconazole is broad and it is fungicidal against a variety of filamentous fungi, including *Aspergillus* [72]. Posaconazole showed better activity (on a milligram to milligram basis) against different *Aspergillus* spp. *in vitro* than itraconazole and voriconazole [73-75]. The efficacy of posaconazole appears to be both dose- and time-dependent. In a model of IA in neutropenic rabbits, posaconazole was more effective than itraconazole and as effective as AMB-DOC [76]. In a temporarily neutropenic mouse model of disseminated aspergillosis, posaconazole was superior to AMB in terms of survival and quantitative organ cultures, and superior to itraconazole when used against infection caused by both itraconazole-sensitive and -resistant strains [77]. A recent study in persistently neutropenic rabbits showed that posaconazole at ≥ 6 mg/kg/day was as effective in the treatment and prevention of invasive pulmonary aspergillosis as AMB-DOC at 1 mg/kg and more effective than itraconazole at ≥ 6 mg/kg/day [78]. In a model of IA in immunocompetent and immunocompromised mice, posaconazole was effective in terms of survival and quantitative lung cultures [79]. Pharmacokinetic evaluations in humans show that posaconazole has a half-life of ~ 25 h and that adequate plasma concentrations are yielded with once daily dosing. Posaconazole has extensive tissue distribution [80]. When the drug is taken with food, the bioavailabity is increased as compared

to the drug taken when fasting and the bioavailability of the suspension is about 40% higher than that of the tablet [81]. A randomised, double-blind, placebo-controlled study in healthy volunteers to evaluate the safety and tolerance of escalating doses of posaconazole for 14 days showed that posaconazole was well tolerated. There was no difference in incidence or type of adverse events reported by either the subjects taking the drug or the placebo [80]. Posaconazole has currently entered Phase III clinical trials, which are largely complete.

#### 4.2.4 Ravuconazole

Ravuconazole (BMS-207147, ER-30346) is a synthetic triazole with structural similarities to voriconazole. Ravuconazole has activity against a number of moulds and yeasts [82,83]. It shows excellent activity against *Aspergillus* spp. [73,83, 84]. Ravuconazole was more effective than the echinocandin anidulafungin and as effective as AMB in a rabbit model of IA [85]. Ravuconazole was developed for oral administration but an intravenous formulation is in development. In a randomised, double-blind study on the safety of ravuconazole in humans, healthy male persons received escalating doses of ravuconazole (a maximum of 800 mg) or placebo. No serious adverse events were reported, with headache and abdominal pain reported most frequently. The bioavailability of ravuconazole was 50% and the half-life 3.5-7.5 days. Ravuconazole showed no induction of the cytochrome P450 isoenzyme CYP3A4. Ravuconazole has currently entered several Phase III trials [86].

#### 4.2.5 Other azoles

Other new azoles with *in vitro* anti-*Aspergillus* activity include R-102557 [87], SS-750, TAK-456 and its prodrug TAK-457 [86], T-8581, SYN-2836, SYN-2869, SYN-2903, SYN-2921 [88] and UR-9825 (low activity) [89]. The therapeutic efficacy of SS-750 was evaluated in murine models of systemic aspergillosis. SS-750 had excellent activity *in vivo*, with equivalence shown for the intravenous and oral administration. SS-750 was more effective than itraconazole [86].

### 4.3 Glucan synthase inhibitors

One of the three components of the fungal cell wall is ß-1,3-glucan [12,15,90,91]. ß -1,3-glucan synthesis is catalysed by the enzyme ß-1,3-glucan synthetase which is inhibited non-competetively by the cyclic lipopetides echinocandins and the papulacandins [17,92,93]. The papulacandins are no longer pursued since their *in vitro* activity is limited to *Candida* species and does not translate to *in vivo* activity [92].

### 4.3.1 Echinocandins

The echinocandins are a novel class of cyclic lipopeptide fungicidal agents which inhibit the synthesis of 1,3-ß-glucan, an essential polysaccharide found in the cell wall of many pathogenic fungi [11,94,95]. The inhibition of fungal cell wall synthesis is highly specific and brief exposure of fungal cells to echinocandins leads to cell death [94]. The echinocandins all have a consistent spectrum of antifungal activity, no cross-resistance to existing antifungal agents and lack of a mechanism-based toxicity [95]. They all possess favourable pharmacokinetics but can only be administered intravenously.

### 4.3.2 Caspofungin

Caspofungin (Cancidas®, MK-991, L-743,872, Merck & Co.) is a semisynthetic echinocandin derivative being developed as an antifungal and antipneumocystic agent. It has activity against a number of clinically important yeasts and fungi, including *Aspergillus* spp. It is fungicidal against *Candida* spp. [96] but fungistatic against *Aspergillus* [72,75,97-100] and inactive against *Fusarium, Rhizopus, Cryptococcus* and *Trichosporon* species [201]. Caspofungin has shown *in vitro* activity against refractory clinical isolates of *Aspergillus* [101]. The *in vivo* activity against *Aspergillus* infections was shown in models of neutropenic [102] and immunocompetent mice [103,104]. Phase I studies were performed in healthy male adult volunteers. The half-life of caspofungin was 9-10 h with an average plasma clearance of 11 ml/min. A 70 mg/day once-daily dosing regimen maintained mean trough plasma levels above the reported minimum inhibitory concentration (MIC) values for most susceptible fungi throughout the treatment period from day 1 forward [105,106]. A Phase II trial was conducted in 128 HIV-positive patients with *Candida oesophagitis*. Caspofungin was as effective as AMB and had less side-effects. No serious adverse events were reported for caspofungin [107]. In a multicentre, non-comparative study of IA, 56 immunocompromised patients were treated with 70 mg dose of caspofungin on the first day, followed by 50 mg once-daily. All patients had refractory IA or were intolerant to AMB, liposomal AMB or azole therapy. A favourable response was noted in 41% of the patients. One serious adverse event was reported and three patients discontinued therapy because of adverse events. Infusion-related reactions and nephrotoxicity were uncommon. Caspofungin is not subject to drug interactions based on CYP3A4 inhibition [108]. The FDA has recently approved caspofungin intravenous infusion, for the treatment of patients who are unresponsive to or cannot tolerate standard therapies for IA. Caspofungin is thereby the first approved drug in a new class of antifungals.

### 4.3.3 Micafungin

Micafungin (FK-463) is a water-soluble semisynthetic echinocandin-like lipopeptide. FK-463 has *in vitro* activity against a number of yeasts and filamentous fungi including *Aspergillus* [109]. However, in contrast to AMB, FK-463 does not exert fungicidal activity against *Aspergillus* spp. [109,110]. The efficacy of treatment with FK-463 in mouse models of aspergillosis was comparable or inferior to that of amphotericin B [111,112]. The safety and pharmacokinetics of FK-463 have been investigated in healthy adult volunteers, in febrile neutropenic paediatric patients and in patients receiving haematopoietic stem cell transplantation [113-115]. In these studies, FK-463 was well-tolerated. Similar to caspofungin and VER-002, FK-463 is not metabolised to a significant extent through the cytochrome P450 enzyme system [95]. Phase II studies with FK-463 have only been performed in HIV-infected patients with oropharyngeal and oesophageal candidiasis [116]. Currently, a non-comparative, open-label trial is performed for assessing the value of FK-463 75 mg/day for the treatment of newly diagnosed IA and IA in patients who have not responded to systemic therapy with other antifungal drugs [117].

### 4.3.4 Anidulafungin

Anidulafungin (VER-002, V-echinocandin, LY-303366) is also a potent non-competitive inhibitor of ß -1,3-glucan synthase [118]. *In vitro*, anidulafungin showed activity against AMB-resistant *A. fumigatus* [119]. The *in vivo* efficacy of anidulafungin was evaluated in a large number of animal models of candidiasis, aspergillosis and pneumocystis carinii pneumonia (PCP). It was shown to be effective in the treatment of *Aspergillus* infections in these experiments [85]. The pharmacokinetics of orally [120,121] and intravenously administered [122,123] anidulafungin were investigated in healthy volunteers. The terminal half-life was reported to be 25-42 h, and the use of anidulafungin was not correlated with serious adverse events. Common side-effects were: dizziness, nausea, headache, facial flushing, dyspnea, increased aspartate aminotransferase [122]. In a non-controlled Phase II trial in patients with oesophageal candidiasis anidulafungin was shown to be effective [124]. Anidulafungin is currently being evaluated in Phase II clinical trials.

### 4.3.5 Other echinocandins

The echinocandin HMR-3270 shows *in vitro* activity against *Aspergillus* [125,126]. Furthermore, this candin was effective in reducing aspergillosis-related mortality in an experimental mouse model [125,126]. The echinocandin-derived A-192411.29, which has good *in vitro* activity against clinically relevant *Candida* spp., showed no activity against *Aspergillus* [127]. Pneumocandin B0 and other related macrocycle agents, seem not to have anti-*Aspergillus* activity.

L-705589, L-733560 and L-692289 are all echinocandin-like lipopeptides with reported anti-*Aspergillus* activity but the development of these entities has been discontinued.

#### 4.3.6 Other ß-1,3-glucan synthase inhibitors

Arborcandins are novel cyclic peptides that inhibit ß-1,3-glucan synthase in the fungal cell wall. The arborcandins are structurally different from echinocandins, they show *in vitro* activity against *Candida* and *Aspergillus* [128]. FR-901469 is a water-soluble, non-echinocandin-type lipopeptide. This agent showed activity against *Aspergillus in vitro* as well as *in vivo* [129].

### 4.4 Mannoprotein-acting compounds

Pradimicins and structurally-related benanomycins act by a calcium-dependent complexing with the saccharide portion of surface mannoproteins of fungal cells; this causes perturbation of the cell membrane, leakage of intracellular contents and, ultimately, cell death [130]. The water soluble pradimicin BMS-181184 has broad spectrum antifungal activity *in vitro* [131,132]. BMS-181184 was shown to be effective in aspergillosis in persistently neutropenic rabbits [95,133]. Due to hepatotoxicity in human volunteers, clinical trials were stopped and this agent is no longer under clinical investigation [95]. Several other water soluble mannoprotein compounds are in very early development [94,95].

### 4.5 Chitin synthase inhibitors

#### Nikkomycin-Z

Chitin is a minor but essential cell wall component of fungi. Chitin synthesis is inhibited by the naturally-occurring nucleoside-peptides polyoxins and by the nikkomycins [15]. Nikkomycins are peptidyl nucleoside antibiotics which selectively inhibit chitin synthase in fungi. Nikkomycin Z is the only agent that was developed beyond the *in vitro* stage. Although nikkomycin Z shows good antifungal activity against several chitin-rich fungi, it has no activity against *A. fumigatus*. Only when combined with itraconazole does nikkomycin Z show inhibitory or fungicidal effects against *Aspergillus* spp. *in vitro* [134,135]. Due to its modest antifungal activity, nikkomycin Z has not been further developed for clinical use.

### 4.6 Allylamines and thiocarbamates

Terbinafine inhibits squalene epoxidase, a key enzyme in ergosterol biosynthesis. Although terbinafine is highly active *in vitro* against *Aspergillus* spp., especially in combination with azoles, clinical use has been associated with failure [136,137].

## 4.7 Sordarins

Sordarins are antifungal agents with a completely new mode of action. Sordarins selectively inhibit fungal protein synthesis by impairing the function of elongation factor 2, thereby selectively inhibiting the fungal protein synthesis machinery. They do not interfere with mammalian protein synthesis, since fungal and mammalian protein translation differs significantly at the molecular level [138]. Although sordarins showed remarkable activity against different yeasts and fungi, they have moderate *in vitro* activity against *Aspergillus* spp. [139]. *In vivo* experiments in murine models of IA also show a limited effect of GM-237354 on the survival of animals [127,140].

## 4.8 Other agents

### 4.8.1 Immunisation/vaccination

A study on the immunisation of mice with a *Candida albicans* membrane fraction, showed that this procedure did not only reduce mortality from *C. albicans* systemic infection but also increased resistance to systemic *Aspergillus* infection. Vaccination of mice with *Aspergillus* vaccine preparations protected the animals from developing IA [141]. Clinical experience with immunotherapy for invasive fungal infections is largely limited to anecdotal case reports and small studies. Nevertheless, the outcomes noted with this treatment modality are encouraging and call for controlled trials to evaluate the efficacy of immunotherapy with and without concomitant antifungal therapy [142].

### 4.8.2 Cationic peptides

Cationic peptides can be a component of the early innate defences against invading microbes. Both naturally-occurring and synthetic cationic peptides bind to ergosterol and cholesterol in fungal cell membranes, which ultimately leads to cell lysis. These peptides have antifungal activity against *Aspergillus* spp., *Candida* spp., *Cryptococcus neoformans* and *Fusarium* spp. [143,144]. Naturally-occurring peptides include cecropins, dermaseptins, indolicins, histatins, bactericidal permeability increasing factor (BPI), lactoferrins and defensins [95,143,144]. The *in vitro* synergistic effect of histatin analogues in combination with AMB was shown against several fungi, including *Aspergillus* [145]. The clinical value of these compounds remains to be determined.

### 4.8.3 Other developments

Synthetic cationic poly-heterocyclic molecules (GL-047296, GL-886217, GL-478057) showed *in vitro* activity against *A. fumigatus* and *Candida* spp. Their activity is based upon high affinity binding with double-stranded DNA [146,147]. A new antifungal target is presented by sphingolipid, an essential membrane com-

ponent of the fungal cell wall. Aureobasidin A is a peptide inhibitor of inositol phosphorylceramide synthase and inhibits sphingolipid synthesis. *Aspergillus* showed low sensitivity for aureobasidin A but the MIC was dramatically decreased when aureobasidin A was combined with the efflux inhibitor verapamil [148]. Recently, it was shown that aureobasidin A alone was an *in vitro* inhibitor of growth of *Aspergillus fumigatus* isolates [149]. Studies on the *in vitro* antifungal activities of a number of dication-substituted carbazole, furans and benzimidazoles show that some of these compounds have activity against *A. fumigatus*. The mechanism of action of these dicationic molecules is probably based on binding of DNA but the exact mechanism still has to be elucidated [150]. The activity of 3-phenyl-5-acyloxymethyl-2H,5H-furan-2-ones was tested *in vitro* against *A. fumigatus* and they were shown to be as effective as AMB [151]. The *in vitro* activity of a novel class of antifungal peptides, bifunctional inhibitors (ATBI) was studied. ATBI showed antifungal activity against several fungi, including *Aspergillus* [152]. Spongistatin 1 is an experimental antineoplastic agent that also has broad spectrum antifungal activity [153]. Spongistatin 1 is an antimicrotubule agent in *Aspergillus nidulans* [154]. The indole alkaloid venenatine exhibited antifungal activity against *Aspergillus flavus in vitro*.

## 5. Conclusion and potential development issues

The currently used agents in the treatment of IA have several limitations and the outcome of patients is generally disappointing. The critical need for antifungal agents with improved efficacy and safety has led to the discovery of many compounds. This paper presents an overview of current and new antifungal agents that hold potential for the management of IA.

Although AMB has been used for IA for many years, new strategies such as other modes of administration and different formulations are all focused on the broadening of the therapeutic window of AMB, which would allow more flexible dosing. The new azoles bring improved potency and a broader spectrum than previous azoles, while clinical experience with voriconazole, ravuconazole and posaconazole has been positive.

With the arrival of the echinocandins, we have the first new class of antifungal agents with a novel mode of action. The echinocandins are fungistatic against *Aspergillus* species. Therefore, their role as a single agent for treatment of proven invasive *Aspergillus* infections in the neutropenic host should be carefully investigated. The echinocandins may prove useful in combination with agents that target the cell membrane, such as AMB or triazoles. Entering the field of agents which are in development for IA are several compounds from different structural classes,

these target different functions within the target cell. At present, there are limited data on the clinical utility of these compounds but some of them definitely hold promise for the future.

## 6. Expert opinion

Discovery of new antifungal agents for filamentous fungal infections and IA has a high priority, as the rate of these infections is increasing and the outcome is poor. Given the limitations of the *in vitro* testing of filamentous fungi, many newly described agents show promising *in vitro* fungicidal activity but few make it through to Phase I investigations. Although the newer described agents have an increased potency, fewer side-effects and a broader antifungal spectrum, the most important intervention in IA is the prevention of infection and the reversal of host risk factors. Two major noteworthy contributions to antifungal chemotherapy will dominate the treatment of IA in the coming years. The licensing of caspofungin brought a new class of potent anti-*Aspergillus* drugs to the market with a lower potential for cross-resistance. Furthermore, the drug seems to be an ideal candidate for combination therapy with fungal cell membrane active drugs. The second major breakthrough involves the promising results shown by the new azole voriconazole, with regard to efficacy and survival in a large worldwide comparative trial in IA.

# Bibliography

Papers of special note have been highlighted as of interest (•) to readers.


1. McNeill MM, Nash SL, Hajjeh RA et al. Trends in mortality due to invasive mycotic diseases in the United States, 1980-1997. Clin Infect Dis 2001;33:641-647.
2. Anaissie E, Bodey GP. Nosocomial fungal infections. Old problems and new challenges. Inf Dis Clin North Am 1989;3(4):867-882.
3. Dixon DM, McNeill MM, Cohen ML, Gellin BG, La Montagne JR. Fungal infections, a growing threat. Public Health Rep 1996;111(3):226-235.
4. Denning DW. Invasive aspergillosis (review). Clin Infect Dis 1998;26:781-805.
5. Denning DW, Marinu A, Cohen J et al. Therapeutic outcome in invasive aspergillosis. Clin Infect Dis 1996;23:608- 615.
6. Leenders ACAP, Daenen S, Jansen RL: Liposomal amphotericin B compared with amphotericin B deoxycholate in the treatment of documented and suspected neutropenia-associated invasive fungal infections. Br J Haematol 1998;103:205-212.
7. Bodey GP, Bueltmann B, Duguid W et al. Fungal infections in cancer patients: an international autopsy survey. Eur J Clin Microbiol Infect Dis 1992;11:99-109.
8. Bolard J. How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochim Biophys Acta 1986; 864:257-304.
9. Brajtburg L, Powderly WG, Kobayashi GS, Medoff G. Amphotericin B: Current understanding of the mechanism of action. Antimicrob Agents Chemother 1990;34:183-188.
10. Hitchcock CA. Cytochrome P450-dependent 14 $\alpha$-sterol demethylase of *Candida* albicans and its interaction with azole antifungals. Biochem Soc Trans 1991;19:782-787.
11. Georgopapadakou NH, Walsh TJ. Antifungal agents: chemotherapeutic targets and immunological strategies. Antimicrob Agents Chemother 1996;40(2):279-291.
    • Minireview on novel approaches to antifungal drug discovery. Provides a good summary of new biochemical targets as well as host-immunity approaches.
12. Cabib E, Bowers B, Sburlati A, Silverman SJ. Fungal cell wall synthesis: the construction of a biological structure. Microbiol Sci 1988;5:370-375.
13. Cabib E, Drgon T, Drgonova J, Ford RA, Kollar R. The yeast cell wall, a dynamic structure engaged in growth and morphogenesis. Biochem Soc Trans 1997;25:200-204.
14. Denning DW. Echinocandins and pneumocandins - a new antifungal class with a novel mode of action. J Antimicrob Chemother 1997;40:611-614.
15. Geogopapadakou NH. Cell-wall active antifungals and emerging targets. In: *Anti-infectives: Recent Advances in Chemistry and Structure-Activity Relationships.* Bentley, O'Hanlon (Eds), The Royal Society of Chemistry, Cambridge, UK (1997):163-175.
16. Kurtz MB, Douglas CM: Lipopeptide inhibitors of fungal glucan synthase. J Med Vet Mycol 1997;35:79-86.
17. Maertens, JA, Boogaerts MA. Fungal cell wall

inhibitors: emphasis on clinical aspects. Curr Pharm Des 2000;6:225-239.

18. Walsh TJ, Giri N. Pradimicins: a novel class of broad-spectrum antifungal compounds. Eur J Clin Microbiol Infect Dis 1997;16:93-97.
19. Dominguez JM, Kelly VA, Kinsman OS, Marriot MS, Gomez de las Herras F, Martin JJ. Sordarins: a new class of antifungal with selective inhibition of the protein synthesis elongation cycle in yeast. Antimicrob Agents Chemother 1998;42(9):2274-2278.
20. Fanos V, Cataldi L. Amphotericin B-induced nephrotoxicity: a review. J Chemother 2000;12:463-470.
21. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-329.
22. Harrell CC, Hanf-Kristufek L. Comparison of nephrotoxicity of amphotericin B products. Clin Infect Dis 2001;32:990-991.
23. Luber AD, Maa L, Lam M, Guglielmo BJ. Risk factors for amphotericin B induced nephrotoxicity. J Antimicrob Chemother 1999;43:267-271.
24. Hiemenz JW, Walsh TJ. Lipid formulations of amphotericin B: Recent progress and future directions. Clin Infect Dis 1996;22:S133-S144.
25. Robinson RF, Nahata MC. A comparative review of conventional and lipid formulations of amphotericin B. J Clin Pharm Ther 1999;24(4):249-257.
26. Wong-Beringer A, Jacobs RA, Guglielmo BJ. Lipid formulations of amphotericin B: clinical efficacy and toxicities. Clin Infect Dis 1998;27(3):603-618.
27. Boogaerts M, Winston D, Bow EJ et al. Intravenous and oral itraconazole versus intravenous amphotericin B deoxycholate as empirical antifungal therapy for persistent fever in neutropenic patients with cancer who are receiving broad-spectrum antibacterial therapy. A randomized, controlled trial. Ann Intern Med 2001;135:412-422.
28. De Beule K, Van Gestel J. Pharmacology of itraconazole. Drugs 2001;61:S27-S37.
29. Wallace T and The Nyotran Study Group: *In vitro* antifungal activity of Nyotran against clinical isolates. 14th Conference of the International Society for Human and Animal Mycology (2000).
30. Mehta RT, Hopfer RL, McQueen T, Juliano RL, Lopez-Berestein G. Toxicity and therapeutic effects in mice of liposome-encapsulated nystatin for systemic fungal infections. Antimicrob Agents Chemother 1987;31(12):1901-1903.
31. Reyes GH, Long LA, Florentino F, Ghannoum MA. Efficacy of Nyotran in the treatment of disseminated candidiasis in a neutropenic guinea pig model. 40th ICAAC (2000).
32. Powles R, Mawhorter S, Williams T. Liposomal nystatin (Nyotran) vs amphotericin B (Fungizone) in empiric treatment of presumed fungal infection in neutropenic patients. 39th ICAAC (1999).
33. Offner FCJ, Herbrecht R, Engelhard D et al. EORTC-IFCG Phase II study on liposomal nystatin in patients with invasive *Aspergillus* infections, refractory or intolerant to conventional/lipid ampho B. 40th ICAAC (2000).
34. Papahadjopoulos D, Vail WJ, Jacobson K, Poste G. Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles. Biochim Biophys Act 1975;394:483-491.
35. Seagram I, Jin T, Hyra DA, Mannino RJ, Zarif

L. Oral administration of amphotericin B with a new AmB-cochleate formulation: tissue distribution after single and multiple oral dosing. 39th ICAAC (1999).

36. Zarif L, Jin T, Scolpino A, Mannino RJ. Are cochleates the new lipid-based carrier for oral drug delivery? 39th ICAAC (1999).
37. Perlin DS, Delmas G, Santangelo R, Paderu P, Mannino RJ. Oral delivery of cochleates containing amphotericin B (CAMB) is highly effective in mouse models of candidiasis and aspergillosis. 40th ICAAC (2000).
38. Davis SS. Biomedical applications of nanotechnology - implications for drug targeting and gene therapy. Trends Biotechnol 1997;15:217-224.
39. Otsubo T, Maesaki S, Hossain MA et al. *In vitro* and *in vivo* activities of NS-718, a new lipid nanosphere incorporating amphotericin B, against *Aspergillus fumigatus*. Antimicrob Agents Chemother 1999;43(3):471-475.
40. Hossain MA, Maesaki S, Razzaque MS et al. Attenuation of nephrotoxicity by a novel lipid nanosphere incorporating amphotericin B, NS-718. J Antimicrob Chemother 2000;46:263-268.
41. Van Etten EW, Stearne-Cullen LE, Ten Kate MT, Bakker-Woudenberg IA. Efficacy of liposomal amphotericin B with prolonged circulation in blood in treatment of severe pulmonary aspergillosis in leukopenic rats. Antimicrob Agents Chemother 2000;44(3):540-545.
42. Otsubo T, Maruyama K, Maesaki S et al. Long-circulating immunoliposomal amphotericin B against invasive pulmonary aspergilosis in mice. Antimicrob Agents Chemother 1998;42(1):40-44.
43. Ruijgrok EJ, Vulto AG, Van Etten EWM. Efficacy of aerosolized amphotericin B desoxycholate and liposomal amphotericin B in the treatment of invasive pulmonary aspergillosis in severely immunocompromised rats. J Antimicrob Chemother 2001;48:89-95.
44. Erjavec Z, Woolthuis GM, De Vries-Hospers HG et al. Tolerance and efficacy of amphotericin B inhalations for prevention of invasive pulmonary aspergillosis in haematological patients. Eur J Clin Microbiol Infect Dis 1997;16:364-368.
45. Hertenstein B, Kern WV, Schmeiser T, et al. Low incidence of invasive fungal infections after bone marrow transplantation in patients receiving amphotericin B inhalations during neutropenia. Ann Hematol 1994;68:21-26.
46. Schwartz S, Behre G, Heinemann V et al. Aerosolized amphotericin B inhalations as prophylaxis of invasive *Aspergillus* infections during prolonged neutropenia: results of a prospective randomized multicenter trial. Blood 1999;93:3654-3661.
47. Van Etten EW, Van Vianen W, Roovers P, Frederik P. Mild heating of amphotericin B-desoxycholate: effects on ultrastructure, *in vitro* activity and toxicity and therapeutic efficacy in severe candidiasis in leukopenic mice. Antimicrob Agents Chemother 2001;44(6):1598-1603.
48. Sedlak M, Buchta V, Kubicova L, Simuringnek P, Holcapek M, Kasparova P. Synthesis and characterisation of a new amphotericin B-methoxypoly(ethylene glycol) conjugate. Bioorg Med Chem Lett 2001;11(21):2833-2835.
49. Clemons KV, Ranney DF, Stevens DA. A novel heparin-coated hydrophilic preparation of

amphotericin B hydrosomes. Curr Opin Invest Drugs 2001;2(4):480-487.

50. Rimaroli C, Bruzesse T. Overview of SPA-S-843 *in vitro* activity against filamentous fungi. Chemotherapy 2000;46:28-35.
51. Bruzesse T, Galmozzi MR, Buffa G, Sala G, Bonabello A. Pharmacokinetics in rats of N-dimethylaminoacetyl-patricin A 2-dimethylaminoethylamide diascorbate (SPK-843). Chemotherapy 2001;47:77-85.
52. Bonabello A, Bruzesse T. Antifungal activity of SPA-S-843, in comparison with amphotericin B and fluconazole, on murine experimental infections. 4th Trends Invas Fungal Infect (1997).
53. Hoffman HL, Ernst EJ, Klepser ME. New triazole antifungal agents. Expert Opin Investig Drugs 2000;9(3):593-605.
54. Thompson DO. Cyclodextrins-enabling excipients: their present and future use in pharmaceuticals. Crit Rev Ther Drug Carrier Syst 1997;14:1-104.
55. Caillot D, Basaris H, Seiferty WF et al. Intravenous itraconazole followed by oral itraconazole in the treament of invasive pulmonary aspergillosis in patients with hematological malignancies, chronic granulomatous disease, or AIDS. Clin Infect Dis 2001;33(8):E83-E90.
56. Boogaerts M, Maertens J. Clinical experience with itraconazole in systemic infections. Drugs 2001;61: S39-47.
57. Abraham OC, Manavaathu EK, Cutright JL et al. *In vitro* susceptibilities of *Aspergillus* species to voriconazole, itraconazole, and amphotericin B. Diagn Microbiol Infect Dis 1999;33:7-11.
58. Murphy M, Bernard EM, Ishimaru T et al. Activity of voriconazole (UK-109,496) against clinical isolates of *Aspergillus* species and its effectiveness in an experimental model of invasive pulmonary aspergillosis. Antimicrob Agents Chemother 1997;41(3):696-698.
59. Oakley KL, Moore CD, Denning DW. *In vitro* activity of voriconazole against *Aspergillus* spp. and comparison with itraconazole and amphotericin B. J Antimicrob Chemother 1998;42:91-94.
60. Wildfeuer A, Seidl HP, Paule I et al. *In vitro* evaluation of voriconazole against clinical isolates of yeasts, moulds and dermatophytes in comparison with itraconazole, ketoconazole, amphotericin B and griseofulvin. Mycoses 1998;41:309-319.
61. Hitchcock CAM, Andrews RJ, Lewis BGH, Troke PF. UK-109,496, a novel wide spectrum triazole derivative for the treatment of fungal infection: antifungal activity in experimental infections with *Aspergillus*. 35th ICAAC (1995).
62. Martin MV, Yates J, Hitchcock CA. Comparison of voriconazole (UK-109,496) and itraconazole in prevention and treatment of *Aspergillus fumigatus* endocarditis in guinea-pigs. Antimicrob Agents Chemother 1997;41(1):13-16.
63. Sabo JA, Abdel-Rahman SM. Voriconazole: a new triazole antifungal. Ann Pharmacother 2000;34:1032-1043.
64. Patterson BE, Coates PE. UK-109,496, a novel wide-spectrum triazole derivative for the treatment of fungal infection: Pharmacokinetics in man. 35th ICAAC (1995).
65. Patterson BE, Roffey S, Jezequel SG et al. UK-109,496, a novel wide-spectrum triazole

derivative for the treatment of fungal infection: Disposition in man. 35th ICAAC (1995).

66. Denning D, Del Favero A, Gluckman E et al. UK-109,496, a novel wide spectrum triazole derivative for the treatment of fungal infection: Clinical efficacy in acute invasive aspergillosis. 35th ICAAC (1995).
67. Dupont B, Denning D, Lode H et al. UK-109,496, a novel wide spectrum triazole derivative for the treatment of fungal infection: Clinical efficacy in chronic invasive aspergillosis. 35th ICAAC (1995).
68. Schwartz S, Milatovic D, Thiel E. Successful treatment of cerebral aspergillosis with a novel triazole (voriconazole) in a patient with acute leukemia. Br J Haematol 1997;97:663-665.
69. Herbrecht R, Denning DW, Patterson TF et al. Open, randomised comparison of voriconazole and amphotericin B followed by other licensed antifungal therapy for primary therapy of invasive aspergillosis. 41st ICAAC (2001).
70. Verweij PE, Brinkman K, Kremers HPH, Kullberg BJ, Meis JFGM. *Aspergillus meningitis*: diagnosis by non-cultured based microbiological methods and management. J Clin Microbiol 1999;37:1186-1189.
71. Walsh TJ, Pappas P, Winston DJ et al. Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. N Engl J Med 2002;346:225-234.
    • A large and rigourous trial that shows proof of efficacy of this new triazole agent.
72. Espinel-Ingroff A. Comparison of *in vitro* activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743872) and LY-303366 against opportunistic filamentous and dimorphic fungi and yeasts. J Clin Microbiol 1998;36(10):2950-2956.
73. Oakley KL, Moore CD, Denning DW. *In vitro* activity of SCH-56592 and comparison with activities amphotericin B against *Aspergillus* spp. Antimicrob Agents Chemother 1997;41(5):1124-1126.
74. Hayette MP, Amadore A, Seidel L et al. Comparative *in vitro* activity of amphotericin B, itraconazole, voriconazole and posaconazole against *Aspergillus fumigatus*. 41st ICAAC (2001).
75. Pfaller MA, Messner SA, Hollis RJ, Jones RN, The Sentry Participants Group. Antifungal activity of posaconazole, ravuconazole and voriconazole compared to that of itraconazole tested against 213 clinical isolates of *Aspergillus* spp. and other filamentous fungi: report from the SENTRY Antimicrobial Surveillance Program, 2000. 41st ICAAC (2001).
76. Patterson TF, Kirkpatrick WR, McAttee RK, Loebenberg D. Efficacy of SCH-6592 in a rabbit model of invasive aspergillosis. 36th ICAAC (1996).
77. Oakley KL, Moore CD, Denning DW. Efficacy of SCH-56592 in a temporarily neutropenic murine model of invasive aspergillosis with an itraconazole-susceptible and an itraconazole resistant isolate of *Aspergillus fumigatus*. Antimicrob Agents Chemother 1997;41:1504-1507.
78. Petraitiene R, Petraitis V, Groll AH et al. Antifungal activity and pharmacokinetics of posaconazole (SCH 56592) in treatment and prevention of experimental invasive pulmonary aspergillosis: correlation with galactomannan antigenemia. Antimicrob

Agents Chemother 2001;45(3):857-869.

79. Cacciapuoti A, Loebenberg D, Corcoran E et al. *In vitro* and *in vivo* activities of SCH 56592 (posaconazole), a new triazole antifungal agent, against *Aspergillus* and *Candida*. Antimicrob Agents Chemother 2000;44(8):2017-2022.
80. Laughlin M, Pai S, Menon S et al. Rising multiple-dose safety, tolerance and pharmacokinetic evaluation in healthy volunteers. 37th ICAAC (1997).
81. Radwanski E, Kosoglou T, Lim J, Laughlin M, Affrime MB, Nomeir AA. SCH-56592: bioavailibility of an oral suspension relative to a solid dosage form in healthy volunteers. 37th ICAAC (1997).
82. Fung-Tomc JC, Huczko E, Minassian B, Bonner DP. *In vitro* activity of a new oral triazole, BMS-207147 (ER-30346). Antimicrob Agents Chemother 1998;2(2):313-318.
83. Hata K, Kimura J, Toyosawa T, Nakamura T, Katsu K. *In vitro* and *in vivo* antifungal actvities of ER-30346, a novel oral triazole with a broad antifungal spectrum. Antimicrob Agents Chemother 1996;40(10):2237-2242.
84. Moore CB, Walls CM, Denning DW. *In vitro* activity of the new triazole BMS-207147 against *Aspergillus* species in comparison with itraconazole and amphotericin B. Antimicrob Agents Chemother 2000;44(2):441-443.
85. Roberts J, Schock K, Marino S, Andriole VT. Efficacies of two new antifungal agents, the triazole ravuconazole and the echinocandin LY-303366, in an experimental model of invasive aspergillosis. Antimicrob Agents Chemother 2000;44(12):3381-3388.
86. Ryan B, Ho H-T, Wu P, Frosco M-B, Dougherty T, Barrett JF. 40th Interscience Conference on Antimicrobial Agents and Chemotherapy. Expert Opin Investig Drugs 2000;9(12):2945-2972.
87. Najvar LK, Bocanegra RA, Luther MF, Graybill JR. New antifungal triazole R-02557 (r-1) in murine aspergillosis and candidiasis. 40th ICAAC (2000).
88. Salama SM, Atwal H, Gandhi A et al. *In vitro* and *in vivo* activities of SYN-2836, SYN-28969, SYN-2903 and SYN-2921: new series of triazole antifungal agents. Antimicrob Agents Chemother 2001;45(9):2420-2426.
89. Capilla J, Ortoneda M, Pastorf J, Guarro J. *In vitro* antifungal activities of the new triazole UR-9825 against clinical important filamentous fungi. Antimicrob Agents Chemother 2001;45(9):2635-2637.
90. Cid VJ, Duran A, Del Rey F, Snyder MP, Nombella C, Sanchez M: Molecular basis of cell wall integrity and morphogenesis in *Saccharomyces cerevisiae*. Microbiol Rev 1995;59:345-386.
91. Smits GJ, Kapteyn JC, Van Den Ende H, Klis FM. Cell wall maintenance in fungi. Trends Microbiol 1995;6:147-150.
92. Georgopapadakou NH, Tkacz JS. The fungal cell wall as a drug target. Trends Microbiol 1995;3:98-104.
93. Kohno S, Masaoka T, Yamaguchi H. A multicenter, open-label clinical study of FK-463 in patients with deep mycoses. 41st ICAAC (2001).
94. Andriole VT. Current and future therapy of invasive fungal infections. In: *Current Clinical Topics in Infectious Diseases. Vol. 18.* Remington J, Schwartz M (Eds) (1998),

Blackwell Sciences, Malden, MA, USA 19-36.

95. Groll AH, Piscitelli SC, Walsh TJ. Clinical pharmacology of systemic antifungal agents: a comprehensive review of agents in clinical use, current investigational compounds, and putative targets for antifungal drug development. Adv Pharmacol 1998;44:343-500.
96. Groll AH, Walsh TJ. MK-0991. Curr Opin Anti-infective Investig Drugs 1999;1(3):334-345.
97. Pfaller MA, Marco F, Messner SA, Jones RN. *In vitro* activity of two echinocandin derivatives LY 303366 and MK-0991 (L-743792), against clinical isolates of *Aspergillus*, *Fusarium, Rhizopus*, and other filamentous fungi. Diagn Microbiol Infect Dis 1998;30(4):251-255.
98. Bartizal K, Smith JG, Gill CJ. Preclinical efficacy of MK-0991 in pancytopenic mouse models of disseminated candidiasis and aspergillosis. 37th ICAAC (1997).
99. Del Poeta M, Schell WA, Perfect JR. *In vitro* antifungal activity of pneumocandin L-743872 against a variety of clinically important molds. Antimicrob Agents Chemother 1997;41(8):1835-1836.
100. Fothergill AW, Sutton DA, Rinaldi MG. Antifungal susceptibility testing of Merck L-743,872 against a broad range of fungi. 36th ICAAC (1996).
101. Sutton DA, Rinaldi MG, Fothergill AW. *In vitro* activity of the echinocandin caspofungin (MK-0991) against refractory clinical isolates of the *Candida* and *Aspergillus* species. 41st ICAAC (2001).
102. Abruzzo GK, Flattery AM, Gill CJ et al. Evaluation of the echinocandin antifungal MK-0991 (L-743,872): efficacies in mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. Antimicrob Agents Chemother 1997;41(11):2333-2338.
103. Bartizal K, Gill CJ, Abruzzo GK et al. *In vitro* preclinical evaluation studies with the echinocandin antifungal MK-0991 (L-743,872). Antimicrob Agents Chemother 1997;41(11):2326-2332.
104. Smith JG, Abruzzo CJ, Flattery AM et al. Evaluation of pneumocandin L- 43872 in neutropenic mouse models of disseminated candidiasis and aspergillosis. 36th ICAAC (1996).
105. Stone, JA, Holland SD, Ju WD et al. Single- and multiple-dose pharmacokinetics of the antifungal agent MK-0991 in man. 38th ICAAC (1998).
106. Stone, JA, Holland SD, Ju WD et al. A Phase I study of caspofungin evaluating the potential for drug interactions with itraconazole, the effect of gender and the use of a loading dose regimen. 40th ICAAC (2000).
107. Sable CA, Villanueva A, Arathon E et al. A randomized, double blind, multicenter trial of MK-0991 (L-743,872) vs amphotericin B in the treatment of *Candida* esophagitis in adults. 38th ICAAC (1998).
108. Maertens J, Raad I, Sable CA et al. Multicenter, non-comparative study to evaluate safety and efficacy of caspofungin in adults with invasive aspergillosis refractory or intolerant to amphotericin B, amphotericin B lipid formulations, or azoles. 40th ICAAC (2000).
    - Proof of clinical efficacy of caspofungin in invasive aspergillosis.
109. Tawara S, Ikeda F, Maki K et al. *In vitro* activities of a new lipopeptide antifungal

agent, FK 463, against a variety of clinically important fungi. Antimicrob Agents Chemother 2000;44(1):57-62.

110. Tomishima M, Ohki H, Yamada A et al. FK-463, a novel water-soluble echinocandin lipopeptide: synthesis and antifungal activity. J Antibiot 1999;52:674-676.

111. Matsumoto S, Wakai Y, Nakai T et al. Efficacy of FK-463, a new lipopeptide antifungal agent, in mouse models of pulmonary aspergillosis. Antimicrob Agents Chemother 2000;44(3):619-621.

112. Ikeda F, Wakai Y, Matsumoto S et al. Efficacy of FK-463, a new lipopeptide antifungal agent, in mouse models of disseminated candidiasis and aspergillosis. Antimicrob Agents Chemother 2000;44(3):614-618.

113. Hiemenz J, Cagnoni P, Simpson D, Devine S, Chao N. Maximum tolerated dose and pharmacokinetics of FK-463 in combination with fluconazole for the prophylaxis of fungal infections in adult bone-marrow or peripheral stem cell transplant patients. 39th ICAAC (1999).

114. Azuma J, Yamamoto I, Ogura M et al. Phase I study of FK-463, a new antifungal agent, in healthy adult male volunteers. 38th ICAAC (1998).

115. Seibel N, Schwartz C, Arrieta A et al. A phase I study to determine the safety and pharmacokinetics of FK-463 in febrile neutropenic pediatric patients. 40th ICAAC (2000).

116. Pettengell K, Mynhardt J, Kluyts T, Soni P. A multicenter study to determine the minimal effective dose of FK-463 for the treatment of esophagal candidiasis in HIV-positive patients. 39th ICAAC (1999).

117. Hunter P. Micafungin: a novel antifungal agent for invasive infections. Inpharma 2001;1300:7-8.

118. Current WL, Tang J, Boylan C et al. Glucan biosynthesis as a target for antifungals: the echinocandin class of antifungal agents. In: *Antifungal Agents: Discovery and Mode of Action.* Dixon GK, Copping LG, Hollomon DW (Eds), Bios Scientific Publishers, Oxford, UK(1995):143-160.

119. Verweij PE, Oakley KL, Morrissey G, Denning DW. Efficacy of LY-303366 against amphotericin B-susceptible and -resistant *Aspergilus fumigatus* in a murine model of invasive aspergillosis. Antimicrob Agents Chemother 1998;42(4):873-878.

120. Lucas R, Desante K, Hatcher B et al. LY-303366 in healthy and HIV-infected volunteers after single-dose oral administration. 36th ICAAC (1996).

121. Ni L, Smith B, Hatcher B et al. Pharmacokinetics of LY-303366 in healthy and HIV-infected volunteers. 38th ICAAC (1998).

122. Brown GL, White RJ, Taubel J. Phase I dose optimization study for V-echinocandin. 40th ICAAC (2000).

123. Rajman I, Desante K, Hatcher B et al. LY-303366 single dose pharmacokinetics and safety in healthy volunteers. 37th ICAAC (1997).

124. Brown GL, White RJ, Turik M. Phase II, randomized, open label study of two intravenous dosing regimens of V-echinocandin in the treatment of esophageal candidiasis. 40th ICAAC (2000).

125. Warn P, Sharp A, Denning D. *In vivo* activity of the echinocandin HMR-3270 against *Aspergillus* spp. 41st ICAAC (2001).

126. Warn P, Sharp A, Morrissey G, Denning D.

The *in vivo* activity of HMR-3270 in temporarily neutropenic murine models of disseminated infection caused by *Aspergillus fumigatus* (2 strains). 41st ICAAC (2001).

127. Nilius AM, Raney PM, Hensey-Rudloff DM, Wang W, Li Q, Flamm RK. *In vitro* activity of A-192411.29, a novel antifungal lipopeptide. Antimicrob Agents Chemother 2000;44(5):1242-1246.
128. Ohyama T, Kutihara Y, Ono Y et al. Arborcandins A, B, C, D, E and F, novel 1,3-glucan synthase inhibitors: production and biological activity. J Antibiot 2000;53(10): 1108-1116.
129. Georgopapadakou NH. Update on antifungals targeted to the cell wall: focus on ß-1,3-glucan synthase inhibitors. Expert Opin Investig Drugs 2001;10(2):269-280.
130. Sawada Y, Numata K, Murakami T, Tanimichi H, Yamamoto S, Oki T. Calcium dependent anticancidal action of pradimicin A. J Antibiot 1990;43:715-721.
131. Fung-Tomc JC, Minassian B, Huczko E, Kolek B, Bonner DP, Kessler RE. *In vitro* antifungal and fungicidal spectra of a new pradimicin derivative, BMS-181184. Antimicrob Agents Chemother 1995;39(2):295-300.
132. Oakley KL, Moore CD, Denning DW. Activity of pradimicin BMS-181184 against *Aspergillus* spp. Int J Antimicrob Agents 1999;12(3):267-269.
133. Gonzalez CE, Groll AH, Giri N et al. Antifungal activity of the pradimicin derivative BMS-181184 in the treatment of experimental pulmonary aspergillosis in persistently neutropenic rabbits. Antimicrob Agents Chemother 1998;42(9):2399-2404.
134. Li RK, Rinaldi MG. *In vitro* antifungal activity of nikkomycin Z in combination with fluconazole or itraconazole. Antimicrob Agents Chemother 1999;43(6):1401-1405.
135. Stevens DA. Drug interaction studies of a glucan synthase inhibitor (LY-303366) and a chitin synthase inhibitor (Nikkomycin Z) for inhibition and killing of fungal pathogens. Antimicrob Agents Chemother 2000;44(9):2547-2548.
136. Moore CB, Walls CM, Denning DW. *In vitro* activities of terbinafine against *Aspergillus* species in comparison with those of itraconazole and amphotericin B. Antimicrob Agents Chemother 2001;45(6):1882-1885.
137. Mosquera J, Sharp A, Moora CB, Warn PA, Denning DW. *In vitro* interaction of terbinafine with itraconazole, amphotericin B and 5-flucytosine against *Aspergillus* spp. 41st ICAAC (2001).
138. Capa L, Medoza A, Lavander JL, Gomes De Las Heras F, Garcia-Bustos JF. Translation elongation factor 2 is part of the target for a new family of antifungals. Antimicrob Agents Chemother 1998;42(10):2694-2699.
139. Herreros E, Martinez CM, Almela MJ, Marriot MS, Gomez De Las Heras F, Gargallo-Viola D: Sordarins: *in vitro* activities of new antifungal derivatives against pathogenic yeasts, *Pneumocystis carinii,* and filamentous fungi. Antimicrob Agents Chemother 1998;42(11):2863-2869.
140. Martinez A, Avilez P, Jimenez E, Caballero J, Gargallo-Viola D. Activities of sordarins in experimental models of candidiasis, aspergillosis, and pneumocystosis. Antimicrob Agents Chemother 2000;44(12):3389-3394.
141. Ito JI, Lyons JM. Protection of corticosteroid

immunosuppressed mice against invasive pulmonary aspergillosis. 41st ICAAC (2001).

142. Casadevall A, Pirofski LA. Adjunctive immune therapy for fungal infections. Clin Infect Dis 2001;33(7):1048-56.
143. De Luca AJ, Bland JM, Jacks TJ, Grimm C, Walsh TJ. Fungicidal and binding properties of the natural peptides cecropin B and dermaseptin. Med Mycol 1998; 36:291-298.
144. Hong SY, Oh JE, Kwon MY et al. Identification and characterization of novel antimicrobial decapeptides generated by combinatorial chemistry. Antimicrob Agents Chemother 1998;42(10):2534-2541.
145. Van't Hof W, Reijnders IM, Helmerhorts EJ et al. Synergistic effects of low doses of histatin 5 and its analogues on amphotericin B antimycotic activity. Antonie Van Leeuwenhoek 2000;78(2):163-169.
146. Botyanszki J, Roberts C, Dyatkina N et al. Discovery and optimization of a new family of DNA targeting compounds that shows antifungal activity towards *Candida* and *Aspergillus* species. 41st ICAAC (2001).
147. Velligan M, Stevens D, Kongpachith A. A new family of DNA targeting compounds are active against pathogenic yeasts and molds. 41st ICAAC (2001).
148. Zhong W, Jeffries MW, Georgopapadakou NH. Inhibition of inositol phosphorylceramide synthase by aureobasidin A in *Candida* and *Aspergillus* species. Antimicrob Agents Chemother 2000;44(3):651-3.
149. Harrigan LA, Wilks JW. Revealing the activity of aureobasidin A (AbA) toward *Aspergillus fumigatus*. 41st ICAAC (2001).
150. Del Poeta M, Schell WA, Dykstra CC. *In vitro* antifungal activities of a series of dication-substituted carbazoles, furans, and benzimidazoles. Antimicrob Agents Chemother 1998;42(10):2503-2510.
151. Pour M, Spulak M, Buchta V. 3-Phenyl-5-acyloxymethyl-2H,5H-furan-2-ones: Synthesis and biological activity of a novel group of potential antifungal drugs. J Med Chem 2001;44(17):2701-2706.
152. Dash C, Ahmad A, Nath D, Rao M. Novel bifunctional inhibitor of xylanase and aspartic protease: Implications for inhibition of fungal growth. Antimicrob Agents Chemother 2001;45(7):2008-2017.
153. Pettit RK, McAllister SC, Pettit GR, Herald CL, Johnson JM, Cichaz ZA. A broad-spectrum antifungal from the marine sponge *Hyrtios erecta*. Int J Antimicrob Agents 1998;9:147-152.
154. Ovechkina YY, Pettit RK, Cichaz ZA, Pettit GR, Oakley BR. Unusual antimicrotubule activity of the antifungal agents spongistatin 1. Antimicrob Agents Chemother 1999;43(8):1993-1999.

## Website

155. http://www/aspergillus.man.ac.uk/secure/temptreatment/FK463text.html Groll AH, Walsh TJ: FK463, a novel echinocandin-like antifungal lipopeptide. *Aspergillus* website (2000).

Chapter 3

# Aerosol delivery of amphotericin B desoxycholate and liposomal amphotericin B: Aerosol characteristics and *in vivo* amphotericin B deposition in Rats

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# Abstract

In the treatment or prophylaxis of invasive pulmonary aspergillosis, it may be attractive to administer the antifungal agent amphotericin B (AMB) directly to the pulmonary route via aerosol inhalation. In this study, we describe the aerosol characteristics of aerosolised amphotericin B desoxycholate (AMB-DOC) and liposomal amphotericin B (L-AMB), and the *in vivo* aerosol deposition.

Aerosols were generated with a Collison nebuliser. Aerosol AMB concentrations and mass median diameters (MMDs) were measured. *In vivo* pulmonary deposition was evaluated by measuring AMB concentrations in lungs of treated animals. Whole body aerosol deposition was determined by measuring radioactivity in tissues of rats after treatment with radiolabeled liposomes. For AMB-DOC and L-AMB, aerosol AMB concentrations were 24.5 ± 4.9 μg/L and 23.8 ± 3.0 μg/L, respectively. MMDs were 1.38 μm and 2.26 μm, for AMB-DOC and L-AMB, respectively. AMB concentrations in lungs after 60 min nebulisation of AMB-DOC or L-AMB were 24.2 ± 6.4 μg/g and 21.7 ± 2.6 μg/g, respectively. After nebulisation of radiolabelled liposomes, no radioactivity was retrieved from other tissues than the lungs or the gastrointestinal tract.

Nebulisation of either AMB-DOC or L-AMB leads to respirable aerosols and results in a substantial lung tissue concentration of AMB and low systemic exposure of AMB. Aerosol administration of either AMB-DOC or L-AMB may be an attractive approach to prevent or treat pulmonary aspergillosis.

# Introduction

*Aspergillus* spp., and especially *Aspergillus fumigatus*, are increasingly recognised as major fungal pathogens in severely immunosuppressed or neutropenic patients [1]. The most encountered form of disease due to *Aspergillus* is invasive pulmonary aspergillosis. The polyene antifungal agent AMB has been the drug of choice since many years in the treatment of many fungal infections, including invasive pulmonary aspergillosis [1]. However, use of this drug is hampered by considerable toxicity following intravenous injection, such as renal toxicity, anemia and hypokalemia [2]. New (lipid based) formulations of AMB, which have an increased therapeutic index as compared to AMB-DOC, are currently available [3]. However, treatment of immunocompromised patients with an established pulmonary infection with *Aspergillus* spp. is to date often unsuccessful and optimisation of antifungal treatment of invasive pulmonary remains a challenge. *Aspergillus* spp. are respiratory pathogens and pulmonary infections leading to invasive pulmonary aspergillosis are usually acquired through inhalation of *Aspergillus* conidia. However, concentrations of AMB after intravenous injection of both non-liposomal as liposomal AMB are mainly seen in liver and spleen [4,5]. This may be the cause of treatment failure often observed in invasive pulmonary aspergillosis. Improvement in the treatment of invasive pulmonary aspergillosis can therefore be sought in the aerosol administration of AMB as such or of lipid formulations of AMB. Aerosol inhalation is an important drug delivery method in pulmonary diseases since it targets the lungs and reduces exposure of other organs to the drug, thus minimising side-effects [6,7].
In this study, a system for aerosol administration of the commercially available products AMB desoxycholate (AMB-DOC) and liposomal AMB (L-AMB) is described in order to evaluate several aspects of stability of nebulised suspensions, output of mass and volume of AMB suspensions and delivered dose of AMB. Furthermore, *in vivo* deposition in lungs and biodistribution in other organs of rats have been evaluated.

# Materials and Methods

## Materials

AMB desoxycholate (AMB-DOC, Bristol Myers-Squibb, Woerden, The Netherlands) and liposomal AMB (L-AMB, NeXstar, San Dimas, CA) were reconstituted in distilled water according to the manufacturer's instructions and further diluted in 5% glucose in water up to a concentration of 4 mg/mL AMB. AMB (activity 916 $\mu$g/g)

for calibration was from Bristol Myers-Squibb, Woerden, The Netherlands. Hydrogenated soy phosphatidylcholine (HSPC) and distearoyl phosphatidylglycerol (DSPG) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol (Chol), dimethylsulphoxide (DMSO) and 8-hydroxyquinone sulfate were from Sigma Chemical Co (St. Louis, MO). $^{67}$Gallium-citrate was from Mallinckrodt Medical BV (Petten, The Netherlands). Desferoxamine mesylate (Desferal) was from Novartis (Basel, Switzerland). All other reagents were of analytical grade.

### Animals

Female RP strain rats (specified pathogen free, 18-25 weeks, 185-225 g) were used. Experiments were approved by the animal experiments ethical committee of the Erasmus *university* Medical Center.

### Nebulisation apparatus and procedure

The aerosol inhalation system is schematically depicted in Figure 1. Compressed air was supplied to the nebuliser with a flow rate of 20 L/min and 37.5 psi as measured

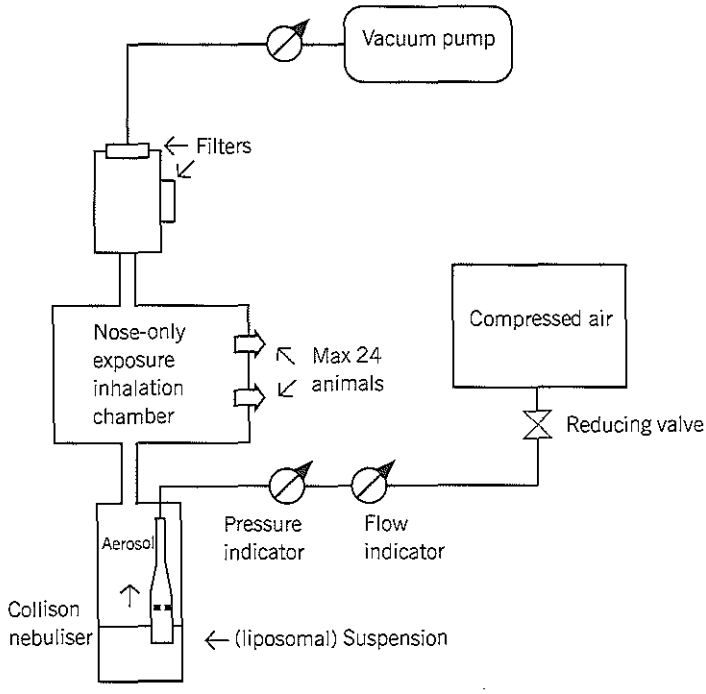



Figure 1. Schematic representation of the aerosol inhalation system.

by a flow indicator (Sho-rate, Brooks Instrument BV, Veenendaal, The Netherlands) and a pressure indicator (ITT Fluid Technology, St. George, SC). Aerosols were generated by a Collison six jet nebuliser (Model CN, BGI Inc., Waltham, MA). A glass nebuliser reservoir with a small fill hole at the jar bottom ensured high efficiency nebulisation of fluids. Generated aerosols were led through a nose-only exposure inhalation chamber (CH Technologies USA Inc., Westwood, NJ). This inhalation chamber is suitable for aerosol treatment of 24 individual animals. A continuous flow was present inside the system, due to a vacuum pump at the end, operating with a flow rate of 23 L/min. In connection with this vacuum pump was a filter house containing two filters, one main filter to extract aerosol droplets from outgoing air, the other filter to compensate for over or under pressure developing during nebulisation. In the *in vivo* experiments, animals were constrained in cone-ended plastic animal holders such, that only their nose was in contact with the aerosol.

### AMB spectrophotometrical analysis and liposomal characterisation

AMB concentrations in the nebuliser reservoir and in the inhalation chamber sample were determined spectrophotometrically. After diluting the samples in DMSO:methanol 1:1 v/v the observed extinctions at 410 nm could be interpolated in a standard calibration curve. Mean particle size of liposomes was determined by dynamic light scattering (4700 system, Malvern Instruments, Malvern UK) [8]. Phospholipid content was determined by phosphorus assay [9].

### Measurement of volume and mass output

Volume output of nebulised liquid was determined gravimetrically by subtracting the weight of the complete reservoir containing the residue after nebulisation from the weight of the reservoir containing the liquid to be nebulised. Mass output of AMB was determined by subtracting the amount of AMB (mg) in the reservoir after nebulisation from the amount of AMB in the reservoir before nebulisation.

### Aerosol concentration measurement

Aerosol samples were extracted from the inhalation chamber at the breathing level of animals as depicted in Figure 2. A 10 mL syringe was used to extract with frequent intervals in total an amount of 1 L during 60 min of nebulisation. This sampling volume provided sufficient drug for spectrophotometrical analysis. The sampled air was led through 10 mL collection medium consisting of a mixture of DMSO:methanol 1:1 v/v, which could thereafter directly be measured according to the method described above.

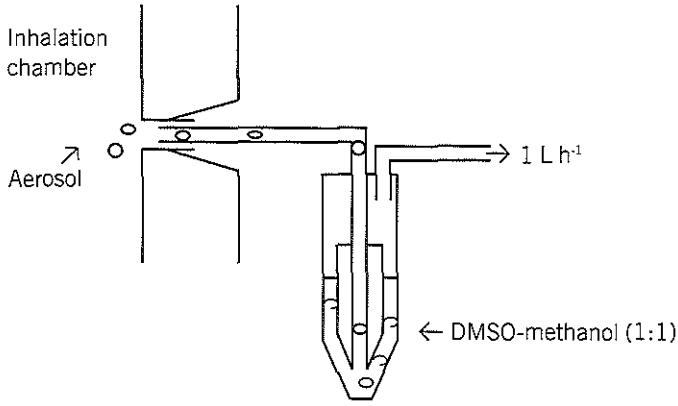



Figure 2. Inhalation chamber sampling procedure.

Droplet size measurements

Droplet size distribution was measured using a laser velocity particle sizer (Aerosol Particle Sizer 3320A, TSI Inc., St. Paul, MN). Aerosols were generated with compressed air at a flow rate of 10 L/min. Distribution of the number of generated aerosol particles was directly measured with this technique. For extrapolation of mass distribution from the number distribution, specific gravity was set at that of the solvent (1 g/cm³) and relative humidity at 60%. From the mass distribution, the parameters mean diameter (MMD), geometric standard deviation (GSD) and percentage of particles with a mass diameter $<5\ \mu m$ were calculated.

Preparation of radiolabelled liposomes

Liposomes were labelled with $^{67}Ga$ according to the method described by Gabizon et al [10]: Liposomes were prepared using the film hydration method. Liposomal membranes consisted of HSPC:DSPG:Chol in a molar ratio of 2:1:0.8, similar to the lipid composition of L-AMB. Lipids were dissolved in 2 mL chloroform:methanol (1:1, v/v). The lipid mixture was evaporated to dryness in a round bottom flask at 65°C. The lipid film was hydrated by vortex mixing with a buffer solution containing 10 mM sodium-succinate, 10% (w/v) sucrose (pH 5.5) and 5 mM desferoxamine. Liposomes were sonicated which resulted in vesicles with an average particle size of 100 nm, as measured by dynamic light scattering. Unencapsulated desferoxamine was removed by eluting the suspension over a Sephadex G-50 column (Pharmacia, Uppsala, Sweden), followed by ultracentrifugation at 280,000 x g for 2 h at 4°C. Phospholipid content was determined by phosphorus assay [8]. $^{67}Ga$-citrate (370 MBq/mL) was

diluted 1:9 in a 0.5 mg/mL 8-hydroxyquinone sulfate solution and incubated for 1 h at 50°C to yield $^{67}$Ga-oxine. The liposome suspension was incubated overnight at 4°C with 3.7 x $10^{-2}$ MBq of $^{67}$Ga-oxine per $\mu$mole of lipid. Unencapsulated $^{67}$Ga-oxine was removed by gelfiltration (Sephadex G-50 column, Pharmacia, Uppsala, Sweden), and liposomes were concentrated by ultracentrifugation as described above.

Deposition of radiolabelled liposomes

Animals were treated with nebulised $^{67}$Ga-labelled liposomal suspension according to the nebulisation method described above. Groups of 2 animals were euthanised with pentobarbital (100 mg/kg i.v.) after 10, 20, 30, 40, 50 and 60 min of nebulisation. Directly after, approximately 0.3 mL blood was sampled via an orbital punction and left lung, right lung, trachea, tongue, oral cavity, lower jaw, snout, stomach and esophagus, intestines, spleen, liver and kidney were dissected. Organs, tissues and blood were weighed and analysed for γ-irradiation with a Minaxi autogamma 5000 gamma counter (Packard Instrument Company, Meriden, CT).

Calculation of percentage deposition of radioactivity

Measured counts per minute (cpm) of $^{67}$Ga-labelled liposomal suspension before and after 60 min of nebulisation were 6.5 x $10^6$ and 1.2 x $10^7$ cpm/mL respectively. From these values, total cpm nebulised and the inhalation chamber concentration can be calculated. Delivered doses at the breathing point of animals can subsequently be derived as follows:

delivered dose (cpm) =
minute volume (L/min) x aerosol chamber concentration (cpm/L)
x duration of nebulization (min) (1)

Minute volume of animals was calculated according to Guyton [11]:
minute volume (L/min) = (body weight (g))$^{0.75}$ x 0.0021 (2)

Measured cpm in organs and tissues divided by the delivered dose gives the relative deposition.

Maximum pulmonary concentration of AMB after nebulisation of AMB-DOC or L-AMB

Directly after 60 min nebulisation of either AMB-DOC or L-AMB, animals were euthanised and lungs were removed. Right and left lung lobes were weighed and homogenised in 20 mL 5% glucose. AMB was extracted from the homogenate with

ethanol in a 4:6 (v/v) ratio. The extracts were centrifuged for 5 min at 13,000 x g and concentrations of AMB in the supernatants were determined by HPLC with a UV detector operating at 382 nm. The mobile phase consisted of a 0.1 M sodium acetate solution (pH 7.2) containing 0.2% (v/v) triethylamine and 60% (v/v) acetonitrile and was pumped through a Guard pre-column (Chromguard, 10 x 3 mm; Chrompack, Middelburg, The Netherlands) followed by a reverse-phase C18 column (Chromspher, 100 x 3 mm, Chrompack, Middelburg, The Netherlands) at a flow rate of 0.5 mL/min. The lower quantity of determination of this assay was 0.2 mg/L.

# Results

### Solute concentration during the process of nebulisation

Table 1 gives mean concentrations of AMB in the nebuliser reservoir before and after nebulisation of AMB-DOC and L-AMB. For L-AMB, total lipid concentration and AMB-lipid ratio ($\mu$g AMB/mmol total lipid) are given. After 60 min of nebulisation, concentrations of AMB in the reservoir were increased with both AMB-DOC and L-AMB. Increase in concentration of AMB with nebulisation of AMB-DOC was more pronounced than with L-AMB ($P = 0.006$). Total lipid concentration in the L-AMB suspension increased linearly with concentration of AMB in L-AMB (Table 1). As a result, AMB-lipid ratio of the liposomal suspension did not change during nebulisation.

### Stability of liposomes to nebulisation

Diameters of liposomes in the L-AMB suspension before nebulisation, in the remnant volume in the reservoir after nebulisation, and of the inhalation chamber sample taken during 60 min of nebulisation are shown in Table 2. A significant increase in mean liposomal size was observed after nebulisation in the residue suspension after nebulisation ($P = 0.004$) as well as in the inhalation chamber sample ($P = 0.013$) as compared to liposomal diameter of the suspension to be nebulised.

### Volume and mass output

The nebulised volumes of glucose 5%, AMB-DOC or L-AMB were similar during a nebulisation period of 60 min (Table 3). Mass output in terms of mg AMB was $35.40 \pm 6.20$ mg for AMB-DOC and $57.13 \pm 10.17$ mg for L-AMB. AMB output after nebulisation of AMB-DOC was significantly lower than after nebulisation of L-AMB (P - 0.031).

Table 1. Amphotericin concentration, total lipid concentration and amphotericin/lipid ratio in the reservoir suspension before and after 60-min nebulisation of AMB-DOC and L-AMB.

| | AMB-DOC AMB conc (mg mL$^{-1}$) | L-AMB AMB conc (mg mL$^{-1}$) | Total lipid ($\mu$mol mL$^{-1}$) | Amphotericin/lipid ($\mu$g mmol$^{-1}$) |
|---|---|---|---|---|
| Before nebulisation | 4.1 ± 0.6[a] | 4.1 ± 0.2 | 38.6 ± 2.2 | 104.1 ± 9.0 |
| After nebulisation | 12.9 ± 1.0 | 8.4 ± 2.2 | 81.6 ± 13.9 | 106.5 ± 11.6 |
| Ratio $conc_{after}$/ $conc_{before}$ | 3.1 ± 0.2[b] | 2.1 ± 0.6 | 2.3 ± 0.4 | 1.0 ± 0.0 |

[a]Each value represents the mean±s.d. of three individual experiments.

[b]$P$=0.006 vs AmBisome.

Table 2. Liposomal diameters of L-AMB before and after nebulisation in the nebuliser reservoir and in the inhalation chamber sample.

| | Liposomal diameter (nm) |
|---|---|
| Before nebulisation | 74.0 ± 6.1[a,b] |
| After nebulisation | 84.1 ± 5.2 |
| Inhalation chamber sample | 86.1 ± 1.4 |

[a]Each value represents the mean±s.d. of at the least three individual experiments.

[b]P=0.004 and 0.013 vs liposomal diameter after nebulisation and of the chamber sample, respectively.

Table 3. Volume output and mass out[put of amphotericin after 60 min nebulisation of AMB-DOC and L-AMB.

| | Glucose (5%) | Fungizone (4 mg mL$^{-1}$) | AmBisome (4 mg mL$^{-1}$) |
|---|---|---|---|
| Volume (mL) | 23.7 ± 1.0[a] | 23.4 ± 1.2 | 23.2 ± 1.3 |
| Mass (mg amphotericin) | - | 35.4 ± 6.2[b] | 57.1 ± 10.2 |

[a]Each value represents the mean±s.d. of three individual experiments.

[b]P=0.031 vs AmBisome.

Aerosol concentration measurement

Aerosol concentrations measured at the breathing-points of animals were the same for AMB-DOC and L-AMB per liter of collected air over one hour period (24.53 ± 4.93 and 23.75 ± 2.97 μg/L, respectively).

Aerosol size distribution

Figure 3 shows the droplet size distribution of aerosol droplets for AMB-DOC, L-AMB and for 5% glucose in water. A similar homogenous distribution for all formulations was observed (median droplet size for AMB-DOC 0.743 μm and 0.758 μm for L-AMB). Figure 4 shows the calculated cumulative mass distribution. Percentages of particles <5 μm mass diameter were 83.20 and 86.60 for nebulised AMB-DOC and L-AMB, respectively. Derived mean mass diameter (MMD) and geometric standard deviation (GSD) for nebulised AMB-DOC were 1.38 μm and 2.26 μm and 2.43 μm and 1.97 μm for L-AMB.

*In vivo* deposition of $^{67}$Ga labelled liposomes

Distribution of delivered dose in relevant organs and tissues after 60 min nebulisation calculated as percentage of the delivered dose is shown in Table 4. The largest part was retrieved from the intestines (26%). 2% and 4% of the delivered dose was retrieved from the left lung and right lung, respectively. Less than 1% was observed in blood, kidneys, liver and spleen. During the 60 min nebulisation period, linear accumulation of radioactivity in the lungs of animals was observed (Figure 5).

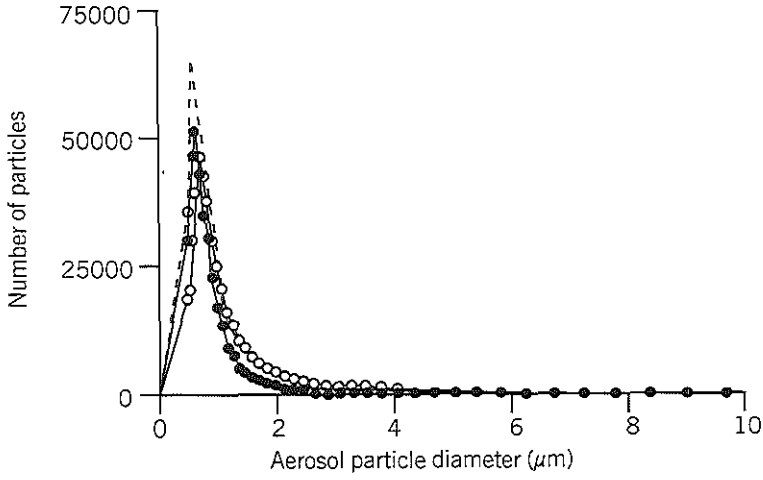



Figure 3. Aerosol particle number distribution of aerosolised AMB-DOC (•), L-AMB (o) and 5% glucose in water (---) as determined by laser diffraction.

Table 4. Recovery of radioactivity (% of delivered dose) in different organs and tissues of rats after aerosolisation (60 min) of $^{67}$Ga-labelled liposomes.

| Organ/tissue | % deposition |
|---|---|
| Left lung | 2.06 ± 0.22 |
| Right lung | 3.84 ± 0.58 |
| Trachea | 1.40 ± 0.25 |
| Tongue | 1.06 ± 0.14 |
| Lower jaw | 1.37 ± 0.82 |
| Snout | 13.36 ± 1.42 |
| Oral cavity | 0.32 ± 0.23 |
| Stomach/oesophagus | 2.69 ± 0.01 |
| Intestines | 25.30 ± 3.82 |
| Liver | 0.27 ± 0.10 |
| Spleen | 0.01 ± 0.00 |
| Kidney | 0.31 ± 0.08 |
| Blood | 0.03 ± 0.02 |

Each value represents the mean±s.d. of three individual experiments.

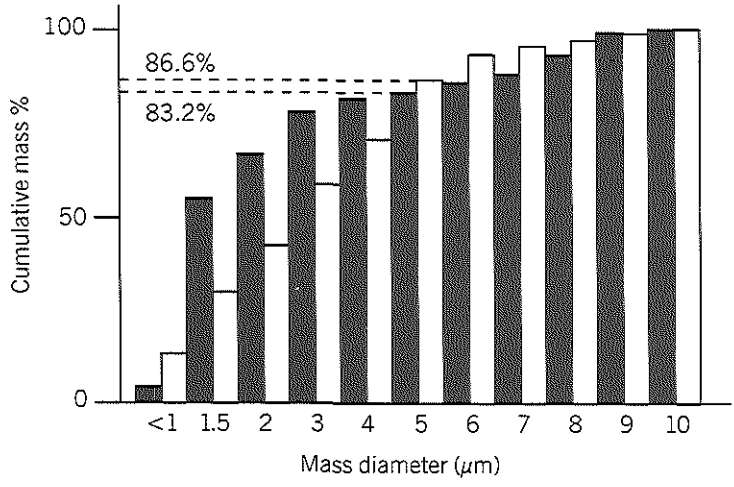



Figure 4. Cumulative mass distribution of aerosol droplets after aerosolisation of AMB-DOC (■) or L-AMB (□).

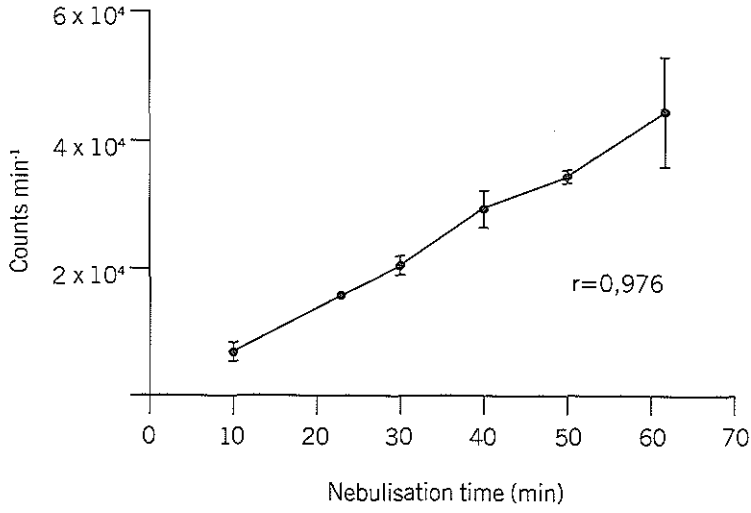



Figure 5. Radioactivity in lungs of rats (n=2) at different times during aerosolisation (60 min) of $^{67}$Ga-labelled liposomes. Each point represents the mean and range.

Deposition of AMB in lungs after nebulisation of AMB-DOC and L-AMB

Table 5 shows the absolute amount of AMB (μg) and the concentration of AMB (μg AMB/g tissue) retrieved from both left and right lung lobes of rats treated with aerosols of AMB-DOC and L-AMB. Both the absolute deposited dose of AMB in μg and the concentration of AMB in lung tissue reached directly after nebulisation were similar for AMB-DOC and L-AMB. Furthermore, concentrations of AMB were similar in both lung lobes in all animals.

Table 5. Amount and concentration of amphotericin B in left and right lungs of rats after 60-min nebulisation of AMB-DOC or L-AMB.

| Formulation | Left lung | | Right lung | |
|---|---|---|---|---|
| | AMB (μg) | AMB concn (μg g$^{-1}$) | AMB (μg) | AMB concn (μg g$^{-1}$) |
| AMB-DOC | 15.3 ± 1.1 | 24.2 ± 6.4 | 21.5 ± 0.2 | 19.9 ± 4.8 |
| L-AMB | 12.5 ± 2.5 | 21.7 ± 2.6 | 20.8 ± 3.8 | 21.6 ± 0.9 |

Each value represents the mean±s.d. of three individual experiments.

# Discussion

Aerosol administration of non-liposomal as well as liposomal AMB in treatment or prophylaxis of fungal infections was reported by others and appeared to be promising [12-17]. With respect to future studies on the efficacy of AMB products in the treatment of invasive pulmonary aspergillosis in a rat model, we describe in this study the nebulisation of non-liposomal (AMB-DOC) as well as liposomal AMB (L-AMB) with a Collison nebuliser.

Before assessing efficacy of nebulised products in an experimental model of pulmonary aspergillosis, it is important to gain insight in several aspects of nebuliser function, since parameters like design, operating conditions and ancillary equipment are pivotal for final delivered dose and product stability [18,19]. The Collison nebuliser has been widely used in aerosol research for many years and is documented in detail [20,21].

Volume output of the Collison nebuliser under the experimental conditions used was the same for both AMB preparations examined. For both AMB-DOC and L-AMB, concentration of AMB in the nebuliser reservoir occurred during the nebulisation process. The phenomenon of concentration of nebulised solution or dispersed material was previously described as a consequence of evaporative loss of water needed for humidification of inflowing air [22,23]. This indicates that determinations of mass output should not be calculated from initial reservoir concentrations. Increase in AMB concentration after nebulisation of AMB-DOC was higher as compared to L-AMB, which was directly correlated with a lower mass output of AMB for nebulised AMB-DOC. Previous work by Sorensen et al. showed that inhalation chamber air AMB concentrations during nebulisation of AMB-DOC decreased as a function of time, which could explain the difference in aerosol concentration between AMB-DOC and L-AMB. Foaming of AMB-DOC due to presence of the detergent desoxycholate has been given as an explanation for this phenomenon [24].

It is an option to calculate AMB aerosol concentrations from solute concentrations before and after nebulisation (mass output). However, without considering inertial impaction and gravimentational sedimentation of aerosol droplets inside the aerosol inhalation system this method results in an inaccurate (overestimation) of delivered dose. More precise estimations of AMB output are made by direct measuring of aerosol concentrations. Despite the observed differences in mass outputs of AMB, we have seen a similar measured aerosol concentration for nebulised AMB-DOC and nebulised L-AMB. This is in correspondence with others [13]. AMB-lipid ratio of L-AMB was not influenced by nebulisation. It can be concluded

from this that AMB remains associated with the liposomal membrane during nebulisation. Liposomal size was, however, affected by the nebulisation process. The mean liposome size increased slightly during 60 min nebulisation, probably because of fusion of a small population of vesicles as result of air pressure upon passage through the nebuliser orifices. This size increase was however small, which indicates that liposomal integrity was hardly influenced. A formulation with similar lipid composition and liposomal size as L-AMB (26 mol% cholesterol) has shown to be relatively stable to nebulisation [25,26]. In addition, an increase in liposomal size will not influence deposition pattern of nebulised L-AMB, since liposomes are still several-fold smaller than average aerosol droplet size.

Droplet size and shape play a significant role in the drug deposition in the respiratory passageways. Preferably, more than 90% of the generated droplet population should be in the 0.5 to 10 $\mu$m range to maximize delivery in pulmonary tissues and fluid. In general, droplets of 1 to 5 $\mu$m are considered optimal for penetrating the lower respiratory tract [27,28]. Particles of this size have been demonstrated to deposit in the lung via gravimentational sedimentation, inertial impaction or by diffusion into terminal alveoli by Brownian motion [29].

Aerosol droplet sizing by laser based analysis offers a few advantages over the impaction approach frequently used to characterise aerosol droplets. The nebuliser cloud can be measured directly after leaving the nebuliser reservoir, and therefore information is collected on the 'true' droplet size which enters the respiratory tract of laboratory animals. Moreover, this technique is much faster than the impaction technique and no chemical analysis of solute is required. Laser diffraction has proven to be robust and reliable for measuring droplet sizes directly after nebulisation [30]. Some care must however be taken with interpreting number distribution results from laser scattering data. It should be kept in mind that with using this method, extrapolation of number distribution to mass distribution must be made in order to predict the final mass deposition in pulmonary regions of interest. For this, the specific gravity of droplets should be known.

As seen from the number distribution, a very homogenous aerosol cloud was generated by the Collison nebuliser under the experimental conditions of this research. A high number (>80%) of droplets with both AMB-DOC and L-AMB was below 5 mm mass diameter. Therefore, a substantial deposition in the alveobronchial region can be expected.

In order to evaluate the biodistribution of nebulised materials in rats, we have used $^{67}$Ga-labelled liposomes. We chose this indirect measure of biodistribution since radiolabelled L-AMB is not available and analysis of AMB in all organs and tissues is difficult to measure accurately. The radiolabelled liposomes however, had a lipo-

some composition similar to L-AMB and results can therefore directly be correlated to the biodistribution of nebulised L-AMB. The percentage deposition was calculated on the basis of the aerosol concentration. Total *in vivo* counts only accounted for 52% of the calculated delivered dose. Two important factors can attribute to the relatively low *in vivo* counts compared to the calculated delivered dose. Impaction and subsequent loss of nebulised material inside the system is not taken into account. Furthermore, the exhaled fraction of aerosol clouds can add up to loss of material. Highest deposition of radioactivity was seen in the gastrointestinal tract (28.0%), due to deposition of inhaled particles inside the oral region and on the snout. Swallowing of deposited material will lead to high intestinal drug levels. Gastrointestinal absorption of AMB is however very small (<5%) and biodistribution through this route will thus not lead to significant systemic drug levels. Deposition of radioactivity in lungs accumulated linearly in time and was 11.3% of total *in vivo* counts. Very little (<1%) radioactivity was found in the internal organs.

As could be expected from the similar aerosol concentrations measured, AMB concentrations reached in lung tissues of treated rats directly after 60 min of nebulisation were similar with AMB-DOC and L-AMB. For *Aspergillus fumigatus*, the pathogen of interest in our animal model, the *in vitro* minimal inhibitory concentration is 0.4 - 0.8 mg/L. Final concentrations of AMB in pulmonary tissues reached with both formulations should be sufficient for *in vivo* killing. The results of this study were obtained in healthy rats. Whether deposition in diseased lungs will be equivalent remains to be examined.

In summary, we have reported on the nebulisation of AMB-DOCl and L- AMB with a Collison nebulizer. AMB-DOC is less efficiently nebulised than L-AMB, when mass output of AMB is considered. However, aerosol concentration of AMB is similar with the two products. The delivered dose of AMB can therefore be considered the same. Aerosols of AMB-DOC and L-AMB are highly respirable and result in sufficient lung tissue concentrations of AMB. Distribution of inhaled AMB to other organs than the lungs or the gastro-intestinal tract is expected to be negligible. Furthermore, liposomes containing AMB are physically stable to nebulisation.

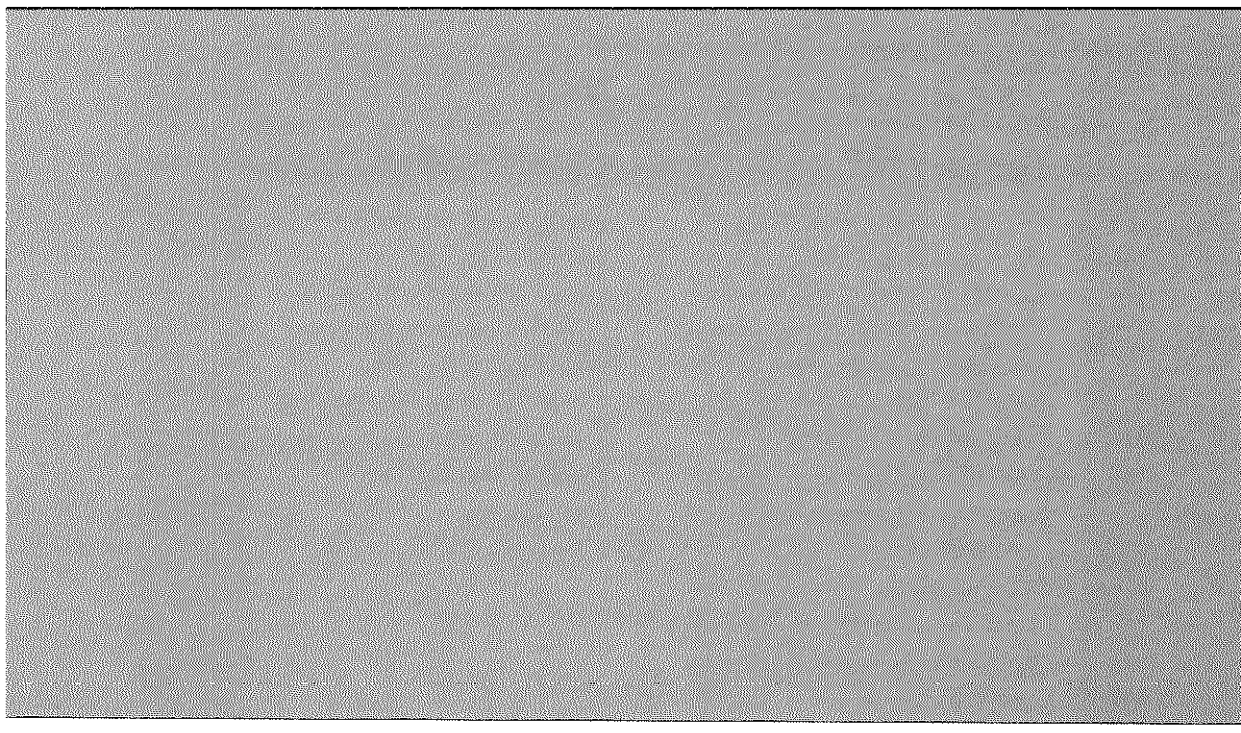
It appears that aerosol administration of AMB formulations is a promising approach to target the lungs and may offer a valuable strategy in the treatment or prevention of invasive pulmonary aspergillosis.

# References


1. Denning, DW. Invasive aspergillosis. Clin Infect Dis 1998;26:781-803.
2. Gallis, HA, Drew, RH, Pickard, WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-329.
3. Hiemenz JW, Walsh TJ. Lipid formulations of amphotericin B: recent progress and future directions. Clin Infect Dis 1996;22:S133-144.
4. Christiansen, KJ, Bernard, EM, Gold, JWM, Armstrong, D. Distribution and activity of amphotericin B in humans. J Infect Dis 1985;152:1037-1043.
5. Janknegt, R, de Marie, S, Bakker-Woudenberg, IAJM, Crommelin, DJA. Liposomal and lipid formulations of amphotericin B - Clinical pharmacokinetics. Clin Pharmacokinet 1992;23:279-291.
6. Lambros, MP, Bourne, DWA, Abbas, SA, Johnson, DL. Disposition of aerosolized liposomal amphotericin B. J Pharm Sci 1997;86:1066-1069.
7. Ramsey, BW, Pepe, MS, Quan, JM, et al. Intermittent administration of inhaled tobramycin in patients with cystic fibrosis. Cystic Fibrosis Inhaled Tobramycin Study Group. N Engl J Med 1999;340:23-30.
8. Chong, CS, Colbow, K. Light scattering and turbidity measurements on lipid vesicles. Biochim Biophys Acta 1976;436:260-82
9. Bartlett, GR. Phosphorus assay in column chromatography. J Biol Chem 1959;234:466-468.
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 Lip Res 1988;1:123-125.
11. Guyton, AC. Measurement of the respiratory volumes of laboratory animals. Am J Physiol 1947;150:70-77.
12. Kilburn, KH. The innocuousness and possible therapeutic use of aerosol amphotericin B. Am Rev Resp Dis 1959;80:441-442.
13. Allen, SD, Sorensen, KN, Nejdl, MJ, Durrant, C, Proffit, RT. Prophylactic efficacy of aerosolized liposomal (L-AMB) and non-liposomal (AMB-DOC) amphotericin B in murine pulmonary aspergillosis. J Antimicrob Chemother 1994;34:1001-1013.
14. Beyer, J, Schwartz, S, Barzen, G et al. Use of amphotericin B aerosols for the prevention of pulmonary aspergillosis. Infection 1994;22:143-148.
15. Hertenstein, B, Kern, WV, Schmeiser, T et al. Low incidence of invasive fungal infections after bone marrow transplantation in patients receiving amphotericin B inhalations during neutropenia. Ann Hematol 1994;68:21-26.
16. Reichenspurner, H, Gamberg, P, Nitschke, M, Valantine, H, Hunt, S, Oyer, PE, Reitz, BA. Significant reduction in the number of fungal infections after lung-, heart-lung, and heart transplantation using aerosolized amphotericin B prophylaxis. Transplant Proc 1997;29:627-628.
17. Trigg, ME, Morgan, D, Burns, TL, et al. Successful program to prevent *aspergillus* infections in children undergoing marrow transplantation: use of nasal amphotericin. Bone Marrow Transplant 1997;19:43-47.
18. Niven, RW, Carvajal, TM, Schreier, H. Nebulization of liposomes. III. The effects of

operating conditions and local environment. Pharm Res 1992;9:515-520.

19. Waldrep, JC, Keyhani, K, Black, M, Knight, V. Operating characteristics of 18 different continuous-flow jet nebulizers with beclomethasone dipropionate liposome aerosol. Chest 1994;105:106-110.
20. May, KM. The Collison nebulizer: description, performance and application. Aeros Sci 1973;4:235-243.
21. Young, HW, Dominik, JW, Walker, JS, Larson, EW. Continuous aerosol therapy system using a modified Collison nebulizer. J Clin Microbiol 1977;5:131-136.
22. Ip, AY, Niven, RW. Prediction and experimental determination of solute output from a Collison nebulizer. J Pharm Sci 1994;83:1047-1051.
23. Roth, C, Gebhart, J, Just-Nubling, G, Von Eisenhart-Rothe, B, Beinhauer-Reeb, I. Characterization of amphotericin B aerosols for inhalation treatment of pulmonary aspergillosis. Infection 1996;24:354-360.
24. Sorensen, KN, Allen, SD, Nejdl, MJ, Proffit, RT. Aerosolization of liposomal (L-AMB) and non-liposomal (AMB-DOC) amphotericin B as treatment for pulmonary fungal infections. J Control Release 1994;28:345-347.
25. Niven, RW, Schreier, H. Nebulization of liposomes. I. Effects of lipid composition. Pharm Res 1990;7:1127-1133.
26. Niven, RW, Speer, M, Schreier, H. Nebulization of liposomes. II. The effects of size and modeling of solute release profiles. Pharm Res 1991;8:217-221.
27. Newman, SP, Clarke, SW. Therapeutic aerosols 1 - Physical and practical considerations. Thorax 1983;38:881-886.
28. Barry, PW, O'Callaghan, C. Inhalational drug delivery from seven different spacer devices. Thorax 1996;51:835-840.
29. Hickey, A. J. (1996) Inhalation Aerosols - Physical and biological basis for therapy. Marcel Dekker, Inc., New York.
30. Clark, AR. The use of laser diffraction for the evaluation of the aerosol clouds generated by medical nebulizers. Int J Pharm 1995;115:69-78.



Chapter 4

# Pulmonary kinetics of nebulised liposomal and non-liposomal amphotericin B in healthy rats and in rats with invasive pulmonary aspergillosis

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# Abstract

The pulmonary kinetics of amphotericin B (AMB) after aerosol administration of amphotericin B desoxycholate (AMB-DOC) and liposomal amphotericin B (L-AMB) were studied in rats. Rats were treated with a single dose of nebulised AMB-DOC or L-AMB. At different times after aerosol treatment, AMB was measured in lungs, broncho-alveolar lavage fluid, blood, liver, spleen and kidneys. Pulmonary deposition of AMB after aerosol treatment was compared with that after treatment with a single dose of 10 mg/kg intravenous L-AMB. Influence of infection on aerosol deposition was determined by analysis of AMB in lungs of rats suffering from pulmonary aspergillosis, as well as by histopathological examination of infected lungs after aerosol treatment with colloidal-gold labelled liposomes. Pulmonary tissue concentrations of AMB directly after aerosol treatment were 26.9 (± 8.5) $\mu$g/g and 46.7 (± 10.5) $\mu$g/g for AMB-DOC and L-AMB, respectively. AMB was retained in the lungs for more than 5 weeks for both formulations. Pulmonary concentrations after a single intravenous dose of 10 mg/kg L-AMB were substantially lower than that after aerosol administration, and AMB was not retained in the lungs after intravenous administration. After nebulisation, no AMB was detected in blood or other organs than the lungs. The amount of deposited nebulised AMB in infected lungs did not significantly differ from that in uninfected lungs. Histopathological examination revealed that nebulised liposomes deposited in the alveoli, close to the fungal mycelium. In conclusion, the pulmonary kinetics of nebulised AMB-DOC and L-AMB are favourable and underscore the possible value of aerosol AMB products for the treatment or the prophylaxis of pulmonary aspergillosis.

# Introduction

Amphotericin B (AMB) is a broad spectrum polyene antifungal agent which is used intravenously for the treatment of most serious fungal infections such as pulmonary aspergillosis. Unfortunately, conventional AMB, amphotericin B desoxycholate (AMB-DOC), has considerable toxicity which can be dose-limiting [1]. Liposome encapsulated AMB (L-AMB) has an increased therapeutic index as compared to that of conventional AMB [2]. However, treatment failure after intravenous administration of L-AMB is still frequently observed in patients with invasive pulmonary aspergillosis [3].
Administration of AMB via the inhalational route might be an attractive alternative approach in the management of pulmonary fungal disease, since aerosol delivery directly targets the lungs and systemic AMB exposure is reduced. Nebulised AMB-DOC or L-AMB has proven to be valuable in the treatment or prophylaxis of fungal pulmonary infections in different animal models [1-7]. Several clinical studies have been performed with nebulised AMB-DOC for prophylaxis of IPA [8-10]. Although deposition of nebulised (liposomal) AMB has been studied in animals little is known about the exact pulmonary deposition and kinetics of AMB after nebulisation [11,12]. Thus, it is not clear what dose or regimen should be used in the management of IPA.
AMB-DOC and L-AMB can both be efficiently nebulised [13]. Pulmonary deposition is dependent on type of nebuliser system and duration and frequency of nebuliser treatments [4,11,14]. Previous studies on nebulised AMB-DOC and nebulised AMB lipid complex show that pulmonary deposited AMB is cleared slowly from the lungs [11,14]. In the present study we have determined AMB concentrations in lungs and other organs after a single dose of nebulised AMB-DOC or L-AMB. We have determined the half-life of AMB in lungs after this single dose, and alveolar deposition by means of histology. In addition, we have determined the influence of infection on the deposition of AMB after aerosol treatment with AMB-DOC and L-AMB.
With this work, we collect support for the profitable pulmonary deposition and retention of AMB after aerosol administration as opposed to that after intravenous administration.

# Materials and Methods

## Materials

AMB-DOC was from Bristol Myers-Squibb (Woerden, The Netherlands) and L-AMB was from NeXstar (San Dimas, CA). Cyclophosphamide, cholesterol and $AuCl_2$ were from Sigma Chemical Co. (St. Louis, MO). Hydrogenated soy phosphatidylcholine (HSPC) and distearoyl phosphatidylcholine (DSPG) were

from Avanti Polar Lipids, Inc. (Alabaster, AL). All other reagents were of analytical grade.

### Animals

Female R-strain albino rats, specified pathogen free, 18- to 25- weeks old (own breed), weighing 185-225 g were used for all experiments. Animals received a normal, pathogen free diet and water *ad libitum*. Experiments were approved by the animal experiments ethical committee of the Erasmus *university* Medical Center.

### Experimental lung infection

Infection of the lung was established according to the method described by Leenders et al. [15]. Granulocytopenia was induced by intraperitoneal administration of 90 mg/kg cyclophosphamide at 5 days before fungal inoculation followed by an additional dose of 60 mg/kg at 1 day before inoculation. Conidia were collected from a clinical isolate of *Aspergillus fumigatus* (MIC 0.4 mg/l). Under general anaesthesia the left main bronchus was intubated. A canula was passed through the tube and the left lung was inoculated with $1.5 \times 10^5$ conidia, resulting in a left sided pneumonia.

### Aerosol administration of antifungal agents

AMB-DOC and L-AMB were reconstituted according to the manufacturer's instructions and further diluted in 5% glucose up to an AMB concentration in the nebuliser reservoir of 2 mg/ml in the case of AMB-DOC and 4 mg/ml in the case of L-AMB. Previous work in our laboratory showed that these concentrations are the optimal concentrations for effective treatment of rats suffering from pulmonary aspergillosis [6]. The nebulisation procedure was described previously [16]. In short: aerosols were generated by a Collison six-jet nebuliser system (Model CN, BGI Inc., Waltham, MA). The nebuliser operated at 20 l/min air flow. Under these conditions, more than 80% of generated aerosol droplets are below 5 $\mu$m, which ensures substantial deposition of material in the alveobronchial region. Rats were exposed to aerosols in a nose-only inhalation system (CH Technologies USA Inc., Westwood, NJ). Animals were exposed to aerosol treatment for a period of 60 minutes. In the case of aerosol treatment of infected rats, treatment was started at 30 h after fungal inoculation.

### Intravenous administration of L-AMB

To compare AMB lung deposition after aerosol and intravenous treatment, L-AMB was reconstituted according to the manufacturer's instructions and further diluted in 5% glucose. Uninfected rats received a dose of 1 x 10 mg/kg intravenous L-AMB.

Concentration of AMB in organs and blood

Animals were treated with nebulised or intravenous AMB-DOC or L-AMB. At different time points after nebulisation, groups of 3 animals were euthanised with intravenous pentobarbital (100 mg/kg). Directly after, blood was sampled via cardiac punction, and lungs, trachea, liver, kidneys and spleen were dissected. Broncho-alveolar lavage (BAL) was performed by washing the lungs via the trachea with 8 ml glucose 5%. Subsequently, the lungs were divided in left and right lung lobe. Organs were weighed and homogenised in 5 ml glucose 5%. AMB was extracted from the homogenate, blood and BAL-fluid with ethanol in a 2:3 (v/v) ratio. The extracts were centrifuged for 5 min at 13,000 x g and concentrations of AMB in the supernatants were determined by HPLC with an UV detector operating at 382 nm. The mobile phase consisted of a 0.1 M sodium acetate solution (pH 7.2) containing 0.2% (v/v) triethylamine and 60% acetonitrile and was pumped through a Guard pre-column (Chromguard, 10 x 3 mm; Chrompack, Middelburg, The Netherlands) followed by a reverse-phase C18 column (100 x 3 mm, Chrompack, Middelburg, The Netherlands) at a flow rate of 0.5 ml/min. The lower limit of quantification of this assay was 0.2 mg/ml.

Determination of half-life of AMB in pulmonary tissue

Pulmonary tissue concentration-data were analysed by a non-compartmental curve-fitting program (TopFit version 2.0, Gustav Fischer, Germany).

Deposition of colloidal gold-labelled liposomes in infected lung tissue

Colloidal-gold labelled liposomes were prepared as described previously by Daemen et al. [17]. Briefly, a lipid film containing HSPC, DSPG and cholesterol, was prepared by film hydration. The lipid film consisted of the same lipids as the liposomal membrane of L-AMB, in the same molar ratio (HSPC:DSPG:cholesterol=2:1:0.8). The lipid film was hydrated at 4°C with a sodium citrate-potassium carbonate buffer containing 0.3% (w/v) $AuCl_2$. Liposomes were prepared by the multiple extrusion of the hydrated lipids through two stacked 100 nm membranes (Nuclepore, Pleasanton, CA). Unencapsulated colloidal gold was removed by gelfiltration of the liposomes over a Sephacryl SF S1000 column (Pharmacia, Uppsala, Sweden) using Hepes/NaCl buffer as the eluens. This method yielded liposomes with an average particle size of 100 nm.

Colloidal-gold labelled liposomes were nebulised at 30 h after fungal inoculation. The nebuliser reservoir lipid concentration was similar to that used for nebulising L-AMB. Directly after nebulisation, left and right lung lobes were excised, washed in 2.5% buffered formaldehyde, fixed in 10% buffered formaldehyde and embedded in paraffin. Five $\mu$m sections were cut longitudinally onto a microtome and

mounted on slides. Sections were deparaffinised with xylene and hydrated. Colloidal gold was silver-enhanced using a silver enhancement-kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Adjacent sections were counterstained with either eosin/haematoxylin or Grocott methenamine stain.

# Results

Pulmonary deposition and clearance of AMB

The pulmonary concentration-time profiles of AMB in uninfected lungs for nebulised AMB-DOC (nebuliser reservoir concentration 2 mg/ml) and nebulised L-AMB (nebuliser reservoir concentration 4 mg/ml) are shown in Figure 1. The concentration AMB in lung tissue directly after nebulisation (C0) was 26.9 (± 8.5) mg/g for AMB-DOC and 46.7 (± 10.5) mg/g for L-AMB. AMB was cleared very slowly from the lungs for both AMB-DOC as for L-AMB ($t_{1/2\beta}$ = 33.7 days and 41.6 days, respectively). AMB was detectable in lung tissue up to 5 weeks after nebulising either formulation. $C_0$ is also shown in Table 1, together with the percentage of AMB thereof

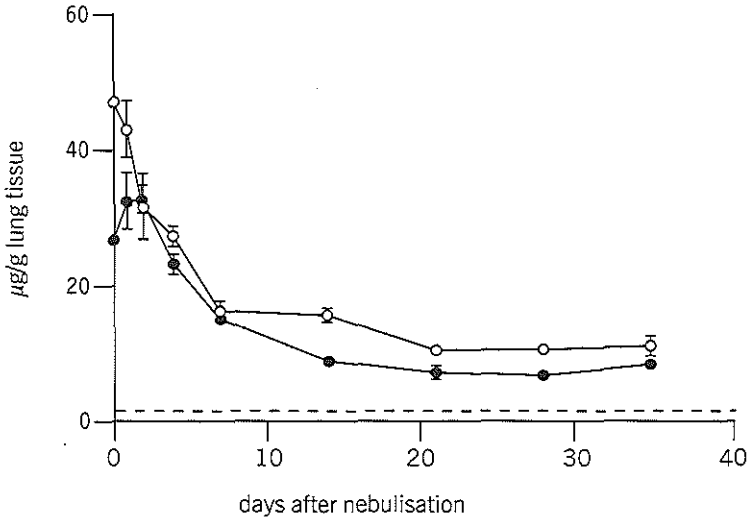



Figure 1. Concentration - time profile of nebulised AMB-DOC (●) and L-AMB (○) in lungs of healthy rats, as determined by HPLC. $t_{1/2\beta}$ is 33.7 days for AMB-DOC and 41.6 days for L-AMB, respectively. Each value plotted is the mean ± SD of three rats. ---- is the lower limit of quantification of AMB.

which was recovered in BAL. The percentage AMB found in BAL after nebulisation of AMB-DOC was higher than that found after nebulisation of L-AMB, which indicates that the liposomal formulation was more rapidly taken up by the lung tissue. Table 2 shows that nebulising AMB-DOC or L-AMB yielded higher AMB concentrations than intravenous administration of 1 x 10 mg/kg of L-AMB. At 48 h after intravenous administration, no AMB was retained in the lungs. Furthermore, no AMB was detected in blood or other organs than lungs after aerosol administration of either AMB-DOC or L-AMB, whereas intravenous administration of L-AMB obviously resulted in systemic exposure of AMB (Table 2).

Table 1. Pulmonary AMB concentrations ($C_0$) directly after aerosol administration of AMB-DOC and L-AMB (nebuliser reservoir AMB concentration 2 and 4 mg/ml, respectively) and % of deposited AMB in broncho-alveolar lavage (BAL)

| | μg AMB/g lungs ($C_0$)[a] | % of AMB in BAL |
|---|---|---|
| AMB-DOC | 26.9 (8.5)[b] | 35.7 (13.0) |
| L-AMB | 46.7 (10.5) | 16.1 (8.7) |

[a]concentrations in lung tissue including bronchoalveolar lavage

[b]values are mean (SD), n=3

Table 2. Deposition of AMB in lungs (μg/g) and blood (μg/ml) directly after (0h), at 24 h and at 48 h after aerosol administration of AMB- DOC and L-AMB and after intravenous administration of 1 x 10 mg/kg L-AMB

| | aerosol AMB-DOC | | | aerosol L-AMB | | | intravenous L-AMB | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 h | 24 h | 48 h | 0 h | 24 h | 48 h | 0 h | 24 h | 48 h |
| lungs[a] | 26.9 (8.5)[b] | 32.4 (8.4) | 32.6 (4.7) | 46.7 (10.5) | 42.8 (10.8) | 31.8 (15.0) | 16.4 (2.4) | nd | nd |
| blood | nd[c] | nd | nd | nd | nd | nd | 48.4 (2.4) | nd | nd |

[a]concentrations in lung tissue including bronchoalveolar lavage

[b]values are mean (SD), n=3

[c]nd is not detectable

Table 3. Pulmonary concentrations of AMB ($C_0$) directly after aerosol administration of AMB-DOC and L-AMB (nebuliser reservoir concentration 2 and 4 mg/ml, respectively) in lungs of uninfected rats and in infected left lung lobes of rats with pulmonary aspergillosis

| | μg AMB/g lungs[a] uninfected lungs | μg AMB/g lungs infected lungs |
|---|---|---|
| AMB-DOC | 26.9 (8.5)[b] | 29.4 (4.2) |
| L-AMB | 46.7 (10.5) | 36.7 (5.9) |

[a]concentrations in lung tissue including bronchoalveolar lavage

[b]values are mean (SD), n=3

Deposition of AMB in infected lungs

Table 3 shows the concentrations of AMB deposited directly after nebulisation of AMB-DOC and L-AMB in infected lungs versus deposition in uninfected lungs, as measured by HPLC. AMB deposition in infected lungs did not differ statistically significant from that in uninfected lungs.

Deposition of colloidal-gold labelled liposomes in infected lungs

Localisation of liposomes in infected left lung lobes was visualised using silver enhancement of nebulised colloidal gold-labelled liposomes (Figure 2). The black clusters are silver-enhanced colloidal-gold labelled liposomes. The liposomes are deposited at the pulmonary epithelium of bronchioles, but also in the alveolar regions of the lungs (Figure 2a). Liposomes deposited in the alveolar sacs and ducts are partially internalised by alveolar macrophages (Figure 2b). Grocott methenamine staining of a section of infected left lung lobes at 30 after inoculation of $1.5 \times 10^5$ *Aspergillus* conidia shows that radially grown hyphae are formed out of conidia (Figure 2c). Combined eosin/haematoxylin staining and silver enhancement of the adjacent section shows that nebulised liposomes are deposited close to the *Aspergillus* hyphae (Figure 2d). (See pages 82-83)

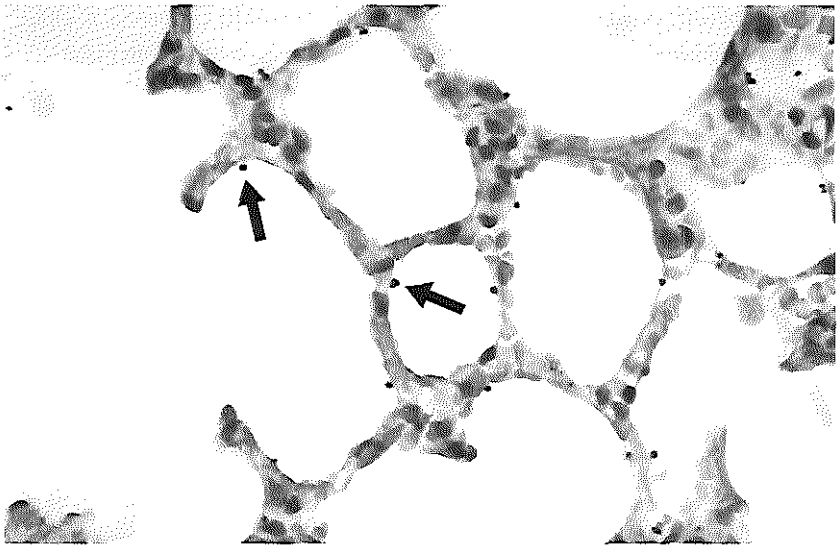
# Discussion

Pharmacokinetic evaluation of nebulised AMB-DOC or L-AMB is essential in order to rationalise aerosol treatment of invasive pulmonary aspergillosis with these agents. We have shown that using the nebuliser system we have described earlier, AMB-DOC as well as L-AMB can be efficiently nebulised in rats [6]. Pulmonary concentrations reached directly after a single dose of nebulised AMB-DOC and L-AMB were 26.9 μg/g and 46.7 μg/g, respectively. These concentrations exceed by far the MIC for *Aspergillus fumigatus* which is 0.4 - 0.8 mg/L for sensitive strains. The pulmonary AMB deposition after aerosol administration exceeded that found after intravenous administration of a single dose of 10 mg/kg L-AMB. After nebulising either AMB-DOC or L-AMB, pulmonary concentrations were retained in the lungs for a long period of time. The slow clearance of AMB from the lungs is probably the result of the fact that AMB binds extensively to tissues. At all times after aerosol treatment, no AMB was detected in blood or other organs than the lungs. This indicates that aerosol administration leads to low systemic exposure of AMB. The substantial pulmonary deposition as well as the extensive pulmonary retention of AMB renders the aerosol delivery of both AMB-DOC as L-AMB very suitable for prophylactic treatment of pulmonary aspergillosis.

The nebuliser reservoir concentration of AMB-DOC was half that of L-AMB. This explains differences in pulmonary concentrations reached directly after treatment. Previous work has shown that these concentrations are optimal to treat rats with severe invasive pulmonary aspergillosis [6]. Clearance of both differs little, indicating that lung retention is determined largely by AMB itself and for a smaller part by the liposomal formulation. Differences between AMB-DOC and L-AMB were seen in the AMB concentrations in the broncho-alveolar lavage fluid, which were relatively lower for the liposomal formulation. Apparently, L-AMB was more rapidly distributed into pulmonary tissue. Background or clinical implications of this finding are not clear.

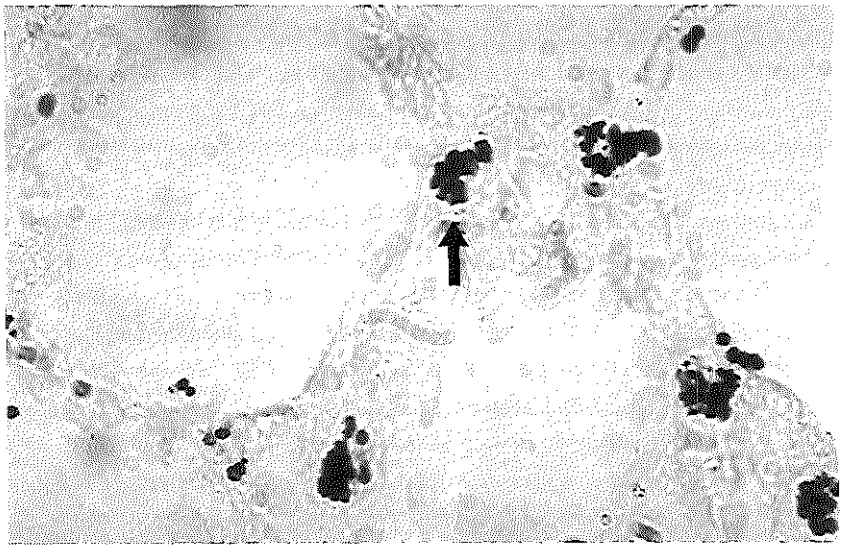
HPLC analysis of infected lung lobes revealed that deposition in infected, impaired lungs is similar to that in uninfected lungs. Aerosol administration can thus be regarded as an attractive treatment option in cases of established fungal infection. However, the method we have used to analyse AMB is a rather crude one, since the complete lung lobe is excised and healthy tissue and infected tissue is mixed. Therefore, we also did a localisation experiment of nebulised liposomes. Colloidal-gold labelled liposomes were utilised as histochemical markers of liposome tissue deposition and cellular uptake. Microscopic evaluation of silver-enhanced colloidal-gold labelled liposomes showed that nebulised liposomes

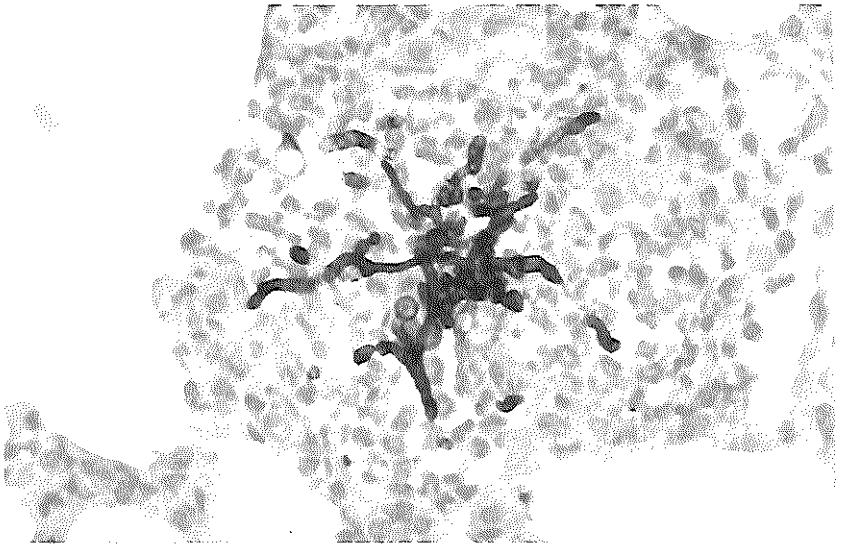
Figure 2. Localisation of nebulised colloidal-gold labelled liposomes in infected lung tissue



a. Localisation of silver-enhanced colloidal gold-labelled liposomes in alveoli is visualised as black dots (arrows). The liposomes are deposited at the alveolar epithelium. Alveolar epithelium consists of alveolar type I and type II epithelium cells which are identified as well as blood vessels. Counterstain haematoxylin/eosin.

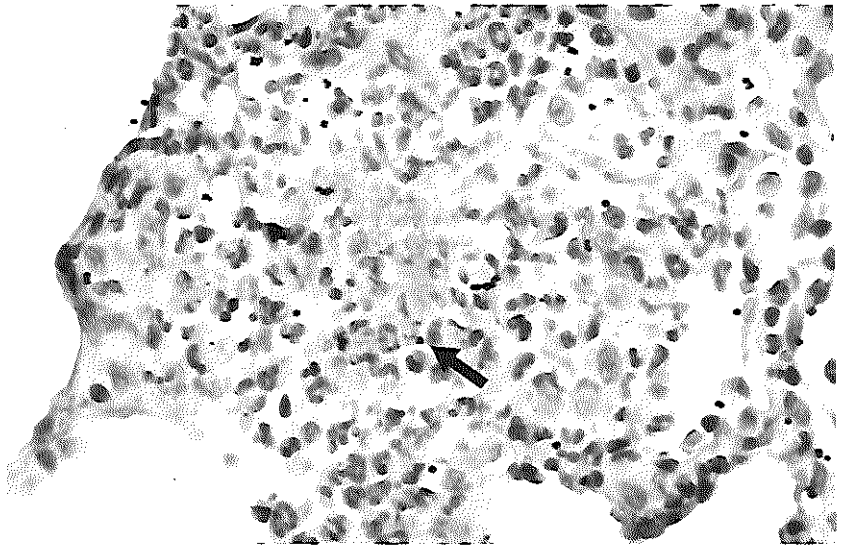
b. Liposomes deposited in the alveolar sacs and ducts are partially internalised by alveolar macrophages (black clusters indicated by arrows).





c. Grocott methenamine stain of pulmonary tissue at 30 h after inoculation of $1.5 \times 10^9$ *Aspergillus* conidia. The conidia are inoculated deep in the left lung lobe, internalised by pulmonary epithelial tissue and germinated into hyphae at this time.

d. Silver-enhanced colloidal-gold liposomes are deposited in and around pulmonary tissue (arrow). Counterstain haematoxylin/eosin. Leukocytes are identified as purple cells. Section adjacent to that shown in Figure 2c.



could be visualised in the infected lung. The prepared liposomes had the same lipid-bilayer as L-AMB, which was stable under the influence of nebulisation [13]. Therefore, we assumed that the colloidal-gold labelled liposomes could also withstand nebulisation. Schiffelers et al. [18] showed that with the described method, intact liposomes and uptake of liposomes by macrophages could be visualised. Liposomes were present throughout the whole respiratory tract, in the large bronchi, small bronchi-bronchioles, and alveolar ducts and sacs. The deposition pattern of colloidal-gold labelled liposomes indicated that nebulised L-AMB was able to penetrate deep in the lower respiratory tract, since the membrane characteristics and particle sizes of both liposomal products were similar. Our data show that the liposomal formulation reached the infected regions of the lung, was deposited close to the mycelium of the fungus and was furthermore partially incorporated by alveolar macrophages. Uptake of liposomes by alveolar macrophages is believed to be succeeded by release of the drug [15].

Unfortunately, we were not able to determine whether the deposited AMB was biologically active, because the agar plate diffusion assays that are employed to this purpose are, in our opinion, not suitable for reliable quantification of AMB activity. However, in other experiments we have seen that IPA can be prevented in part by nebulised AMB-DOC or L-AMB administered up to weeks before fungal inoculation (unpublished results). This observation might be seen as an indication for the biological prolonged activity of single-dose nebulised AMB.

Nebulising AMB-DOC or L-AMB did not result in systemic AMB levels. From the point of toxicity, this is a major advantage. However, *Aspergillus* usually disseminates from the lungs to other organs and pulmonary administered AMB will not be able to prevent this. It is our opinion that optimal therapy of an established infection of invasive pulmonary aspergillosis therefore combines the pulmonary route with intravenous administration of adequate dosages of AMB-DOC or L-AMB.

In summary, aerosol administration of AMB-DOC or L-AMB efficiently delivered AMB to healthy or infected lungs, while avoiding systemic exposure and accumulation of AMB in other organs. Absence of systemic exposure makes it highly unlikely that the aerosol route will cause extrapulmonary toxicity. AMB was eliminated very slowly from the lungs, with a half life of more than 30 days. The pharmacokinetic characteristics of nebulised AMB imply long term protection against IPA. The fact that AMB deposition was visualised in the consolidated regions of infected lungs, suggests that not only prophylaxis but also treatment of *Aspergillus* pneumonia, when combined with systemic administration of AMB, can benefit from aerosol treatment.

# References


1. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-29.
2. Hiemenz JW, Walsh TJ. Lipid formulations of amphotericin B: recent progress and future directions. Clin Infect Dis 1996;22:S133-144.
3. Denning DW. Invasive aspergillosis (review). Clin Infect Dis 1998;26:781-805.
4. Allen SD, Sorensen KN, Nedl MJ, Durrant C, Proffit RT. Prophylactic efficacy of aerosolised liposomal (AmBisome) and non-liposomal (Fungizone) amphotericin B in murine pulmonary aspergillosis. J Antimicrob Chemother 1994;34:1001-1013.
5. Gilbert BE, Wyde PR, Wilson SZ. Aerosolised liposomal amphotericin B for treatment of pulmonary and systemic *Cryptococcus neoformans* infections in mice. Antimicrob Agents Chemother 1992;36:1466-1471.
6. Ruijgrok EJ, Vulto AG, van Etten, EWM. Aerosol delivery of amphotericin B desoxycholate (Fungizone) and liposomal amphotericin B (AmBisome): aerosol characteristics and in-vivo amphotericin B deposition in rats. J Pharm Pharmacol 2000;52:619-627.
7. Schmitt HJ, Bernard EM, Häuser H, Armstrong D. Aerosol amphotericin B is effective for prophylaxis and therapy in a rat model of pulmonary aspergillosis. Antimicrob Agents Chemother 1988;32:1676-1679.
8. Erjavec Z, Woolthuis GM, de Vries - Hospers HG, et al. Tolerance and efficacy of amphotericin B inhalations for prevention of invasive pulmonary aspergillosis in haematological patients. Eur J Clin Microbiol Infect Dis 1997;16:364-8.
9. Hertenstein BW,Kern V, Schmeiser T, et al. Low incidence of invasive fungal infections after bone marrow transplantation in patients receiving amphotericin B inhalations during neutropenia. Ann Hematol 1994;68:21-6.
10. Schwartz S, Behre G, Heinemann H. Aerosolized amphotericin B inhalations as prophylaxis of invasive aspergillus infections during prolonged neutropenia: results of a prospective randomized multicenter trial. Blood 1999;93:3654-3661.
11. Niki Y, Bernard EM, Schmitt HJ, Tong WP, Edwards FF, Armstrong D. Pharmacokinetics of aerosolized amphotericin B in rats. Antimicrob Agents Chemother 1990;34:29-32.
12. Lambros MP, Bourne DWA, Abbas SA, Johnson DL. Disposition of aerosolized liposomal amphotericin B. J Pharm Sci 1997;86:1066-1069.
13. Ruijgrok EJ, Vulto AG, van Etten EWM. Efficacy of aerosolized amphotericin B desoxycholate and liposomal amphotericin B in the treatment of invasive pulmonary aspergillosis in severely immunocompromised rats. J Antimicrob Chemother 2000;48:89-95.
14. Cicogna CE, White MH, Bernard EM, et al. Efficacy of prophylactic aerosol amphotericin B lipid complex in a rat model of pulmonary aspergillosis. Antimicrob Agents Chemother 1997;41:259-61.
15. Lasic D. Liposomes: synthetic lipid microspheres serve as multipurpose vehicles for the delivery of drugs, genetic material and cosmetics. Am Sci 1992;80:20-31.
16. Leenders ACAP, de Marie S, ten Kate MT,

Bakker-Woudenberg IAJM, Verbrugh HA. Liposomal amphotericin B (AmBisome) reduces dissemination of infection as compared with amphotericin B deoxycholate (Fungizone) in a rat model of pulmonary aspergillosis. J Antimicrob Chemother 1996;38:215-225.

17. Daemen T, Velinova M, Regts J. Different intrahepatic distribution of phosphatidylglycerol and phosphatidylserine liposomes in the rat. Hepatol 1997;26:416-423.

18. Schiffelers RM. 2001. Ph.D. Thesis. Liposomal targeting of antimicrobial agents to bacterial infections. Erasmus University Rotterdam, Rotterdam, The Netherlands.

Chapter 5

# Efficacy of aerosolised amphotericin B desoxycholate and liposomal amphotericin B in the treatment of invasive pulmonary aspergillosis in severely immunocompromised rats

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# Abstract

The effects of treatment with aerosolised amphotericin B desoxycholate (AMB-DOC) and aerosolised liposomal amphotericin B (L-AMB) were evaluated in severely immunosuppressed rats with invasive pulmonary aspergillosis. Aerosol treatment with AMB-DOC consisted of a single dose (60 min) with amphotericin B concentrations in the nebuliser reservoir of 1, 2 and 4 mg/mL, respectively. For liposomal amphotericin B, aerosol treatment consisted of single, double or quadruple doses with a nebuliser reservoir concentration of 4 mg/mL of AMB. Treatment, started at 30 h after inoculation, with aerosolised AMB-DOC (nebuliser reservoir concentration 2 mg/mL) significantly prolonged survival of rats as compared with placebo-treated rats, whereas treatment with aerosolised AMB-DOC with nebuliser reservoir concentration of 1 or 4 mg/mL did not have a significant effect on survival. Treatment with aerosolised L-AMBsignifcantly prolonged survival with all treatment regimens when compared with placebo-treated animals. Aerosol treatment did not prevent dissemination of the infection. The effects of AMB-DOC and L-AMB on pulmonary surfactant function were also evaluated *in vitro*. AMB-DOC inhibited surfactant function in a dose-dependent fashion. L-AMB had no detrimental effect on surface activity of surfactant. These results indicate that aerosol administration of AMB, especially the liposomal formulation, could be an additional approach to optimising treatment of invasive pulmonary aspergillosis.

# Introduction

An increase in the number of immunosuppressed patients at risk of developing fungal infections due to increasing advances in transplantation medicine has led to a substantial increase in the number of cases of invasive pulmonary aspergillosis (IPA) in the last few decades[1]. Standard treatment of IPA with intravenous amphotericin B desoxycholate (AMB-DOC) is often unsuccessful and complicated by severe, dose-limiting toxicity. Newly developed lipid-based modalities of AMB have an increased therapeutic index as compared with AMB-DOC [2]. However, treatment of established infection with *Aspergillus* spp. in patients with persisting granulocytopenia is correlated with high failure rates. Thus, there is a continuing need for optimisation of antifungal treatment in pulmonary aspergillosis. The portal of entry of *Aspergillus* is often the respiratory tract, since the spores of this fungus are ubiquitous and easily inhaled.
Spores descend to the lowest regions of the lungs and invasive disease subsequently develops. Further improvement in the treatment of IPA can therefore be sought in the administration of AMB products via the pulmonary route. With this mode of administration, the therapeutic agent targets the lungs directly, and systemic toxicity is reduced. Improvement in efficacy as well as reduction of systemic side effects could be anticipated.
Few clinical studies exist on the use of aerosolised AMB in the prophylaxis or treatment of fungal disease. These studies do not demonstrate a convincing beneficial effect of aerosolised AMB, probably because of a lack of power or adequate study design [3–5]. Preclinical animal work therefore has to be performed in order to determine the value of aerosolised conventional and lipid preparations of AMB. Aerosol administration of different formulations of AMB has been described and shown to be effective in the prophylaxis of pulmonary aspergillosis in animal models [6–8]. However, in these studies only temporary or mild immunosuppression was applied in the experimental models. In the clinical situation however, patients are generally persistently granulocytopenic and diagnosis of IPA is often late in the course of infection. An important feature of the experimental design of the present study is therefore the treatment of an established infection under severe, persisting immunosuppression.
Results from our laboratory and from others show that after aerosol administration of liposomal amphotericin B, little or no drug was deposited in organs other than the lungs, which suggests that systemic toxicity following aerosol administration would be minimal [6,9–11]. Pulmonary toxicity of inhaled AMB however, could be an important detrimental effect of inhalation therapy. There are many *in vivo* and *in vitro* parameters that could be indicative of damaged pulmonary tissue or impaired

pulmonary function. In this study we chose pulmonary surfactant function as a toxicity indicator, since this is an important basic physiological function. Surface activity of pulmonary surfactant is relatively easily measured *in vitro*, and provides relevant information on the possible harmful influence of inhaled AMB formulations. In the present study, we describe the efficacy of aerosolised AMB desoxycholate and aerosolised L-AMB in the treatment of established IPA in rats with severe, persistent immunosuppression. Furthermore, we describe the influence of AMB products on surfactant function *in vitro*.

# Materials and methods

### Materials

AMB-DOC (containing 50 mg AMB and 41 mg desoxycholate per vial) was from Bristol Myers-Squibb (Woerden, The Netherlands) and L-AMB was from NeXstar (San Dimas, CA, USA). Hydrogenated soybean phosphatidylcholine (HSPC) and distearoylphosphatidylglycerol (DSPG) were from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol and cyclophosphamide were from Sigma Chemical Co. (St Louis, MO, USA). Sabouraud dextrose agar (SDA) was from Oxoid (Basingstoke, UK).

### Animals

Female R-strain albino rats, specified pathogen free, 18–25 weeks old (own breed), weighing 185–225 g were used for all experiments. Animals received a normal, pathogen-free diet and water ad libitum. Experiments were approved by the animal experiments ethics committee of the Erasmus *university* Medical Center.

### Aspergillus strain

A clinical isolate of *Aspergillus fumigatus* from an immunocompromised patient with IPA was used. The MIC and minimal fungicidal concentration (MFC) of AMB for this strain were 0.4 and 0.8 mg/L, respectively [12]. This strain was stored under oil on SDA. At least once every 2 months, the strain was passed through a rat to maintain its virulence. For inoculation, conidia were harvested and suspended in sterile phosphate-buffered saline (PBS), as described previously [12].

### Immunosuppression and supportive care

Granulocytopenia was induced by i.p. administration of 90 mg/kg cyclophosphamide 5 days before fungal inoculation, followed by additional dosages of 60 mg/kg every 4 days throughout the study. This treatment resulted in a persis-

tent granulocytopenia (<0.5 x $10^9$ cells/L) from the time of A. fumigatus inoculation up to the end of the study [12].To prevent bacterial superinfection, strict hygienic care was applied, and animals received ciprofloxacin (660 mg/L) and polymyxin E (100 mg/L) in their drinking water throughout the experiment. Furthermore, im administration of amoxicillin (40 mg/kg/day) was added to this regimen. Shortly before and after inoculation, gentamicin (6 mg/kg) was administered i.m.

### Experimental lung infection

Infection of the lung was established according to the method described by Leenders et al [12]. Briefly, under general anaesthesia the left main bronchus was intubated. A canula was passed through the tube and the left lobe of the lung was inoculated with 0.02 mL of a suspension containing 2 x $10^4$ conidia. This resulted in a left-sided pneumonia.

### Antifungal treatment

AMB-DOC and L-AMB were reconstituted according to the manufacturers' instructions and further diluted in 5% glucose up to an AMB concentration in the nebuliser reservoir of 4 mg/mL in the case of L-AMB or 1, 2 or 4 mg/mL in the case of amphotericin B desoxycholate. Since calculation of the actual dose delivered is a rather complicated measure, in this study nebuliser reservoir concentration is given as an indirect dose indication. Treatment was started 30 h after fungal inoculation, at which time mycelial growth was established by histopathological examination (periodic acid-Schiff stain). The aerosolisation procedure for L-AMB and AMB-DOC was as described previously.[13] In short: infected rats were constrained in cone-ended plastic tubes and placed in a nose-only inhalation apparatus (CH Technologies USA Inc., Westwood, NJ, USA). Aerosols were generated by a Collison six-jet nebuliser system (Model CN; BGI Inc., Waltham, MA, USA). The nebuliser operated at 20 L/min air flow. Under these conditions, >80% of the aerosol droplets that are generated are <5 $\mu$m mass diameter, which ensures substantial deposition of material in the alveobronchial region. Animals were exposed to aerosol treatment for one or more periods of 60 min.

### Liposome preparation

Placebo liposomes with similar lipid composition (HSPC: DSPG:Chol, 2:1:0.8) and similar liposomal diameter to L-AMB were prepared by the film hydration method. Lipids were dissolved in 2 mL chloroform:methanol (1:1, v/v). The lipid mixture was evaporated to dryness in a round bottom flask at 65°C. The lipid film was

hydrated by vortex mixing with a buffer solution containing 10 mM sodium succinate, 10% (w/v) sucrose (pH 5.5). Liposomes were sonicated, which resulted in vesicles with an average particle size of 100 nm, as measured by dynamic light scattering. The liposome suspension was concentrated by ultracentrifugation at 280,000 x g for 2 h at 4°C in a Beckman ultracentrifuge L-70 (Beckman, Palo Alto, CA, USA) and further diluted in buffer to a concentration of 40 $\mu$mol/mL of lipid (similar to the lipid concentration in aerosolised liposomal amphotericin B). Phospholipid content was determined by phosphorus assay [14].Placebo liposomes were aerosolised as described above during a single treatment period of 60 min.

Efficacy of aerosolised AMB-DOC and aerosolised liposomal amphotericin B

Groups comprised of 15 infected rats each were treated with aerosolised AMB-DOC or aerosolised liposomal amphotericin B. For AMB-DOC, treatment consisted of a single nebulisation period of 60 min with a nebuliser reservoir concentration of 1, 2 or 4 mg/mL amphotericin B. For liposomal amphotericin B, treatment consisted of a single, double (q 24 h) or quadruple (q 24 or q 12 h) nebulisation period. Controls received aerosolised glucose 5% or placebo liposomes. In one experiment, pure AMB suspended in glucose 5% (nebuliser reservoir concentration 4 mg/mL) was nebulised in order to evaluate survival after treatment with this agent alone. Animals were checked twice daily and mortality was recorded for the 12 days following fungal inoculation, after which time post-mortem studies were conducted on all animals. The left lung, right lung and liver were dissected and homogenised in 20 mL PBS for 45 s at 20 000 rpm in a VirTis homogeniser (VirTis, Gardiner, NY, USA). Volumes of 0.2 and 2 mL and the remainder of each homogenate were spread on to or poured into SDA plates. Plates were incubated for 24 h at 37°C followed by 24 h at 25°C.

Surfactant function experiments

Freeze-dried natural surfactant prepared from bovine lavages was a gift from the Department of Anaesthesiology of the Erasmus *university* Medical Center Rotterdam. It consisted of c. 90-95% phospholipids, 1% hydrophobic proteins (surfactant proteins B and C) and 1% free fatty acids. This surfactant is highly surface active at low concentrations. Influence of different AMB formulations on surfactant function was determined by means of a modified Wilhelmy balance system (E. Biegler GmbH, Mauerbach, Austria). This system records the surface tension of an air-liquid film over several cycles of mechanical compression and expansion of this film. The lower the surface tension at minimal surface area, the higher the surface activity of the applied film. The trough of the Wilhelmy balance was filled

with warm saline (37°C) and calibrated. After calibration, 100 $\mu$L of surfactant (1 mg/mL total lipids) alone or in the presence of various concentrations of AMB formulations was applied on to the saline hypophase and allowed to spread for 2 min. The surface area was compressed and expanded with a cycling speed of one cycle per 3 min and an area reduction from 100% to 20%. Minimal surface tension ($\gamma_{min}$) was measured after three cycles at 20% surface area, and is expressed as mN/m. Inhibition of surfactant function of natural surfactant by AMB formulations resulted in increased surface tension at minimal surface area.

### Statistical analysis

Survival curves were generated by the method of Kaplan and Meier. Statistical evaluation of differences in the survival curves was performed by the log rank test. This test examines the decrease in survival with time as well as the final percentage survival.
Differences in proportions of animals with *A. fumigatus* in the left lung and dissemination to the right lung and liver at the time of death were examined by Fisher's exact test. Data of surface activity measurements were compared using the two-sided t-test. P values of 0.05 were considered significant.

## Results

### Effect of aerosolised AMB-DOC and aerosolised L-AMB on survival

The effect of aerosol treatment on survival of rats with pulmonary aspergillosis is shown in Figures 1 and 2. In this model of pulmonary aspergillosis placebo-treated rats died between days 4 and 9 after inoculation. At a nebuliser reservoir concentration of 2 mg/mL administration of aerosolised AMB-DOC 30 h after inoculation led to a significantly prolonged survival (P = 0.006) as compared with controls (Figure 1). Administration of aerosolised AMB-DOC with a nebuliser reservoir concentration of 1 or 4 mg/mL however, showed no beneficial effect on the survival of rats as compared with controls. Administration of a single dose of aerosolised L-AMB (nebuliser reservoir concentration 4 mg/mL) resulted in significantly prolonged survival (P = 0.002) as compared with glucose controls as well as empty liposome controls (Figure 2a). Aerosolised L-AMB adminstered as a double (q = 24 h) or quadruple (q = 24 and 12 h) dose also significantly prolonged survival (P 0.005) as compared with glucose-treated animals (Figure 2b). Intensification of treatment yielded no further improvement in survival as compared with single dose treatment. No statistically significant differences were

Figure 1. Effect of a single dose of aerosolised AMB-DOC with a reservoir concentration of 1 mg/mL (□), 2 mg/mL (●) or 4 mg/mL (▣) on survival of severely immunosuppressed rats with pulmonary aspergillosis (Kaplan–Meier plot). Each group consisted of 15 animals. Control animals received aerosolised 5% glucose (----). Treatment was started 30 h after inoculation, at which time mycelial growth was established. *P = 0.006 versus control rats.

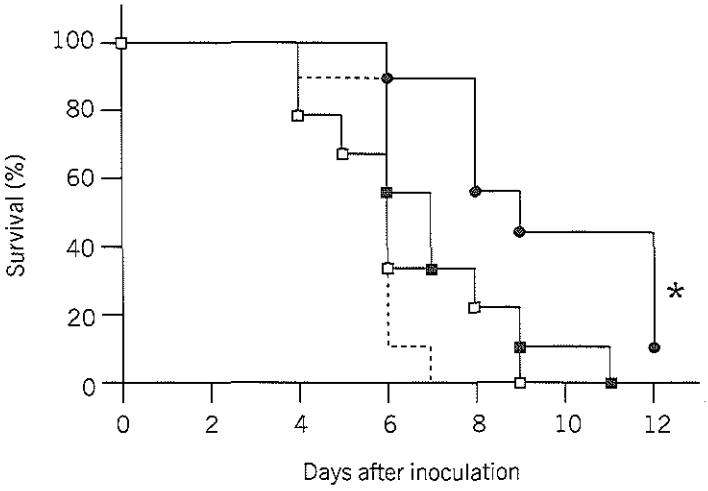
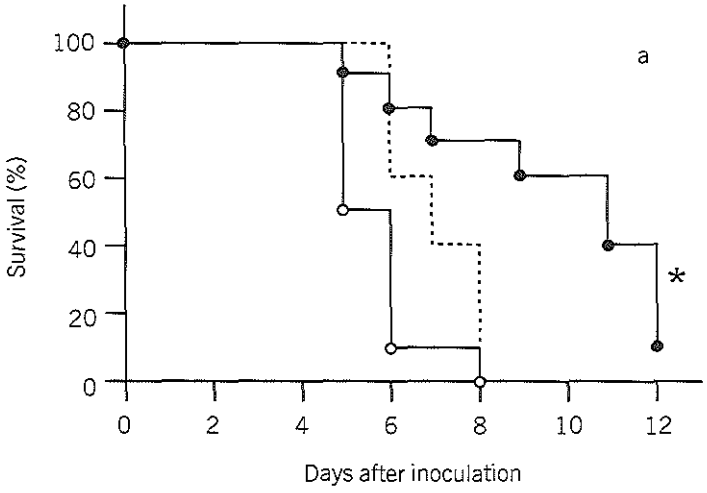
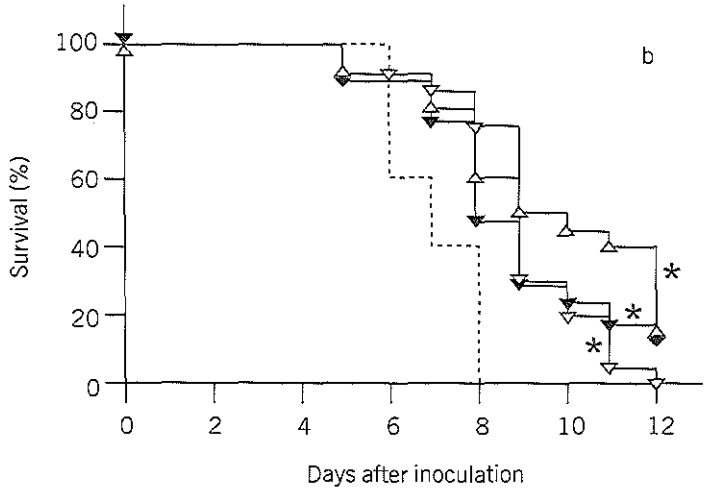


Figure 2. Effect of single (a) or multiple (b) dose aerosolised L-AMB versus glucose or empty liposome controls on survival of severely immunosuppressed rats with pulmonary aspergillosis (Kaplan–Meier plot). Groups of 15 animals each were treated with aerosolised L-AMB (reservoir concentration 4 mg/mL) as a single dose (●), as a double dose (q 24 h) (△) or as a quadruple dose, either delivered every 24 h (▽) or every 12 h (▼). Controls were treated with placebo liposomes (○), or 5% glucose (----). *P < 0.05 versus placebo liposomes or glucose controls.



observed between aerosolised AMB-DOC and the four different regimens of aerosolised L-AMB.

Effect of aerosolised AMB-DOC and aerosolised L-AMB on the presence of viable *A. fumigatus* in the left lung and on dissemination to the right lung and liver

The results of post-mortem quantitative cultures of *A. fumigatus* from lungs and liver after treatment are shown in Table 1. Cultures revealed that at the time of death the infection had disseminated to the right lung and liver in the majority of rats. Aerosol treatment had no significant beneficial effect on prevention of dissemination to the right lung or the liver.

Table 1. Effect of aerosolised AMB-DOC and L-AMB versus aerosolised glucose 5% controls on the presence of viable *A. fumigatus* in the left lung and dissemination to the right lung and liver at the time of death[a]

| Aerosol treatment | AMB nebuliser reservoir concentration (mg/mL) | Dosing frequency and interval | % culture positive organs | | |
|---|---|---|---|---|---|
| | | | Left lung | Right lung | Liver |
| Glucose 5% | | 1 x 60 min | 100 | 60 | 53 |
| AMB-DOC | 1 | 1 x 60 min | 100 | 53 | 40 |
| AMB-DOC | 2 | 1 x 60 min | 80 | 40 | 60 |
| AMB-DOC | 4 | 1 x 60 min | 100 | 87 | 87 |
| Placebo liposomes | | 1 x 60 min | 100 | 53 | 53 |
| L-AMB | 4 | 1 x 60 min | 100 | 40 | 40 |
| L-AMB | 4 | 2 x 60 min, q = 24 h | 100 | 47 | 73 |
| L-AMB | 4 | 4 x 60 min, q = 24 h | 100 | 47 | 67 |
| L-AMB | 4 | 4 x 60 min, q = 12 h | 100 | 33 | 53 |

[a]Leukopenic rats (n = 15) were inoculated in the left lung at time zero with $2 \times 10^4$ conidia *A. fumigatus*. Start of treatment was at 30 h.

Influence of AMB products on surfactant function

Table 2 shows the mean $\gamma_{min}$ of surfactant alone or in combination with different concentrations of AMB alone, AMB-DOC, L-AMB and desoxycholate. The natural surfactant was highly surface active at the concentrations examined ($\gamma_{min}$ 1.97 ± 1.23). Minimal surface tensions after mixing of surfactant with AMB-DOC or L-AMB in different concentrations yielded similar low values. Addition of increasing concentrations of AMB-DOC to natural surfactant resulted in significantly increased $\gamma_{min}$, indicating a loss of surface activity of the mixtures. An increase in $\gamma_{min}$ of natural surfactant was also seen with addition of increased concentrations of desoxycholate.

Table 2. Mean minimal surface tension ($\gamma_{min}$) of surfactant (1 mg/mL) together with saline, AMB, AMB-DOC, L-AMB or DOC

| | Concentration (mg/ml) | | Minimal surface tension |
|---|---|---|---|
| | AMB | DOC | (mN/m)[a] |
| Saline | - | - | 1.97 ± 1.23 |
| AMB | 0.02 | | 2.81 ± 0.63 |
| | 0.2 | | 2.65 ± 0.47 |
| | 2 | | 2.70 ± 0.53 |
| AMB-DOC[b] | 0.02 | 0.016 | 7.53 ± 1.51[c] |
| | 0.2 | 0.16 | 14.76 ± 1.85[c] |
| | 2 | 1.6 | 47.53 ± 0.89[c] |
| L-AMB | 0.02 | | 2.44 ± 0.41 |
| | 0.2 | | 1.11 ± 0.72 |
| | 2 | | 1.42 ± 0.83 |
| Desoxycholate | | 0.008 | 1.94 ± 0.77 |
| | | 0.08 | 5.59 ± 1.80[c] |
| | | 2.1 | 23.13 ± 1.16[c] |
| | | 21 | 45.21 ± 2.08[c] |

[a]each value represents the mean ± sd of three individual experiments

[b]In AMB-DOC, 54% (g/g) is AMB, 46% is DOC, corresponding with 0.02 mg/ml AMB and 0.016 mg/ml DOC

[c]$p \leq 0.05$

# Discussion

The importance of invasive aspergillosis has progressively increased and it is now a major direct or contributory cause of death at leukaemia treatment centres and bone marrow and solid organ transplantation centres [1]. In the present study, the efficacy of aerosolised AMB-DOC and aerosolised L-AMB were evaluated in a rat model of severe pulmonary aspergillosis. In this aspergillosis model, rats received cyclophosphamide injections before fungal inoculation and throughout the whole experiment. This resulted in persistent deep granulocytopenia, in order to closely mimic a highly relevant, difficult to treat clinical situation. Treatment was started at 30 h after fungal inoculation, at which time hyphal formation was confirmed by histological examination of pulmonary tissue.

Significant prolongation of survival was observed after aerosol treatment with AMB-DOC with a nebuliser reservoir concentration of 2 mg/mL. Lack of efficacy with a lower dose of AMB-DOC was probably due to inadequate concentrations of AMB in the lung tissue, whereas lack of efficacy with the high dose could be a consequence of pulmonary toxicity that overshadowed antifungal efficacy. Prolonged survival was also seen in animals treated with a single dose of aerosolised L-AMB (nebuliser reservoir concentration 4 mg/mL). Similar results were obtained after double or quadruple dosing with aerosolised L-AMB, therefore it appears that intensifying the aerosol regimens does not lead to improvement in efficacy in terms of survival. The lack of a dose-effect relationship after aerosol administration is probably due to inadequate deposition of bioactive AMB at the site of the infection, which is not ameliorated by giving extra doses. The survival of rats after treatment with the optimal dose of aerosolised AMB-DOC and with aerosolised L-AMB seems to be similar to survival seen after 10 day treatment with iv L-AMB (10 mg/kg) in the same animal model [12].

The observations in terms of survival in the present study resemble those found in clinical practice, where, despite intravenous antifungal treatment, survival is <10–20% in persistently immunosuppressed patients with an established infection. However, the observed prolongation of survival after aerosol treatment may be useful in cases of temporary granulocytopenia. When leucocyte counts are restored early enough in the course of infection, improvements in the outcome of treatment can be expected, since it is known from clinical experience that bone marrow recovery and increasing numbers of circulating leucocytes are crucial factors in the favourable outcome of treatment [1]. Aerosol treatment with AMB could be an alternative to current intravenous regimens, which offer good prognosis with neutrophil recovery.

Treatment of animals with a suspension of 4 mg/mL pure AMB in glucose 5% had no effect on survival as compared with controls (data not shown). The lack of efficacy may be the result of poor aerosolisation characteristics and/or poor aqueous solubility and therefore poor availability of the drug at the site of deposition.
Aerosol treatments with either AMB-DOC or L-AMB had no effect on dissemination of *A. fumigatus* to the right lung or liver. It was shown previously that intravenous administration of L-AMB in the same rat model of IPA does reduce dissemination [12]. This difference can be explained by the different pharmacokinetic profile of aerosol administration as compared with intravenous administration. Previous work in our laboratory has shown that aerosol treatment results in negligible systemic exposure to AMB and subsequent negligible tissue concentrations in organsother than the lungs in contrast to the extensive tissue distribution following intravenous administration. Considering this, it would be interesting to study the efficacy of high dose aerosol treatment in combination with low dose systemic administration of AMB in order to optimise drug delivery to the lungs and reduce dissemination at the same time.
Although limited data are available, inhaled L-AMB seems to be well-tolerated by humans [15]. With inhaled AMB-DOC side effects such as cough, nausea and mild bronchospasms are reported. These pulmonary side effects were in some cases dose limiting or even necessitated discontinuation of inhalation therapy [16-18]. The major side effect, which is cough, is presumed to be caused by desoxycholate rather than by AMB [18]. In our study, no acute toxicities in terms of death or increased breathing frequencies were observed in animals during aerosol treatment with either AMB-DOC or liposomal amphotericin B. *In vitro* surfactant function tests were performed in order to evaluate the disturbance of surfactant function after disposition of the AMB-DOC and L-AMB. The lowest AMB concentrations and therefore the corresponding desoxycholate concentrations were based upon our observed lung deposition results, which were c. 20 $\mu g/g$ lung tissue for AMB-DOC and for L-AMB. L-AMB alone (data not shown) showed high surface activity, similar to that of the surfactant, which can be explained by the presence in the formulation of phospholipids that are surface active components. As expected, L-AMB did not influence surface activity of the surfactant. However, there was a dose-dependent inhibition of surfactant function by AMB-DOC. This was due to the detrimental effects of desoxycholate on surfactant function, since this agent alone showed high influence on surface activity of natural surfactant in comparison with AMB without desoxycholate. These results are in accordance with those found by Griese et al [19]. Our results suggest that inhalation of AMB-DOC but not L-AMB may lead to surfactant dysfunction and this should be considered when choosing a formulation to be nebulised.

In this refractory model of IPA, survival of infected animals was prolonged after even a single dose of aerosolised AMB-DOC or L-AMB. Despite the uncertainties about pathogenesis and the precise antifungal activities of administered agents, these results encourage further investigations of the potential of aerosolised AMB products in other settings such as prophylaxis or empirical treatment. Furthermore, it would be interesting to determine efficacy for other lipid formulations of AMB that are currently on the market, since these products have different structural properties. In summary, both aerosolised AMB-DOC and L-AMB are effective in prolonging survival of persistently leucopenic rats infected with *A. fumigatus*. L-AMB demonstrated no negative effect on pulmonary surfactant, as opposed to AMB-DOC, and therefore would be the formulation of choice for aerosol administration in patients suffering from invasive pulmonary aspergillosis.

# References


1. Denning, DW. Invasive aspergillosis. Clin Infect Dis 1998;26,781–803.
2. Hiemenz, JW, Walsh, TJ. Lipid formulations of amphotericin B. J Lipos Res 1998;8,443-67.
3. Erjavec, Z, Woolthuis, GM, De Vries-Hospers, HG, et al. Tolerance and efficacy of amphotericin B inhalations for prevention of invasive pulmonary aspergillosis in haematological patients. Eur J Clin Microbiol Infect Dis 1997;16,364-8.
4. Hertenstein, B, Kern, WV, Schmeiser, T, et al. Low incidence of invasive fungal infections after bone marrow transplantation in patients receiving amphotericin B inhalations during neutropenia. Ann Hematol 1994;68,21-6.
5. Schwartz, S, Behre, G, Heinemann, V, et al. Aerosolized amphotericin B inhalations as prophylaxis of invasive aspergillus infections during prolonged neutropenia: results of a prospective randomized multicenter trial. Blood 1999;93,3654-61.
6. Allen, SD, Sorensen, KN, Nejdl, MJ, Durrant, C, Proffit, RT. Prophylactic efficacy of aerosolized liposomal (AmBisome) and non-liposomal (Fungizone) amphotericin B in murine pulmonary aspergillosis. J Antimicrob Chemother 1994;34,1001-13.
7. Cicogna, CE, White, MH, Bernard, EM, et al. Efficacy of prophylactic aerosol amphotericin B lipid complex in a rat model of pulmonary aspergillosis. Antimicrob Agents Chemother 1997;41,259-61.
8. Schmitt, HJ, Bernard, EM, Hauser, M, Armstrong, D. Aerosol amphotericin B is effective for prophylaxis and therapy in a rat model of pulmonary aspergillosis. Antimicrob Agents Chemother 1988;32,1676-9.
9. Koizumi, T, Kubo, K, Kaneki, T, et al. Pharmacokinetic evaluation of amphotericin B in lung tissue: lung lymph distribution after intravenous injection and airspace distribution after aerosolization and inhalation of amphotericin B. Antimicrob Agents Chemother 1998;42, 1597-600.
10. Lambros, MP, Bourne, DWA, Abbas, SA, Johnson, DL. Disposition of aerosolized liposomal amphotericin B. J Pharm Sci 1997;86,1066–9.
11. Niki, Y, Bernard, EM, Schmitt, HJ, Tong, W P, Edwards, FF, Armstrong, D. Pharmacokinetics of aerosol amphotericin B in rats. Antimicrob Agents Chemother 1990;34,29-32.
12. Leenders, ACAP, De Marie, S, Ten Kate, MT, Bakker-Woudenberg, IAJM, Verbrugh, HA. Liposomal amphotericin B (Ambisome) reduces dissemination of infection as compared with amphotericin B deoxycholate (Fungizone) in a rat model of pulmonary aspergillosis. J Antimicrob Chemother 1996;38,215-25.
13. Ruijgrok, EJ, Vulto, AG, Van Etten, EWM. Aerosol delivery of amphotericin B desoxycholate (Fungizone) and liposomal amphotericin B (AmBisome): aerosol characteristics and in-vivo amphotericin B deposition in rats. J Pharm Pharmacol 2000;52,619-27.
14. Bartlett, GR. Phosphorus assay in column chromatography. J Biol Chem 1959;234,466–8.
15. Purcell, IF, Corris PA. Use of nebulised liposomal amphotericin B in the treatment of *Aspergillus fumigatus* empyema. Thorax 1995;50,1321-3.

16. Diot, P, Rivoire, B, Le Pape, et al. Deposition of amphotericin B aerosols in pulmonary aspergilloma. Eur Resp J 1995;8,1263-8.
17. Dubois, J, Bartter, T, Gryn, J, Pratter, MR. The physiologic effects of inhaled amphotericin B. Chest 1995;108,750-3.
18. Gryn, J, Goldberg, J, Johnson, E, Siegel, J, Inzerillo, J. The toxicity of daily inhaled amphotericin B. Am J Clin Oncol 1993;16,43-6.
19. Griese, M, Schams, A, Lohmeier, KP. Amphotericin B and pulmonary surfactant. Eur J Med Res 1998;3,383-6.

Chapter 6

# Intravenous amphotericin B desoxycholate and liposomal amphotericin B in combination with aerosolised liposomal amphotericin B is effective in the treatment of rats with severe invasive pulmonary aspergillosis

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# Abstract

The efficacy of aerosolised liposomal amphotericin B (L-AMB) in addition to standard intravenous amphotericin B desoxycholate (AMB-DOC) or intravenous L-AMB was evaluated in persistently leukopenic rats with invasive pulmonary aspergillosis. Endpoints were survival of animals and dissemination of infection to the liver. In this model, untreated rats die between day 4 and day 9 after fungal inoculation. Intravenous treatment was started at 16 h after fungal inoculation and continued for 10 days and consisted of AMB-DOC (1 mg/kg), L-AMB (10 mg/kg) or a combination of both. Aerosol treatment consisted of a single period of 60 minutes nebulisation of L-AMB at 16 after fungal inoculation. Aerosols were generated by a Collison nebuliser. Treatment with intravenous AMB-DOC, L-AMB or a combination of these, significantly prolonged survival as compared to controls. However, addition of aerosol L-AMB to these intravenous regimens further improved survival. Aerosol treatment with L-AMB alone yielded similar results as the intravenous regimens.

The intravenous regimens reduced dissemination of the infection to the liver as compared to controls as well as the regimens were intravenous treatment was combined with aerosol treatment. Aerosol treatment alone did not reduce dissemination.

The results of the present study show that addition of aerosol treatment with L-AMB to the standard intravenous treatment with AMB-DOC or L-AMB can be valuable in optimising treatment of invasive pulmonary aspergillosis.

# Introduction

Invasive pulmonary aspergillosis (IPA) is a serious life-threatening infection with a high mortality in leukopenic patients. Ever since the first description of IPA in 1953, there has been a substantial increase in the frequency of the disease. This is due to growing numbers of patients at risk, such as those treated with cytotoxic chemotherapeutic agents or aggressive immunosuppressants [1]. Intravenous amphotericin B desoxycholate (AMB-DOC) remains the standard treatment for most serious fungal infections, including invasive pulmonary aspergillosis. However, the use of AMB-DOC is often limited due to toxic side effects such as nephrotoxicity [2]. The liposomal formulation of AMB (L-AMB) has been developed in order to diminish the side effects of AMB-DOC. Consequently, L-AMB can be administered in higher dosages than AMB-DOC [3,4].
Although intravenous administration of AMB-DOC or L-AMB leads to a reduction in mortality due to IPA, treatment of such an established infection in patients with prolonged persistent leukopenia is correlated with high failure rates [5]. Therefore, there is still a critical need to optimise treatment of invasive aspergillosis.
Failure of treatment of invasive pulmonary aspergillosis with intravenous AMB is probably the result of several factors. Due to the nature of the infection, the fungus resides predominantly in the airways and can spread to other body tissues. Inadequate pulmonary deposition of AMB after intravenous administration of AMB-DOC or L-AMB can be a reason for failure of treatment. It has been shown in preclinical work, that only a small percentage of intravenously administered AMB is actually delivered to the lungs [6,7]. By administration of drugs via the inhalation route the lungs are directly targeted which results in high pulmonary drug concentrations. We have shown in a rat model of IPA that administration of a single dose of aerosolised AMB, either liposomal or non-liposomal, yields substantial, long lasting pulmonary AMB concentrations and improves survival in rats as compared to controls [8]. Addition of aerosolised AMB to intravenously administered AMB could be an attractive way to enhance pulmonary deposition of AMB.
The hypothesis of the present study is that aerosol treatment additive to standard intravenous treatment can result in a treatment which has a higher success rate in terms of improvement of survival in experimental IPA. The study describes the combination of intravenous AMB-DOC and/or L-AMB with aerosolised L-AMB in the treatment of leukopenic rats suffering from IPA. Therapeutic endpoints were survival as well as dissemination of infection in treated animals as compared to untreated controls.

# Materials and Methods

## Materials

AMB-DOC was from Bristol Myers-Squibb (Woerden, The Netherlands) and L-AMB was from NeXstar (San Dimas, CA). Cyclophosphamide was from Sigma Chemical Co. (St. Louis, MO). Sabouraud dextrose agar (SDA) was from Oxoid (Basinstoke, England).

## Animals

Female R-strain albino rats, specified pathogen free, 18- to 25- weeks old (own breed), weighing 185-225 g were used for all experiments. Animals received a normal, pathogen free diet and water ad libitum. Experiments were approved by the animal experiments ethical committee of the Erasmus *university* Medical Center Rotterdam.

## Aspergillus strain

A clinical isolate of *Aspergillus fumigatus* from an immunocompromised patient with invasive pulmonary aspergillosis was used. MIC and minimal fungicidal concentration of AMB for this strain are 0.4 and 0.8 mg/l, respectively [9]. The strain was stored under oil on SDA. At least once every two months, the strain was passed through a rat to maintain its virulence. For inoculation, conidia were harvested and suspended in sterile phosphate buffered saline, as previously described [9].

## Immunosuppression and supportive care

Leukopenia was induced by intraperitoneal administration of 90 mg/kg cyclophosphamide at 5 days before fungal inoculation followed by additional dosages of 60 mg/kg every 4 days throughout the study. This treatment resulted in a persistent leukopenia ($< 0.5 \times 10^9$/l) from the time of *A. fumigatus* inoculation up to the end of the study [10]. To prevent bacterial superinfection, strict hygienic care was applied, and animals received ciprofloxacin (660 mg/l) and polymyxin E (100 mg/l) in their drinking water during the whole experiment. Furthermore, intramuscular administration of amoxicillin (40 mg/kg/day) was added to this regimen. Shortly before and after inoculation, gentamicin (6 mg/kg) was administered intramuscularly.

## Experimental lung infection

Infection of the lung was established according to the method described by Bakker-Woudenberg et al. [10], and adapted for *Aspergillus fumigatus* by Leenders et al. [9]. Briefly, under general anaesthesia the left main bronchus was intubated. A canula was passed through the tube and the left lobe of the lung was

inoculated with 0.02 ml of the conidial suspension containing 1.5 x $10^5$ conidia. This resulted in a left sided pneumonia.

Antifungal treatment

Treatment was started at 16 h after fungal inoculation. Histopathologic examination (Periodic Acid-Schiff stain) confirms that mycelial outgrowth begins at 16 h. Treatment regimens are given in Table 1. Groups of 15 animals received monotherapy (only intravenous or only aerosol treatment) or combination therapy (intravenous and aerosol treatment). AMB-DOC and L-AMB were reconstituted according to the manufacturers instructions and further diluted in 5% glucose. Intravenous treatment started at 16 h or directly after aerosol treatment when combination treatment was given. The intravenous regimens showed optimal efficacy in the treatment of IPA in rats [9,11]. For aerosol treatment, L-AMB was diluted with 5% glucose up to an AMB concentration in the nebuliser reservoir of 4 mg/ml AMB. The aerosol procedure of L-AMB and AMB-DOC was as previously described [13]. In short: infected rats were constrained in cone ended plastic tubes and placed in a nose-only inhalation apparatus (CH Technologies USA Inc., Westwood, NJ). Aerosols were generated by a Collison six-jet nebuliser system (Model CN, BGI Inc., Waltham, MA). The nebuliser operated at 20 l/min air flow. Under these conditions, more than 80% of the aerosol droplets that are generated are below 5 $\mu$m mass diameter, which ensures substantial deposition of material in the alveobronchial region. Animals were exposed to aerosol treatment for a period of 60 minutes.

Table 1. Treatment regimens

| Treatment | |
|---|---|
| Intravenous[a] | Aerosol[b] |
| AMB-DOC[c] | - |
| AMB-DOC[c] | L-AMB |
| - | L-AMB |
| L-AMB[d] | - |
| L-AMB[d] | L-AMB |
| AMB-DOC[c] + L-AMB[d] | - |
| AMB-DOC[c] + L-AMB[d] | L-AM |

[a]Intravenous treatment during 10 days.
[b]Aerosol administration of a single period of 60 minutes.
[c]Daily dosages of 1 mg/kg.
[d]Daily dosages of 10 mg/kg.
[e]Single dose of 1 mg/kg at day 1.

### Efficacy of antifungal treatments

Two endpoints were chosen: survival and fungal dissemination to the liver. Animals were checked twice daily and mortality was recorded for 12 days after fungal inoculation. Surviving animals were sacrificed after day 12. Post mortem, the left lung and liver were dissected and homogenised in 20 ml phosphate buffered saline for 45 sec at 20,000 rpm in a VirTis homogeniser (The VirTis Co. Inc., Gardiner, NY, USA). Volumes of 0.2 ml and 2 ml and the remainder of each homogenate were spread onto or poured into Sabouraud dextrose agar plates. Plates were incubated for 24 h at 37 °C followed by 24 h at 25 °C. After incubation, plates were checked to exclude bacterial superinfection and second to that, colonies of viable *Aspergillus* were counted in order to determine dissemination to the liver.

### Statistical analysis

Survival curves were generated by the method of Kaplan and Meier. Statistical evaluation of differences in the survival curves was performed by the logrank test. This test examines the length of survival as well as the percentage of survival. Differences in proportions of animals with *A. fumigatus* in the left lung and dissemination to the right lung and liver at the time of death were examined by the Fischer's exact test.

## Results and Discussion

In the present study, the efficacy of different AMB treatment modalities was evaluated in leukopenic rats with IPA. IPA was studied in rats under severe persistent leukopenia, in order to closely mimic a difficult-to-treat clinical situation. In this model, untreated rats die between day 4 and day 9 after fungal inoculation. Treatment was started at 16 h after fungal inoculation, at which time hyphal outgrowth begins. The efficacy endpoints were survival and dissemination of infection to the liver. The effect of different treatment regimens on survival is shown in Figure 1. The control group did not receive any treatment. Previous work has demonstrated that sham treatment resulted in similar results as no treatment at all [8]. We have chosen the liposomal formulation of AMB for nebulisation since L-AMB showed no toxicity in-vitro to the pulmonary surfactant as opposed to AMB-DOC, which was detrimental to the surfactant and may lead to inhalation toxicity [8,12]. The nebuliser reservoir concentration of 4 mg/ml was chosen because it has been shown that nebulising higher, more viscous, concentrations is technically not possible with the Collison nebuliser [13].

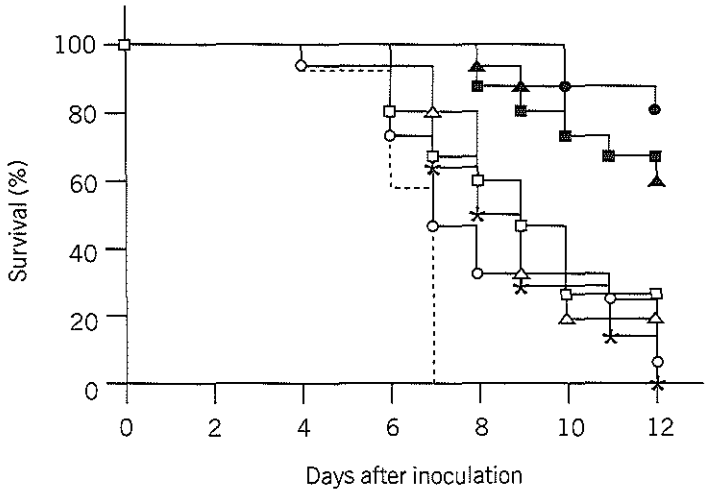


Figure 1. Effect of different treatment regimens on survival of persistently leukopenic rats with invasive pulmonary aspergillosis. (Kaplan-Meier plot). Each group consisted of 15 animals. Control animals received no treatment (---). Treatment was started at 16 h after fungal inoculation at which time mycelial growth begins. Animals were treated with intravenous AMB-DOC (○), intravenous AMB-DOC in combination with aerosolised L-AMB (●), aerosolised L-AMB (*), intravenous L-AMB (□), intravenous L-AMB in combination with aerosolised L-AMB (■), intravenous AMB-DOC plus L-AMB (△), intravenous AMB-DOC plus L-AMB in combination with aerosolised L-AMB (▲).

Treatment with monotherapy of a high dose of intravenous AMB-DOC (daily 1 mg/kg) resulted in significantly prolonged survival as compared to controls ($P = 0.03$). These results are in agreement with those of Leenders et al [9]. Addition of aerosol treatment with L-AMB to this intravenous regimen resulted in a prolonged survival of rats as compared to controls ($P = 0.0001$) but also as compared to the intravenous AMB-DOC alone ($P = 0.0001$). Thus, the addition of just a single aerosol treatment to the intravenous regimen of AMB-DOC, yields better results in terms of survival. Treatment of animals with monotherapy of aerosolised L-AMB was as effective as monotherapy with intravenous AMB-DOC.
Treatment with a high dose of intravenous L-AMB (daily 10 mg/kg) resulted in a significantly prolonged survival as compared to controls ($P = 0.0001$). The results are not different from that obtained after administration of intravenous AMB-DOC, which is in agreement with Leenders et al. [9]. Addition of a single dose aerosol treatment to this regimen also improved survival as compared to controls ($P = 0.0001$), but also as compared to the intravenous L-AMB ($P = 0.0001$).

Administration of intravenous L-AMB (daily 10 mg/kg) plus a single dose of intravenous AMB-DOC (1 mg/kg) at the first day of treatment resulted in a prolonged survival as compared to controls ($P = 0.0001$). Addition of aerosol L-AMB to this regimen also resulted in an improved survival as compared to controls ($P = 0.0001$) and as compared to treatment with the intravenous regimen alone ($P = 0.0001$). We found no difference between the intravenous regimen of L-AMB plus AMB-DOC and the intravenous monotherapies ($P > 0.05$), which is in discrepancy with Becker et al. [11], who showed that the efficacy of the combination of L-AMB with a single injection of AMB-DOC was superior to that of one of both agents alone. The discrepancy between these results may be explained by the difference in severity of leukopenia resulting from a different cyclophosphamide regimens in both studies. The effect of the different treatment regimens on dissemination is shown in Table 2. Results are expressed as percentage culture positive organs. Cultures revealed that at the time of death the infection had disseminated to the liver of all untreated rats. Monotherapy with intravenous AMB-DOC, L-AMB or L-AMB plus AMB-DOC, resulted in a reduction of dissemination to the liver. Combination of intravenous

Table 2. Effect of different treatment regimens on the presence of viable *A. fumigatus* in the left lung and dissemination to the liver at the time of death[a]

| Treatment | % culture positive organs | |
|---|---|---|
| | Left lung | Liver |
| None | 100 | 100 |
| I.v. AMB-DOC[b] | 100 | 7[e] |
| I.v. AMB-DOC[b] + aerosol L-AMB | 100 | 13[e] |
| Aerosol L-AMB | 100 | 87 |
| I.v. L-AMB[c] | 100 | 0[e] |
| I.v. L-AMB[c] + aerosol L-AMB | 100 | 0[e] |
| I.v. AMB-DOC[d] + i.v. L-AMB[c] | 100 | 0[e] |
| I.v. AMB-DOC[d] + i.v. L-AMB[c] +aerosol L-AMB | 100 | 0[e] |

[a]Leukopenic rats (n = 15) were inoculated in the left lung at time zero with $1.5 \times 10^5$ conidia *A. fumigatus*. Start of intravenous treatment was at 16 h after inoculation and continued for 10 days. Aerosol administration of a single dose of 60 min at 16 h after fungal inoculation.

[b]Daily dosages of 1 mg/kg.

[c]Daily dosages of 10 mg/kg.

[d]Single dose of AMB-DOC 1 mg/kg at day one.

[e]$P < 0.01$ as compared to untreated controls.

with aerosol treatment also showed a significant reduction in dissemination to the liver as compared to untreated rats. Treatment with only aerosol L-AMB did not prevent dissemination to the liver. It seems that intravenous treatment is crucial to prevent dissemination from the infection from the lungs to other organs such as the liver. Aerosol treatment deposits AMB in the lungs and not systemically and can therefore not prevent spreading of the infection via the bloodstream.
The positive results in terms of survival of animals which are shown in the present work, prove the benefit of adding local treatment to intravenous treatment.
The added value of local treatment is that AMB is deposited directly at the site of the infection and thus can prevent further outgrowth of hyphae and thus further damage to pulmonary tissue. Furthermore, pulmonary deposited AMB can prevent reinfection of the lungs from foci elsewhere.
There are no clinical data on the safety of a treatment with intravenous AMB-DOC combined with intravenous L-AMB, nor on the combination of aerosolised L-AMB combined with an intravenous regimen of AMB-DOC or L-AMB. Administration of intravenous AMB-DOC in a dose of 1 mg/kg, can lead to nephrotoxicity [14]. Treatment with intravenous L-AMB can be given at a dose of 10 mg/kg without significant toxicity [15]. L-AMB showed no *in vitro* toxicity on pulmonary surfactant when administered as an aerosol [8]. Furthermore, L-AMB administered via an aerosol is not systemically absorbed and will thus not exert systemic toxicity.
In conclusion, we have described the efficacy of intravenous AMB, either liposomal or non-liposomal, combined with aerosolised liposomal AMB in the treatment of leukopenic rats with IPA. The combination of systemic and local administration of AMB is superior to that of either systemic or local treatment alone. Intravenous treatment is crucial for preventing dissemination of the infection from the lungs. This finding is clinically relevant as current treatment strategies of aspergillosis in leukopenic patients are still disappointing and there is a critical need for new antifungal therapies which are both effective and have little toxicity. We suggest that L-AMB delivered via the pulmonary route, additive to the current standard of care (intravenous administration of AMB), can be a major step forward in the optimisation of treatment of invasive pulmonary aspergillosis.

# References


1. Denning DW. Invasive aspergillosis. Clin Infect Dis 1998;26:781-803.
2. Fanos V, Cataldi L. Amphotericin B-induced nephrotoxicity: a review. J Chemother 2000;12:463-470.
3. Hiemenz JW, Walsh TJ. Lipid formulations of amphotericin B. J Liposome Res 1998;8:443-467.
4. Mills W, Chopra R, Linch DC, Goldstone AH. Liposomal amphotericin B in the treatment of fungal infections in neutropenic patients: a single centre experience of 133 episodes in 116 patients. Brit J Haematol 199486:754-760.
5. Andriole VT. 1998. Current and future therapy of invasive fungal infections. In: Current Clinical Topics in Infectious Diseases, Vol. 18 (Remington, J. & Schwartz, M. Eds), 19-36. Blackwell Sciences, Malden, MA.
6. 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 leukopenic mice infected with *Candida albicans*. J Antimicrob Chemother 1995;35:509-19.
7. Lambros MP, Bourne DWA, Abbas SA, Johnson DL. Disposition of aerosolized amphotericin B. J Pharm Sci 1997;86:1066-1069.
8. Ruijgrok EJ, Vulto AG, van Etten EWM. Efficacy of aerosolized amphotericin B desoxycholate and liposomal amphotericin B in the treatment of invasive pulmonary aspergillosis in severely immunocompromised rats. J Antimicrob Chemother 2000;48:89-95.
9. Leenders ACAP, de Marie S, ten Kate MT, Bakker-Woudenberg IAJM, Verbrugh HA. Liposomal amphotericin B (AmBisome) reduces dissemination of infection as compared to amphotericin B deoxycholate (Fungizone) in a newly developed animal model of one-sided pulmonary aspergillosis. J Antimicrob Chemother 1996;38:215-225.
10. 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;22:1042-1050.
11. Becker MJ. de Marie S, Fens MHAM, Hop WCJ, Verbrugh HA, Bakker-Woudenberg IAJM. Enhanced antifungal efficacy by combining Ambisome with Fungizone in experimental invasive pulmonary aspergillosis as assessed by several parameters of antifungal response. J Antimicrob Chemother 2002;45, in press.
12. Griese M, Schams A, Lohmeier KP. Amphotericin B and pulmonary surfactant. Eur J Med Res 1998;3:383-386.
13. Ruijgrok EJ, Vulto AG, van Etten, EWM. Aerosol delivery of amphotericin B desoxycholate (Fungizone) and liposomal amphotericin B (AmBisome): aerosol characteristics and in-vivo amphotericin B deposition in rats. J Pharm Pharmacol 2000;52:619-627.
14. Luber AD, Maa L, Lam M, Guglielmo BJ. Risk factors for amphotericin B-induced nephrotoxicity. J Antimicrobial Chemotherapy 1999;43:267-271.
15. Walsh TJ, Anaissie EJ, Goodman JL, Pappas P, Bekersky I, Buell DN. High dose liposomal amphotericin B in patients infected with aspergillosis and other filamentous fungi. 39th ICAAC, 1999.

Chapter 7

# Prophylaxis of invasive pulmonary aspergillosis with aerosolized amphotericin B desoxycholate and liposomal amphotericin B in severely immunocompromized rats

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# Abstract

The efficacy of nebulised amphotericin B desoxycholate (AMB-DOC) and liposomal amphotericin B (L-AMB) in the empirical and prophylactic treatment of invasive pulmonary aspergillosis (IPA) was studied in rats. A model of IPA in persistently leukopenic rats was used. The endpoint in this model is survival of animals. In this model, untreated rats die between day 4 and day 9 after fungal inoculation. Rats were treated with nebulised AMB-DOC or L-AMB (nebuliser reservoir concentration 2 mg/ml and 4 mg/ml, respectively) at 16 h after (empirical treatment), or 2 h, 1 day, 1 week, 2 weeks, 4 weeks or 6 weeks before (prophylactic treatment) fungal inoculation. Aerosol treatment consisted of a single period of 60 minutes of nebulisation. Aerosols were generated by a Collison nebuliser. Treatment started at 16 h after fungal inoculation, resulted in a significantly prolonged survival for both formulations. There was no difference between the efficacy of nebulised AMB-DOC and nebulised L-AMB. Aerosol treatment with both formulations given at 2 h, 1 day, 1 week, or 2 weeks before fungal inoculation prolonged survival as compared to untreated controls. Aerosol treatment given at 4 weeks before fungal inoculation also resulted in prolonged survival for both formulations, however, the nebulised L-AMB was more effective than nebulised AMB-DOC. When aerosol treatment was given at 6 weeks before fungal inoculation, only L-AMB resulted in prolonged survival of rats as compared to controls. These results suggest that nebulised AMB-DOC and L-AMB can be used in the empirical and prophylactic treatment of IPA. The duration of protection to IPA is longer for the liposomal formulation.

# Introduction

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. Invasive pulmonary aspergillosis (IPA) is the second most common mycosis, after candidiasis, encountered in patients with cancer, particularly in those with haematological malignancies. Major predisposing factors include absolute neutrophil count and the duration of neutropenia [1,2]. Standard treatment for IPA is intravenous administration of amphotericin B desoxycholate (AMB-DOC). Newly developed lipid-based modalities of amphotericin B (AMB) show an increased therapeutic index as compared to conventional AMB-DOC [3]. However, up to now, treatment of an established infection with *Aspergillus* spp. in neutropenic patients is still correlated with high mortality rates. There are no rapid, accurate diagnostic tests that can confirm with certainty the presence of invasive fungal disease. 30% of cases of IPA remain undiagnosed and untreated at death [4]. The fact that diagnosis of IPA during life is a significant problem complicates adequate antifungal treatment. Preventing the development of IPA by giving antifungal prophylaxis may be the best approach to improve outcome. The route of infection of IPA is by inhalation of the infective particles, the conidia, from the environment. Administration of AMB via the pulmonary route can be an attractive alternative approach in the treatment or prophylaxis of pulmonary aspergillosis. Little clinical data are available on the efficacy of inhaled (liposomal) AMB [5,6]. Studies with inhaled AMB in humans describe partial prevention of pulmonary aspergillosis. However, up to date, significant beneficial effects have not been proven, probably because the published studies lack adequate power or design. Animal studies have shown that AMB administration via the pulmonary route can be effective in preventing or treating IPA [7-10]. However, these studies all applied temporary or mild immunosuppression in the experimental model. In the clinical situation however, patients are generally persistently granulocytopenic and diagnosis of IPA is late in the course of infection which emphasises the need for effective treatment of IPA. An important feature of the experimental set-up of our study is therefore that we use an established *Aspergillus* infection under severe, persisting immunosuppression.

In the present study, we use an animal model of IPA in rats which are severely immunosuppressed. In this clinically relevant model, we have recently shown that treatment of an established infection with nebulised AMB-DOC or with L-AMB results in significant beneficial effect on survival of treated animals versus controls.

To explore other ways to optimise outcome in IPA, we investigated in this study the possibilities of empirical and prophylactic treatment in a rat model of severe IPA.

# Materials and Methods

## Materials

AMB-DOC was from Bristol Myers-Squibb (Woerden, The Netherlands) and L-AMB was from NeXstar (San Dimas, CA). Cyclophosphamide was from Sigma Chemical Co. (St. Louis, MO). Sabouraud dextrose agar (SDA) was from Oxoid (Basinstoke, England).

## Animals

Female R-strain albino rats, specified pathogen free, 18- to 25-weeks old (own breed), weighing 185-225 g were used for all experiments. Animals received a normal, pathogen free diet and water *ad libitum*. Experiments were approved by the animal experiments ethical committee of the Erasmus *university* Medical Center.

## Aspergillus strain

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Granulocytopenia was induced by intraperitoneal administration of 90 mg/kg cyclophosphamide at 5 days before fungal inoculation followed by dosages of 60 mg/kg every 4 days throughout the study. This treatment resulted in a persistent granulocytopenia ($<0.5 \times 10^9$/L) from the time of *A. fumigatus* inoculation up to the end of the study [11]. To prevent bacterial superinfection, strict hygienic care was applied, and animals received ciprofloxacin (660 mg/L) and polymyxin E (100 mg/L) in their drinking water during the whole experiment. Furthermore, daily intraperitoneal administration of amoxicillin (40 mg/kg/day) was added to this regimen. Shortly before and after inoculation, gentamicin (6 mg/kg) was administered intramuscularly.

Experimental lung infection

Infection of the lung was established according to the method described by Leenders et al [11]. Briefly, under general anaesthesia, the left main bronchus was intubated. A canula was passed through the tube and the left lobe of the lung was inoculated with 0.02 mL of the conidial suspension containing $1.5 \times 10^5$ conidia. This resulted in a left sided pneumonia.

Aerosol treatment

The nebulisation procedure was previously described [12]. Aerosols were generated by a Collison 6-jet nebuliser system (Model CN, BGI Inc, Waltham, MA). The nebuliser operated at 20 L/min airflow. Under these conditions, more than 80% of the aerosol droplets that are generated are below 5 $\mu$m mass diameter, which ensures substantial deposition of aerosol in the alveobronchial region. Rats were constrained in cone ended plastic tubes and placed in a nose-only inhalation apparatus (CH Technologies USA Inc., Westwood, NJ). Animals were exposed to aerosol treatment for one (single dose) or two periods (double dose) of 60 minutes.

Antifungal treatment

AMB-DOC and L-AMB were reconstituted according to the manufacturers instructions and further diluted in 5% glucose up to an AMB concentration in the nebuliser reservoir of 2 mg/ml in the case of AMB-DOC and 4 mg/ml in the case of L-AMB. These nebuliser reservoir concentrations were chosen based upon results of previous work with this animal model. Aerosol treatment with AMB-DOC is limited to a nebuliser reservoir concentration of 2 mg/ml because higher dosages are not tolerated. The nebuliser reservoir concentration of L-AMB is limited not because of *in vivo* tolerability, but because of technical limitations, since the liposomal suspension can not be nebulised efficiently when the concentration is above 4 mg/ml. The treatment regimens are given in Table 1. Empirical aerosol treatment with AMB-DOC or L-AMB was started at 16 h after fungal inoculation. Histopathological examination (Periodic Acid-Schiff stain) confirms that mycelial outgrowth begins at 16 h. When rats were treated twice, the second aerosol treatment was given at 40 h after fungal inoculation. Prophylactic aerosol treatment with AMB-DOC or L-AMB was given at 2 h, 1 day, 1 week, 2 weeks, 4 weeks or 6 weeks before fungal inoculation. Aerosol treatment consisted of a single dose of 60 minutes.

Efficacy of nebulised AMB-DOC and L-AMB

Groups of 15 rats each were treated with nebulised AMB-DOC or L-AMB. Efficacy, or lack thereof, in each experiment was assessed by the rate of survival of treated

Table 1. Treatment regimens of nebulised AMB-DOC and nebulised L-AMB

| Start of aerosol treatment | Treatment regimen |
|---|---|
| 16 hours after fungal inoculation | single dose |
| 16 hours after fungal inoculation | double dose |
| 2 hours before fungal inoculation | single dose |
| 1 day before fungal inoculation | single dose |
| 1 week before fungal inoculation | single dose |
| 2 weeks before fungal inoculation | single dose |
| 4 weeks before fungal inoculation | single dose |
| 6 weeks before fungal inoculation | single dose |

animals as compared with that of controls. Control rats received nebulised glucose 5%. Animal were checked twice daily and mortality was recorded for 12 days after fungal inoculation. Surviving animals were sacrificed after day 12. To check for bacterial superinfections, left and right lung lobes and liver were dissected and homogenised in 20 mL phosphate buffered saline and cultured in SDA agar as described above.

### Statistical analysis

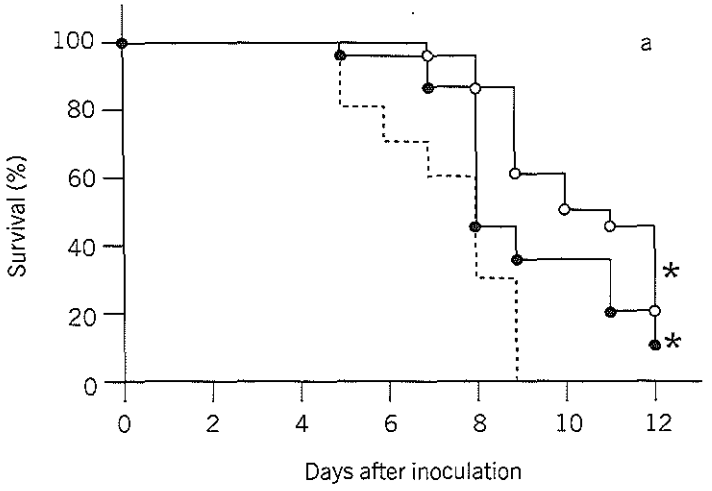
Survival curves were generated by the method of Kaplan and Meier. Statistical evaluation of differences in the survival curves was performed by the log rank test. This test examines the degree in survival with time as well as the final percentage of survival.

## Results

### Effect of nebulised AMB-DOC and nebulised L-AMB on survival

The effect of aerosol treatment on survival of rats with pulmonary aspergillosis is shown in Figures 1 and 2. In this model of pulmonary aspergillosis untreated rats die between day 4 and day 9 after fungal inoculation. Empirical aerosol treatment started at 16 h after fungal inoculation with AMB-DOC or L-AMB showed a statistically significant prolonged survival as compared to controls ($P < 0.05$) (Fig. 1a). The efficacy of AMB-DOC did not differ from that of L-AMB. Administration of a double dose of both agents also showed a statistically significant prolonged survival as compared to controls ($P < 0.05$) (Fig. 1b). However, this effect was not different from the single dose treatment.

Figure 1. Effect of a single (a), or double dose (b) of nebulised AMB-DOC (●) and nebulised L-AMB (○) on survival of severely immunosuppressed rats with pulmonary aspergillosis (Kaplan-Meier plot). Nebuliser reservoir concentrations were 2 mg/mL in the case of nebulised AMB-DOC and 4 mg/mL in the case of L-AMB. Each group consisted of 15 animals. Control animals received nebulised 5% glucose (---). Treatment was started 16 h after fungal inoculation, at which time mycelial growth begins. *P < 0.05 versus controls.



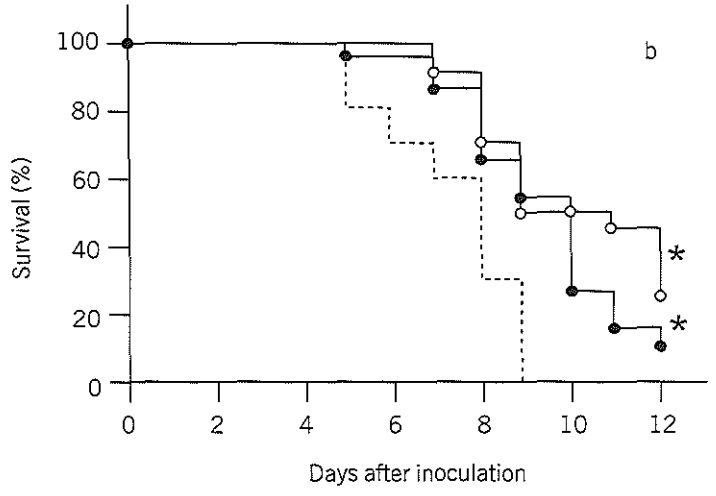
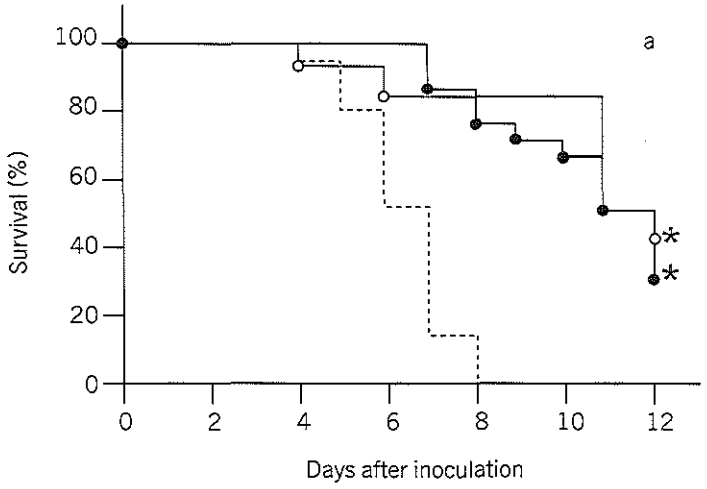
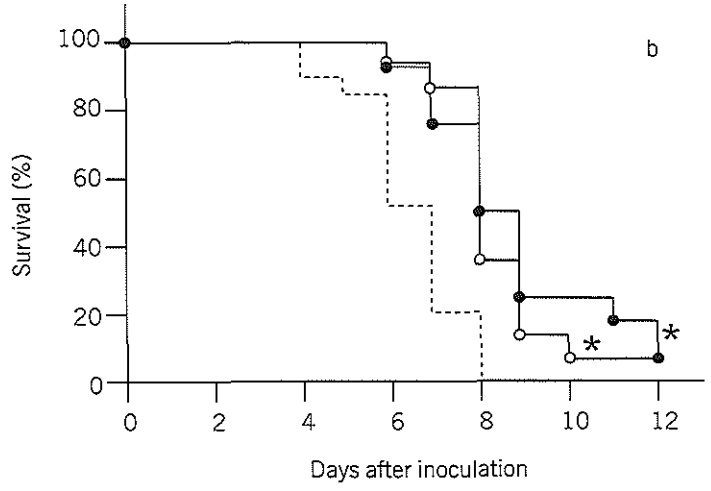
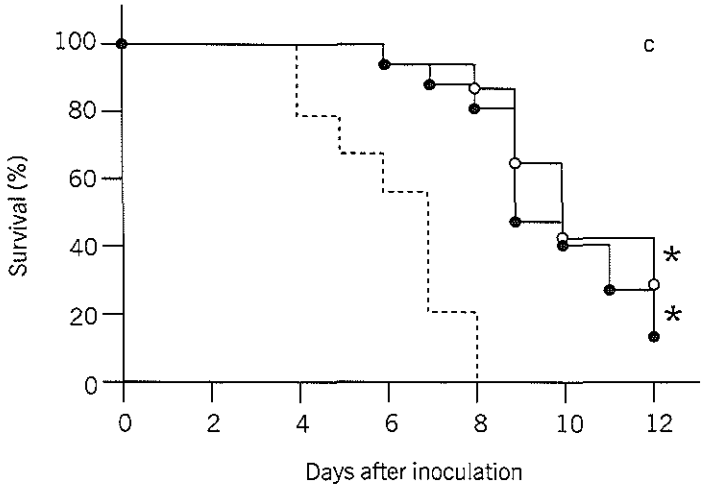



Figure 2. Effect of single dose of nebulised AMB-DOC (●) and nebulised L-AMB (○) on survival of severely immunosuppressed rats with pulmonary aspergillosis (Kaplan-Meier plot). Treatment was started at 2 hours (a), 1 day (b), 1 week (c), 2 weeks (d), 4 weeks (e) or 6 weeks (f) before fungal inoculation. Nebuliser reservoir concentrations were 2 mg/mL in the case of AMB-DOC and 4 mg/mL in the case of L-AMB. Each group consisted of 15 animals. Control animals received aerosolised 5% glucose (---). *P < 0.05 versus controls. †P < 0.05 versus AMB-DOC.

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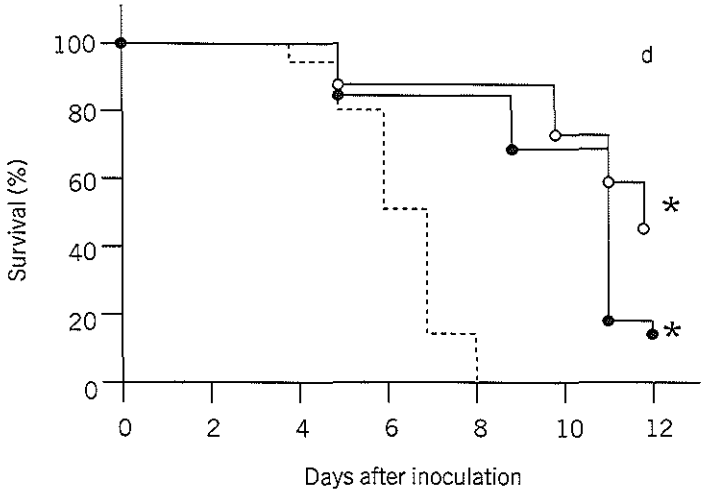
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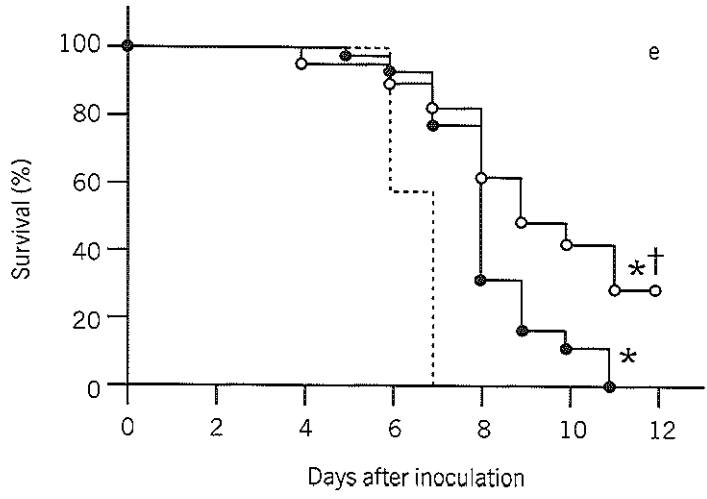
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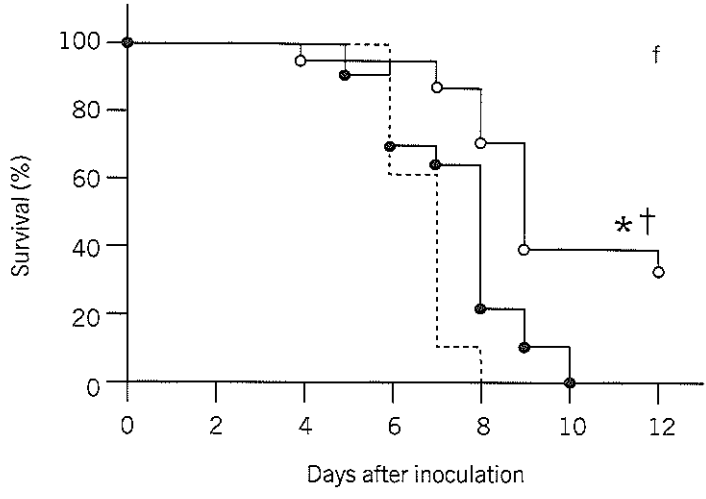
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The effects of single dose prophylactic aerosol treatment started at different time points before fungal inoculation are shown in Fig. 2a to Fig. 2f. Administration of a single aerosol dose directly preceding inoculation (2 h) resulted in a statistically significant prolonged survival ($P < 0.05$) for both the non-liposomal as the liposomal formulation (Fig. 2a). When aerosol treatment was started at 1 day, 1 week or 2 weeks before fungal inoculation, the beneficial effect on survival remains for both formulations (Fig. 2b-d). At these time points, no difference is seen between AMB-DOC and L-AMB. Administration of nebulised AMB-DOC and L-AMB at 4 weeks before fungal inoculation results in statistically significant prolonged survival for both products ($P < 0.05$) (Fig. 2e & 2f). With this regimen, the liposomal formulation resulted in a statistically significant prolonged survival as compared to the non-liposomal formulation ($P < 0.05$). When aerosol treatment was given at 6 weeks before fungal inoculation, only L-AMB resulted in a significantly prolonged survival ($P < 0.05$).

# Discussion

Pulmonary aspergillosis is a serious opportunistic disease in patients with immunosuppression. Aerosol administration of antifungal agents directly deposits the drug at the site of the intended action and could therefore be an attractive therapeutic approach. Empirical or prophylactic measures would be highly beneficial because treatment often fails after infection is diagnosed. In the present study, we have investigated the possibilities of empirical and prophylactic aerosol administration of AMB-DOC and L-AMB in a rat model of severe pulmonary aspergillosis. We have used a difficult-to-treat, animal model of IPA. This model is highly clinically relevant since it is characterised by profound and persistent neutropenia and shows hyphal invasion of pulmonary tissue and vessels. The Collison nebuliser and inhalation system for treatment of rats with nebulised AMB-DOC and L-AMB was validated in a previous study [12]. Nebulisation of AMB-DOC and L-AMB with this device leads to respirable aerosols and yields substantial lung tissue concentrations of AMB (above the minimal inhibitory concentration of *A. fumigatus*) and negligible systemic exposure. We have shown previously that aerosol treatment of an established infection results in prolonged survival in the same rat model as used in the present study [13]. However, in view of sub-optimal treatment after the infection has fulminated, we suggested that early empirical treatment or prophylactic treatment would be an interesting approach to further optimise outcome of pulmonary aspergillosis. Empirical treatment with maximal dosages of AMB-DOC

and L-AMB at 16 hours after fungal inoculation, the time at which the hyphal outgrowth of the fungus begins, resulted in a prolonged survival of animals as compared to controls. No difference is seen between the liposomal and the non-liposomal formulation. Administering an additional dose at 40 hours after fungal inoculation did not further improve efficacy.

When a single dose of nebulised AMB-DOC or L-AMB was given directly before fungal inoculation, a significantly prolonged survival is seen as compared to controls. Administering the optimal nebulised dose of either agent just before inoculating the conidial suspension is close to an *in vitro* experiment. Surprisingly, the mortality rate in this experiment is still high, with only 30-40% of the rats surviving. This observation illustrates the severity of the animal model we have used for our experiments. The Kaplan Meier curves for the prophylaxis given at 1 day, 1 week or 2 weeks before fungal inoculation are similar to that seen at 2 hours before inoculation, whites demonstrates that a single dose of nebulised AMB, either liposome-encapsulated or not, will be retained in the lungs for a long period of time. A long half-life of AMB in pulmonary tissue after lung deposition was already described [14]. The extensive retention is probably due to extensive tissue binding as a result of a high affinity of AMB for cholesterol. In this study, we have shown that the deposited AMB remains biologically active. This suggests that prophylactic administration of only a single dose of AMB can be beneficial for several weeks for neutropenic patients at risk for acquiring aspergillosis.

Single dose prophylaxis, given at 4 weeks before fungal inoculation, still results in a beneficial effect on survival for both formulations. With aerosol administration at 6 weeks before inoculation, only L-AMB is effective. We see here that the effect of L-AMB is more pronounced than that of AMB-DOC. Liposomal encapsulation may result in an extended half life of AMB in the lungs, as compared to non-liposomal AMB.

An additional advantage of administering the liposomal formulation is that *in vitro* data show that it will probably be less irritating for pulmonary mucosa than the desoxycholate formulation [13].

Next to the effect of treatment on survival of animals we have also investigated the effect of aerosol treatment of AMB-DOC and L-AMB on number of viable aspergilli in order to examine the direct antifungal effect of administered agents (unpublished data). We have seen that number of colony forming units did not seem to be corresponding to the severity of the infection. Possibly, nuclear multiplication of inoculated conidia and mycelial growth are not expressed as increase in the number of colony forming units. We therefore decided not to use the parameter of the viable fungal load to determine antifungal efficacy of administered agents.

However, this measure is used in a number of studies to express antifungal activity of investigational drugs [7,14].
In summary, the present study shows that administering a single dose of nebulised liposomal or non-liposomal AMB to animals before they develop fulminant aspergillosis, is effective in prolonging survival. The effect of the liposomal formulation is longer lasting than that of the conventional formulation of AMB. These results suggest that a single dose of nebulised AMB-DOC, but preferentially L-AMB, could prevent pulmonary aspergillosis in patients at risk.

# References


1. Denning DW. Therapeutic outcome in invasive aspergillosis. Clin Infect Dis 1996;23:608-15.
2. Denning DW. Invasive aspergillosis. Clin Infect Dis 1998;26:781-803.
3. Hiemenz JW, Walsh TJ. Lipid formulations of amphotericin B. J Liposome Res 1998;8:443-467.
4. Vogeser M, Wanders A, Haas A, Ruckdeschel G. A four-year review of fatal aspergillosis. Eur J Clin Microbiol Infect Dis 1999;18:42-45.
5. Purcell, I. F., and P. A. Corris. 1995. Use of liposomal amphotericin B in the treatment of *Aspergillus fumigatus* empyema. Thorax 50:1321-1323
6. Schwartz S, Behre G, Heinemann V, et al. Aerosolized amphotericin B inhalations as prophylaxis of invasive *aspergillus* infections during prolonged neutropenia: Results of a prospective randomized multicenter trial. Blood 1999;93:3654-3661.
7. Allen SD, Sorensen KN, Nejdl MJ, Durrant, and R. T. Proffit. 1994. Prophylactic efficacy of aerosolized liposomal (AmBisome) and non-liposomal (Fungizone) amphotericin B in murine pulmonary aspergillosis. J Antimicrob. Chemother. 34:1001-1013.
8. Cicogna CE, White MH, Bernard EM, Ishimura T, Sun M, Tong WP, Armstrong D. Efficacy of prophylactic aerosol amphotericin B lipid complex in a rat model of pulmonary aspergillosis. Antimicrob Agents Chemother 1997;41:259-261.
9. Schmitt HJ, Bernard EM, Hauser M, Armstrong D. Aerosol amphotericin B is effective for prophylaxis and therapy in a rat model of pulmonary aspergillosis. Antimicrob Agents Chemother 1988;32:1676-9.
10. Sorensen KN, Allen SD, Nejdl MJ, Proffit RT. Aerosolisation of liposomal (Ambisome) and non-liposomal (Fungizone) amphotericin B as treatment for pulmonary fungal infections. J Control Release 1994;28:346-347.
11. Leenders ACAP, de Marie S, ten Kate MT, Bakker-Woudenberg IAJM, Verbrugh HA. Liposomal amphotericin B (AmBisome) reduces dissemination of infection as compared to amphotericin B deoxycholate (Fungizone) in a newly developed animal model of one-sided pulmonary aspergillosis. J Antimicrob Chemother 1996;38:215-225.
12. Ruijgrok EJ, Vulto AG, van Etten, EWM. Aerosol delivery of amphotericin B desoxycholate (Fungizone) and liposomal amphotericin B (AmBisome): aerosol characteristics and in-vivo amphotericin B deposition in rats. J Pharm Pharmacol 2000;52:619-627.
13. Ruijgrok EJ, Vulto AG, van Etten EWM. Efficacy of aerosolized amphotericin B desoxycholate and liposomal amphotericin B in the treatment of invasive pulmonary aspergillosis in severely immunocompromised rats. J Antimicrob Chemother 2000;48:89-95.
14. Lambros MP, Bourne DWA, Abbas SA, Johnson DL. Disposition of aerosolized amphotericin B. J Pharm Sci 1997;86:1066-1069.
15. Niki Y, Bernard EM, Schmitt HJ, Tong WP, Edwards FF, Armstrong D. Pharmacokinetics of aerosol amphotericin B in rats. Antimicrob Agents Chemother 1990;34:29-32.

Chapter 8

# Nebulisation of four commercially available amphotericin B formulations in persistently leukopenic rats with invasive pulmonary aspergillosis: evidence for long-term biological activity

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# Abstract

The nebulisation of amphotericin B desoxycholate (AMB-DOC), liposomal amphotericin B (L-AMB), amphotericin B lipid complex (ABLC) and amphotericin B colloidal dispersion (ABCD) was investigated. Particle sizes of generated aerosol droplets were measured. Pulmonary amphotericin B (AMB) deposition and AMB concentration in blood directly after nebulisation and at 6 weeks after nebulisation was measured in healthy rats. The efficacy of nebulised AMB formulations was evaluated in persistently leukopenic rats with invasive pulmonary aspergillosis (IPA). Aerosols were generated with a Collison nebuliser. Treatment consisted of a single inhalation period of 60 min. The nebuliser reservoir AMB concentration was 4 mg/mL for the lipid preparations and 2 mg/mL for AMB-DOC. Treatment was given either at 16 h after fungal inoculation, or at 1 week, 2 weeks or 6 weeks before fungal inoculation. The endpoint was survival of animals. Aerosol particle sizes, expressed as the values for the mass median diameter were 1.38, 2.43, 0.90 and 2.29 $\mu$m for AMB-DOC, L-AMB, ABLC and ABCD, respectively. For all formulations, AMB concentrations in the lungs directly after nebulisation exceeded the MIC of *Aspergillus fumigatus* and AMB was still detected in lungs of rats at 6 weeks after nebulisation. Treatment, started at 16 h after fungal inoculation, resulted in a significantly prolonged survival as compared to sham-treated rats for all 4 formulations. Prophylactic treatment at 1 week before fungal inoculation resulted in a significantly prolonged survival for all 4 formulations. Aerosol treatment given at 2 weeks before inoculation was effective only for AMB-DOC and L-AMB, whereas treatment given at 6 weeks resulted in a significantly prolonged survival only for L-AMB.

All commercially available AMB preparations can be nebulised efficiently and may be of value in the prophylactic treatment of IPA.

# Introduction

An increase in the number of immunosuppressed patients in the last decades has led to an increase in the number of invasive fungal infections such as invasive pulmonary aspergillosis (IPA) [1,2]. For many years, conventional amphotericin B, in the form of amphotericin B desoxycholate (AMB-DOC), has been considered as the treatment of choice for IPA, due to its potent fungicidal activity [3]. Its use, however, is limited by its narrow therapeutic index. A number of toxic side effects, especially nephrotoxicity, have made this drug difficult for patients to tolerate [4]. Lipid-based formulations of AMB were developed in order to diminish side effects of AMB. Lipid-based AMB formulations have a broader therapeutic index as compared to AMB-DOC, which permits higher dosing schedules. Currently, there are 3 marketed lipid formulations of AMB. Liposomal amphotericin B (L-AMB) consists of small unilamellar vesicles with AMB incorporated within the bilayer membrane of phopholipids. Amphotericin B lipid complex (ABLC) has AMB complexed to two phospholipids in a ribbonlike structure. Amphotericin B colloidal dispersion (ABCD) is composed of disklike structures of cholesteryl sulphate complexed with AMB. In the empirical treatment of systemic fungal infections, all 3 lipid-formulations of AMB are at least as effective as AMB-DOC and show less toxicity [5]. Little direct comparative data are available on the relative efficacy of the different lipid formulations or on the comparative efficacy against AMB-DOC. Despite the choice of several available AMB formulations, mortality rates from IPA are still unacceptably high and most neutropenic patients with proven IPA die from this infection [2,6]. These severe life-threatening features of IPA stress the critical need for optimising management of this infection.

Failure of treatment of IPA with intravenous AMB is probably the result of several factors. The fungus resides predominantly in the airways and can spread to other body tissues. Inadequate penetration of AMB in the airways after intravenous administration of any AMB formulation can contribute to failure of treatment. It has been shown that only a small percentage of intravenously administered AMB is actually delivered to the lungs [7,8]. By administration of drugs via the inhalation route, the lungs are directly targeted which results in high pulmonary drug concentrations. Nebulised AMB-DOC, L-AMB, and ABLC in different animal models of IPA resulted in substantial, long lasting pulmonary AMB concentrations and significantly improved survival of rats [9-12]. However, data on the comparative value of the AMB formulations are scarce.

Aerosol administration of AMB formulations can be valuable in the optimisation of the management of IPA. Which formulation is most suitable for this approach has not yet been investigated. This study presents a head-to-head comparison of the biodistribution, efficacy and toxicity of nebulised AMB formulations.

# Materials and Methods

## Materials

AMB-DOC was from Bristol Myers-Squibb (Woerden, The Netherlands), L-AMB was from Nexstar (San Dimas, CA), ABLC was from The Liposome Company (Princeton, NJ) and ABCD was from Alza Corporation (Palo Alto, CA). Cyclophosphamide was from Sigma Chemical Co. (St. Louis, MO). Sabouraud dextrose agar (SDA) was from Oxoid (Basingstoke, UK).

## Animals

Female R-strain albino rats, specified pathogen free, 18- to 25- weeks old (own breed), weighing 185-225 g were used for all experiments. Animals received a normal, pathogen free diet and water ad libitum. Experiments were approved by the animal experiments ethical committee of the Erasmus *university* Medical Center Rotterdam.

## Aspergillus strain

A clinical isolate of *Aspergillus fumigatus* from an immunocompromised patient with IPA was used. MIC and minimal fungicidal concentrations of AMB for this strain are 0.4 and 0.8 mg/L, respectively [13]. This strain was stored under oil on SDA. At least once every two months, the strain was passed through a rat to maintain its virulence. For inoculation, conidia were harvested and suspended in sterile phosphate buffered saline, as previously described [13].

## Immunosuppression and supportive care

Leukopenia was induced by intraperitoneal administration of 90 mg/kg cyclophosphamide at 5 days before fungal inoculation followed by additional dosages of 60 mg/kg every 4 days throughout the study. This treatment resulted in a persistent leukopenia ($< 0.5 \times 10^9$/L) from the time of *A. fumigatus* inoculation up to the end of the study [13]. To prevent bacterial superinfection, strict hygienic care was applied, and animals received ciprofloxacin (660 mg/L) and polymyxin E (100 mg/L) in their drinking water during the whole experiment. Furthermore, intramuscular administration of amoxicillin (40 mg/kg/day) was added to this regimen. Shortly before and after inoculation, gentamicin (6 mg/kg) was administered intramuscularly.

Experimental lung infection

Infection of the lung was established according to the method described by Bakker-Woudenberg et al. [14]. Briefly, under general anaesthesia the left main bronchus was intubated. A canula was passed through the tube and the left lobe of the lung was inoculated with 0.02 mL of the conidial suspension containing $1.5 \times 10^5$ conidia. This resulted in a left sided pneumonia.

Nebulisation procedure

AMB-DOC, L-AMB, ABLC and ABCD were reconstituted according to the manufacturers instructions and further diluted in 5% glucose. L-AMB, ABLC and ABCD were diluted with glucose up to an AMB concentration in the nebuliser reservoir of 4 mg/mL. AMB-DOC was diluted up to a nebuliser reservoir concentration of 2 mg/mL. Previous work in our laboratory describes the rationale for these concentrations [12]. In short: for AMB-DOC, 2 mg/mL is the maximum dose which is tolerated by rats, whereas the dose of the lipid formulations is limited to 4 mg/mL because of technical limitations to nebulisation. The aerosol procedure was as previously described [15]: rats were constrained in cone ended plastic tubes and placed in a nose-only inhalation apparatus (CH Technologies USA Inc., Westwood, NJ). Aerosols were generated by a Collison six-jet nebuliser system (Model CN, BGI Inc., Waltham, MA). The nebuliser operated at 20 L/min air flow. Animals were exposed to aerosol treatment for one or more periods of 60 minutes.

Droplet size measurements

The droplet size distribution of aerosols was measured using a laser velocity particle sizer (Aerosol Particle Sizer 3320 A, TSI, Inc., St Paul, MN). Aerosols were generated with compressed air at a flow rate of 10 L/min. Distribution of the number of generated aerosol particles was directly measured with this technique. For extrapolation of mass distribution from the number distribution, specific gravity was set at that of the solvent (1 g/cm$^3$) and relative humidity at 60%. From the mass distribution, the mass median diameter and the percentage of particles $< 5\ \mu$m were calculated.

Deposition of AMB in lungs after nebulisation of AMB-DOC, L- AMB, ABLC, or ABCD

Deposition experiments were performed in healthy rats. Directly after and at 6 weeks after nebulisation of AMB-DOC, L-AMB, ABLC or ABCD, rats were killed and blood was sampled via a cardiac punction. Right and left lung lobes were removed and weighed. Lungs were homogenised in 5 mL glucose 5%. AMB was extracted from blood and homogenate with ethanol in a 2:3 (v/v) ratio.

The extracts were centrifuged for 5 min at 13,000 x g and concentrations of AMB in the supernatants were determined by HPLC with an UV detector operating at 382 nm. The mobile phase consisted of 0.1 M sodium acetate solution (pH 7.2) containing 0.2 % (v/v) triethylamine and 60% acetonitrile and was pumped through a guard pre-column (Chromguard, 10 x 3 mm; Chrompack, Middelburg, The Netherlands) followed by a reverse-phase ODS 2 C18 column (100 x 3 mm ID; particle size 5 mm; Chrompack, Middelburg, The Netherlands) at a flow rate of 0.5 mL/min. The recovery was 70% for blood and 75% for lung tissue. The lower limit of quantification of this assay was 0.2 mg/mL.

Effect of AMB-DOC, L-AMB, ABLC and ABCD on survival of rats

Groups of 15 infected rats each were treated with a single dose of nebulised AMB-DOC, L-AMB, ABLC or ABCD at different times after or before fungal inoculation. Treatment given after fungal inoculation started at 16 h from inoculation. Histopathological examination (Periodic Acid-Schiff stain) confirmed that mycelial outgrowth begins at 16 h. Prophylactic treatment was given at 1 week, 2 weeks, or 6 weeks before fungal inoculation. The endpoint was survival of treated animals as compared to controls. Controls received nebulised glucose 5%. Animals were checked twice daily and mortality was recorded for 12 days after fungal inoculation. Surviving animals were sacrificed after day 12. To check for bacterial superinfections, the left lung, right lung and liver were dissected post mortem and homogenised in 20 ml phosphate buffered saline for 45 sec at 20,000 rpm in a VirTis homogeniser (The VirTis Co. Inc., Gardiner, NY, USA). Volumes of 0.2 ml and 2 ml and the remainder of each homogenate were spread onto or poured into Sabouraud dextrose agar plates. Plates were incubated for 24 h at 37 °C followed by 24 h at 25 °C.

Influence of AMB products on surfactant function

Freeze dried natural surfactant prepared from bovine lavages was a gift of the Department of Anaesthesiology of the Erasmus *university* Medical Center Rotterdam. It consisted of approximately 90-95% phospholipids, 1% hydrophobic proteins (surfactant protein B & C) and 1% free fatty acids. This surfactant is highly surface active at low concentrations. Influence of different AMB formulations on surfactant function was determined by means of a modified Wilhelmy balance |system (E. Biegler GmbH, Mauerbach, Austria). This system records surface tensions of an air-liquid film over several cycles of mechanical compression and expansion of this film. The lower the surface tension at minimal surface area, the higher the surface activity of the applied film.

The trough of the Wilhelmy balance was filled with warm saline (37°C) and calibrated. After calibration, 100 $\mu$L of surfactant (1 mg/mL total lipids) alone or in the presence of AMB formulations was applied onto the saline hypophase and allowed to spread for 2 min. The surface area was compressed and expanded with a cycling speed of 1 cycle per 3 min and an area reduction from 100% to 20%. Minimal surface tension ($\gamma_{min}$) was measured after 3 cycles at 20% surface area, and is expressed as mN/m. Inhibition of surfactant function of natural surfactant by AMB formulations results in increased surface tension at minimal surface area.

### Statistical analysis

Survival curves were generated by the method of Kaplan and Meier. Statistical evaluation of differences in the survival curves was performed by the log rank test. This test examines the length of survival as well as the percentage of survival.

## Results

### Aerosol droplet size

Aerosol droplet sizes of all 4 formulations defined as the mass median diameter and the percentage of particles < 5 $\mu$m are given in Table 1. For all formulations, more than 80% of the particles was below 5 $\mu$m mass diameter.

### Deposition of AMB in lungs after nebulisation of AMB-DOC, L- AMB, ABLC, ABCD

The pulmonary concentrations of AMB in uninfected lungs for aerosolised AMB-DOC (nebuliser reservoir concentration 2 mg/mL) and aerosolised L-AMB, ABLC and ABCD (nebuliser reservoir concentration 4 mg/mL) are given in Table 2. The concentrations were determined directly after and at 6 weeks after a single aerosol dose of either formulation. For all formulations, substantial AMB concentrations were deposited in the lungs directly after nebulisation. There was no statistically

Table 1. Mass median diameter and % of aerosol particles < 5 $\mu$m as determined by laser diffraction analysis

| | Mass median diameter ($\mu$m) | % particles < 5 $\mu$m |
|---|---|---|
| AMB-DOC | 1.38 | 83.2 |
| L-AMB | 2.43 | 86.6 |
| ABLC | 0.90 | 91.2 |
| ABCD | 2.29 | 86.7 |

Table 2. Deposition of AMB in lungs (μg/g) directly after (0 h) and 6 weeks after aerosol administration (60 minutes) of 4 different AMB formulations

| | AMB conc (mg/ml) in nebuliser | AMB in lungs (μg/g) at 0 h | AMB in lungs (μg/g) at 6 wk |
|---|---|---|---|
| AMB-DOC | 2 | 26.9 (8.5)[a] | 11.4 (1.3) |
| L-AMB | 4 | 46.7 (15.5) | 11.1 (2.9) |
| ABLC | 4 | 24.9 (11.4) | 7.7 (0.4) |
| ABCD | 4 | 30.9 (5.1) | 14.8 (2.5) |

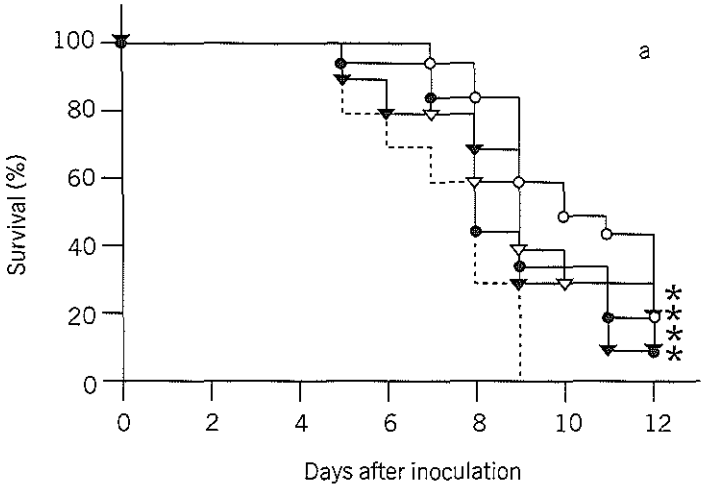
[a]values are mean (SD), n=3

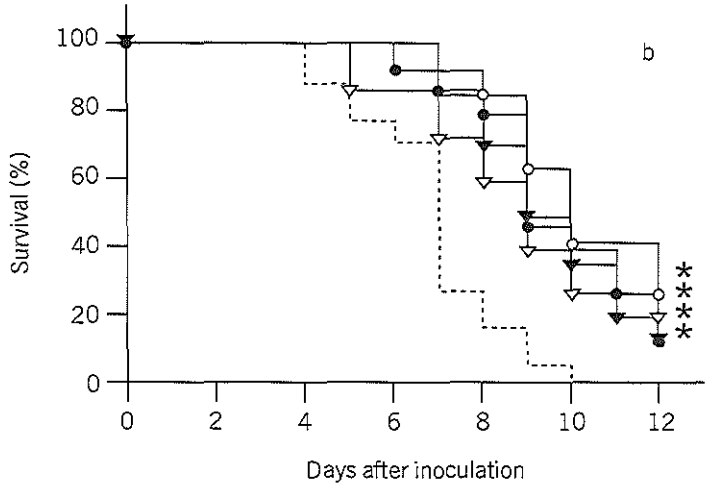
significant difference in the deposited AMB between the 4 formulations. With all 4 products, AMB was still detected in lungs at 6 weeks after nebulisation, which indicates a very slow clearance of AMB from the lungs. The amount of AMB in blood was below the limit of detection in all samples.

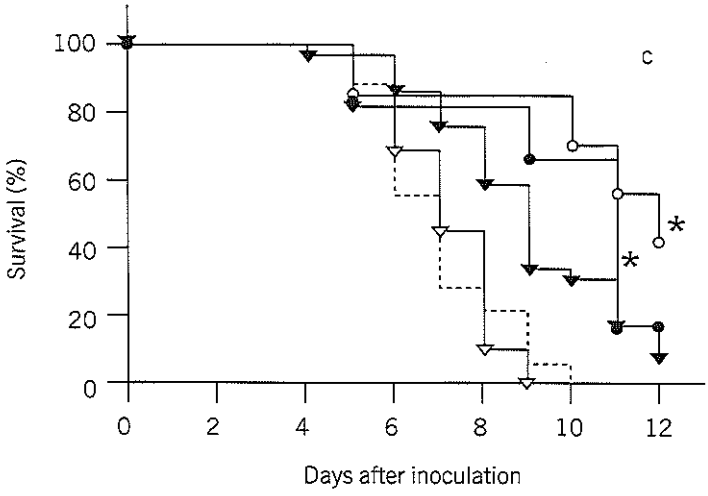
Effect of nebulised AMB-DOC, L-AMB, ABLC and ABCD on survival of rats

The effect of aerosol treatment on survival is shown in Figure 1. In this model of IPA, control rats die between day 4 and day 9 after fungal inoculation. Empirical treatment started at 16 h after fungal inoculation, the time at which hyphal outgrowth begins. This treatment resulted in a significantly prolonged survival as compared to controls for all 4 regimens ($P < 0.05$) (Fig. 1a), with no difference between the 4 AMB-formulations. Prophylactic treatment was started at 1 week, 2 weeks or 6 weeks before fungal inoculation. Treatment started at 1 week before inoculation resulted in a significantly prolonged survival as compared to controls for all 4 regimens ($P < 0.05$) (Fig. 1b), again with no difference between the 4 AMB formulations. Treatment started at 2 weeks before fungal inoculation resulted in a significantly prolonged survival for AMB-DOC and L-AMB ($P < 0.05$), whereas ABLC showed borderline significance ($P = 0.06$). Treatment with ABCD did not result in a significantly prolonged survival ($P = 0.77$). Only for L-AMB did treatment at 6 weeks before inoculation result in a significantly prolonged survival as compared to controls ($P < 0.05$).

Figure 1. Effect of a single dose of aerosolized AMB-DOC (●), L-AMB (○), ABLC (▽) and ABCD (▼) on survival of persistently leukopenic rats with pulmonary aspergillosis (Kaplan-Meier plot). Treatment was started at 16 hours after (a) or 1 week (b), 2 weeks (c) or 6 weeks (d) before fungal inoculation. Nebuliser reservoir AMB concentrations were 4 mg/mL, except for AMB-DOC, where the nebuliser reservoir concentration was 2 mg/mL. Each group of 15 animals was exposed for 60 minutes. Control animals received nebulised 5% glucose (---). *P < 0.05 versus controls.

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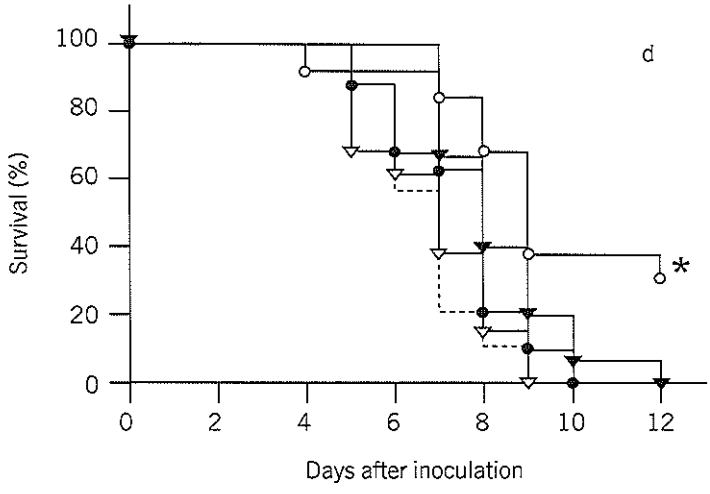
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Table 3. Mean minimal surface tension ($\gamma_{min}$) of surfactant (1 mg/mL) together with saline, AMB, desoxycholate, AMB-DOC, L-AMB, ABLC and ABCD

| | Concentration (mg/ml) | | Minimal surface tension (mN/m)[a] |
|---|---|---|---|
| | AMB | DOC | |
| Saline | - | - | 1.97 ± 1.23 |
| AMB | 2 | | 2.70 ± 0.53 |
| Desoxycholate | | 2.1 | 23.13± 1.16[c] |
| AMB-DOC[b] | 2 | 1.6 | 47.53± 0.89[c] |
| L-AMB | 2 | | 1.42 ± 0.83 |
| ABLC | 2 | | 1.45 ± 0.39 |
| ABCD | 2 | | 3.33 ± 0.39 |

[a]each value represents the mean ± sd of three individual experiments

[b]In AMB-DOC, 54% (g/g) is AMB, 46% is DOC, corresponding with 2 mg/ml AMB and 1.6 mg/ml DOC

[c]$p<0.05$ as compared to saline

Influence of AMB products on surfactant function

Table 3 shows the mean minimal surface tensions ($\gamma_{min}$) of surfactant alone or in combination with AMB alone, desoxycholate, AMB-DOC, L-AMB, ABLC and ABCD. The natural surfactant was highly surface active at the examined concentrations ($\gamma_{min}$ 1.97 ± 1.23). Minimal surface tensions after mixing of surfactant with AMB alone, L-AMB, ABLC or ABCD yielded similar low values. Addition of AMB-DOC to natural surfactant resulted in significantly increased minimal surface tensions, indicating a loss of surface activity of the mixtures. An increase in minimal surface tension of natural surfactant was also seen with addition of desoxycholate alone.

# Discussion

IPA is a serious opportunistic disease in patients with immunosuppression. A difficult diagnosis, which is often confirmed late in the course of the infection, hampers an adequate treatment of IPA. In order to treat IPA effectively, multiple high doses of intravenous AMB-DOC are needed, but these can usually not be given due to nephrotoxicity of AMB. Using aerosol administration of AMB formulations, the drug is directly deposited at the intended site of action, which could be highly attractive both in terms of avoidance of toxicity as in terms of efficacy. In the present study, we have examined the value of aerosol administration of 4 commercially available

AMB formulations in a model of IPA in persistently leukopenic rats.

The amount of deposition of an aerosol in the respiratory tract is mainly determined by the particle size distribution of the aerosol droplets. Large particles with a diameter of > 5 mm are deposited in the upper airways and will not be distributed to the peripheral regions of the lungs. Particles with a diameter of < 0.5 $\mu$m are exhaled [16]. Inhaled *Aspergillus fumigatus* spores, with an average particle size of 1-2 $\mu$m, are deposited in the peripheral (alveolar) region of the lungs and invasive disease develops from thereon. It is suggested that the optimal range of particle sizes for peripheral deposition for AMB aerosols is between 1 and 5 $\mu$m. We have shown before that AMB-DOC and L-AMB aerosols are characterised by an adequate particle size [15]. Although the 4 tested AMB formulations have distinct different physico-chemical properties, all formulations were efficiently nebulised by the nebuliser used in the present study. Although the mass median diameter is different for the 4 formulations, this is probably not relevant for their peripheral deposition. The median particle size of all 4 AMB formulations is between 1 and 5 $\mu$m, and >80% of generated particles are below 5 $\mu$m. A substantial peripheral deposition of AMB can therefore be expected for all formulations.

We previously described pulmonary kinetics of aerosolised AMB-DOC and L-AMB [17]. In this work, we showed the favourable AMB deposition and retention in the lungs of rats after nebulisation of AMB-DOC or L-AMB. In the present study, we show that for all 4 AMB formulations, the pulmonary concentrations of AMB directly after nebulisation are substantial and exceed the MIC for *Aspergillus fumigatus*, which is 0.4 - 0.8 mg/L for non-lipid associated AMB. The deposition data of the different formulations directly after nebulisation were not in discrepancy with the differences in aerosol particle sizes. The pulmonary deposition seemed not to differ significantly between the different products. Furthermore, we show that AMB can still be detected in pulmonary tissue at 6 weeks after nebulisation of all 4 formulations. No AMB was detected in blood at all times. This indicates that aerosol administration leads to very low systemic exposure of AMB. The substantial deposition as well as the extensive pulmonary retention and low systemic exposure of AMB renders the aerosol delivery of either product very suitable for empirical as well as prophylactic treatment of pulmonary aspergillosis.

Few clinical data are available on the nebulisation of AMB formulations in patients with pulmonary fungal infections [18-20]. Until now, the exact value of this approach still has to be determined. In models of aspergillosis in mice and rats with corticosteroid induced immunosuppression, prophylaxis or treatment of IPA with nebulised AMB-DOC or L-AMB was evaluated [9,10]. In these studies, the nebulised formulations were effective in decreasing the number of viable

*Aspergillus fumigatus* counts in the lungs and in increasing survival of animals. L-AMB was more effective than AMB-DOC [9]. In rats with corticosteroid induced immunosuppression, prophylactically nebulised ABLC resulted in improved survival of IPA [11]. These studies were all focused on the prophylaxis of IPA, and temporary or mild immunosuppression was applied in the experimental model. In the clinical situation however, patients with severe IPA are generally persistently leukopenic. An important feature of the experimental set-up of the present study is therefore the persistently immunosuppressed state. In this model, we have previously described the therapeutic efficacy of nebulised AMB-DOC versus L-AMB, with the start of treatment at 30 h after fungal inoculation [12]. In that study we showed that nebulised AMB-DOC as well as nebulised L-AMB resulted in a statistically significant prolonged survival in rats with established IPA. In the present study start of treatment was at 16 h after and at different times before inoculation. Histopathological examination confirms that at 16 h after fungal inoculation hyphal outgrowth begins. We have seen that aerosol treatment started at 16 h with all 4 formulations resulted in a significantly prolonged survival as compared to control rats. However, we showed before that aerosol administration of AMB formulations does not prevent dissemination of the infection. [12] We concluded therefore, that aerosol administration of AMB will be of limited value as treatment of IPA.

The deposition data show that long term protection could be expected from the nebulised AMB products, since at 6 weeks after a single dose of nebulised AMB-DOC, L-AMB, ABLC or ABCD, AMB is still present in lung tissue. The measured AMB concentrations exceeded the MIC value of *Aspergillus fumigatus* strain for AMB. Whether this AMB is indeed biologically active, remains to be proven by the efficacy data. We showed prophylactic efficacy for all 4 formulations when aerosols were given at 1 week before fungal inoculation. The efficacy seemed not to differ between the 4 products. When aerosol treatment was given at 2 weeks before fungal inoculation, we see that nebulised AMB-DOC and L-AMB still showed prophylactic efficacy, whereas nebulised ABCD did not and ABLC did not conclusively. When given at 6 weeks before only nebulised L-AMB showed prophylactic efficacy. The differences between the 4 formulations in the efficacy experiments are not reflected by the aerosol particle size or the deposition data. We can therefore only speculate about the reason for the observed discrepancy between the chemical presence and the biological activity of AMB. It is known that AMB tightly binds to cell membranes and sterol receptors which may render AMB inactive over time, however, still extractable with organic solvents as we did.

It is tempting to suggest that the liposomal formulation acts as a slow release

pool, which results in long term efficacy as is shown in the survival data.
The toxicity of aerosolised AMB formulations was evaluated in an in-vitro experiment in which their influence on surfactant function was determined. It appeared that AMB-DOC, and not the lipid formulations, had a detrimental effect on surfactant. This was due to the detrimental effects of desoxycholate on surfactant function, since this agent alone showed high influence on surface activity of natural surfactant as opposed to AMB without desoxycholate. The detrimental effect of deoxycholate is probably caused by its deterging capacities. The lipid formulations showed no detrimental effects, which was as expected because the lipid formulations consist in a large part of phospholipids, which are the surface active components of pulmonary surfactant. Therefore, it is assumed that the lipid formulations will be more safe to administer to the respiratory tract and that administration of aerosols of AMB-DOC is more likely to lead to local toxic effects.
In summary, we have described the nebulisation of 4 commercially available AMB formulations. All formulations were nebulised efficiently in terms of aerosol particle size and deposition in the lungs. Nebulisation of all 4 AMB formulations did not lead to systemic AMB exposure and therefore side effects which are frequently related to intravenous administration of AMB formulations are expected to be minimal. Local toxicity on the lungs is expected to be minimal with the lipid formulations. All nebulised formulations showed promising efficacy when administered prophylactically. However, L-AMB is superior as it is effective for a relatively long period of time compared to the other formulations. The presented work provides support for more effective strategies to prevent the threat of *Aspergillus* infections during periods of prolonged immunosuppression. These positive results from the presented work have instigated a clinical trial with nebulised L-AMB in our institution.

# References


1. Bodey GP. The emergence of fungi as major hospital pathogens. J Hospital Infect 1988;11(suppl A):411-426.
2. Denning DW. Invasive aspergillosis. Clin Infect Dis 1998;26:781-803.
3. Gallis HA, Drew RH, Pickard WW. Amphotericin B: 30 years of clinical experience. Rev Infect Dis 1990;12:308-329.
4. Luber AD, Maa L, Lam M, Guglielmo BJ. Risk factors for amphotericin B-induced nephrotoxicity. J Antimicrob Chemother 1999;43:267-271.
5. Meis JFGM, Verweij PE. Current management of fungal infections. Drugs 2001;61(Suppl.1):13-25.
6. Leenders ACAP, Daenen S, Jansen RL. Liposomal amphotericin B compared with amphotericin B deoxycholate in the treatment of documented and suspected neutropenia-associated invasive fungal infections. Br J Haematol 1998;103:205-12.
7. 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 leukopenic mice infected with *Candida albicans*. J Antimicrob Chemother 1995;35:509-19.
8. Lambros MP, Bourne DWA, Abbas SA, Johnson DL. Disposition of aerosolized amphotericin B. J Pharm Sci 1997;86:1066-1069.
9. Allen SD, Sorensen KN, Nejdl MJ, Durrant, and R. T. Proffit. 1994. Prophylactic efficacy of aerosolized liposomal (AmBisome) and non-liposomal (Fungizone) amphotericin B in murine pulmonary aspergillosis. J. Antimicrob. Chemother. 34:1001-1013.
10. Schmitt HJ, Bernard EM, Hauser M, Armstrong D. Aerosol amphotericin B is effective for prophylaxis and therapy in a rat model of invasive pulmonary aspergillosis. Antimicrob Agents Chemother 1988;32:1676-1679.
11. Cicogna CE, White MH, Bernard EM, Ishimura T, Sun M, Tong WP, Armstrong D. Efficacy of prophylactic aerosol amphotericin B lipid complex in a rat model of pulmonary aspergillosis. Antimicrob Agents Chemother 1997;41:259-261.
12. Ruijgrok EJ, Vulto AG, van Etten EWM. Efficacy of aerosolized amphotericin B desoxycholate and liposomal amphotericin B in the treatment of invasive pulmonary aspergillosis in severely immunocompromised rats. J Antimicrob Chemother 2001;48:89-95.
13. Leenders ACAP, de Marie S, ten Kate MT, Bakker-Woudenberg IAJM, Verbrugh HA. Liposomal amphotericin B (AmBisome) reduces dissemination of infection as compared to amphotericin B deoxycholate (Fungizone) in a newly developed animal model of one-sided pulmonary aspergillosis. J Antimicrob Chemother 1996;38:215-225.
14. Bakker-Woudenberg IAJM, van den Berg JC, Michel MF. Therapeutic activities of cefazolin, cefotaxime, and ceftazidime against experimentally induced Klebsiella pneumoniae in rats. Antimicrob Agents Chemother 1982;22:1042-1050.

15. Ruijgrok EJ, Vulto AG, van Etten EWM. Aerosol delivery of amphotericin B desoxycholate (Fungizone) and liposomal amphotericin B (AmBisome): Aerosol characteristics and in-vivo deposition in rats. J Pharm Pharmacol 2000;52,1-10.
16. Hickey AJ. 1996. Inhalation aerosols - Physical and biological basis for therapy. Marcel Dekker Inc., New York.
17. Ruijgrok EJ, Fens MHAM, van Vianen W, Vulto AG, van Etten EWM. Pulmonary kinetics of aerosolized liposomal and non-liposomal amphotericin B in healthy rats and in rats with severe invasive aspergillosis. 2002 submitted for publication.
18. Erjavec Z, Woolthuis GM, de Vries - Hospers HG, et al. Tolerance and efficacy of amphotericin B inhalations for prevention of invasive pulmonary aspergillosis in haematological patients. Eur J Clin Microbiol Infect Dis 1997;16:364-8.
19. Hertenstein BW,Kern V, Schmeiser T, et al. Low incidence of invasive fungal infections after bone marrow transplantation in patients receiving amphotericin B inhalations during neutropenia. Ann Hematol 1994;68:21
20. Schwartz S, Behre G, Heinemann V, et al. Aerosolized amphotericin B inhalations as prophylaxis of invasive *Aspergillus* infections during prolonged neutropenia: Results of a prospective randomized multicenter trial. Blood 1999;11:3654-3661.

Chapter 9

# Summarising discussion

The aim of the work described in this thesis was to evaluate the merits of inhalational amphotericin B formulations in the prevention and treatment of experimental invasive pulmonary aspergillosis. In this last chapter, the sum of results of the in vitro experiments and the preclinical studies, described in the previous chapters, are discussed and put in perspective.

# Background

The incidence of invasive pulmonary aspergillosis has increased in the last decades and is still increasing in patients with impaired immunity such as patients who are treated with aggressive chemotherapeutic agents, transplant recipients and HIV patients. Although potent antifungal agents are available, morbidity and mortality from invasive pulmonary aspergillosis remains unacceptably high. The golden standard fungicidal drug, amphotericin B (AMB), has been formulated in lipid carriers to allow more flexible, higher dosing of this toxic agent. However, due to the delay in diagnosis of invasive pulmonary aspergillosis, treatment is often started too late in the course of the infection. Therefore, we are still faced with the severe life-threatening features of invasive pulmonary aspergillosis. Several strategies are under investigation to reduce the incidence of invasive aspergillosis or to enhance therapeutic outcome of an established infection:

- As long as simple and adequate treatment is not available, prevention of invasive aspergillosis, for patients who are at particular risk, would be the strategy of choice for decreasing aspergillosis related mortality and morbidity. HEPA-filtration of air in rooms of patients who are at risk does reduce the incidence of invasive fungal disease. However, HEPA-filtration of room air does not completely eliminate the incidence of invasive aspergillosis in these settings. Another option is to prevent disease by prophylaxis with antifungal agents. Results of studies that have been performed with prophylactic systemic itraconazole or (liposomal) AMB, are controversial and this strategy is currently not recommended or practised.
- Under development are better diagnostic techniques that would allow us to better select patients who have early stages of invasive pulmonary aspergillosis and to start antifungal treatment earlier in the course of infection. These new techniques include the detection of circulating galactomannan in serum or broncho-alveolar lavage fluid, the use of high resolution CT scans and the use of amplification methods to detect fungal DNA in patient samples. These techniques have shown to be of value in the detection of invasive aspergillosis.

Their value in terms of reduction of mortality and morbidity as a result of earlier indication of fungal infection still has to be determined.

- Many research efforts are focused at new antifungal entities and new treatment strategies with existing antifungal agents to achieve better therapeutic outcome. Chapter 2 of this thesis describes the chemical entities that have proven *in vitro* or *in vivo* activity against *Aspergillus* spp. Some of these agents, including new azoles such as voriconazole and peptides such as echinocandins and pneumocandins, are currently entering the market. Their value as promising new antifungals will become clear in the coming years. The lipid formulations of AMB, liposomal AMB (L-AMB, AmBisome®), AMB lipid complex (ABLC, Abelcet®) and AMB colloidal dispersion (ABCD, Amphocil®, Amphotec®), clearly have an improved therapeutic index as compared to AMB-DOC (AMB desoxycholate, AMB-DOC, Fungizone®). Because of this improved therapeutic index, these lipid formulations can be administered in a higher dose. For liposomal amphotericin B, this higher dosing has resulted in a better efficacy in clinical trials than AMB-DOC. As yet, this has not been shown for the two other lipid-formulations. Future clinical practice will elucidate the differential value of these lipid formulations as compared to AMB-DOC as well as compared to each other.

Administration of (lipid-associated) AMB via the inhalational route, either as prophylactic measure or as treatment of invasive pulmonary aspergillosis is an attractive approach for the following reasons:

- Development of invasive pulmonary aspergillosis starts with the inhalation of *Aspergillus* spores and subsequent deposition of these spores in the bronchial tree. Inhalation of an aerosol of (lipid associated) AMB targets the drug to the bronchial tree, to the site where the infection originates.
- Systemic toxicity, which can be a dose-limiting consequence of intravenously administered AMB formulations, is minimised with aerosol administration.

In Chapter 3 to 8 of this thesis, the inhalational route has been studied as an alternative administration route of AMB formulations. This work presents an evaluation of the aerosol-characteristics, pharmacokinetics, toxicity and prophylactic and therapeutic efficacy of aerosolised AMB formulations in an experimental aspergillosis model in immunocompromised rats.

# The feasibility of aerosol administration of AMB

## Aerosol characteristics

Before the efficacy of nebulised AMB or either of the lipid formulations can be evaluated, the aerosol procedure must first be validated. In chapter 3 we describe the characteristics of aerosols of AMB-DOC and of liposomal amphotericin B. It was shown in this work that AMB-DOC as well as the liposomal formulation can be efficiently nebulised with the experimental nebuliser system such that adequate AMB concentrations are deposited in the respiratory tract of rats. A large portion of inhaled AMB is deposited elsewhere than in the respiratory tract. Measurement of radiolabelled aerosol deposition shows that the largest part of the aerosol is deposited in the oral cavity and in the gastro-intestinal tract. Since AMB is poorly absorbed after oral administration, this amount of extrapulmonary AMB does not lead to systemic concentrations, and thus not to systemic toxicity or antifungal activity additive to activity from pulmonary deposited AMB. This finding is an obvious distinction from intravenous administration, where the severe side effects due to the systemic distribution limit the use of AMB.

## Pulmonary kinetics

Pharmacokinetic evaluation of nebulised AMB-DOC and L-AMB (Chapter 4) revealed that the amount of AMB deposited in infected lungs of rats, apparently supersedes the MIC values of susceptible *Aspergillus* strains, to be fungicidal to conidia or hyphae that are present at that site. With regard to the correlation of measured AMB tissue concentrations with *Aspergillus* MIC the following comments are of importance:

- AMB was quantified with a chemical analysis, preceded by a vigorous extraction procedure. This quantification method does not allow to equate the chemically measured AMB with actually bioactive AMB. We have tried to determine bioactive AMB with a diffusion-agar plate assay. However, this method appeared erratic, since this assay was not reproducible and not of use for quantitative determination of AMB. Efficacy experiments aimed at biological activity are needed to provide the proof whether these chemical data correlate with *in vivo* bioactive AMB.
- Total lunglobes (lungtissue, alveoli and bronchioles) were excised and analysed for AMB. Consequently, there is no understanding on where the aerosol was exactly deposited inside the respiratory tract. The aerosol droplet size, which has been measured for all formulations, ensures that the largest part is deposited in the peripheral regions of the lungs, that is in the terminal

bronchioli and the alveolar sacs and ducts. Furthermore, the results of the experiments with nebulised gold-labelled liposomes (Chapter 4) indicate that the liposomes are indeed deposited in the alveoli and at the site of the infection. It is therefore plausible that the nebulisation of AMB products leads to presence of AMB at the site of intended action.

The absence of detectable AMB in blood or other organs than the lungs (Chapter 4) indicates that no systemic toxicity is expected from this mode of administration. This is very interesting, since the side effects of AMB, especially on the kidneys, limit the intravenous use of this agent.

After a single dose of aerosol of either AMB formulation, AMB was retained in the lungs for up to 6 weeks. Assuming that deposition is associated with bioactivity, this implies that a single dose will be effective for weeks. The strikingly long retention time would, therefore, allow infrequent administration schedules, which are welcome to patients and their caretakers.

## Treatment of confirmed invasive pulmonary aspergillosis with nebulised conventional and liposomal AMB: Effective and safe?

The treatment of an established infection of IPA is correlated with high failure rates. This failure rate is the result of a combination of the troublesome diagnosis of invasive pulmonary aspergillosis resulting in diagnostic delay and the limited efficacy of intravenous antifungal regimens. To determine whether nebulised AMB-DOC and L-AMB were effective in the treatment of IPA, an animal model of invasive pulmonary aspergillosis was used. In this model, which was developed in our laboratory, the fungal infection was induced in deeply leukopenic rats. Leukopenia was induced by cyclophosphamide in order to mimic the chemotherapy-induced neutropenia in haematological oncology patients. This model is a severe infection model with all untreated rats dying between day 4 and day 9 after inoculation with the fungal spores of *Aspergillus fumigatus*. Prolonged survival of treated animals as compared to controls was chosen as primary endpoint of efficacy of antifungal regimens. When a single aerosol dose of conventional and L-AMB was administered to rats with an established infection of invasive pulmonary aspergillosis, i.e. with hyphal outgrowth of *Aspergillus* conidia in the lungs, both products yielded a prolonged survival as compared to non-treated control rats (Chapter 5). When compared to controls of intravenous treatment with conventional or L-AMB in the same rat

model, there was no difference in efficacy between the intravenous or the aerosol treatment. Aerosol treatment did not however, prevent dissemination of the infection to the liver.
The use of inhalation treatment of AMB formulations is not presumed to lead to systemic toxicity. However, local toxicity on the lung epithelium must be taken into account. The toxicity of aerosolised AMB formulations was evaluated in an *in vitro* experiment in which their influence on the surfactant function was determined. It appeared that AMB-DOC, and not the lipid formulations, had a detrimental effect on surfactant. Therefore, it was assumed that the lipid formulations are more safe to administer to the respiratory tract than AMB-DOC, which is likely to lead to local toxic effects.
In conclusion, aerosol administration of lipid formulations of AMB is safe and has an effect in the treatment of an established experimental infection. Aerosolised AMB-DOC might lead to local side effects, but is as effective as the lipid-preparations. Nevertheless, all regimens did not prevent spreading of the infection from the lungs to other organs. Therefore, the therapeutic value of this application is probably limited.

## Efficacy of standard intravenous treatment can be improved when a single dose of nebulised L-AMB is added

Treatment of invasive pulmonary aspergillosis with only a single dose of aerosolised AMB, conventional or liposomal, shows a limited efficacy. Combining aerosol treatment with a regimen that does prevent dissemination, (intravenous treatment with AMB), could enhance outcome of treatment of confirmed invasive pulmonary aspergillosis.
In chapter 7 we describe the combination of intravenous and aerosol therapy in the treatment of an established infection. It was shown, that this regimen was much more effective than mono-therapy with either intravenous or aerosol therapy. Furthermore, this combination therapy prevented dissemination of the infection. This shows that systemic AMB concentrations resulting from intravenous administration, are crucial to prevent bloodstream dissemination of *Aspergillus fumigatus*. The efficacy in this combination therapy is remarkable when the results were compared to that of other treatment regimens studied by us. These results encourage the use of aerosol AMB as additive therapy to currently used intravenous AMB treatment in the management of invasive pulmonary aspergillosis.

# Prophylaxis of invasive pulmonary aspergillosis with nebulised AMB: Long term protection

Given the troublesome diagnosis in combination with the high refractory rate of invasive pulmonary aspergillosis to systemic antifungals, the best way to overcome aspergillosis related mortality and morbidity is to prevent the infection. HEPA filtration of air in rooms of patients does reduce the incidence of invasive pulmonary aspergillosis, but patients can be colonised with *Aspergillus* before hospital admission, which emphasises an additional need for chemoprophylaxis. At present, there is not an intravenous antifungal regimen which has been shown to be effective in preventing invasive pulmonary aspergillosis.

Prophylaxis of invasive pulmonary aspergillosis with aerosolised AMB has some appealing aspects. Inhalation of AMB (either conventional or lipid-associated) targets the drug to the lungs where spores may already reside and/or where spores may be acquired during hospitalisation when inhaled. After deposition in the lungs, we have shown that the drug will be present in the pulmonary tissue for weeks. This allows infrequent aerosol administration. Furthermore, we have shown that aerosolised AMB (lipid-associated or conventional) does not lead to systemic toxicity.

The hypothesis that aerosolised AMB formulations can have prophylactic efficacy in invasive pulmonary aspergillosis, was tested in the same rat model which was used for the experiments to study established invasive pulmonary aspergillosis. Commercially available AMB formulations were evaluated (Chapter 7 & 8). It was investigated whether a single dose, given at different times before the rats were infected, could prevent, completely or in part, mortality that resulted from the subsequent infection. All formulations showed prophylactic efficacy, they prolonged survival of treated rats as compared to controls. The effect was present even when the aerosol was administered at 2 weeks before fungal infection was applied. The activity of the liposomal formulation existed even for 6 weeks. This finding demonstrates that the AMB concentrations measured in the deposition experiments (Chapter 4) had a therapeutic significance and were indicative for antifungal activity. More interesting even, was the fact that these results convincingly show the usefulness of aerosolised AMB formulations in the prophylaxis of invasive pulmonary aspergillosis.

# Conclusions and final remarks

Considering the aim of the work presented in these studies, we have shown the value of nebulised AMB formulations in the treatment or prevention of invasive pulmonary aspergillosis. We have shown that the commercially available AMB formulations can be nebulised and that a single aerosol dose leads to adequate, long-lasting concentrations of bioactive AMB in the lungs.

Systemic toxicity after aerosol administration is unlikely, as AMB was undetectable in blood or other organs than the lungs. Local toxicity is probably absent for the lipid-associated products. AMB-DOC had detrimental effects *in vitro* on the pulmonary surfactant system.

Administration of aerosolised AMB formulations had only a limited efficacy in the treatment of established invasive pulmonary aspergillosis, because spreading of the infection with this mode of administration is not prevented. Combining aerosolised AMB formulations with intravenous AMB was more efficacious than either aerosolised or intravenous treatment alone, with no dissemination of the infection. Since current intravenous treatment regimens still lack sufficient efficacy, further clinical investigation of this approach of combination therapy is warranted. The long retention of AMB after aerosol administration of either formulation resulted in a long-lasting prophylactic efficacy after single dose aerosol treatments. We propose therefore that this elegant concept is also investigated in the clinical situation.

In conclusion, we have shown the potential of inhalational AMB when administered both in a therapeutic and prophylactic setting with regard to invasive pulmonary aspergillosis. Our results provide a strong case for further studies in a clinical setting. Up to now, treatment outcome of invasive pulmonary aspergillosis is often disappointing, both due to limitations in diagnosis and in therapy. Our studies provide evidence that can change this scene. Inhalational AMB may enhance therapeutic efficacy of treatment of established invasive pulmonary aspergillosis. Moreover, the favourable retention behaviour and low toxicity of inhaled L-AMB make prophylaxis feasible and therefore the preferred approach.

# Samenvatting voor niet-ingewijden

In dit proefschrift worden de resultaten weergegeven van proefdieronderzoek dat gedaan is naar de verbetering van de behandeling van de schimmelinfectie Invasieve Pulmonale Aspergillose. Invasieve Pulmonale Aspergillose is een infectie in de longen die wordt veroorzaakt door de schimmelsoort *Aspergillus*. Invasieve Pulmonale Aspergillose komt vrijwel uitsluitend voor bij mensen die een ernstig verzwakte afweer tegen infecties hebben. Dit zijn bijvoorbeeld patienten die behandeld worden met langdurige en intensieve chemokuren tegen kanker of patienten die een beenmerg- of orgaantransplantatie hebben ondergaan en ten gevolge daarvan geneesmiddelen krijgen toegediend die afstoting van het transplantaat tegengaan. Deze mensen hebben een verzwakte afweer, waardoor ze meer vatbaar zijn voor infecties met allerlei micro-organismen, waaronder de schimmel *Aspergillus*.
*Aspergillus* is een schimmel die normaal in onze omgeving veel voorkomt. De schimmel groeit in de vorm van schimmeldraden, waarbij aan het uiteinde schimmelsporen (conidia) ontstaan. Door hun kleine diameter kunnen deze sporen zich gemakkelijk verspreiden door de lucht. Daardoor kunnen ze vervolgens door mens en dier worden ingeademd. Bij gezonde personen is het afweersysteem voldoende om deze sporen op te ruimen, echter mensen met een verzwakte afweer zijn daartoe slecht in staat, waardoor de schimmel zich kan gaan nestelen, uitgroeien en vervolgens kan leiden tot een infectie.
De diagnose van Invasieve Pulmonale Aspergillose is gecompliceerd, omdat de aandoening weinig specifieke verschijnselen heeft. Daardoor is het moeilijk onderscheid te maken tussen Invasieve Pulmonale Aspergillose en andere infecties. Het moment dat de diagnose met zekerheid kan worden gesteld laat hierdoor vaak lang op zich wachten, met als gevolg dat de schimmel de kans krijgt om uit te groeien in de longen en vervolgens naar andere delen van het lichaam. De late diagnose bemoeilijkt dan ook een adequate behandeling met het huidige arsenaal aan anti-schimmelmiddelen met als gevolg een hoge sterfte ten gevolge van Invasieve Pulmonale Aspergillose. Omdat de slaagkans van de behandeling teleurstellend klein is, is preventie van Invasieve Pulmonale Aspergillose de beste strategie.
De standaardbehandeling van mensen met een (vermoedelijke) Invasieve Pulmonale Aspergillose bestaat uit het geven van anti-schimmelmiddelen via de bloedbaan (intraveneus). Hierbij heeft men de keuze uit twee geneesmiddelen met een werking tegen *Aspergillus*: itraconazol of amfotericine B. Vanwege de krachtige werking wordt amfotericine B het meest gebruikt. Amfotericine B wordt na inname via de mond niet opgenomen het bloed, waardoor het noodzakelijk is het middel intraveneus te geven als een infectie in het lichaam moet worden voorkomen of behandeld. Het gebruik van intraveneus amfotericine B is echter wel beperkt vanwege de ernstige bijwerkingen, het is met name schadelijk voor de nieren. Ter verbetering van

de behandeling zijn er in de laatste jaren nieuwe formuleringen van amfotericine B ontwikkeld. Deze nieuwe formuleringen hebben gemeenschappelijk dat amfotericine B is gekoppeld aan bepaalde vetmoleculen, fosfolipiden, waardoor de bijwerkingen van amfotericine B verminderd zijn. Er is een drietal van deze amfotericine B lipide-formuleringen op de markt: *amfotericine B liposomen* (vetbolletjes), *amfotericine B lipiden-complex* en *amfotericine B colloidale dispersie*.
Hoewel deze nieuwe, minder schadelijke amfotericine B lipide-formuleringen, waarmee hoger gedoseerd kan worden ten opzichte van het conventionele (niet lipide-gebonden) amfotericine B, op de markt zijn gekomen en inmiddels bij patienten worden toegepast, zien we nog steeds een hoge sterfte bij patienten met Invasieve Pulmonale Aspergillose. Daarom is men nog steeds naarstig opzoek naar mogelijkheden om Invasieve Pulmonale Aspergillose te voorkómen of de slaagkans van de behandeling van de infectie te verbeteren.
Eén van de aantrekkelijke alternatieven voor de huidige intraveneuze behandelingen zou zijn de toepassing van de antischimmelmiddelen via de luchtwegen ofwel via inhalatie ervan. Dit heeft een aantal voordelen: de medicatie komt direct op de plek waar de schimmelsporen zijn neergestreken (de sporen komen immers ook binnen via inhalatie). De rest van het lichaam hoeft niet belast te worden met het geneesmiddel met als gevolg minder bijwerkingen. Daarnaast wordt aan mensen niet meer een infuus gegeven, maar inhaleren ze, wat mogelijk een meer patientvriendelijke toepassing is. Bovendien blijkt uit de literatuur van andere onderzoekers dat het inhaleren van lipiden zoals die van de amfotericine B-lipide formuleringen, goed verdragen wordt en bovendien resulteert in een langdurig verblijf in de longen, met als gevolg een langdurig effect.
Het doel van het onderzoek dat beschreven wordt in dit proefschrift was inzicht te verkrijgen in de haalbaarheid en de waarde van inhalatie van amfotericine B formuleringen voor de preventie of behandeling van invasieve pulmonale aspergillose. Het onderzoek werd uitgevoerd in een diermodel.

De inleiding op het onderzoek is beschreven in Hoofdstuk 1. Hoofdstuk 2 is een beschrijving van alle bestaande antischimmel-middelen, en middelen die in de pijplijn zitten, die gebruikt kunnen gaan worden bij de preventie of behandeling van Invasieve Pulmonale Aspergillose. Dit hoofdstuk schetst hoe het landschap er nu uit ziet, welke middelen we nu hebben, en wat er in de toekomst mogelijk aan extra middelen ter beschikking komt. Vanwege de ernst van Invasieve Pulmonale Aspergillose en de teleurstellende resultaten van behandeling met het huidige arsenaal aan anti-schimmelmiddelen, is men zoals gezegd wereldwijd naarstig op

zoek naar betere alternatieven. Dit heeft geleid tot de ontwikkeling van een aantal potentieel bruikbare nieuwe stoffen met werking tegen Aspergillus. Een aantal van de nieuw ontwikkelde stoffen hebben geheel andere werkingsmechanismen dan amfotericine B of itraconazol. Hierdoor kunnen we mogelijk van deze stoffen een meer potente werking, minder (of andere) bijwerkingen ten opzichte van de huidige middelen verwachten. Sommige middelen zoals voriconazol of caspofungin zijn al in een ver gevorderd stadium van ontwikkeling en hebben hun activiteit bij patienten reeds bewezen. Deze middelen zijn binnenkort op de Nederlandse markt te verwachten. De exacte plaats van de nieuwe middelen ten opzichte van de standaardbehandelingen is nog niet vastgesteld, maar zeker is wel dat we in de toekomst een aantal waardevolle alternatieve anti-schimmelmiddelen erbij krijgen. Om de waarde van inhalatie van amfotericine B formuleringen bij de preventie of behandeling van Invasieve Pulmonale Aspergillose te kunnen beoordelen, is het van belang om eerst te onderzoeken hoe het vernevelen van amfotericine B formuleringen in zijn werk gaat en of het wel mogelijk is via inhalatie het amfotericine B in adequate hoeveelheden in de longen te krijgen. Daarom wordt in Hoofdstuk 3 de verneveling beschreven van amfotericine B formuleringen bij ratten. Vernevelen is het proces waarbij een vloeistof wordt omgevormd in een mist (aerosol), die kan worden ingeademd. In dit hoofdstuk wordt de vernevelprocedure beschreven die wordt gebruikt in de rest van het onderzoek. De grootte van de neveldruppels is gemeten. Het bleek dat de neveldruppels klein genoeg waren om diep in de longen te penetreren. Bovendien bleek dat de neveldruppels ongeveer dezelfde grootte hebben als de sporen van *Aspergillus*. Dit is gunstig aangezien dan de neveldruppels vermoedelijk op dezelfde plek terecht komen als de geinhaleerde schimmelsporen. Daarnaast is er gemeten hoeveel AMB er in de longen van de ratten, maar ook in andere delen van het lichaam van de rat, na verneveling terecht was gekomen. De gemeten hoeveelheid amfotericine B die in de longen terecht komt zou in theorie voldoende moeten zijn om de schimmel te doden en dus lijkt de gevolgde procedure de juiste. Verder bleek dat er veel neveldruppels terecht komen in de mondkeelholte en in het maagdarmkanaal. Dit is te verwachten omdat de neveldruppels een lange weg van het vernevelvaatje naar de longen moeten afleggen en de kans dat ze ergens 'uit de bocht vliegen' groot is. Het amfotericine B dat in de mond-keelholte of het maagdarmkanaal terecht komt wordt niet in het bloed opgenomen, maar verdwijnt via het spijsverteringskanaal weer uit het lichaam en levert geen bijdrage aan de effectiviteit of bijwerkingen.
In Hoofdstuk 4 wordt meer uitgebreid beschreven wat er met de neveldruppels en amfotericine B gebeurt na inhaleren. Hier bleek dat de hoeveelheid amfotericine B die na vernevelen in de longen terecht komt na eenmalige verneveling niet alleen hoog genoeg is (in theorie) om de schimmel te doden, maar ook dat het amfotericine

B gedurende zeer lange tijd, nl enkele weken, in de longen aanwezig bleef. Amfotericine B wordt blijkbaar erg langzaam uit de longen verwijderd. Dit gold ook voor ratten die met opzet waren geïnfecteerd met *Aspergillus*. Foto's van het longweefsel van behandelde ratten bevestigden dat amfotericine B liposomen daadwerkelijk op de plek in de longen waar de schimmelsporen zich bevinden terecht komen. In het bloed en organen anders dan de longen werd geen amfotericine B gemeten waaruit bleek dat er vanuit de longen geen of onmeetbaar weinig, amfotericine B terecht komt in het bloed. Dit betekent dat de bijwerkingen die gezien worden bij intraveneuze behandeling met amfotericine B producten, zeer waarschijnlijk niet zullen optreden bij de inhalatietoepassing van amfotericine B. Concluderend uit de voorgaande twee hoofdstukken kunnen we zeggen dat het vernevelen van amfotericine B formuleringen voor inhalatietoepassing, zeer wel bruikbaar is voor zowel preventie als behandeling van Invasieve Pulmonale Aspergillose. Het eerste onderzoek naar de effectiviteit van verneveld AMB staat beschreven in Hoofdstuk 5. In dit hoofdstuk hebben we onderzocht of verneveling van AMB in de conventionele vorm of in de vorm van liposomen een gunstig effect heeft op het verloop van een kunstmatig aangebrachte Invasieve Pulmonale Aspergillose. We hebben daarvoor een diermodel gebruikt waarbij de ratten eerst een sterk verminderde afweer hebben, analoog aan mensen die met chemotherapie zijn behandeld. Vervolgens werd aan de ratten een bepaalde hoeveelheid *Aspergillus*-sporen toegediend in de luchtwegen. Zo ontstond er een infectie als gevolg waarvan de dieren, net als patienten, ernstig ziek werden en uiteindelijk overleden ten gevolge van de infectie. Dit ziekteproces verloopt vergelijkbaar als bij de mens. Getest kan worden of een experimentele behandeling effectief is door te bezien of behandelde dieren langer leven dan dieren die niet met een anti-schimmelmiddel worden behandeld. Wanneer we dieren die Invasieve Pulmonale Aspergillose hebben verneveld conventioneel amfotericine B of amfotericine B liposomen gaven, zagen we dat ze statistisch significant langer leefden. Met andere woorden, de behandeling had een gunstig effect. Het maakte niet uit of het conventionele amfotericine B werd gebruikt of amfotericine B liposomen, beide formuleringen vertoonden dezelfde effectiviteit. In hetzelfde onderzoek is nagegaan of er een schadelijk effect optrad in de luchtwegen. Dit hebben we gedaan door te onderzoeken of de amfotericine B formuleringen een schadelijke uitwerking hadden op het pulmonaal surfactant. Pulmonaal surfactant is het laagje vloeistof dat de longen bekleedt en zorgt dat de longen voldoende elasticiteit hebben bij het in- en uitademen. Het bleek dat het conventionele amfotericine B schadelijk was voor dit surfactant terwijl de lipide formuleringen niet schadelijk bleken te zijn. Vervolgens is onderzocht wat de waarde is van toevoeging van vernevelde amfotericine B liposomen aan de

intraveneuze behandeling met ofwel conventioneel amfotericine B ofwel amfotericine B liposomen (Hoofdstuk 6). Het bleek dat de effecten dan zeer gunstig uitvallen: het effect van de combinatie van verneveld en intraveneus amfotericine B is beter dan een van de behandelstrategieen alleen. Hieruit kan men concluderen dat het zeer zinvol lijkt de huidige behandeling van Invasieve Pulmonale Aspergillose, nl de intraveneuze behandeling met ofwel conventioneel amfotericine B, ofwel met amfotericine B liposomen, te combineren met een éénmalige behandeling met verneveld amfotericine B liposomen. Preventie van Invasieve Pulmonale Aspergillose is nog altijd beter dan de behandeling ervan vanwege de geringe slaagkans van behandeling. In Hoofdstuk 7 en Hoofdstuk 8 wordt de mogelijkheid van preventieve behandeling met ver-nevelde amfotericine B formuleringen beschreven. In deze hoofdstukken worden het conventionele amfotericine B en de drie bestaande lipide-formuleringen met elkaar vergeleken. Alle formuleringen lieten zien dat de overleving van behandelde dieren statistisch significant langer was dan die van controle dieren indien de verneveling éénmalig voorafgaande aan het aanbrengen van de infectie werd uitgevoerd. Alle vier de formuleringen lieten dit gunstig effect zien, ook wanneer de verneveling twee weken voor de infectie geschiedde. Behandeling met amfotericine B liposomen was zelfs nog effectief wanneer dit 6 weken voor het aanbrengen van de infectie wordt gegeven.

Terugkomend op het doel van het onderzoek zoals dat staat beschreven aan het begin van deze samenvatting, kunnen we concluderen dat we de technische haalbaarheid en de therapeutische waarde van de toepassing van inhalatie van amfotericine B formuleringen hebben aangetoond. De resultaten zijn zeer bemoedigend en vragen erom deze manier van toedienen van amfotericine B te onderzoeken in de klinische situatie. Tot nu toe is het resultaat van de behandeling van Invasieve Pulmonale Aspergillose vaak teleurstellend, door een complexe diagnose die vaak veel tijd kost en een tekortschietende behandeling. Ons onderzoek laat zien dat dit mogelijk kan veranderen. Inhalatie van amfotericine B formuleringen gecombineerd met standaard intraveneuze behandeling verhoogt de slaagkans van de behandeling. Daarnaast lijkt de inhalatie van amfotericine B formuleringen zeer zinvol om toe te passen ter preventie van Invasieve Pulmonale Aspergillose. Het klinisch patientenonderzoek naar dit laatste is in volle gang en het is spannend om af te wachten of de veelbelovende resultaten behaald bij proefdieren net zo uit zullen pakken bij de patienten. Indien dat het geval is, hebben we niet alleen veel geleerd van de, in dit onderzoek uitgevoerde, experimenten, maar hebben ze ook betekenis voor de kwaliteit van de behandeling van patienten.

# List of publication


EJ Ruijgrok, GJM Burer, DM Burger. Vrouwen & AIDS. Behandeling van HIV-infectie en AIDS bij vrouwen. Rapport Wetenschapswinkel Geneesmiddelen Utrecht, november 1994.

EJ Ruijgrok, GJM Burer, DM Burger, H Wolschrijn, PP Koopmans, WJM Underberg en JH Beijnen. Nieuwe ontwikkelingen in de behandeling en de complicaties van HIV-infectie. 4. HIV-infectie en AIDS bij vrouwen. Pharm Weekbld 1995;130(45):1234-44.

EJ Ruijgrok en AM Soeterboek. Paclitaxel (Taxol,): Geneesmiddelprofiel. Pharm Weekbld 1996;132(22):732-737.

Al de Vos, K Nooter, J Verweij, WJ Loos, E Brouwer, P de Bruijn, EJ Ruijgrok, MEL van der Burg, G Stoter and A Sparreboom. Differential modulation of cis-platin accumulation in leukocytes and tumor cell lines by the paclitaxel vehicle Cremophor EL. Annals of Oncology 1997;8:1145-1150.

EJ Ruijgrok, AG Vulto and EWM van Etten. Sterically stabilized liposomes containing gentamicin: limitations to gentamicin encapsulation. J Lipos Res 1999;9(2),291-300.

EJ Ruijgrok, AG Vulto and EWM van Etten. Aerosol delivery of amphotericin B desoxycholate (Fungizone) and liposomal amphotericin B (AmBisome): aerosol characteristics and *in vivo* amphotericin B deposition in rats. J Pharmacy Pharmacol 2000;52:619-627.

EJ Ruijgrok, J Denyer, P Robberts, AG Vulto, HAWM. Tiddens. Nebulisation of liposomal amphotericin B: targeting the peripheral airways. Eur Resp J 2000;16(S31):295.

DFS Kehrer, A Sparreboom, J Verweij, P de Bruijn, C Nierop, J van de Schraaf, EJ Ruijgrok, MJA de Jonge. Modulation of irinotecan induced diarrhea by co-treatment with neomycin in cancer patients. Clin Cancer Res 2001;7(5):1136-41.

EJ Ruijgrok, AG Vulto and EWM van Etten. Efficacy of aerosolized amphotericin B desoxycholate and liposomal amphotericin B in the treatment of invasive pulmonary aspergillosis in severely immunocompromized rats. J Antimicrob Chemother 2001;48:89-95.

EJ Ruijgrok and JFGM Meis. Pharmacological agents in development for invasive aspergillosis. Expert Opin Invest Drugs 2002;in press.

EJ Ruijgrok, MAHM Fens, W van Vianen, AG Vulto and EWM van Etten. Pulmonary kinetics of aerosolised amphotericin B desoxycholate and liposomal amphotericin B in rats with invasive pulmonary aspergillosis. submitted for publication

EJ Ruijgrok, MAHM Fens, IAJM Bakker-Woudenberg, EWM van Etten and AG Vulto. Intravenous amphotericin B desoxycholate and liposomal amphotericin B in combination with aerosolised liposomal amphotericin B is effective in the treatment of rats with severe invasive pulmonary aspergillosis. submitted for publication

EJ Ruijgrok, MAHM Fens, AG Vulto, HA Verbrugh and EWM van Etten. Prophylaxis of invasive pulmonary aspergillosis with aerosolised amphotericin B desoxycholate and liposomal amphotericin B in severely immunocompromised rats. submitted for publication

EJ Ruijgrok, MAHM Fens, IAJM Bakker-Woudenberg, EWM van Etten and AG Vulto. Nebulisation of four commercially available amphotericin B formulations in persistently leukopenic rats with invasive pulmonary aspergillosis. submitted for publication

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# List of abbreviations

| | |
|---|---|
| ABCD | amphotericin B colloidal dispersion |
| ABLC | amphotericin B lipid complex |
| AMB | amphotericin B |
| AMB-DOC | amphotericin B desoxycholate |
| BAL | broncho-alveolar lavage fluid |
| BMT | bone marrow transplant |
| cfu | colony forming units |
| $C_0$ | concentration at time zero |
| Chol | cholesterol |
| cpm | counts per minute |
| CT | computed tomography |
| DOC | desoxycholate |
| DMPC | dimyristoyl phosphatidylcholine |
| DMPG | dimyristoyl phosphatidylglycerol |
| DSPC | distearoyl phosphatidylcholine |
| DSPE | distearoyl phosphatidylethanolamine |
| DSPG | distearoyl phosphatidylglycerol |
| ymin | minimal surface tension |
| $^{67}Ga$ | $^{67}$gallium |
| GSD | geometric standard deviation |
| HPLC | high performance liquid chromatography |
| HSPC | hydrogenated soy phosphatidylglycerol |
| i.p. | intraperitoneal |
| IPA | invasive pulmonary aspergillosis |
| i.v. | intravenous |
| L-AMB | liposomal amphotericin B |
| MFC | minimal fungicidal concentration |
| MIC | minimal inhibitory concentration |
| MMD | mass median diameter |
| PEG | polyethylene glycol |
| spp. | species |

Elisabeth Johanna Ruijgrok is geboren in Wassenaar op 20 september 1970. Na het behalen van het VWO diploma aan "Het Nederlandsch Lyceum" te Den Haag, is zij in 1988 begonnen met de studie Farmacie aan de Faculteit Farmacie van de Universiteit Utrecht. In het kader van het bijvakonderzoek heeft zij in 1992-1993 gedurende zeven maanden gewerkt aan de Faculty of Pharmaceutical Sciences van de Kumamoto University, in Kumamoto, Japan.
Daar heeft zij bij de vakgroep "Biopharmaceutical Sciences" een onderzoek uitgevoerd met als titel "Binding of protizinic acid to human serum albumin" (begeleiding Prof. Dr. Masaki Otagiri).
In 1993 behaalde zij haar doctoraalexamen.
De post-doctorale apothekersopleiding rondde zij in 1995 met succes af. Zij werkte aansluitend gedurende 5 maanden als projectapotheker in de Stichting Apotheek der Haarlemse Ziekenhuizen.

In 1996 is zij begonnen aan het zgn Zapico-traject (opleiding ziekenhuisapotheker in combinatie met onderzoek) aan het Erasmus MC te Rotterdam.
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In juli 2002 zal zij de opleiding tot ziekenhuis-apotheker afronden.