Journal of Infection (2006) **53**, 131–139

ELSEVIER

www.elsevierhealth.com/journals/jinf

Efficacy of amphotericin B or amphotericin B–intralipid in combination with caspofungin against experimental aspergillosis

Edward Sionov [a], **Sonia Mendlovic** [b], **Esther Segal** [a,*]

[a] *Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Ramat Aviv, Tel-Aviv 69978, Israel*
[b] *Institute of Pathology, Assaf Harofe Medical Center, Zerifin, Israel*

Accepted 17 October 2005
Available online 19 December 2005

KEYWORDS
Aspergillosis;
Amphotericin B;
Amphotericin B–intralipid;
Caspofungin;
Combination therapy

Summary *Objective:* Infections caused by *Aspergillus* species are increasing in importance, especially among immunocompromised hosts. The objective of this study was to evaluate the efficacy of combination treatment consisting of the polyene amphotericin B (AMB) or amphotericin B–intralipid admixture (AMB–IL) and the echinocandin caspofungin (CAS) in experimental murine systemic aspergillosis. Inhibition of synthesis of a major component of the fungal cell wall and an effect on the cell membrane, by combining echinocandin and a polyene, may result in a synergistic interaction in vitro and in vivo against *Aspergillus fumigatus*.
Methods: ICR mice were immunosuppressed by intraperitoneal (ip) administration of cyclophosphamide (CY). Three days post-CY administration the mice were inoculated intravenously (iv) with *A. fumigatus* conidia. Infection and treatment were evaluated during an observation period of 30 days in terms of mortality (survival rate and mean survival time) and morbidity (quantitative determination of fungal burden, histopathology, and detection of serum galactomannan).
Results: The data showed that combined CAS and AMB or AMB–IL treatment increased the survival of the mice (up to 69.2%) as compared to those treated with each agent alone (44.4, 40.7 and 50%, respectively), and prolonged their mean survival time to 22.5 days. These combinations also resulted in reduction of fungal burden in organs, and decrease in serum galactomannan.
Conclusion: The successful results obtained in the experimental animal model of this study may possibly open the way to more effective management of aspergillosis in humans.

* Corresponding author. Tel.: +972 3 6409870; fax: +972 3 6422494.
E-mail address: segale@post.tau.ac.il (E. Segal).

0163-4453/$30
doi:10.1016/j.jinf.2005.10.015

Introduction

Infections caused by *Aspergillus* species are increasing in importance, especially among immunocompromised hosts. The most commonly affected patients are with hematological malignancies undergoing intensive chemotherapy, bone marrow and stem cells transplant recipients who developed graft-versus-host disease.[1,2] Additional several predisposing factors for development of invasive aspergillosis include more widespread use of solid organ transplantation, the use of adrenal corticosteroids, the acquired immunodeficiency syndrome (AIDS).[3] The most common species of *Aspergillus* causing human infections is *Aspergillus fumigatus*, although other species can also cause invasive disease.[4,5]

The survival of patients with invasive aspergillosis is poor because of difficulties in early diagnosis and lack of effective treatment options.[6–8] Despite the use of amphotericin B (AMB) and its lipid formulations, itraconazole, voriconazole and caspofungin mortality rate is still high.[2,9] Because of the poor outcome, combination antifungal therapy may possibly contribute to improvement of treatment outcomes for aspergillosis.

AMB is still a major antifungal drug for treatment of invasive aspergillosis, but it has severe side effects, particularly nephrotoxicity. The currently commercially available less toxic lipid formulations of AMB are very expensive and therefore, generally not at the first line of treatment. Our laboratory has shown previously[10] that an admixture of AMB and intralipid (AMB–IL) obtained by vigorous prolonged shaking produced a stable non-toxic preparation. This admixture demonstrated in vitro activity against various *Candida* spp. and *A. fumigatus*, and was significantly more effective than conventional AMB in an experimental model of systemic candidiasis and aspergillosis[10–13] demonstrating pharmacokinetics similar to other lipid–AMB formulations.[14] The echinocandin lipopeptide caspofungin (CAS) is the first of a novel class of antifungal compounds that inhibits synthesis of 1,3-β-D-glucan, the important component of the cell wall of most pathogenic fungi. Inhibition of 1,3-β-D-glucan synthesis causes severe damage to *A. fumigatus* at the sites of hyphal growth.[15,16]

We hypothesized that inhibition of the synthesis of a major component of the fungal cell wall and an effect on the cell membrane, by combining echinocandin and a polyene, may result in a synergistic interaction in vitro and in vivo. Therefore, in the present study we investigated the combination therapy of caspofungin and AMB or AMB–IL against experimental murine aspergillosis induced in transiently immunocompromised mice. The data obtained in this study are reported herewith.

Materials and methods

Organism

A. fumigatus (ATCC 64026) was used throughout the study. The strain was grown on Sabouraud dextrose agar (SDA) slants at 28 °C for 72–96 h. Conidia were harvested by rinsing the surface of the slant with sterile saline and dislodging the conidia by gentle rubbing with a sterile quadloop. The conidial suspension was adjusted to the required concentration by counting in a hemacytometer. The number of conidia was verified by plating on Sabouraud dextrose agar plates for determination of colony counts.

Antifungal susceptibility testing

The in vitro activities of antifungal agents against *A. fumigatus* were determined by a modified technique based on the NCCLS M38-A protocol[17] using a broth microdilution system. The test broth medium was Yeast Nitrogen Base (YNB) supplemented with glucose (1%) and asparagine (0.15%). Minimal inhibitory concentrations (MIC) and minimal effective concentration (MEC) of the drugs were determined after 48 h of incubation. The MIC was taken as the lowest drug concentration with no visible growth. The microplates were scanned microscopically for determination of MEC with an inverted microscope at low magnification (×40). The MEC was the lowest drug concentration resulting in reduced hyphal growth.[18]

Drug interactions were assessed by checkerboard assays to determine the fractional inhibitory concentrations (FIC) of the combination of caspofungin and AMB or AMB–IL. The FIC of each drug was calculated by using both MIC endpoints as described previously,[17] namely, the ratio of the concentration of the drug in combination that achieves the MIC endpoint to the MIC of the drug alone by using that endpoint. The FIC index (FICI) value was calculated by adding the FIC of CAS to the FIC of AMB or AMB–IL. Drug interactions were classified as follows: $FICI \leq 0.5$, synergistic; $0.5 < FICI \leq 1$, additive; $1 < FICI \leq 4$, indifferent; $FICI > 4$, antagonistic.

Animals

Female ICR mice, 4–5 weeks old, weighing 23–28 g were used in all experiments. Animals were kept under conventional conditions and were given food and water ad libitum. The ethics committee of the Faculty of Medicine of Tel-Aviv University granted permission for the animal experiments described in the work.

Induction of an immunosuppressed state

A transiently compromised state in mice was achieved by intraperitoneal (ip) injection of the immunosuppressive agent cyclophosphamide (CY) at a dose of 200 mg/kg, 3 days prior to infection. At this time point the mice were at an immunocompromised state, as previously determined by decrease in the number of total white blood cells and neutrophils.[19]

Experimental aspergillosis

Experimental systemic aspergillosis was obtained by intravenous (iv) inoculation via the lateral tail vein of immunocompromised mice with 0.8×10^6 conidia/mouse of *A. fumigatus*, administered on day 3 post-CY treatment. Infection was followed up for 30 days and evaluated in terms of mortality and morbidity.

Mortality was assessed by number of mice that succumbed to infection out of total number of animals inoculated with the fungus at the end of the observation period (30 days), expressed as 30 day percentage of mortality, and mean survival time (MST). Morbidity was assessed by evaluation of fungal colonization of viscera (kidneys, spleen, and lungs) by quantitative determination of fungal burden (see below), histopathology, and by detection of serum galactomannan.

Drugs

A stock solution of conventional AMB (Fungizone, Bristol–Myers Squibb, France) (5 mg/ml) was prepared in 5% dextrose. AMB–IL was prepared by a 25-fold dilution of Fungizone in the lipid emulsion intralipid 20% (Kabi Pharmacia, Stockholm, Sweden) to a final AMB concentration of 0.2 mg/ml, and then agitated vigorously at 24 °C for 18 h on a controlled environmental incubator shaker (New Brunswick Scientific Co., Edison, NJ, USA) at 280 rpm, as described previously.[10] Caspofungin acetate (Merck & Co., Ink., Whitehouse Station, NJ, USA) was dissolved in 0.9% sodium chloride for injection.

Drug administration

Infected mice received the following treatments: AMB (1 mg/kg/day, iv), AMB–IL (1 mg/kg/day, iv), CAS (0.5 mg/kg/day, iv), and combination therapy of 2 agents, i.e., CAS + AMB or CAS + AMB–IL, at the same doses, as above. Treatment began 2 h after fungal inoculation and consisted of 5 consecutive daily injections, as described previously[13] and as based on studies of other investigators.[20] Assessment of activity of the combination therapy was based on similar criteria of evaluating mortality and morbidity as in the model of experimental aspergillosis in comparison to untreated animals and to animals treated with each agent alone.

Three experiments were performed, 7–9 mice/therapy group/experiment.

Cultures from organ homogenates

Quantitative measurement of infection was performed by enumeration of *Aspergillus* colony forming units (CFU) in tissue homogenates of kidneys, spleen and lungs samples at days 1, 2, 3, 5, 7, 14, 21, and 28. The kidneys, spleen and lungs were removed aseptically and homogenized in 1 ml of sterile saline. The tissue homogenates were diluted and plated on SDA, and the plates were incubated at 28 °C for 48 h. The number of CFU per organ was calculated. Duplicate determinations were performed for each time point per organ. There were 20 mice/group.

Galactomannan assay

Pooled blood (from 2 to 3 mice) was collected for each sample at days 1, 2, 3, 5, 7, 14, 21, and 28 for the determination of serum galactomannan concentrations (20 mice/group). Serum galactomannan concentrations were determined by the Platelia Aspergillus EIA – a commercial sandwich ELISA kit, and were carried out according to the protocol recommended by the producer (Platelia Aspergillus 62797, Immunoenzymatic detection of galactomannan antigen of *Aspergillus* in serum, Sanofi Diagnostic Pasteur). Briefly, 300 μl of each serum sample was mixed with 100 μl of treatment solution (4% EDTA), and the mixture was subsequently boiled for 3 min. After centrifugation the supernatant was used for further testing. Fifty microlitres of conjugate was added to each well of an anti-galactomannan monoclonal antibody coated microtiter plate, followed by the addition of 50 μl of the treated sample. The plates were incubated at 37 °C for 90 min and then washed 5 times

Table 1 MIC, MEC and FICI data of combinations of CAS + AMB, CAS + AMB—IL for *A. fumigatus* (ATCC 64026)

Endpoint (μg/ml)	CAS + AMB					CAS + AMB—IL				
	CAS (alone)	CAS (with AMB)	AMB (alone)	AMB (with CAS)	FICI	CAS (alone)	CAS (with AMB—IL)	AMB—IL (alone)	AMB—IL (with CAS)	FICI
MIC	125	15.6	1.56	0.78	0.63	125	1.95	0.78	0.39	0.52
MEC	0.78	—	—	—		0.78	—	—	—	

with washing solution. Then, 200 μl of substrate buffer was added to each well, and the plates were incubated for 30 min at room temperature in darkness. The reaction was stopped and the optical density (OD) of the samples was measured at 450 and 620 nm by a microplate spectrophotometer. Enzyme immunoassay data were expressed as a serum galactomannan index (GMI). The GMI for each test serum is equal to the OD of a sample divided by the OD of a threshold serum provided in the test kit. Sera with GMIs of less than 1 were considered negative. Sera with GMIs of greater than 1.5 were considered positive.

Histopathology

Organ samples (kidneys and lungs) were fixed in 4% buffered formalin overnight, processed and sectioned by routine histologic techniques after paraffin embedding. Sections were then stained with either hematoxylin and eosin or Grocott's methenamine silver stain (GMS).

Statistical analysis

Survival data were analyzed by Kaplan—Meier test and comparisons between groups were performed by the one-way analysis of variance (ANOVA) multiple comparisons test throughout the course of the experiments, using the Graphpad Prism 4 software package (Graphpad Software Inc., San Diego, CA, USA). *P* values of <0.05 were considered significant in these analyses.

Results

In vitro activities of AMB/AMB—IL and CAS combinations

In vitro combinations of AMB with CAS and AMB—IL with CAS were tested against *A. fumigatus* by the checkerboard assay. Since the MIC endpoint to be used in caspofungin susceptibility testing is not well established, the MEC endpoint was determined for this agent. The MICs, MECs and FICIs obtained from checkerboard tests at 48 h are shown in Table 1. The MICs of AMB and AMB—IL for *A. fumigatus* were 1.56 and 0.78 μg/ml, respectively. The MIC of CAS was significantly higher (125 μg/ml) than that of AMB or AMB—IL; in contrast, the MEC of this agent was far below the MIC — 0.78 μg/ml. The FICI values were calculated by using both MIC endpoints. AMB + CAS and AMB—IL + CAS exhibited an additive effect against *A. fumigatus*.

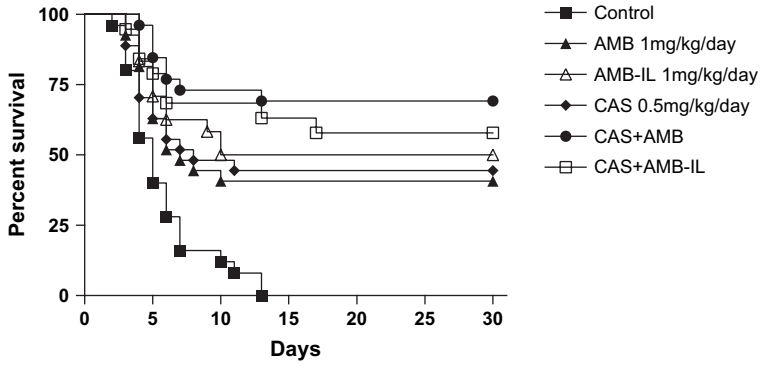

Figure 1 Survival of *Aspergillus*-infected mice treated with single or combination therapy. Mice were infected with 0.8×10^6 conidia of *A. fumigatus* on day 3 post-CY treatment. Survival was followed up for 30 days. Treatment began 2 h after fungal inoculation and consisted of 5 consecutive daily injections. The combined treatment of CAS and AMB resulted in significantly higher survival rate ($P < 0.001$), in comparison to groups treated by each agent alone (Kaplan—Meier test).

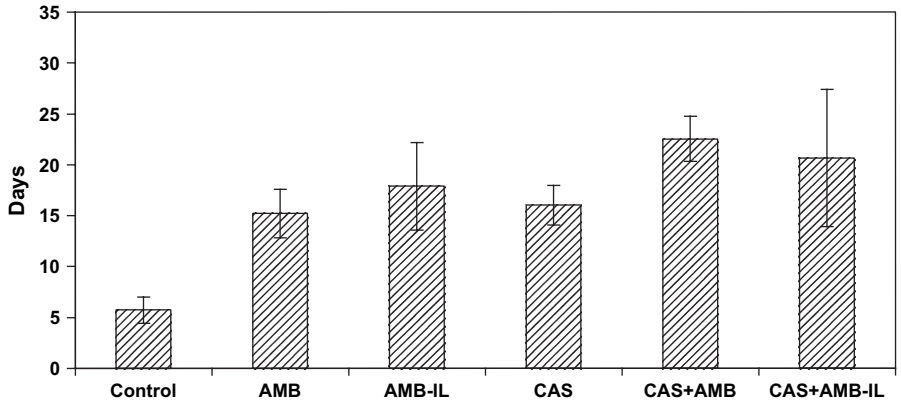

Figure 2 Mean survival time of mice treated with single agents or combination therapy. The mean survival times of mice treated with combination therapies were prolonged to 20.6—28 days, in comparison to untreated controls or to animals treated by single antifungal therapy (5.7—18 days).

Survival of animals treated by single and combination antifungal therapy

Treatment with AMB or AMB—IL at a dose of 1 mg/kg/day, or with CAS at a dose of 0.5 mg/kg/day significantly increased ($P < 0.001$) the survival rate of infected mice in comparison to the untreated control group (40.7, 50 and 44.4% for AMB, AMB—IL and CAS-treated groups, respectively, vs. 0% for the untreated group) (Fig. 1). The MSTs for AMB, AMB—IL and CAS-treated mice were 15.2, 17.9 and 16.03 days, respectively, vs. 5.72 days for untreated controls. The data indicated that treatment with AMB—IL was more effective than treatment by conventional AMB or CAS.

We also examined the combination treatment of experimental aspergillosis with two antifungal agents: AMB + CAS or AMB—IL + CAS. The drugs in the combination protocol were administered at the same doses mentioned above. Survival rates of animals treated by combined therapy with AMB + CAS or AMB—IL + CAS were considerably higher in comparison to groups treated by each agent alone (Fig. 1). The MSTs were also prolonged for combined AMB + CAS or AMB—IL + CAS-treated groups (22.5 and 20.6 days, respectively) (Fig. 2).

Serum galactomannan detection

The detection of galactomannan, as an important indication of therapeutic response, was carried out in serum samples from mice with systemic aspergillosis (Fig. 3). Serum samples (20 animals/group) from untreated controls and CAS-treated mice showed high galactomannan levels throughout the experiment. Serum samples from all treated groups demonstrated progressive galactomannan antigenemia till day 3 of the therapy. The serum GMI started to decline on day 5 in animals treated by the combination of CAS + AMB—IL. No galactomannan was detected after day 7 of the

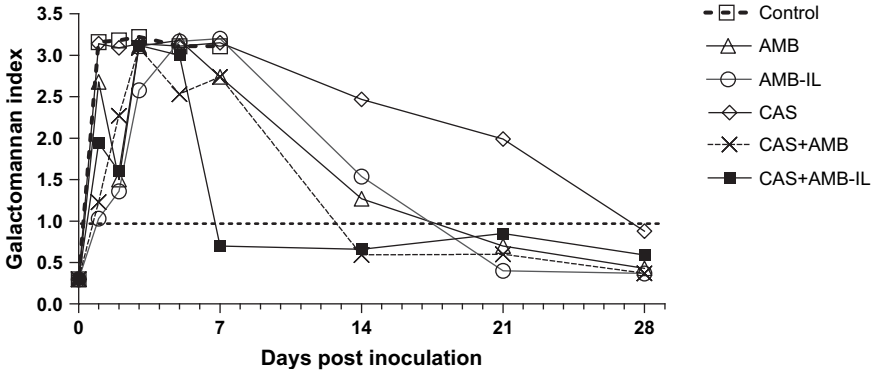

Figure 3 Galactomannan levels in *Aspergillus*-infected mice treated with single or combination therapy. Serum galactomannan concentrations were determined as described in Materials and methods. Galactomannan level was negative (GMI < 1) after day 7 in serum of CAS + AMB—IL-treated mice, and after day 14 in CAS + AMB-treated animals, compared with those that received single agents or untreated controls.

Table 2 Fungal burden in kidneys

Group	Number of CFU/organ (mean ± SD)[a]							
	24 h	48 h	72 h	5 Days	7 Days	14 Days	21 Days	28 Days
Control	345 ± 80	332 ± 156	540 ± 175	277 ± 178	182 ± 55	n.d.[b]	n.d.	n.d.
AMB	295 ± 117	127 ± 60	32 ± 22	50 ± 36	7 ± 9	0	0	0
AMB—IL	200 ± 39	47 ± 29	30 ± 18	32 ± 26	0	0	0	0
CAS	337 ± 105	210 ± 86	65 ± 31	40 ± 29	38 ± 31	0	0	0
CAS + AMB	293 ± 116	80 ± 26	38 ± 17	0	0	0	0	0
CAS + AMB—IL	367 ± 73	55 ± 26	70 ± 14	0	0	0	0	0

[a] Determined as described in Materials and methods. Mean value of 2—3 mice/group for each time point.
[b] n.d. — Not done, because the animals succumbed.

experiment in serum of CAS + AMB—IL-treated mice, and after day 14 in CAS + AMB-treated animals.

Fungal burden in organs

In the present experiment organ clearance was analyzed by determination of fungal burden in kidneys, spleen and lungs of the animals, as shown in Tables 2—4, by enumeration of *Aspergillus* CFU in tissue homogenates of these organs at various dates post-infection (CFU per organ was calculated).

(a) *Kidneys*. As shown in Table 2, in animals treated by combination therapies, the number of CFU in the kidneys was reduced in comparison to untreated controls or to groups treated by a single drug. Clearance of fungal colonization was noted from day 5 or 7. It is also noticeable that the combinations of CAS + AMB and CAS + AMB—IL were particularly effective in reducing the fungal burden.

(b) *Spleen*. High amounts of *A. fumigatus* CFU were found in the spleen in all groups at days 1 and 2 of the experiment (Table 3). The number of CFU in the spleen of animals that received combination therapies was greatly reduced on day 3. *Aspergillus* was absent in all groups at day 7, except in the untreated controls and CAS-treated group. There were no fungi found in the spleen on days 14, 21 and 28 in any treatment group.

(c) *Lungs*. *Aspergillus* CFU were detected in the lungs in all groups at 24 and 48 h post-infection. *A. fumigatus* was absent in most treated groups and untreated controls from day 5 of the experiment (Table 4).

Histopathology

Histopathology was performed on lungs and kidneys of mice. Kidneys from untreated controls showed foci of acute pyelonephritis within the medulla. In one of such foci fungal hyphae are demonstrated (Fig. 4A). A small abscess surrounds this focus. Lung tissues of untreated mice show diffuse areas of massive bronchopneumonia, involving also pleura (Fig. 5A). Occasional abscesses are scattered within lung tissue, surrounded by edematous tissue and showing foci of necrosis.

Fig. 4B presents a kidney from a mouse that received CAS, showing a focus of chronic and acute inflammatory infiltrate within the kidney pelvic mucosa. No evidence of inflammation in kidneys from mice treated with AMB, AMB—IL or with combinations of CAS + AMB and CAS + AMB—IL was

Table 3 Fungal burden in spleen

Group	Number of CFU/organ (mean ± SD)[a]							
	24 h	48 h	72 h	5 Days	7 Days	14 Days	21 Days	28 Days
Control	2212 ± 225	860 ± 211	567 ± 154	185 ± 56	117 ± 27	n.d.[b]	n.d.	n.d.
AMB	718 ± 237	467 ± 208	25 ± 19	0	0	0	0	0
AMB—IL	1327 ± 304	518 ± 146	25 ± 23	0	0	0	0	0
CAS	1965 ± 415	1727 ± 276	637 ± 55	128 ± 31	45 ± 23	0	0	0
CAS + AMB	1017 ± 346	107 ± 33	17 ± 17	0	0	0	0	0
CAS + AMB—IL	1167 ± 263	115 ± 45	127 ± 44	0	0	0	0	0

[a] Determined as described in Materials and methods. Mean value of 2—3 mice/group for each time point.
[b] n.d. — Not done, because the animals succumbed.

Table 4 Fungal burden in lungs

Group	Number of CFU/organ (mean ± SD)[a]							
	24 h	48 h	72 h	5 Days	7 Days	14 Days	21 Days	28 Days
Control	898 ± 194	525 ± 155	102 ± 86	0	0	n.d[b]	n.d.	n.d.
AMB	495 ± 82	65 ± 34	20 ± 18	0	0	0	0	0
AMB—IL	320 ± 89	42 ± 12	22 ± 22	0	10 ± 14	0	0	0
CAS	668 ± 99	170 ± 76	45 ± 23	0	0	0	0	0
CAS + AMB	467 ± 217	20 ± 18	10 ± 8	0	0	0	0	0
CAS + AMB—IL	730 ± 213	37 ± 22	20 ± 14	0	0	0	0	0

[a] Determined as described in Materials and methods. Mean value of 2—3 mice/group for each time point.
[b] n.d. — Not done, because the animals succumbed.

noted (Fig. 4C—F). Medulla and cortex seem to be well preserved. A diffuse heavy bronchopneumonia accompanied by congestion of blood vessels is seen in lung tissue of a mouse treated with CAS alone (Fig. 5B). In the lungs of AMB or AMB—IL-treated mice small foci of very mild neutrophilic infiltrate within parenchyma are present (very mild bronchopneumonia) (Fig. 5C, D). In lungs of mice that received combination therapies of CAS + AMB and CAS + AMB—IL few areas are involved by bronchopneumonia, and no necrosis of tissue is evident (Fig. 5E, F).

Discussion

Combination antifungal therapy has become of interest in view of the poor outcomes in treatments with the available antifungal agents in immunocompromised patients. In the present study we examined combination therapy with the polyene amphotericin B or amphotericin B—intralipid admixture and the echinocandin caspofungin in treatment of experimental systemic aspergillosis in transiently immunosuppressed mice.

The polyene—echinocandin combination demonstrated significantly increased efficacy in comparison to either drug alone or to untreated controls, as expressed in increased or prolonged survival of infected mice. In addition these therapies led to a significant reduction in the fungal burden or even absence of *A. fumigatus* in tissues of organs, particularly in the kidneys and lungs, and caused a rapid decline of the galactomannan level, a criterion of *Aspergillus* morbidity, in the serum of the animals, compared to those treated with single therapy or no treatment.

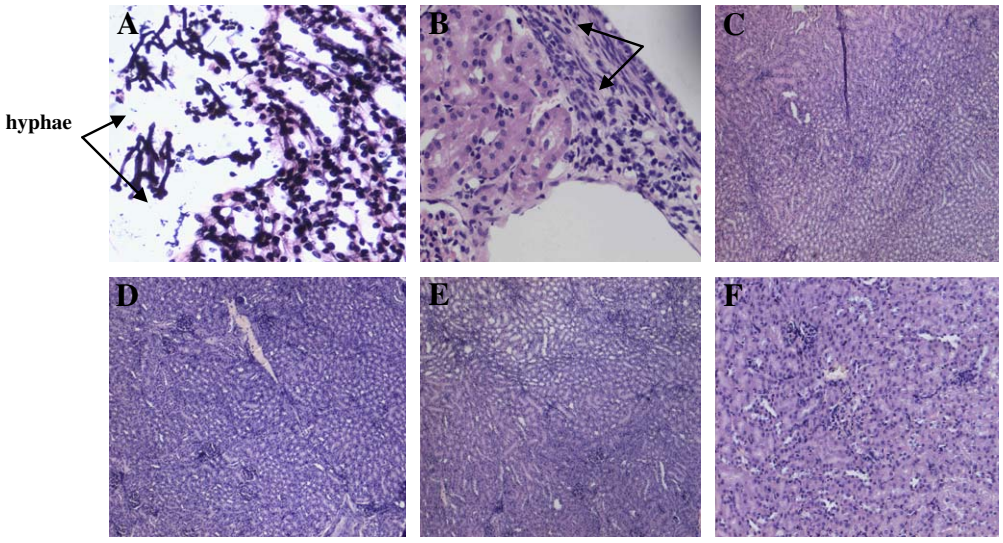

Figure 4 Histopathology of kidneys. *A. fumigatus* hyphae in the kidney tissue of untreated control stained with GMS (A) (original magnification, ×200). In kidney from mouse that received CAS one focus of chronic and acute inflammatory infiltrate is seen within the kidney pelvic mucosa (B) (original magnification, ×200). No evidence of inflammation of kidneys from mice treated with AMB, AMB—IL or with combinations of CAS + AMB and CAS + AMB—IL (C—F) (original magnification, ×100).

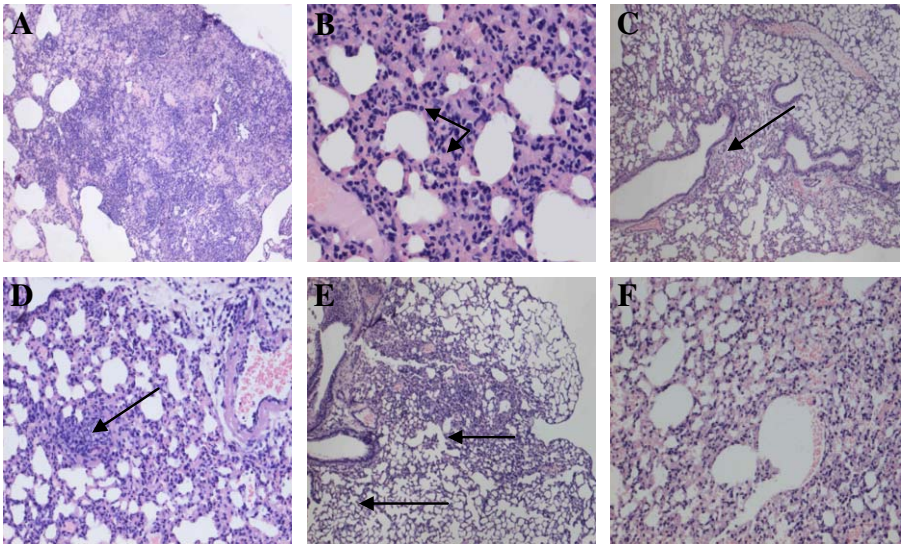

Figure 5 Histopathology of lungs. Lung tissue of untreated mouse shows diffuse areas of massive bronchopneumonia, involving also pleura (A) (original magnification, ×100). Diffuse heavy bronchopneumonia in lung of mouse treated with CAS alone (B) (original magnification, ×200). Small foci of very mild neutrophilic infiltrate within parenchyma in the lungs of AMB or AMB—IL-treated mice (C, D) (original magnification, ×100). In lungs of mice that received combination therapies of CAS + AMB and CAS + AMB—IL few areas are involved by bronchopneumonia (E, F) (original magnification, ×100).

The additive effect of the polyene and the echinocandin against *Aspergillus* is apparently due to simultaneous effect of the AMB/AMB—IL on the fungal cell membrane and inhibition by CAS of synthesis of 1,3-β-D-glucan in the cell wall. Combinations involving polyene and azole in treatment of aspergillosis[21] or candidiasis[22] have been reported to be antagonistic.

In the murine aspergillosis model used in our study fungal organ colonization was assessed by histology and quantitatively by enumeration of *Aspergillus* CFU. Fungi could be detected histologically in the kidneys and not in the lungs. Our data are compatible with those of Kretschmar et al.[23] and Wallace et al.[24] who also stated that no *Aspergillus* hyphae were noted in lung tissue while present in the kidneys in their animal models. Furthermore, our experimental model represents, primarily, systemic disseminated aspergillosis, being induced by iv inoculation of the fungus, a model used by various investigators, as summarized in the reviews of Latge[5] and Schmidt.[25] Quantitative assessment of colonization by *Aspergillus* CFU determination showed that all organs tested were colonized. Clearance was obtained by day 14 in mice treated with antifungals. Quantitative assessment of colonization is a method widely used in experimental studies,[23,26,27] including those assessing caspofungin efficacy,[27] despite possible limitations due to the filamentous nature of the fungus and the recent development of molecular technologies, such as quantitative PCR.[28] Furthermore, recent studies indicate as well that either CFU determination or the PCR technology may be valuable equally in drug assessment.[29]

It may be concluded from our study that treatment of experimental aspergillosis by combinations of antifungal agents is significantly more effective than single therapy. This finding merits further investigation in humans.

References

1. Denning DW. Invasive aspergillosis. *Clin Infect Dis* 1998;**26**:781—803.
2. Kontoyiannis DP, Bodey GP. Invasive aspergillosis in 2002: an update. *Eur J Clin Microbiol Infect Dis* 2002;**21**:161—72.
3. Kontoyiannis DP, Mantadakis E, Samonis G. Systemic mycoses in the immunocompromised host: an update in antifungal therapy. *J Hosp Infect* 2003;**53**:243—58.
4. Denning DW. *Aspergillus* species. In: Mandell GL, Bennett JE, Dolin R, editors. 5th ed., *Principles and practice of infectious diseases*, vol. 2 Churchill Livingstone; 2000. p. 2674—85.
5. Latge JP. *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* 1999;**12**:310—50.
6. Denning DW. Therapeutic outcome in invasive aspergillosis. *Clin Infect Dis* 1996;**23**:608—15.
7. Kontoyiannis DP, Lewis RE. Combination chemotherapy for invasive fungal infections: what laboratory and clinical studies tell us so far. *Drug Resist Updat* 2003;**6**:257—69.
8. Lin SJ, Schranz J, Teutsch SM. Aspergillosis case-fatality rate: systematic review of the literature. *Clin Infect Dis* 2001;**32**:358—66.
9. Patterson TF, Kirkpatrick WR, White M, Hiemenz JW, Wingard JR, Dupont B, et al. Invasive aspergillosis. Disease

spectrum, treatment practices, and outcomes. I3 *Aspergillus* study group. *Medicine (Baltimore)* 2000;**79**:250—60.
10. Shadkhan Y, Segal E, Bor A, Gov Y, Rubin M, Lichtenberg D. The use of commercially available lipid emulsions for the preparation of amphotericin B—lipid admixtures. *J Antimicrob Chemother* 1997;**39**:655—8.
11. Shadkhan Y, Segal E. Antifungal activity of amphotericin B—lipid admixtures in experimental systemic candidosis in naive mice. *J Antimicrob Chemother* 1999;**44**:787—90.
12. Shadkhan Y, Segal E. Treatment of experimental candidosis with amphotericin B—intralipid admixtures in immunocompromised mice. *J Antimicrob Chemother* 2001;**48**: 245—51.
13. Sionov E, Segal E. Treatment of murine systemic aspergillosis with polyene—intralipid admixtures. *Med Mycol* 2004;**42**: 73—80.
14. Schadkchan Y, Zaslavsky Z, Segal E. Pharmacokinetics of amphotericin B in serum and tissues in mice treated with amphotericin B—intralipid. *Med Mycol* 2003;**41**:15—9.
15. Bowman JC, Hicks PS, Kurtz MB, Rosen H, Schmatz DM, Liberator PA, et al. The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus* in vitro. *Antimicrob Agents Chemother* 2002;**46**:3001—12.
16. Groll AH, Walsh TJ. Caspofungin: pharmacology, safety and therapeutic potential in superficial and invasive fungal infections. *Expert Opin Investig Drugs* 2001;**10**:1545—58.
17. National Committee for Clinical Laboratory Standards. *Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi; approved standard. NCCLS document M38-A*. Wayne, PA: NCCLS; 2000.
18. Arikan S, Lozano-Chiu M, Paetznick V, Rex JH. In vitro synergy of caspofungin and amphotericin B against *Aspergillus* and *Fusarium* spp. *Antimicrob Agents Chemother* 2002;**46**: 245—7.
19. Sionov E, Segal E. Polyene and cytokine treatment of experimental aspergillosis. *FEMS Immunol Med Microbiol* 2003; **39**:221—7.
20. Ikeda F, Wakai Y, Matsumoto S, Maki K, Watabe E, Tawara S, et al. Efficacy of FK463, a new lipopeptide antifungal agent, in mouse models of disseminated candidiasis and aspergillosis. *Antimicrob Agents Chemother* 2000;**44**:614—8.
21. Schaffner A, Bohler A. Amphotericin B refractory aspergillosis after itraconazole: evidence for significant antagonism. *Mycoses* 1993;**36**:421—4.
22. Sugar AM, Liu XP. Interactions of itraconazole with amphotericin B in the treatment of murine invasive candidiasis. *J Infect Dis* 1998;**177**:1660—3.
23. Kretschmar M, Buchheidt D, Hof H, Nichterlein T. Galactomannan enzyme immunoassay for monitoring systemic infection with *Aspergillus fumigatus* in mice. *Diagn Microbiol Infect Dis* 2001;**41**:107—12.
24. Wallace TL, Paetznick V, Cossum PA, Lopez-Berestein G, Rex JH, Anaissie E. Activity of liposomal nystatin against disseminated *Aspergillus fumigatus* infection in neutropenic mice. *Antimicrob Agents Chemother* 1997;**41**:2238—43.
25. Schmidt A. Animal models of aspergillosis — also useful for vaccination strategies? *Mycoses* 2002;**45**:38—40.
26. Sivak O, Bartlett K, Wasan KM. Heat-treated fungizone retains amphotericin B antifungal activity without renal toxicity in rats infected with *Aspergillus fumigatus*. *Pharmacol Res* 2004;**21**:1564—6.
27. Sivak O, Bartlett K, Risovic V, Choo E, Marra F, Batty Jr DS, et al. Assessing the antifungal activity and toxicity profile of amphotericin B lipid complex (ABLC; Abelcet) in combination with caspofungin in experimental systemic aspergillosis. *J Pharm Sci* 2004;**93**:1382—9.
28. Wiederhold NP, Kontoyiannis DP, Chi J, Prince RA, Tam VH, Lewis RE. Pharmacodynamics of caspofungin in a murine model of invasive pulmonary aspergillosis: evidence of concentration-dependent activity. *J Infect Dis* 2004;**190**: 1464—71.
29. Singh G, Imai J, Clemons KV, Stevens DA. Efficacy of caspofungin against central nervous system *Aspergillus fumigatus* infection in mice determined by TaqMan PCR and CFU methods. *Antimicrob Agents Chemother* 2005;**49**:1369—76.