EXPERIMENTAL TREATMENTS
OF SYSTEMIC ASPERGILLOSIS
IN ANIMAL MODELS

Thesis submitted for the degree "Doctor of Philosophy"

By

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This work was carried out under the supervision of

Professor Esther Segal
This work is dedicated

To my wife Marianna, who supported me along the way

To my parents Boris and Tamara, who always stand by me

And to my sons Doron and Eithan
I would like to convey special thanks to my supervisor professor Esther Segal for her very much appreciated help and support throughout my work
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Abstract

Background

Invasive fungal infections, especially those caused by opportunistic molds and yeasts, have emerged as a leading cause of morbidity and mortality in immunocompromised patients. The predisposing factors that contribute to development of these diseases include profound immunosuppression associated with modern high-intensity chemotherapy regimens, acquired immunodeficiency syndrome, more widespread use of solid organ, bone marrow or haematopoietic stem cell transplantations, use of aggressive supportive care in high-risk populations, and other debilitating factors. The most frequently encountered systemic fungal infections of immunocompromised patients are those caused by *Candida* or *Aspergillus* species.

Despite significant advances in the management of immunosuppressed patients, invasive aspergillosis remains an important life-threatening complication. *Aspergillus fumigatus* is the most common species causing disease, being responsible for approximately 90% of invasive cases in humans. The majority of patients have pulmonary disease, which may be classified into three main syndromes: allergic bronchopulmonary aspergillosis, aspergilloma (fungus ball), and invasive aspergillosis.

Antifungal therapies that are currently available are suboptimal, and a complete response to treatment depends in many instances on correction of the underlying immune deficiency, which often can not be achieved. Until recently, there were primarily two agents with activity against *Aspergillus*, amphotericin B deoxycholate (AMB) and itraconazole, and these compounds were often ineffective and frequently toxic or unpredictable. In spite of the development of newer antifungals, including new formulations of older drugs (i.e., cyclodextrin-itraconazole, polyenes in lipid formulations) and entirely new classes of drugs...
with novel targets (echinocandins), an optimal approach to the treatment of aspergillosis has yet to be determined.

**Aims**

Thus, the main goal of this study was to find novel antifungal formulations, antifungal combinations, or drug and immunomodulator combinations with efficacy against systemic aspergillosis in an experimental animal model. The specific objectives included:

a) Evaluation of efficacy of the novel polyene-intralipid admixtures, amphotericin B-intralipid (AMB-IL) and nystatin-intralipid (Ny-IL), in experimental systemic aspergillosis

b) Assessment of efficacy of the polyenes or polyene-intralipid admixtures in combination with recombinant growth factor G-CSF against experimental murine aspergillosis

c) Assessment of the efficacy of the echinocandin caspofungin (CAS) in combination with immune therapy (cytokine G-CSF) in treatment of experimental systemic aspergillosis

d) Evaluation of combination antifungal therapy with the echinocandin CAS and the polyene AMB or AMB-IL in treatment of experimental systemic aspergillosis

e) Evaluation of a combination therapy containing three agents – G-CSF, caspofungin and amphotericin B/amphotericin B-intralipid – against systemic murine aspergillosis

f) Assessment of in vitro antifungal effect against *Aspergillus* and possible mode of activity of a compound isolated from the marine sponge *Dysidea herbacea*

**Experimental Design and Experimental Data**

**Experimental systemic murine aspergillosis**

The initial experiments were devoted to the adaptation of a suitable animal model. We chose ICR female mice, animals used previously in our laboratory. A compromised state in the mice was achieved by intraperitoneal injection of 200 mg/kg of the immunosuppressive agent cyclophosphamide (CY). The mice were examined for assessment of an immunosuppressed
state by blood analysis for determination of total number of white blood cells (WBC) and differential analysis for number of neutrophils. The blood analyses showed that administration of CY led to a decrease in the number of WBC, most evident on day 3 post CY treatment. The differential blood analyses revealed that the effect was particularly marked on the neutrophils, and their count decreased to a low level of 105/µl on day 3 after CY administration. Therefore, day 3 post CY pretreatment was chosen as a model representing an immunocompromised state. At this time point the animals were inoculated iv with *A. fumigatus* (ATCC 64026) with an inoculum ranging from 0.5 x 10^6 to 1 x 10^6 conidia/mouse. The optimal inoculum was determined to be 0.8-1 x 10^6 conidia per mouse. This concentration caused systemic aspergillosis that led to 100% mortality within 3-10 days. Systemic aspergillosis was determined by demonstration of fungal elements in visceral organs.

*Treatment of murine systemic aspergillosis with polyene-intralipid admixtures*

Following the adaptation of a suitable experimental model the animals were treated with various doses of AMB-IL or Ny-IL admixtures administered iv for 5 consecutive days, starting 2 hrs after infection. The mean survival rate (MSR) and mean survival time (MST) were evaluated during an observation period of 30 days in comparison to untreated infected mice and to animals treated with conventional formulations of these drugs.

The experiments showed that AMB-IL increased significantly the survival rate of the animals. Specifically, the MSR ranged in dependence of dose, between 48 and 65.7% vs 0% of the untreated (*P*<0.001) and 39.7% of AMB-treated mice (*P*<0.01). The MSR of the Ny-IL-treated mice ranged between 16.2-40% in comparison to 0% of the untreated group (*P*<0.001). Treatment with both admixtures prolonged the MST of the mice (AMB-IL: 17.3-23.07 days; Ny-IL: 8.61-16.8 days) in comparison to either untreated (6.13 days) or AMB treated animals (15.23 days). The data of this part of the study show that both AMB-IL and Ny-IL
formulations, particularly AMB-IL at the highest dose, were effective in the treatment of experimental systemic aspergillosis.

**Polyene and cytokine treatment of experimental aspergillosis**

In an attempt to increase the therapeutic efficacy of the antifungals we undertook combination therapy with G-CSF and the polyene agents. Survival rates of animals treated by combined therapies – G-CSF + AMB, G-CSF + AMB-IL and G-CSF + Ny-IL – were significantly higher ($P < 0.001$) (87.3%, 83.3%, 38.53%, respectively) than in the groups treated with G-CSF, AMB, AMB-IL and Ny-IL alone (14.65, 41.9, 51.48 and 29%, respectively). The combination treatment also prolonged meaningfully ($P < 0.01$) the survival time of animals in comparison to treatment with each agent alone. The MSTs were 8.47, 28.83, 26.25 and 15.35 days for the G-CSF, G-CSF + AMB, G-CSF + AMB-IL and G-CSF + Ny-IL-treated groups, respectively. The follow-up of the course of infection indicated that combined therapies, particularly treatment by G-CSF + AMB and G-CSF + AMB-IL, markedly delayed the mortality of mice, contrary to untreated controls and to treatment with either drug alone.

The results obtained from these experiments clearly showed that treatment of murine systemic aspergillosis by the cytokine G-CSF and AMB, G-CSF and AMB-IL or G-CSF and Ny-IL was superior to treatment by each agent alone.

**Polyene-echinocandin combination and immunotherapy in the treatment of experimental systemic aspergillosis**

In the next step of our study we examined the combination therapy of CAS and AMB or AMB-IL admixture against disseminated murine aspergillosis induced in transiently immunocompromised mice. Furthermore, we also explored the effect of supplementing the antifungal combination with the cytokine G-CSF.
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). Combination of CAS+G-CSF increased survival of infected mice up to 77.7% and prolonged their mean survival time to 25 days. Addition of G-CSF to the combinations of CAS+AMB or CAS+AMB-IL did not increase further the survival rate nor did prolong survival time. These combinations also resulted in reduction of fungal burden in organs, and decrease in serum galactomannan. These successful results obtained in the experimental animal model may possibly open the way to more effective management of aspergillosis in humans.

In vitro antifungal activity of a compound from the marine sponge Dysidea herbacea

Development of new antifungal drugs, particularly from natural sources with novel mechanisms of action, is of significance in view of the increased frequency of fungal infections, particularly in compromised hosts, and the problems associated with available antifungal agents. In our study we examined the in vitro activity of series of extracts of marine sponges against the fungal pathogens Candida and Aspergillus species, focusing primarily on C.albicans and A.fumigatus. The extract of Dysidea herbacea was among the few that exhibited activity. Bioassay guided separation of the extract led to the active compound, which was determined to be 3,5-dibromo-2-(3,5-dibromo-2-methoxyphenoxy) phenol. This compound exhibited in vitro activity against the fungal pathogens.

For determination of the possible mode of activity of the compound we tested the effect on fungal cellular morphology (light, scanning and transmission electron microscopy) and possible site of activity in the fungal cells, such as cell membrane (ion leakage kinetics) as well as toxicity (cytotoxicity tests).

The experiments on the mode of activity revealed that there are significant changes in fungal cell morphology, as demonstrated by scanning and transmission electron microscopy, and that
the compound, apparently, affects the fungal cell membrane, expressed primarily in leakage of potassium ions from the fungal cells.

Summary, conclusions and significance

1. The data presented in this study show that AMB-IL was more effective than conventional AMB in treatment of experimental systemic aspergillosis, especially when high doses of AMB-IL were used
2. Ny-IL may also be a possible additional therapeutic option against aspergillosis that should be considered
3. Combination of polyenes and G-CSF, particularly G-CSF with AMB or AMB-IL, was synergistic in experimental aspergillosis and significantly increased the survival rate and prolonged the survival time of the animals in comparison to treatment with either polyene alone
4. The polyene-echinocandin combination (CAS + AMB or CAS + AMB-IL) demonstrated significantly increased efficacy in comparison to either drug alone or to untreated controls, as expressed in increased or prolonged survival of infected mice, reduction of CFU in organs and reduced galactomannan antigenemia
5. Combination of CAS with G-CSF was clearly more effective than CAS alone in treatment of experimental systemic aspergillosis
6. Combined therapies consisting of three agents (CAS, G-CSF and either AMB or AMB-IL) markedly increased the survival of animals compared to those treated with single therapy or no treatment, but were not better than combinations involving two agents – antifungal drug and G-CSF
7. The compound isolated from the marine sponge *Dysidea herbacea* exhibited in vitro activity against *Aspergillus* and *Candida* species by apparently affecting the fungal cell membrane
In conclusion, our promising data pointing to the efficacy of lipid formulations of polyenes, polyene-echinocandin combinations and adjunct immunotherapy in the treatment of disseminated experimental aspergillosis warrant further clinical investigations.
Summary

Invasive fungal infections, especially those caused by opportunistic molds and yeasts, have emerged as a leading cause of morbidity and mortality in immunocompromised patients. The most frequently encountered systemic fungal infections of immunocompromised patients are those caused by *Candida* or *Aspergillus* species. Despite significant advances in the management of immunosuppressed patients, invasive aspergillosis remains an important life-threatening complication. Antifungal therapies that are currently available are suboptimal, and a complete response to treatment depends in many instances on correction of the underlying immune deficiency, which often can not be achieved.

Thus, the main goal of this study was to find novel antifungal formulations, antifungal combinations, or drug and immunomodulator combinations with efficacy against systemic aspergillosis in an experimental animal model.

The initial experiments were devoted to the adaptation of a suitable animal model. A compromised state in ICR mice was achieved by intraperitoneal injection of 200 mg/kg of the immunosuppressive agent cyclophosphamide (CY). The blood analyses showed that administration of CY led to a decrease in the number of white blood cells, most evident on day 3 post CY treatment. At this time point the animals were inoculated iv with *A. fumigatus* (ATCC 64026) with an inoculum of 0.8-1x10^6 conidia/mouse. This concentration caused systemic aspergillosis that led to 100% mortality within 3-10 days. Systemic aspergillosis was determined by demonstration of fungal elements in visceral organs.

Following the adaptation of a suitable experimental model the animals were treated with various doses of amphotericin B-intralipid (AMB-IL) or nystatin-intralipid (Ny-IL) admixtures administered iv for 5 consecutive days, starting 2 hrs after infection. The mean survival rate and mean survival time were evaluated during an observation period of 30 days in comparison to untreated infected mice and to animals treated with conventional formulations of these
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Experimental treatments of systemic aspergillosis in animal models  
Professor Esther Segal

drugs. The data showed that both AMB-IL and Ny-IL formulations, particularly AMB-IL at the highest dose, were effective in the treatment of experimental systemic aspergillosis.

In an attempt to further increase the therapeutic efficacy of the antifungals we undertook combination therapy with the cytokine G-CSF and the polyene agents. Survival rates of animals treated by combined therapies, particularly of those treated by G-CSF+AMB and G-CSF+AMB-IL, were significantly higher than in the groups treated with each agent alone. The results obtained from these experiments clearly showed that treatment of murine systemic aspergillosis by the cytokine G-CSF and AMB, G-CSF and AMB-IL or G-CSF and Ny-IL was superior to treatment by each agent alone.

In the next step of our study we examined the combination therapy of caspofungin (CAS) and AMB or AMB-IL admixture against disseminated murine aspergillosis induced in transiently immunocompromised mice. Furthermore, we also explored the effect of supplementing the antifungal combination with the cytokine G-CSF. These combinations increased survival rates, reduced the fungal burden in organs and galactomannan level in serum of the animals.

Development of new antifungal drugs, particularly from natural sources with novel mechanisms of action, is of significance in view of the increased frequency of fungal infections. In our study we examined the \textit{in vitro} activity of series of extracts of marine sponges against the fungal pathogens \textit{C.albicans} and \textit{A.fumigatus}. The extract of \textit{Dysidea herbacea} was among the few that exhibited \textit{in vitro} activity against the fungal pathogens. The experiments on the mode of activity revealed that there are significant changes in fungal cell morphology, as demonstrated by scanning and transmission electron microscopy, and that the compound, apparently, affects the fungal cell membrane and is expressed primarily in leakage of potassium ions from the fungal cells.
In summary, the results of the present study indicate that:

1. AMB-IL was more effective than conventional AMB in treatment of experimental systemic aspergillosis, especially when high doses of AMB-IL were used
2. Ny-IL may also be a possible additional therapeutic option against aspergillosis that should be considered
3. Combination of polyenes and G-CSF, particularly G-CSF with AMB or AMB-IL, was synergistic in experimental aspergillosis and significantly increased the survival rate and prolonged the survival time of the animals in comparison to treatment with either polyene alone (AMB, AMB-IL or Ny-IL)
4. The polyene-echinocandin combination (CAS + AMB or CAS + AMB-IL) demonstrated significantly increased efficacy, as expressed in increased or prolonged survival of infected mice, reduction of CFU in organs and reduced galactomannan antigenemia
5. Combination of CAS with G-CSF was clearly more effective than CAS alone in treatment of experimental systemic aspergillosis
6. Combined therapies consisting of three agents (CAS, G-CSF and either AMB or AMB-IL) markedly increased the survival of animals, but were not better than combinations involving two agents – antifungal drug and G-CSF
7. The compound isolated from the marine sponge *Dysidea herbacea* exhibited in vitro activity against *Aspergillus* and *Candida* species by, apparently, affecting the fungal cell membrane

In conclusion, our promising data pointing to the efficacy of lipid formulations of polyenes, polyene-echinocandin combinations and adjunct immunotherapy in the treatment of disseminated experimental aspergillosis warrant further clinical investigations.
Introduction

The last two decades have seen significant changes in the pattern of fungal infections in humans. These diseases have much greater importance due to their increasing incidence in persons with haematological malignances, in recipients of bone marrow, solid organ or haematopoietic stem cell transplants, in persons with the acquired immunodeficiency syndrome (AIDS) and in other debilitated or immunocompromised individuals (1,2). Systemic fungal infections have emerged as a leading cause of morbidity and mortality in heavily immunosuppressed patients (3-5). The most frequently encountered pathogens are Candida and Aspergillus species. (4,6,7). Despite significant advances in the management of immunosuppressed patients, invasive aspergillosis remains an important life-threatening infectious disease (8,9). The mortality among untreated immunocompromised patients with invasive aspergillosis is approximately 100% (3).

Molds of the genus Aspergillus have worldwide distribution and grow in soil, on plants and on decomposing organic matter. These molds are often found in air, in water, in dust and on food (10,11). Although more than 200 species within the genus Aspergillus have been described to date, fewer than 20 can cause disease in humans. Aspergillus fumigatus is the most common species causing disease, being responsible for approximately 90% of invasive cases in humans (10,12,13). A.flavus is the second most frequent pathogen, and less common species include A.nidulans, A.niger and A.terreus.

Risk groups and predisposing factors for aspergillosis

Invasive aspergillosis has emerged as a major problem in several groups of immunocompromised patients. Those at greatest risk include persons with prolonged, profound neutropenia due to hematological and lymphoreticular malignancies.
(incidence range 5-25%), particularly in patients with acute leukemia and lymphomas undergoing intensive chemotherapy; those who have received allogeneic bone marrow transplantation (BMT) or hematopoietic stem cell transplantation (5-30%); solid organ transplant recipients (1-15%); patients with functional neutrophil deficits, such as those seen in chronic granulomatous disease (CGD) or with acquired immunodeficiency syndrome at the most advance stages (25-40%) (14-17). A major risk factor is also the use of glucocorticosteroids that disregulates the immunity towards fungal infections (17a). In transplant patients corticosteroid therapy is usually linked to graft-versus-host disease (GVHD) and/or rejection in transplantation (18). In addition, aspergillosis has been described increasingly in other groups of patients as well. Invasive aspergillosis was found to be a relatively common cause of death in patients with systemic lupus erythematosus and in patients with multiple myeloma who receive high-dose steroids (19,20).

The occurrence rate of aspergillosis in neutropenic cancer patients is variable and is dependent on the underlying disorder and medical intervention that impairs neutrophil function or quantity. Most at risk are those patients who remain neutropenic for 2 weeks or longer (11).

Among solid organ transplant recipients, those with renal transplantation are least at risk; in contrast, lung transplant recipients have a high risk of invasive aspergillosis (16). Among the risk factors that have been identified are graft rejection, tracheobronchitis or bronchial infections, cytomegalovirus infection and increased immunosuppression. It should be also added that there are reports indicating that patients receiving anti-TNF antibodies in treatment of autoimmune diseases, such as rheumatoid arthritis, may be under risk of developing aspergillosis (20a,20b). This points to the importance of TNF as component of the immunity to this infection.
**Pathogenesis**

**Organism factors**

Inhalation of *Aspergillus* conidia is the usual mode of infection in humans. The clinical manifestations of aspergillosis are both the result of the degree to which the organism invades and destroys tissues, and the state of the host’s immune response (13). *A. fumigatus* is one of the most ubiquitous fungi with airborne conidia (21). It sporulates plentifully, with every conidial head producing thousands of conidia. The conidia released into the atmosphere have a very small size (2-3 µm), which enables them to penetrate deep into the lungs (8,12). The hydrophobic protein coat layer of conidia may help protect them from host defenses (22). *A. fumigatus* binds laminin and fibrinogen more efficiently than other species of *Aspergillus* (23,24), probably allowing greater adhesion to epithelia in the airways before invasion. Hyphal factors thought to be important in contributing to pathogenicity include antioxidant defenses, such as superoxide dismutases and catalases (25,26) that may protect the organism from damage due to single oxygen, hydrogen peroxide and other free radicals produced by phagocytes. *A. fumigatus* produces different enzymes, such as phospholipases and various proteases – serine alkaline protease, aspartic protease, metalloprotease – that function to suppress immune response and destroy tissues (27-30).

*A. fumigatus* produces several toxic secondary metabolites. The most intensively studied is gliotoxin that inhibits macrophage and neutrophil phagocytosis, induces apoptosis in macrophages and blocks T- and B-cell activation and the generation of cytotoxic cells (31-33).
In addition, some putative virulence factors, such as cell wall pigments with antioxidant activity or production of RNase and hemolysin with toxic effects, may contribute to pathogenicity of *A.fumigatus* (34-36).

It should also be pointed out that the species *A.flavus* and *A.terreus* that recently increased in importance as etiological agents of aspergillosis are more resistant to antifungal drugs, particularly amphotericin B (36a).

**Host defense factors**

Besides the mucous layer and ciliary action, the first line of defense against *Aspergillus* in the lungs are macrophages, which are capable of attaching to, ingesting, and killing conidia (37,38). Efficacy may be enhanced by conidial opsonization with complement or collectins, such as mannose-binding lectin and surfactant proteins (39,40). Hyphae are damaged and sometimes killed by neutrophils, with some contribution from monocytes and possibly macrophages (37,41). Neutrophils adhere to the surface of the hyphae, since hyphae are too large to be ingested, and therefore, killing proceeds extracellularly. The killing by neutrophils depends on reactive oxygen intermediates aided by the action of nonoxidative killing systems such as neutrophil defensins (42).

Both innate and acquired T-cell immunity contribute to resistance to aspergillosis. Development of protective acquired immunity is usually associated with the activation of Th1 cells producing increased levels of IL-2, gamma interferon (IFN-\(\gamma\)) and macrophages producing IL-12 (12,43). In contrast, Th2-type response is usually correlated with a minimal cellular response and increase in IL-4 and IL-10 levels and a lower level of IFN-\(\gamma\), which is associated with progression of the disease (44,45). IFN-\(\gamma\) neutralization resulted in increased pathological findings and expression of IL-
10 mRNA (46). On the other hand, administration of IFN-γ, TNF-α, or soluble IL-4 receptor rescued infected animals (44).

TNF-α is a critical component of pulmonary host defense against *A.fumigatus*. High level production of TNF-α was observed in mice resistant to invasive pulmonary aspergillosis (47). Increased mortality correlated with cytokine neutralization in both immunocompromised and immunocompetent infected mice, while improved resistance was observed upon administration of a TNF-α agonist peptide (48).

Granulocyte colony stimulating factor (G-CSF) enhanced neutrophil oxidative responses and antifungal activity against *A.fumigatus* hyphae (49). G-CSF is a glycoprotein that regulates the production and release of functional neutrophils from the bone marrow, and also increases their microbicidal activity (50,51). G-CSF administered to human volunteers increased the fungicidal activity through enhanced respiratory burst of their PMNs against *Aspergillus* conidia by 4-fold (52). Human G-CSF induces function of granulocytes and has been shown to have a protective effect in murine models of aspergillosis. Prophylaxis with human G-CSF and amphotericin B or itraconazole showed some additive effect in treatment of invasive aspergillosis in neutropenic (cyclophosphamide-treated) mice; however, corticosteroid-treated mice were protected only when simultaneous G-CSF and itraconazole were administrated (53). In addition, a synergistic effect was found when G-CSF was added to posaconazole therapy in experimental aspergillosis (54).

Human macrophages treated by granulocyte-macrophage colony stimulating factor (GM-CSF) or macrophage colony stimulating factor (M-CSF) exhibited enhanced conidial phagocytosis, oxygen radical production, and hyphal damage (41,55). GM-CSF has increased proliferation of spleen cells in mice, indicating potent stimulation of hematopoietic cells (56). In addition, concanavalin A-stimulated spleen cells from
GM-CSF treated mice significantly increased IFN-γ production in comparison to control spleen cells (56). M-CSF modulates mononuclear phagocyte function such as H₂O₂ production and phagocytosis, and enhances production of IL-1, IFN-γ and TNF-α (57).

**Clinical presentation of aspergillosis**

The main portal of entry and site of infection for *Aspergillus* is the respiratory tract, but some other sites of infection can be involved in the normal or immunocompromised host, such as the skin, peritoneum, kidneys, bones, eyes and gastrointestinal tract (8,12). The majority of patients (80%-90%) have pulmonary disease, which may be classified into three main syndromes: allergic bronchopulmonary aspergillosis (ABPA), aspergilloma (fungus ball), and invasive aspergillosis (IA).

ABPA is severe allergic pulmonary complication caused by *Aspergillus* species. It occurs frequently in patients suffering from cystic fibrosis and bronchial asthma (13,58). ABPA follows the same course as classic asthma, which is a mixture of type I, III and IV allergic reactions, and eosinophilia (59). Clinically, this syndrome manifests as asthma with transient pulmonary infiltrates that can eventually lead to proximal bronchiectasis and lung fibrosis (60).

Aspergilloma, or "fungus ball", occurs in pre-existing lung lesions, which become secondarily infected with *Aspergillus* species. Common pre-existing lung cavities include tuberculosis, sarcoidosis, or other bullous lung disorders (61,62). Aspergilloma consists of a spheroid mass of hyphae protected within a matrix of fibrin and cellular debris. A common symptom of aspergilloma is hemoptysis. Hemoptysis results from the disruption of blood vessels in the wall of the cavity.
occupied by the fungus (63). In addition to occurring within pre-existing cavities, fungal balls may appear in previously normal lung (64).

Invasive aspergillosis (IA) has become a major cause of death, mainly among hematology patients, but it also occurs in patients with nonhematogenous underlying conditions. IA is increasingly reported in AIDS patients and it is also a common infectious complication of CGD (8,65). Four types of IA have been described (66):

1. acute or chronic pulmonary aspergillosis – the most common form of IA
2. tracheobronchitis and obstructive bronchial disease with various degrees of invasion of the mucosa
3. acute invasive rhinosinusitis
4. disseminated disease commonly involving the brain, heart, liver, kidneys, thyroid, spleen, skin (59).

Disseminated systemic aspergillosis occurs in approximately one third of the neutropenic patients suffering from IA (15). CNS-aspergillosis appears to be the main manifestation of hematogenous spread of Aspergillus (67). The gastrointestinal tract is reported to present the second most prominent target of dissemination, particularly with involvement of the esophagus and the bowel (15,68). The general symptoms of IA, similarly to the other forms of aspergillosis, are variable and nonspecific, typically involving fever, hemoptysis, cough, chest pain, malaise, and weight loss (12,13). Early diagnosis of aspergillosis remains a challenge.

**Animal models of aspergillosis**

A major aim of an experimental study of aspergillosis with animal models is to gain insight into the pathogenesis of human aspergillosis. However, experimental models have also been developed and used for the evaluation of drug efficacy in the treatment of aspergillosis, assessment of new diagnostic methods and immunological analysis of
the infection (12,69). A large number of studies have been conducted, using a variety of experimental models of the disease. Aspergillosis has been established in mice, rabbits, rats, guinea pigs, chickens, turkeys, ducks, and monkeys (70-72). However, the experimental models of Aspergillus infection have been most frequently produced in the three different species of rodents that have been mentioned above: mice, rabbits and rats (69).

In general, animal experiments are under public pressure, and FRAME (Fund for the Replacement of Animals in Medical Experiments) suggests three strategies: refinement, reduction and/or replacement of animal testing in biomedical research (73,74). These aspects have to be taken into consideration in the performance of animal testing in the study of infections. On the other hand, it has to be emphasized that due to a poor in vitro/in vivo correlation in the case of the evaluation of antimycotic treatment strategies, animal models can never be totally replaced.

The most frequently used animal model of aspergillosis is that produced in the mice (12,69). Infections can be established by intravenous, intranasal and intraperitoneal routes. Intravenous (i.v.) inoculations show an excellent infectious-dose to mortality ratio, and do not always require a preceding immunosuppression of the animals (75). This model is extremely valuable and produces a more uniform pattern of disease (12,75). In most cases five animals are sufficient per test group to assess significant differences in the efficacy of test substances, and this is of real benefit in accordance with the "reduction" of animal experiments (75). Mice lethally infected by i.v. inoculation with a conidial suspension (usually inoculum 4x10^6 conidia per animal) have been commonly used to evaluate the efficacy of novel antifungal drugs on the basis of mortality and organ colonization, as well as to test the virulence of different strains of Aspergillus (70).
Although intranasal (i.n.) inoculation mimics the natural route of infection, this model is difficult to standardize. The major drawback of the model is the highly variable response of animals to a given inoculum (about $10^8$ conidia per mouse), necessitating the use of large groups within each experiment (up to 25 animals per group) in order to obtain almost reproducible results (12,75). This is generally not in accordance with the strategies suggested by FRAME (75).

The intraperitoneal (i.p.) routes of inoculation in mice are only rarely performed. Mice are primarily resistant towards an intraperitoneal administration of *A. fumigatus* conidia; this infection model requires that *Aspergillus* conidia have to be pregerminated before i.p. administration (75).

In order to have the animal models more analogous to clinical situations, the inoculum size can be reduced if the severity of the immunosuppression is increased by using cyclophosphamide in several cycles (76). However, susceptibility of the immunosuppressed mice to other infections necessitates keeping the mice in a sterile environment.

Next to the mouse, the rabbit has been used most frequently to generate a model of *Aspergillus* infection. Rabbit models are advantageous over murine or rat models in that a rabbit is much larger than the other species of laboratory animals. This makes it possible to perform serial sampling of serum from an infected animal, detailed histopathological observation of pulmonary lesions, and examination of bronchoalveolar lavage specimens (77,78). In this sense, rabbits infected by respiratory tract injection with conidial suspension are considered the usable model of IA (69).

The rat is another species of laboratory animal which is often used for experimental investigations of aspergillosis because of its suitable size to produce primary
pulmonary infection (79). Rat model of IA, which was produced by intratracheal injection of agarose beads containing *A. fumigatus* conidia, which enabled delivery to the alveoli, was developed by Shibuya et al. (80). The histopathological findings of the pulmonary lesions obtained with this rat model are very similar to those for IA in humans.

**Treatment of aspergillosis**

Antifungal therapies that are currently available are suboptimal, and a complete response to treatment depends mainly on correction of the underlying immune deficiency, which often can not be achieved. Until recently, there were primarily two agents with activity against *Aspergillus*, amphotericin B deoxycholate (AMB) and itraconazole, and those compounds were often ineffective and frequently toxic or unpredictable (3). In spite of the development of newer antifungals, including new formulations of older drugs (i.e., cyclodextrin-itraconazole, polyenes in lipid formulations) and entirely new classes of drugs with novel targets (echinocandins), an optimal approach to the treatment of aspergillosis has yet to be determined (2).

**Polyenes**

The oldest antifungal class is the polyene macrolides, amphotericin B and nystatin, which bind to ergosterol, the major sterol in fungal cytoplasmic membranes. This binding creates channels in the cell membrane that increase permeability and cause cell death through leakage of essential nutrients (6). These drugs also have oxidant activity and disrupt cellular metabolism in the fungal cell (81).

AMB, initially approved for use in 1958, until recently has been considered the gold standard antifungal therapy for any form of invasive disease, particularly in people who are immunocompromised and at risk for extrapulmonary invasion (13). AMB has remained the reference standard for treatment of IA (82) as well as the standard of
comparison for all newer antifungal agents (9). AMB has two major drawbacks. First – insolubility in water. To resolve this problem in the conventional AMB formulation (Fungizone), a mixture of AMB with the detergent deoxycholate in the ratio 3:7 is used (83). The second drawback – tolerance of AMB is limited by its acute and chronic toxicities. In addition to fungal ergosterol, the drug also interacts with cholesterol in human cell membranes, which probably accounts for its toxicity (84). Up to 80% of patients receiving AMB develop either infusion-related toxicity or nephrotoxicity (synergistically promoted in presence of cyclosporine A) (83).

In addition to conventional AMB, three fundamentally different lipid-associated formulations have been developed: AMB-lipid complex (Abelcet), AMB-colloidal dispersion (Amphocil), and liposomal AMB (Ambisome). These formulations are available for iv use and reduce the toxicity associated with conventional AMB. Most clinical data have been obtained with the use of these preparations at 5 mg/kg/day (9). However, these AMB formulations are extremely expensive and, therefore, generally not in the first line of treatment in many of the countries. However, in some others, such as the US, the lipid formulations have been recently considered as new "gold standard" in antifungal therapy (84a).

Lipid emulsions for parenteral nutrition, such as Intralipid or Lipofundin, which are known for their relative low toxicity, gained interest as possible vehicles for lipophilic drugs (85,86). In view of this carrier system studies exploring the mixtures of AMB with lipid emulsions have been reported since the 1990-s (87,88). One of these was the study of Chavanet and colleagues (88) who reported that comparison of conventional AMB deoxycholate with AMB deoxycholate – fat emulsion (Intralipid 20%) in patients with oral candidiasis revealed, due to better tolerance, that higher
doses of AMB could be used, resulting in greater efficacy. Later studies confirmed the observations regarding the reduced toxicity of AMB with fat emulsions (89,90).

Our laboratory has been involved in investigation of AMB-Intralipid admixtures. Shadkhan et al. (91) have shown that admixtures of Fungizone and Intralipid (AMB-IL) obtained by vigorous prolonged agitation yielded stable non-toxic preparations. These admixtures exhibited in vitro activity against various Candida species, and were significantly more effective than conventional AMB in an experimental animal model of systemic candidiasis (91-93).

Nystatin was the first polyene antifungal. Nystatin was licensed for use in 1951 against superficial Candida infections; however, problems with solubility and toxicity with parenteral use limited nystatin to topical use (94). The new liposomal nystatin (Nyotran) is a multilamellar liposomal formulation of dimyristol phosphatidylcholine, dimyristol phosphatidyl glycerol, and nystatin (7:3:1) (95). The liposomal formulation enables the use of nystatin intravenously with good evidence of activity against aspergillosis and reduced toxicity (96-98). However, this formulation has not been further developed to the stage of clinical use.

**Azoles**

Another antifungal drug, which is used as a second-line agent in patients having aspergillosis that is refractory to or intolerant of AMB, is the triazole itraconazole (Sporanox). The free azole nitrogen competes for oxygen with the catalytic heme iron atom of cytochrome P-450 enzymes. Inhibition of the cytochrome P-450 14-α-demethylase prevents the synthesis of ergosterol in fungal membranes (12). Itraconazole binds very weakly to mammalian cytochrome P-450, which reduces considerably the toxicity of the drug in humans.
Itraconazole is poorly water-soluble, is not reliably absorbed from the gastrointestinal tract in its capsule formulation, and has high protein binding (99). To overcome problems of variable absorption, itraconazole has now been solubilized in hydroxypropyl-β-cyclodextrin, with substantial improvement as an oral solution (100). A new iv formulation of itraconazole has also been developed, which provides an alternative formulation for those patients who cannot take oral medications (6,101). In addition, the iv formulation can achieve high and steady-state plasma concentrations less than 48 hours after starting treatment (102), a feature that is of particular importance in the treatment of systemic fungal infections.

Voriconazole (VFend) is a new second-generation triazole synthetic derivative of fluconazole. Both fungicidal and fungistatic activities against Aspergillus have been demonstrated (103,104). In addition to its actions on the 14-α-demethylase, like other azoles, voriconazole also inhibits 24-methylene dehydrolanosterol demethylation, explaining why it is active against a mold such as Aspergillus when fluconazole is not (105). The largest prospective clinical trial of voriconazole involved 392 patients at 92 centers in 19 countries over 3 years and compared initial randomized therapy with voriconazole versus AMB (106). Patients who initially received voriconazole had statistically significantly better complete or partial response (53% of patients), compared with those initially receiving AMB (32%). Survival rates also improved to 71% for voriconazole, versus 58% for those initially receiving AMB. Voriconazole has also side effects, including reversible visual disturbances (increased brightness, blurred vision), occasional elevated hepatic transaminases with increasing doses, and occasional skin reactions likely due to photosensitization (2). In addition, the use of iv voriconazole is contraindicated in patients with creatinine clearance of less than 50
ml/min. This is because of concerns regarding accumulation of voriconazole's renally excreted carrier, sulfobutyl ether β-cyclodextrin sodium (16).

Posaconazole is also a second-generation triazole and is a derivative of itraconazole having very low water solubility (2). It is fungicidal in vitro against *Aspergillus* spp., and its activity is slightly superior at 48 hours to that of itraconazole and voriconazole, yet inferior to that of AMB (107). Posaconazole has a long half-life of at least 18-24 hours in humans and probably time-dependent killing (9). Presently it is in clinical trials as an iv formulation (unpublished data).

Ravuconazole is structurally similar to fluconazole and voriconazole. Ravuconazole is available in oral and parenteral formulations and revealed considerable activity in a rabbit model of IA as well as decrease in tissue fungal burden comparable to the effect of AMB (108). No published efficacy data on ravuconazole in humans are available.

**Echinocandins**

Echinocandins are a new class of antifungal drugs derived from several fungal species. These drugs are cell-wall-active agents that presumably act through non-competitive inhibition of 1,3-β-D-glucan synthase, an enzyme present in fungi but absent in mammalian cells (109,110). The 1,3-β-glucan, an essential cell wall polysaccharide, provides structural integrity for the fungal cell wall. Echinocandins are generally fungicidal in vitro, although not as rapidly as AMB, but appear to be more fungistatic against *Aspergillus* (111,112). These agents inhibit hyphal branch point growth, converting the mycelium to small clamps of cells, but the older septated cells with little glucan synthesis are not killed (113).

Caspofungin (Cancidas), a water-soluble semisynthetic derivative of the natural product pneumocandin B, was the first echinocandin to be approved for the treatment
of IA in patients whose disease does not respond to or intolerant of other antifungal therapies (114). The drug, like all echinocandins, has pharmokinetic properties favorable for iv administration and represent a new mechanism in antifungal therapy having considerably less toxicity than AMB does. However, unlike AMB, caspofungin has a relatively narrow antifungal spectrum, and is not active against *Cryptococcus neoformans, Trichosporon, Rhizopus* and *Fusarium* species. Therefore, a potential difficulty with use of caspofungin in the empirical treatment of patients with suspected IA is the possibly unsuspected infection with an organism resistant to the drug (16).

Micafungin is another echinocandin lipopeptide compound with a half-life of ~12 hours and, like all echinocandins, is fungistatic in vitro against *Aspergillus* (115). Micafungin has been approved for clinical use in Japan as salvage therapy for IA in patients refractory to AMB, but the number of published reports are limited (116).

Anidulafungin is an additional echinocandin that currently is in advanced stages of clinical development. Because the clinical experience with this agent is limited, its toxic effects are unknown, but its activity is similar to that of the other echinocandins (2).

**Combination therapy**

As stated above, antifungal treatment is often complicated by high toxicity, low tolerability, or narrow spectrum of activity. The recent introduction of previously non-available antifungals that act on novel targets in the fungal cell, such as the echinocandins, and the aforementioned problems with the currently available antifungals have promoted recent efforts to determine the efficacy of combination therapy in the treatment of invasive fungal infections (117). Potential benefits of using combination therapy include broader spectrum of efficacy, greater potency than either
of the drugs used in single therapy, improved safety and tolerability, and reduction in
the number of resistant organisms (118). *Aspergillus* infections have become the most
important area of research in the use of combination antifungal therapy due to their
poor outcome and high cost of treatment (5). Unfortunately, a few studies have been
performed to determine the efficacy of combination therapy against aspergillosis. For
example, combination of triazole (e.g. voriconazole, posaconazole) and an
echinocandin has revealed effective therapy in animal models of IA (119,120).
Recently, separate clinical studies have demonstrated that combinations of
voriconazole and caspofungin, or liposomal AMB and caspofungin were effective
against aspergillosis in hematopoietic stem cell transplant recipients and in
neutropenic patients with acute lymphoblastic leukemia (121,122).
Combination antifungal therapy is now a conceptually adopted treatment strategy.
However, results of preclinical experiments cannot yet be translated into clinical
decisions because of methodology problems and the complexity of clinical studies (5).
Organized clinical trials are desirable, and, in addition, newer antifungal
combinations, possibly with cytokine therapy, are needed.

**Other investigational antifungal agents**

The sordarin drugs affect the translocation step of the elongation cycle of protein
synthesis by inhibition elongation factor 2, an essential factor for fungal protein
synthesis (123). The sordarins have minimal in vitro activity against *Aspergillus* and
in experimental murine model have shown poor survival efficacy (124,125).
The pradimicins and structurally similar benanomycins disrupt the fungal cell
membrane by binding to the terminal D-mannosides (126). They have shown some in
vitro and in vivo activity against *Aspergillus* spp. (126,127), but further study is
needed to investigate their potential use.
Search for novel antifungal compounds

Considering the fact that the available antifungal agents originate from a limited number of sources, and that most of them have similar modes of activity, it is very important to explore additional sources for substances with potential antifungal activity, which could possibly have different modes of activity or affect different sites in the fungal cell.

The oceans are the source of a large number of unique natural products that were mainly isolated from sponges, soft corals and tunicates. Many of these secondary metabolites possess interesting biological activities (128). Extensive research on marine natural products has revealed that marine sponges are most prolific sources of novel and diverse metabolites (129). One of these sources is the marine sponge *Dysidea herbacea* that occurs, according to its secondary metabolites, in two general chemotypes (130): one produces sesquiterpenes and polychlorinated amino acid derivatives, while the other produces only polybrominated diphenyl ethers. The production of the chlorinated metabolites and the polybrominated diphenyl ethers has been reported to be due to the cyanobacteria (131), which live in symbiosis with the sponges. These compounds may play a role in the chemical defense of the sponge against potential predators and bacterial invasion (128). Polybrominated diphenyl ethers were reported to inhibit enzymes, show antibacterial, antifungal and cytotoxic activities (132). The fact that marine organisms contain secondary metabolites different from their land counterparts in structure and biological activity has led to the hypothesis that marine organisms may contain effective antifungal compounds with different modes of action and selective antifungal activity compared with human cells (133). This line of research was also pursued by us in the present study.
In conclusion, because of the difficulty in diagnosing, rapid progression and severity of disease, aspergillosis remains a devastating opportunistic infection. Despite the currently available treatment, these factors emphasize the need for new treatment strategies of aspergillosis.
Aims of the study

The main goal of this study was to find novel antifungal formulations, antifungal combinations, or drug and immunomodulator combinations with efficacy against systemic aspergillosis in an experimental animal model.

The specific aims included:

- Evaluation of efficacy of the novel polyene-intralipid admixtures, amphotericin B-intralipid and nystatin-intralipid, in experimental systemic aspergillosis
- Assessment of efficacy of the polyenes or polyene-intralipid admixtures in combination with recombinant growth factor G-CSF against experimental murine aspergillosis
- Assessment of the efficacy of the echinocandin caspofungin in combination with immune therapy (cytokine G-CSF) in treatment of experimental systemic aspergillosis
- Evaluation of combination antifungal therapy with the echinocandin caspofungin and the polyene AMB or AMB-IL in treatment of experimental systemic aspergillosis
- Evaluation of a combination therapy containing three agents – G-CSF, caspofungin and amphotericin B/amphotericin B-intralipid – against systemic murine aspergillosis
- Assessment of in vitro antifungal effect and possible mode of activity of a compound isolated from the marine sponge *Dysidea herbacea* against *Aspergillus*
Overview of the articles

1. Treatment of murine systemic aspergillosis with polyene-intralipid admixtures
   *(Medical Mycology, 42: 73-80, 2004)*

The experiments toward the evaluation of the activity of admixtures of AMB-IL and Ny-IL in comparison to conventional formulation of AMB against experimental systemic aspergillosis in immunocompromised mice were described in this article. ICR mice were transiently immunosuppressed by intraperitoneal administration of cyclophosphamide (CY). Three days post CY administration the mice were inoculated intravenously (iv) with *Aspergillus fumigatus* conidia. The animals were treated with various doses of AMB-IL or Ny-IL admixtures administered iv for 5 consecutive days.

The experiments showed that AMB-IL increased significantly the mean survival rate, dependent on drug dose, up to 48-65.7% vs 0% of the untreated and 39.7% of AMB-treated animals. Survival rate of the Ny-IL-treated mice ranged between 16.2-40% in comparison to 0% of the untreated group. Treatment with either admixture prolonged the mean survival time of the mice (AMB-IL: 17.3-23.07 days; Ny-IL: 8.61-16.8 days) in comparison to untreated (6.13 days) or AMB treated animals (15.23 days).

The data obtained in this study showed that both AMB-IL and Ny-IL formulations, particularly AMB-IL at the highest dose, were effective in the treatment of experimental systemic aspergillosis.

2. Polyene and cytokine treatment of experimental aspergillosis *(FEMS Immunology and Medical Microbiology, 39: 221-227, 2003)*

In an attempt to increase the therapeutic efficacy of the polyene agents and their intralipid admixtures we undertook combination therapy with the cytokine G-CSF in treatment of experimental murine aspergillosis. Combination therapy, particularly G-
CSF with AMB or AMB-IL, significantly increased the survival rate (up to 87.3%) and prolonged the mean survival time (MST) (up to 28.8 days) in comparison to untreated controls (0% survival, MST – 6.7 days) and to treatment with polyenes alone (up to 51.5% survival, MST – 18.4 days).

The experiments in this article indicate that combination therapy consisting of antifungal drug and an immunomodulator could be beneficial for management of disseminated aspergillosis in humans.

3. Experimental systemic murine aspergillosis: treatment with polyene and caspofungin combination and G-CSF (Journal of Antimicrobial Chemotherapy, Accepted for publication, 2005)

Following the polyene-cytokine treatment we examined the combination therapy of the echinocandin caspofungin (CAS) with AMB or AMB-IL against disseminated murine aspergillosis induced in transiently immunocompromised mice. Furthermore, we also explored the effect of supplementing the antifungal combination with the cytokine G-CSF.

The data in this article show that combined CAS and AMB or AMB-IL treatment increased the survival of mice (up to 69.2%) as compared to those treated with each agent alone (44.4%, 40.7% and 50%, respectively). Combination of CAS+G-CSF or addition of G-CSF to the combinations of CAS+AMB or CAS+AMB-IL increased the survival rate of infected mice up to 78.9% and prolonged their mean survival time to 25 days. These combinations also resulted in reduction of fungal burden in organs, and decrease in serum galactomannan.

The successful results obtained in the experimental animal model may possibly open the way to more effective management of aspergillosis in humans.

Development of new antifungal drugs, particularly from natural sources with novel mechanisms of action, is of significance in view of the increased frequency of fungal infections, particularly in compromised hosts, and the problems associated with the available antifungal agents. Therefore, the in vitro activity of a series of extracts of marine sponges were examined in this study against the fungal pathogens Candida and Aspergillus species, focusing primarily on C.albicans and A.fumigatus. The extract of Dysidea herbacea was among the few that exhibited activity. Bioassay guided separation of the extract led to the active compound that exhibited in vitro activity against the fungal pathogens.

For determination of the possible mode of activity of the compound we tested the effect on fungal cellular morphology (light, scanning and transmission electron microscopy) and possible site of activity in the fungal cells, such as cell membrane (ion leakage kinetics) as well as toxicity (cytotoxicity tests).

The experiments on the mode of activity revealed that there are significant changes in fungal cell morphology, as demonstrated by scanning and transmission electron microscopy, and that the compound, apparently, affects the fungal cell membrane, expressed, primarily, in leakage of potassium ions from the fungal cells.
Articles – order of appearance


2. Sionov E and Segal E. Polyene and cytokine treatment of experimental aspergillosis. FEMS Immunology and Medical Microbiology 2003; 39: 221-227.


Treatment of murine systemic aspergillosis with polyene-intralipid admixtures

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The objective of this research was the evaluation of the activity of admixtures of amphotericin B (AMB) – intralipid (AMB-IL) and nystatin (Ny) – intralipid (Ny-IL) against experimental systemic aspergillosis in immunocompromised mice. ICR mice were transiently immunosuppressed by intraperitoneal (i.p.) administration of cyclophosphamide (CY). Three days post CY administration the mice were inoculated intravenously (i.v.) with Aspergillus fumigatus conidia. The animals were treated with various doses of AMB-IL or Ny-IL admixtures administered i.v. for 5 consecutive days, starting 2 h after infection. The mean survival rate (MSR) and mean survival time (MST) were evaluated during an observation period of 30 days in comparison to untreated infected mice and to animals treated with conventional formulations of these drugs. These experiments showed that AMB-IL increased significantly the MSR. Specifically, the MSR ranged in dependence of dose, between 48 and 65.7% vs. 0% of the untreated (P < 0.001, anova analysis) and 39.7% of AMB-treated animals (P < 0.01). The MSR of the Ny-IL-treated mice ranged between 16.2 and 40% in comparison to 0% of the untreated group (P < 0.001). Treatment with both admixtures prolonged the MST of the mice (AMB-IL: 17.3–23.07 days; Ny-IL: 8.61–16.8 days) in comparison to either untreated (6.13 days) or AMB treated animals (15.23 days). The data obtained in this study show that both AMB-IL and Ny-IL formulations, particularly AMB-IL at the highest dose, were effective in the treatment of experimental systemic aspergillosis.

Keywords Amphotericin B, Aspergillus fumigatus infection, nystatin

Introduction
Aspergillosis is the most common life-threatening invasive mould infection worldwide, and a major infectious complication in patients with prolonged neutropenia, including leukaemia or lymphoma, and bone marrow transplantation [1–5]. In the last 20 years, a significant increase of the incidence of invasive aspergillosis in this population has been observed [3,4]. The mortality among untreated patients with invasive aspergillosis is approximately 100% [6]. A. fumigatus is the most common species of the genus Aspergillus that is responsible for approximately 90% of human invasive aspergillosis [7].

There are currently several commercially available and investigative antifungal agents with activity against Aspergillus, such as the triazoles itraconazole and voriconazole or the lipopeptide, caspofungin. Nevertheless, Amphotericin B (AMB) is still a major antifungal drug for therapy of invasive aspergillosis. However, AMB treatment has severe side-effects, particularly nephrotoxicity. Three lipid formulations of AMB are now commercially available: AMB-lipid complex (Abelcet), AMB colloidal dispersion (Amphotec) and liposomal AMB (Ambisome) [8], which show a considerable reduction of toxicity and ability to use much higher dosages in comparison to conventional AMB [9]. However, these AMB formulations are
extremely expensive and therefore generally not the first choice of treatment. Lipid emulsions for parenteral nutrition, such as Intralipid or Lipofundin, which are known for their relative low toxicity, gained interest as possible vehicles for lipophilic drugs [10,11]. In view of this carrier system studies on use of mixtures of AMB with lipid emulsions have been reported since the 1990s [12,13]. One of these was the study of Chavanet et al. [13], who reported that comparison of conventional AMB deoxycholate with AMB deoxycholate–fat emulsion (Intralipid 20%) in patients with oral candidiasis revealed that, due to better tolerance, higher doses of AMB could be used, resulting in greater efficacy. Later studies by Nucci et al. [14] and Egoto et al. [15] confirmed the observations regarding the reduced toxicity of AMB with fat emulsions.

We have shown previously [16] that admixtures of Fungizone and Intralipid (AMB-IL) obtained by vigorous prolonged agitation yielded stable non-toxic preparations. These admixtures exhibited in-vitro activity against various Candida species [16], and were significantly more effective than conventional AMB in an experimental animal model of systemic candidiasis [17,18]. Nystatin is another polyene, which due to its toxicity is not suitable for intravenous therapy [19], but development of a liposomal formulation of nystatin (Nystrom) enables the use of nystatin intravenously with good evidence of activity against aspergillosis and reduced toxicity [19–21]. However, this formulation has not been further developed to the stage of clinical use.

Based on this information, our present study focused on the investigation of efficacy of the AMB-IL admixtures in vitro against A. fumigatus and in experimental systemic aspergillosis induced in transiently immunocompromised mice. In addition, we prepared a novel formulation of nystatin with Intralipid (Ny-IL) and assessed its activity in vitro and in vivo. This article describes the data obtained in that study.

Materials and methods

Organism

Aspergillus fumigatus (ATCC 64026) was used throughout the study. The strain was grown on Sabouraud dextrose agar 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 haemocytometer. The counts were verified by plating on Sabouraud dextrose agar plates for determination of colony counts.

In vitro susceptibility testing

The in-vitro activities of antifungal agents against A. fumigatus were determined by a modified technique based on the NCCLS M38-P protocol [22] 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) of the drugs were determined after 48 h incubation. The MIC was taken as the lowest drug concentration with no visible growth.

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. There were 49 mice per experimental group in experiments with AMB/AMB-IL (except the group AMB-IL 2 mg/kg/day: 12 mice), and 24–28 mice per group in experiments with Ny-IL (except the group Ny-IL 6 mg/kg/day: five mice).

Immunosuppression

A transiently compromised state in mice was achieved as described in previous studies [23], by intraperitoneal (i.p.) 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 determined by decrease in the number of white blood cells (WBC) and reduction in the body weight.

Experimental aspergillosis

Experimental systemic aspergillosis was obtained by inoculation via lateral tail vein of transiently immunocompromised mice with 0.5–1.0 × 10⁶ conidia per mouse of A. fumigatus. 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 the 30-day percentage of mortality, and the mean survival time (MST). Morbidity was assessed by determination of fungal colonization of viscera (kidneys, lungs). Fungal colonization.
was demonstrated in calcofluor-stained tissue homogenates by fluorescence microscopy by detection of fungal elements. Quantitative measurement of infection was provided by enumeration of Aspergillus colony forming units (c.f.u.) in tissue homogenates from infected animals. Toward this, end kidneys and lungs were aseptically removed and homogenized in 1 ml sterile saline. The tissue homogenates were diluted and plated on Sabouraud dextrose agar. The plates were incubated at 28°C for 48 h and the enumerated colonies were expressed as c.f.u./ml/organ.

**Antifungal agents**

A stock solution of conventional AMB (Fungizone, Bristol-Myers Squibb, France) (5 mg/ml) was prepared in 5% dextrose. Nystatin (ICN Biomedicals) was prepared by dissolving in dimethyl sulfoxide (DMSO) (Sigma Chemical, USA) and diluting with 0.9% saline to give a final nystatin concentration of 1 mg/ml in a 5% DMSO solution [19].

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, Edison, NJ, USA) at 280 r.p.m., as described previously [16]. Ny-IL was prepared, similarly to AMB-IL preparation, by a 25-fold dilution of dissolved nystatin in Intralipid 20% to a final nystatin concentration of 0.6 mg/ml.

**Drug administration**

Infected mice received daily i.v. injections of AMB (1 mg/kg/day), AMB-IL (1 or 2 mg/kg/day) or Ny-IL (2, 4, 6 or 8 mg/kg/day) via the tail vein for 5 consecutive days. Treatment began 2 h after fungal inoculation. Assessment of activity of the AMB-IL and Ny-IL was based on similar criteria of evaluating mortality and morbidity as in the model of experimental aspergillosis (described above), in comparison to untreated animals and to animals treated with conventional AMB (expressed as survival rate and MST). The follow up period consisted of 30 days.

**Statistical analysis**

Statistical analysis of survival data was assessed by one-way analysis of variance (anova) multiple comparisons test. Significance level was defined as $P < 0.05$.

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**Results**

**In-vitro susceptibility testing of antifungal agents**

The in-vitro activities of AMB, AMB-IL, Nystatin and Ny-IL were examined against A. fumigatus (ATCC 64026) (Table 1). AMB-IL was more active than AMB and Ny-IL demonstrated increased activity in comparison to nystatin. Minimal fungicidal concentration (MFC) values were higher than those of MIC in all tested groups. However, the MFC values of AMB-IL and Ny-IL were lower than those of AMB and nystatin, respectively.

**Experimental murine aspergillosis**

Transiently compromised mice were inoculated i.v. with A. fumigatus on day 3 after pretreatment with CY, with inocula ranging from $0.5 \times 10^6$ to $1 \times 10^6$ conidia per mouse. The optimal inoculum was determined to be $1 \times 10^6$ conidia per mouse. This concentration caused systemic aspergillosis that led to 100% mortality within 3–10 days (MST was 6.04 ± 3.53). Organs (kidneys and lungs) of dead mice examined macroscopically revealed presence of microabscesses. Microscopic observation of organ homogenates stained with calcofluor demonstrated the presence of hyphae.

**Treatment of experimental systemic aspergillosis with AMB and AMB-IL**

Infected mice were treated either with conventional AMB (Fungizone) at a concentration of 1 mg/kg or with AMB-IL at doses of 1 or 2 mg/kg for 5 consecutive days. It was found in previous studies in our laboratory that treatment of systemic candidiasis with higher doses of AMB-IL (2 mg/kg/day) is possible [17,18].

The data presented in Fig. 2 indicate that both preparations (AMB and AMB-IL) at a concentration

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**Table 1 In-vitro activity of Amphotericin B intralipid (AMB-IL) and nystatin intralipid (Ny-IL) against Aspergillus fumigatus (ATCC 64026) in a study on the treatment of murine systemic aspergillosis with polyene-intralipid admixtures**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>MIC (µg/ml)</th>
<th>MFC (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>AMB</td>
<td>1.26 ± 0.58</td>
<td>2.34 ± 1.35</td>
</tr>
<tr>
<td>AMB-IL</td>
<td>0.58 ± 0.22</td>
<td>1.04 ± 0.45</td>
</tr>
<tr>
<td>Nystatin</td>
<td>2.92 ± 0.98</td>
<td>5.73 ± 2.38</td>
</tr>
<tr>
<td>Ny-IL</td>
<td>1.95 ± 0.78</td>
<td>4.16 ± 1.8</td>
</tr>
</tbody>
</table>

*Data are the mean of four experiments ± SD. MIC, minimal inhibitory concentrations; MFC, minimal fungicidal concentrations.
of 1 mg/kg/day increased significantly ($P < 0.0001$) the survival rate of infected mice in comparison to the untreated control group (39.75% and 48.07% for AMB and AMB-IL-treated groups, respectively, vs 0% for the untreated group). Furthermore, AMB-IL at the higher concentration of 2 mg/kg/day was more effective (65.7% of treated mice survived). Fig. 2 presents the course of murine aspergillosis in untreated and treated animals. It is evident that the untreated mice succumb to infection earlier than the treated, with death starting on day 3 post fungal inoculation, and by day 7 all of the animals in this group succumbed to infection. Among the mice treated with the standard formulation of AMB death started on day 4 and the survival rate remained unchanged as of day 8. Treatment with AMB-IL delays the mortality, particularly with the higher dose of 2 mg/kg/day (mortality started on day 8). The MSTs for the untreated, Fungizone-treated and AMB-IL-treated mice at doses of 1 and 2 mg/kg/day were 6.04, 15.23, 17.3 and 23.07, respectively (Fig. 1). These experiments indicated that treatment of systemic aspergillosis by AMB-IL is more effective than treatment by conventional AMB, especially at the highest total dose of 10 mg/kg. In addition, in mice treated with either AMB or AMB-IL, the number of c.f.u. in the kidneys and lungs was greatly reduced or absent, in contrast to untreated animals (Table 2).

Treatment of experimental systemic aspergillosis by Ny-IL

In-vivo experiments were carried out with Ny-IL, administered in a similar regimen as for AMB and AMB-IL, namely, over 5 consecutive days by i.v. injection of different doses: 2, 4, 6 or 8 mg/kg/day. The concentration of 8 mg/kg/day was toxic and caused immediate death of animals after i.v. administration. In further experiments this dose was not used, therefore. All groups treated by Ny-IL at different doses were compared to untreated controls. The data obtained from these experiments revealed that the survival rates at day 30 were 0, 16.2, 29.06 and 40% for the untreated and Ny-IL-treated groups at doses of 2, 4 and 6 mg/kg/day, respectively (Fig. 4). The follow-up of the course of infection indicates that the mortality began later among animals treated with Ny-IL, especially with the higher dose of 6 mg/kg/day (day 5), in contrast to the untreated group (Fig. 4). Survival rate in this group remained unchanged from day 11. The MST was 6.23, 8.61, 12.38 and 16.8 for the untreated and Ny-IL-treated groups at doses of 2, 4 and 6 mg/kg/day, respectively (Fig. 3). Likewise, Ny-IL treatment at doses of 4 and 6 mg/kg/day significantly reduced the amount of *Aspergillus* c.f.u. in the kidneys and resulted in absence of fungi in the lungs, compared to untreated controls and to groups treated with lower dose of Ny-IL (Table 2). The results obtained from these experiments demonstrated that Ny-IL treatment at the higher dose (6 mg/kg/day) was statistically superior to treatment by Ny-IL at lower doses ($P < 0.01$).

Comparison of AMB-IL and Ny-IL treatments

The comparison of the treatment of the 2 polyenes by

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** Mean survival time of mice treated with Amphotericin B (AMB) or Amphotericin B intralipid (AMB-IL) in a study on the treatment of murine systemic aspergillosis with polyene-intralipid admixtures. All data are mean values from five independent experiments, (except the group AMB-IL 2 mg/kg/day: 2 experiments). All groups consisted of 49 mice, 8–10 mice per experiment (except the group AMB-IL 2 mg/kg/day: 12 mice).
group at a concentration of 1 mg/kg/day is higher than that of the Ny-IL-treated group at a concentration of 6 mg/kg/day (48.07 vs 40%), albeit the difference is statistically not significant ($P = 0.626$). However, the survival rate of animals treated with AMB-IL at the concentration of 2 mg/kg/day is statistically greater in comparison to survival of Ny-IL-treated animals at the higher dose of 6 mg/kg/day (65.7 vs 40%) ($P = 0.006$). In addition, treatment with AMB-IL at doses of 1 and 2 mg/kg/day prolongs the survival time of animals, in contrast to treatment by Ny-IL at the tested doses. Thus, the comparative data described above indicate that AMB-IL is more effective for treatment of experimental aspergillosis than is Ny-IL.

**Discussion**

The rationale of the present study was based on the information from literature [13,24] and our own studies on *Candida* [17,18] that AMB-fat emulsions are less toxic than conventional AMB and can be administered at higher doses, thus are more effective in treating *Candida* and *Cryptococcus* human or experimental infections. Hence, the aim was to evaluate the efficacy of admixtures of amphotericin B-intralipid and nystatin-intralipid in experimental systemic aspergillosis. Toward this end, we used an experimental animal model consisting of transiently compromised mice pretreated with CY.

The data presented in this study show that AMB-IL

<table>
<thead>
<tr>
<th>Group</th>
<th>No. c.f.u. (mean ±SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right kidney</td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>1120 (±72)</td>
</tr>
<tr>
<td>AMB</td>
<td>20 (±12)</td>
</tr>
<tr>
<td>AMB-IL 1 mg/kg/day</td>
<td>35 (±15)</td>
</tr>
<tr>
<td>AMB-IL 2 mg/kg/day</td>
<td>18 (±8)</td>
</tr>
<tr>
<td>Ny-IL 2 mg/kg/day</td>
<td>400 (±28)</td>
</tr>
<tr>
<td>Ny-IL 4 mg/kg/day</td>
<td>52 (±15)</td>
</tr>
<tr>
<td>Ny-IL 6 mg/kg/day</td>
<td>38 (±7)</td>
</tr>
</tbody>
</table>

*Data shown on c.f.u. are those of control group day 4, and day 7 for all treated groups. Mean value of four mice per group.
is more effective than conventional AMB in treatment of experimental systemic aspergillosis. In particular, AMB-IL at the higher dose of 2 mg/kg/day significantly increases survival rate and MST of infected mice in comparison to AMB ($P < 0.05$). Previous studies involving systemic candidiasis [17,18] revealed that treatment with higher doses of AMB-IL led to approximately 100% survival of the treated animals. The results obtained in our experimental aspergillosis model seem to be compatible with clinical and epidemiological observations of *Aspergillus* infections, which are considered more severe complications in immunocompromised patients with higher levels of mortality and more difficult therapy [1,2,6], hence the better outcome of treatment of candidiasis with AMB-IL in comparison to treatment of aspergillosis.

Physicochemical characterization [16] of our AMB-IL admixtures prepared by overnight vigorous agitation revealed that the preparations contained particles of similar sizes as before mixing with Fungizone (350.6±6.1 nm) and about 90% of AMB remained in emulsion particles, as determined following ultracentrifugation. The characteristics of the AMB-IL admixtures remained unchanged for a month, pointing to their...

![Fig. 3](image3.png)

**Fig. 3** Mean survival time of mice treated with nystatin intralipid (Ny-IL) in a study on the treatment of murine systemic aspergillosis with polyene-intralipid admixtures. All data are mean values from four independent experiments, (except the group Ny-IL 6 mg/kg/day: 1 experiment). The groups consisted of 24–28 mice, 6–7 mice per experiment (except the group Ny-IL 6 mg/kg/day: 5 mice).

![Fig. 4](image4.png)

**Fig. 4** Course of infection in mice treated with nystatin intralipid (Ny-IL) in a study on the treatment of murine systemic aspergillosis with polyene-intralipid admixtures. Mice were inoculated i.v. with *Aspergillus fumigatus* on day 3 post CY administration. Ny-IL treatment started 2 h after fungal inoculation. ●, control (no treatment); ■, Ny-IL 2 mg/kg/day; ▲, Ny-IL 4 mg/kg/day; ○, Ny-IL 6 mg/kg/day.
stability. This was different in preparations obtained by gentle mixing, which separated spontaneously into several phases. In addition, the AMB-IL admixture caused significant lower haemolysis of RBC and reduced K⁺ leakage in comparison to Fungizone.

Comparative pharmacokinetic studies [25] of AMB, AMB-IL and liposomal AMB (AmBisome) have revealed that the blood and tissues (kidneys, liver, spleen, lungs) distribution of AMB-IL has a similar pattern to that of AmBisome. It was shown that AMB blood levels were higher in animals administered AMB-IL than in those with AMB. In addition, it was found that the level in liver was higher and in kidneys lower.

Nystatin is similar in structure to AMB. The mode of action of these drugs has been attributed to the binding to ergosterol in the fungal membrane, creating transmembrane channels resulting in membrane permeability and leakage of monovalent cations (K⁺), sugars and metabolites [26]. But, unlike AMB, nystatin is not suitable for i.v. administration. Therefore, development of alternative formulations of nystatin, that could enable intravenous therapy, was needed. Liposomal formulation of nystatin was developed and resulted in good efficacy against fungal infections [19,20,27], but this antifungal agent has not yet reached clinical use. A novel admixture of nystatin with intralipid (Ny-IL), which was prepared similarly to the AMB-IL formulation, was examined in our study. The results obtained in the experiments indicate that Ny-IL at the higher concentration of 6 mg/kg/day improved the survival rate and prolonged the MST of infected mice, compared to untreated controls and to Ny-IL-treated groups at lower doses, albeit the group treated with the higher dose of Ny-IL included relatively a small number of mice.

Comparison of data for both formulations (AMB-IL and Ny-IL) revealed that survival rate and MST of animals treated by AMB-IL at the dose of 2 mg/kg/day was significantly higher (P < 0.05) than those of animals treated by Ny-IL. It seems that in our system the Ny-IL is less potent than AMB-IL. It is of interest, that Denning and Warn [28] found that liposomal nystatin at a dose of 5 mg/kg/day was superior to liposomal AMB at the same dose. High doses of liposomal nystatin (10 mg/kg/day) were toxic. In our study the highest non-toxic dose of Ny-IL was 6 mg/kg/day.

In conclusion, the data in this work provide evidence that AMB-IL admixture is very effective against experimental systemic aspergillosis; Ny-IL may also be a possible additional therapeutic option that should be considered. However, it should be pointed out that so far, Intralipid, which is used for nutritional support therapy, has not been approved as a vehicle for antifungal drug therapy.

References


Polyene and cytokine treatment of experimental aspergillosis

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Abstract

The aim of the present study was to assess the efficacy of amphotericin B (AMB), AMB-intralipid admixture (AMB-IL) or nystatin-IL formulation (Ny-IL) in combination with granulocyte colony stimulating factor (G-CSF) in treatment of experimental murine aspergillosis. ICR mice were immunosuppressed by cyclophosphamide and 3 days later inoculated intravenously (i.v.) with Aspergillus fumigatus. Treatment was initiated 2 h later and administered for 5 consecutive days (polyenes, i.v.; G-CSF, intraperitoneally). Combination therapy, particularly G-CSF with AMB or AMB-IL, significantly increased the survival rate (up to 87.3%) and prolonged the mean survival time (MST) (up to 28.8 days) in comparison to untreated controls (0% survival, MST 6.7 days) and to treatment with polyenes alone (up to 51.5% survival, MST 18.4 days). These data indicate that combination therapy could be beneficial for management of disseminated aspergillosis in humans.

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Keywords: Aspergillosis; Polyene; Granulocyte colony stimulating factor

1. Introduction

Aspergillosis is a life-threatening invasive mold infection that affects severely immunosuppressed patients with neutropenia, such as cancer patients of the hematopoietic system, bone marrow and organ transplant recipients. During the last decade a significant rise in the incidence of invasive aspergillosis has been observed, due to new intensive anticancer chemotherapy, a worldwide increase in the number of bone marrow and solid organ transplant recipients and other risk factors [1–3]. Aspergillosis occurs in 10–30% of patients with hematologic malignancies, in whom the mortality rate is very high (up to 80–90%) [4–6].

Aspergillus fumigatus is the most virulent species of the genus Aspergillus that is responsible for approximately 90% of human invasive aspergillosis, although other species can also cause human infections [7,8].

Innate immunity plays a very important role in host defenses against Aspergillus spp. [9]. Pulmonary alveolar macrophages ingest and kill resting conidia using non-oxidative mechanisms for their conidiocidal activity. Neutrophils attack and cause damage to hyphae that escape from macrophages through oxygen-dependent mechanisms [10,11]. It was shown that granulocyte colony stimulating factor (G-CSF) enhanced antifungal activity of human neutrophils against A. fumigatus hyphae in vitro [12]. Human G-CSF is a glycoprotein that regulates the production and release of functional neutrophils from the bone marrow, and also increases their microbicidal activity [13,14]. In addition, G-CSF may contribute to better efficacy of antifungal drugs. Synergistic activity of G-CSF and voriconazole was found in vitro against Candida albicans and A. fumigatus, when added to neutrophils [15,16]. Additive effects or synergism were found between G-CSF and fluconazole or between G-CSF and posaconazole in experimental candidiasis or aspergillosis, respectively [17,18].

In previous studies [19,20] we found that amphotericin B-intralipid (AMB-IL) admixtures were effective and less toxic than the standard formulation of AMB in experimental candidiasis. We also found (unpublished data) that AMB-IL and nystatin-IL admixtures (Ny-IL) were effective in experimental aspergillosis. In the present study we investigated the activity of G-CSF in combination with AMB, AMB-IL or Ny-IL admixtures against experimental murine aspergillosis induced in immunocompromised mice.
2. Materials and methods

2.1. Organism

*A. fumigatus* (ATCC 64026) was used throughout the study. The strain was grown on Sabouraud dextrose agar at 28°C for 72–96 h.

2.2. 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 this study.

2.3. Induction and determination of the immunosuppressive state

A compromised state in mice was achieved by intraperitoneal (i.p.) injection of 200 mg kg⁻¹ of the immunosuppressive agent cyclophosphamide (CY). The mice were examined for assessment of an immunosuppressive state by blood analysis using Technicon H1 System Coulter (Miles Inc. Diagnostic Division, NY, USA) for determination of total number of white blood cells (WBC) and differential analysis for number of neutrophils. Tests were performed at the Kimron Veterinary Institute (Beit-Dagan, Israel). Analyses were performed daily during a follow-up of 13 days (see results).

2.4. Experimental aspergillosis

Experimental systemic aspergillosis was obtained by intravenous (i.v.) inoculation via the lateral tail vein of immunocompromised mice with 1×10⁶ spores per 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 the number of mice that succumbed to infection out of the 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 the mean survival time (MST). Morbidity was assessed by determination of fungal colonization of viscera (kidneys, lungs). Fungal colonization was demonstrated by detection of fungal elements in calcofluor-stained tissue homogenates using fluorescence microscopy. Quantitative measurement of infection was provided by enumeration of *Aspergillus* colony forming units (CFU) in tissue homogenates from infected animals. Toward this end kidneys and lungs were aseptically removed and homogenized in 1 ml of sterile saline. The tissue homogenates were diluted and plated on Sabouraud dextrose agar. The plates were incubated at 28°C for 48 h and the enumerated colonies were expressed as CFU per organ.

2.5. Drugs

A stock solution of conventional AMB (Fungizone, Bristol-Myers Squibb, France) (5 mg ml⁻¹) was prepared in 5% dextrose. Ny (ICN Biomedicals Inc., USA) was prepared by dissolving in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., USA) and diluting with 0.9% saline to give a final Ny concentration of 1 mg ml⁻¹ in a 5% DMSO solution.

AMB-IL was prepared by a 25-fold dilution of Fungizone in the lipid emulsion Intralipid 20% (Kabi Pharmaecia, Stockholm, Sweden) to a final AMB concentration of 0.2 mg ml⁻¹, 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 [21]. Ny-IL was prepared, similarly to the AMB-IL preparation, by a 25-fold dilution of dissolved Ny in Intralipid 20% to a final Ny concentration of 0.6 mg ml⁻¹.

Recombinant methionyl human G-CSF (Filgrastim) was the product of Roche (F. Hoffmann-La Roche Ltd, Basel, Switzerland). G-CSF was diluted for in vivo experiments in 5% dextrose solution to a final G-CSF concentration of 15 μg ml⁻¹.

2.6. Drug administration

Infected mice received the following treatments: AMB (1 mg kg⁻¹ day⁻¹, i.v.), AMB-IL (1 mg kg⁻¹ day⁻¹, i.v.), Ny-IL (4 mg kg⁻¹ day⁻¹, i.v.), G-CSF (150 μg kg⁻¹ day⁻¹, i.p.) and combination therapy of each of these antifungal agents with G-CSF, i.e., AMB+G-CSF, AMB-IL+G-CSF and Ny-IL+G-CSF, at the same doses. Treatment began 2 h after fungal inoculation and consisted of five consecutive daily injections. Assessment of activity of the combination therapy was based on similar criteria of evaluating mortality and morbidity as in the model of experimental aspergillosis (described above), in comparison to untreated animals and to animals treated with each agent alone (expressed as survival rate and MST). The follow-up period consisted of 30 days.

2.7. Statistical analysis

Analyses of minimal inhibitory concentration (MIC) values and survival data were performed by using Student’s *t*-test. Significance level was defined as *P* < 0.05.

3. Results

3.1. Compromised mice

The blood analyses showed that administration of the immunosuppressive agent CY at a dose of 200 mg kg⁻¹ led to a decrease in the number of WBC, most evident on
day 3 post CY treatment (Fig. 1). The differential blood analyses revealed that the effect was particularly marked on the neutrophils. As evident from Fig. 1, the mean count of neutrophils decreased to a low level of 10^5 W_l^3 on day 3 after CY administration. Therefore, day 3 post CY pretreatment was chosen as a model representing an immunocompromised state.

3.2. Effect of G-CSF

On the day with the lowest number of neutrophils the mice received G-CSF treatment at a dose of 150 μg kg^-1 day^-1, administered during 5 consecutive days by i.p. injections. As shown in Fig. 2A, already after three G-CSF injections (day 6 post CY administration) the total number of WBC significantly increased and was >23 000 μl^-1; and the neutrophils rose to approximately 18 000 μl^-1 (Fig. 2B). The high levels of WBC (from 11 000 to 24 000 μl^-1) remained till day 8 after the start of the G-CSF treatment (day 11 of the experiment).

These data demonstrate that G-CSF at a dose of 150 μg kg^-1 day^-1 caused in neutropenic mice a marked raise in WBC numbers, especially peripheral blood neutrophils. The increase was noticed already 24 h after the first dose of G-CSF. The peak was reached after 72 h (three doses).

3.3. Antifungal therapy

Compromised mice were inoculated i.v. with _A. fumigatus_ at a concentration of 1 × 10^6 spores per mouse on day 3 after CY administration. This concentration caused 100% mortality among untreated mice within 3–7 days (MST was 6.7 ± 1.68 days).

Treatment with either conventional AMB or AMB-IL at a dose of 1 mg kg^-1 day^-1 increased significantly (P < 0.001) the survival rate of infected mice in compar-

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean survival rate (%)a (± S.D.)</th>
<th>MST (days)b (± S.D.)</th>
<th>Number of CFU (mean ± S.D.)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>0</td>
<td>6.7 (± 1.68)</td>
<td>1010 (± 52)</td>
</tr>
<tr>
<td>AMB 1 mg kg^-1 day^-1</td>
<td>41.9 (± 7.95)</td>
<td>15.76 (± 2.7)</td>
<td>23 (± 11)</td>
</tr>
<tr>
<td>AMB-IL 1 mg kg^-1 day^-1</td>
<td>51.48 (± 15.14)</td>
<td>18.4 (± 4.27)</td>
<td>26 (± 5)</td>
</tr>
<tr>
<td>Ny-IL 4 mg kg^-1 day^-1</td>
<td>29.06 (± 0.8)</td>
<td>12.38 (± 0.8)</td>
<td>69 (± 15)</td>
</tr>
</tbody>
</table>

aMean survival rate (%) at day 30.
bMST; follow-up for 30 days.
cCFU data shown are those of day 4 for control group, and day 7 for all treated groups. Mean value of four mice per group.
ison to the untreated control group (41.9 and 51.48% for AMB- and AMB-IL-treated groups, respectively, vs. 0% for the untreated group) (Table 1). The MST for the Fungizone- and AMB-IL-treated mice at the same dose were 15.76 and 18.4 days, respectively. These data indicated that treatment of experimental systemic aspergillosis by AMB-IL was more effective than treatment by conventional AMB. In addition, we showed that treatment by another polyene-IL admixture, Ny-IL, achieved a higher survival rate (29%) and prolonged MST (12.4 days) than those of untreated controls.

3.4. Antifungal and G-CSF therapy

In an attempt to increase the therapeutic efficacy of the antifungals we undertook combination therapy with G-

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Fig. 2. A: Effect of G-CSF on total WBC counts in ICR mice. B: Effect of G-CSF on neutrophil counts in ICR mice. Legend: G-CSF administration started on day 0 (3 days post CY pretreatment) and continued for 5 consecutive days.
CSF and the polyene agents. Survival rates of animals treated by combined therapy with G-CSF+AMB, G-CSF+AMB-IL and G-CSF+Ny-IL were significantly higher ($P < 0.001$) (87.3, 83.3, 38.53%, respectively) than in the groups treated with G-CSF, AMB, AMB-IL and Ny-IL alone (14.65, 41.9, 51.48 and 29%, respectively) (Fig. 3). The combination treatment also prolonged meaningfully ($P < 0.01$) the survival time of animals in comparison to treatment with each agent alone (Fig. 4). The MST were 8.47, 28.83, 26.25 and 15.35 days for the G-CSF-, G-CSF+AMB-, G-CSF+AMB-IL- and G-CSF+Ny-IL-treated groups, respectively. The follow-up of the course of infection (Fig. 5) indicated that combined therapies, particularly treatment by G-CSF with AMB and G-CSF with AMB-IL, markedly delayed the mortality of mice, contrary to untreated controls and to treatment with either drug alone. All untreated animals succumbed to infection between days 3 and 7, and the majority of G-CSF-
treated mice (85%) died between days 4 and 11. Among mice treated with G-CSF and Ny-IL mortality began later (day 7) in contrast to the untreated group and to those treated with Ny-IL alone (day 3).

As shown in Table 2, in animals treated by combination therapies, the number of CFU in the kidneys and lungs was greatly reduced or absent in comparison to untreated controls.

The results obtained from these experiments clearly showed that treatment of murine systemic aspergillosis by cytokine G-CSF and AMB, G-CSF and AMB-IL or G-CSF and Ny-IL was superior to treatment by each agent alone.

4. Discussion

The main goal of the present study was to evaluate the efficacy of the recombinant growth factor G-CSF in combination with polyenes or polyene-IL admixtures. Towards this aim we adapted an experimental model of systemic aspergillosis in mice immunosuppressed by CY pretreatment. Administration of this agent at a dose of 200 mg kg\(^{-1}\) led to a neutropenic state in mice (neutrophils < 500 W\(_{1}\)). Such state is analogous to clinical situations that are associated with high risk of development of aspergillosis. Reduction in circulating neutrophils leads to decreased resistance to opportunistic pathogens, such as Aspergillus \[18\]. Our data indicated that immunosuppressed mice receiving G-CSF presented increased numbers of neutrophils, already after 24 h, with the effect peaking after 3 days. This cytokine is known for its ability to restore the number of neutrophils, and to return apparently their antifungal activity \[11,22\].

In our study we found a synergistic effect of G-CSF with AMB or the polyene-IL admixtures, AMB-IL or G-CSF and Ny-IL was superior to treatment by each agent alone.

The results obtained from our experiments indicated that G-CSF alone was ineffective in increasing significantly the survival or delaying mortality of infected animals.

![Course of infection in mice treated with antifungals or combination therapy](image)

Fig. 5. Course of infection in mice treated with antifungals or combination therapy. Legend: Mice were inoculated i.v. with \(A.\) *fumigatus* on day 3 post CY administration. G-CSF, antifungal or combination therapies started 2 h after fungal inoculation. ●, control (no treatment); ○, G-CSF; ▲, AMB 1 mg kg\(^{-1}\) day\(^{-1}\); ■, AMB-IL 1 mg kg\(^{-1}\) day\(^{-1}\); □, Ny-IL 4 mg kg\(^{-1}\) day\(^{-1}\); △, AMB+G-CSF; □, AMB-IL+G-CSF; ○, Ny-IL+G-CSF.

<table>
<thead>
<tr>
<th>Group/Group</th>
<th>Right kidney (CFU, mean ± S.D.)^a</th>
<th>Left kidney (CFU, mean ± S.D.)</th>
<th>Lungs (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>840 (± 254)</td>
<td>782 (± 297)</td>
<td>210 (± 47)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>280 (± 75)</td>
<td>325 (± 62)</td>
<td>60 (± 22)</td>
</tr>
<tr>
<td>AMB+G-CSF</td>
<td>0</td>
<td>7 (± 5)</td>
<td>0</td>
</tr>
<tr>
<td>AMB-IL+G-CSF</td>
<td>15 (± 10)</td>
<td>20 (± 14)</td>
<td>0</td>
</tr>
<tr>
<td>Ny-IL+G-CSF</td>
<td>23 (± 11)</td>
<td>20 (± 10)</td>
<td>0</td>
</tr>
</tbody>
</table>

^aCFU data shown are those of day 4 for control and G-CSF groups, and day 7 for other treated groups. Mean value of four mice per group.
although a reduction in the CFU counts in organ tissues below that of the untreated mice was noted. It also was reported that G-CSF alone was no benefit on survival of experimental cryptococcal meningitis [24].

We did not evaluate the effect of free Ny on aspergillosis, as in this form Ny is not suitable for systemic administration. However, the admixture of Ny and IL is a formulation, which can be used systemically. Thus we assessed the efficacy of the combination of G-CSF with Ny-IL. Treatment of experimental aspergillosis with Ny-IL was less effective than with AMB-IL, but nevertheless had a therapeutic effect in comparison to the untreated group. When G-CSF was administered with Ny-IL at a dose of 4 mg kg\textsuperscript{-1} day\textsuperscript{-1} the survival rate of the mice increased to 38.5% in comparison to 29% in Ny-IL-treated animals. However, this therapy also was inferior to the combination treatment of G-CSF with AMB or G-CSF with AMB-IL.

In conclusion, the present study indicates that addition of the cytokine G-CSF to polylene or polylene-IL therapy has a synergistic effect in experimental aspergillosis and improves the outcome.

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Experimental Systemic Murine Aspergillosis: Treatment with Polyene and Caspofungin Combination and G-CSF

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Synopsis

Objectives. Infections caused by *Aspergillus* species are increasing in importance, especially among immunocompromised hosts. 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*. Supplementing the cytokine G-CSF to the antifungal combination may further contribute to therapy of aspergillosis.

Methods. In the present study we examined the combination therapy of caspofungin (CAS) and amphotericin B (AMB) or amphotericin B-intralipid admixture (AMB-IL) against disseminated murine aspergillosis induced in transiently immunocompromised mice. Furthermore, we also explored the effect of supplementing the antifungal combination with the cytokine G-CSF.

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). Combination of CAS+G-CSF or addition of G-CSF to the combinations of CAS+AMB or CAS+AMB-IL increased the survival rate of infected mice up to 78.9% and prolonged their mean survival time to 25 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.

Key words: aspergillosis, caspofungin, amphotericin B, amphotericin B-intralipid, G-CSF, combination therapy
Introduction

The survival of patients with invasive aspergillosis, which affects primarily immunocompromised patients, is poor because of difficulties in early diagnosis and lack of effective treatment options.\textsuperscript{6-8} Despite of the use of amphotericin B (AMB) and its lipid formulations, itraconazole, voriconazole and caspofungin mortality rate is still high.\textsuperscript{1,9} Thus, combination antifungal therapy seems to be warranted aiming improvement outcome.

AMB is still a major antifungal drug, however is associated with severe adverse effects, particularly nephrotoxicity, while the commercially available less toxic lipid formulations of AMB are very expensive and generally not the first line of treatment. Our laboratory has shown previously\textsuperscript{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 \textit{A.fumigatus}, and was significantly more effective than conventional AMB in an experimental model of systemic aspergillosis.\textsuperscript{11} The echinocandin lipopeptide caspofungin (CAS) is the first of a novel class of antifungal compounds that inhibit synthesis of 1,3-\(\beta\)-D-glucan, causing severe damage to \textit{A.fumigatus} at the sites of hyphal growth.\textsuperscript{12,13}

Human granulocyte colony stimulating factor (G-CSF) is a glycoprotein that regulates the production and release of functional neutrophils from the bone marrow, and may contribute to better efficacy of antifungal drugs.\textsuperscript{16,17}

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-IL, supplement by the cytokine G-CSF in treatment of experimental murine aspergillosis.
induced in transiently immunocompromised mice. The data obtained in this study are reported herewith.
Materials and methods

Organism

Aspergillus fumigatus (ATCC 64026) was used throughout the study. The strain was grown on Sabouraud dextrose agar (SDA) slants (28°C for 72-96h). Conidia were harvested with sterile saline; the conidial suspension was adjusted to the required concentration by counting in a hemacytometer and verified by plating on SDA plates for determination of colony counts.

Animals, induction of an immunosuppressive state and experimental aspergillosis

Female ICR mice, 4-5 weeks old (23-28 g), kept under conventional conditions, were used. The ethics committee of the Faculty of Medicine of Tel-Aviv University granted permission for the animal experiments described in the work.

A transiently compromised state in the mice was achieved by intraperitoneal (ip) injection of the cyclophosphamide (CY) (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. Experimental systemic aspergillosis was obtained by intravenous (iv) inoculation via the lateral tail vein of immunocompromised mice with 0.8 x 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 (survival rate and mean survival time – MST) and morbidity (quantitative determination of fungal burden, histopathology, and detection of serum galactomannan).

Drugs and administration

Amphotericin B-intralipid was prepared by a 25-fold dilution of conventional amphotericin B (Fungizone, Bristol-Myers Squibb, France) in the lipid emulsion Intralipid 20% (Kabi Pharmacia, Stockholm, Sweden) to a final amphotericin B
concentration of 0.2 mg/ml, and then agitated vigorously at 24°C for 18h on a controlled environmental incubator shaker (New Brunswick Scientific Co., Edison, NJ, USA) at 280 rpm.\textsuperscript{10} Caspofungin acetate (Merck & Co., Ink., Whitehouse Station, NJ, USA) was dissolved in 0.9% sodium chloride for injection. Recombinant methionyl human G-CSF (Filgrastim) was the product of Roche (F.Hoffmann-La Roche Ltd, Basel, Switzerland). G-CSF was diluted for in vivo experiments in 5% dextrose solution to a final G-CSF concentration of 30 \( \mu \text{g}/\text{ml} \).

Infected mice received the following single treatments - AMB (1 mg/kg/day, iv), AMB-IL (1 mg/kg/day, iv), CAS (0.5 mg/kg/day, iv), G-CSF (300 \( \mu \text{g/kg/day, ip} \)), and combination therapies - CAS+G-CSF, CAS+AMB-IL+G-CSF (same doses as in single agent treatment). Treatment began 2 h after fungal inoculation and consisted of 5 consecutive daily drug injections. 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 protocol group/experiment.

\textbf{Cultures from organ homogenates}

Enumeration of \textit{Aspergillus} colony forming units (CFU) in tissue homogenates of kidneys and lungs was performed at days 1, 2, 3, 5, 7, 14, 21, and 28. Kidneys' and lungs' tissue homogenates were prepared, diluted and plated on SDA (incubation at 28°C for 48h) for CFU determination.

\textbf{Galactomannan assay}

Serum galactomannan concentrations were determined by the Platelia Aspergillus EIA (commercial sandwich ELISA kit) on days 1, 2, 3, 5, 7, 14, 21, 28, and was carried out according to the protocol recommended by the producer (Platelia Aspergillus).
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**

Analysis of survival data was plotted by Kaplan-Meier test and comparisons between groups were performed by the one-way analysis of variance (ANOVA) multiple comparisons test 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

Survival of animals treated by single and combination therapy

Treatment with AMB-IL or with CAS significantly increased ($P < 0.001$) the survival rate of infected mice in comparison to the untreated controls (50 and 44.4% for AMB-IL and CAS-treated groups, respectively, vs. 0% for the untreated group) (Figure 1a). The MSTs for AMB-IL and CAS-treated mice were 17.9 and 16.03 days, respectively.

We also examined the efficacy of combination treatment. We have previously shown that combination of the polyene agent AMB-IL with G-CSF had greatly increased survival of *Aspergillus*-infected mice.$^{24}$ Hence, in the present study we attempted to combine caspofungin with G-CSF. Combination of CAS+G-CSF resulted in significantly higher survival rate (77.7%, $P < 0.001$) of the thus treated animals as compared to mice treated with each agent alone (44.4 and 7.4%, respectively) (Figure 1b). The survival time of mice was prolonged to 25 days ($P < 0.01$). In addition, combination therapy of three agents jointly – CAS+AMB-IL+G-CSF – was also examined. This combination caused a significant rise ($P < 0.01$) in the survival rates of the animals (78.9%) as compared to therapy by each drug alone.

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 (Figures 3a). Serum samples (20 animals per group) from untreated controls, G-CSF 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 3 in animals treated by the combination of CAS+G-CSF, and on day 5 in those treated with CAS+AMB-
IL+G-CSF. No galactomannan was detected after day 14 of the experiment in serum of CAS+G-CSF and CAS+AMB-IL+G-CSF-treated animals.

**Fungal burden in organs**

**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 combination of CAS+AMB-IL+G-CSF was particularly effective in reducing the fungal burden.

**Lungs.** *Aspergillus* CFU were detected in the lungs in all groups at 24 and 48h post infection, and in most groups at day 3. Combination treatment with three agents – CAS+AMB-IL+G-CSF – resulted in absence of *Aspergillus* infection in lungs from day 3 throughout the whole experiment (Table 4). *A.fumigatus* was not detected in most treated groups and untreated controls from day 5 of the experiment.

**Histopathology**

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

Figure 4c 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 CAS+AMB-IL+G-CSF was noted (Figure 4). 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 (Figure 5b). In lungs of mice that received combination therapy of CAS+AMB-IL+G-CSF few areas are involved by bronchopneumonia, and no necrosis of tissue is evident (Figure 5).
Discussion

In the present study we examined combination therapy with the polyene amphotericin B-intralipid admixture and the echinocandin caspofungin, supplemented with the cytokine G-CSF, in treatment of experimental systemic aspergillosis in transiently immunosuppressed mice.

To the best of our knowledge the evaluation of efficacy of CAS and G-CSF as well as combination of two antifungals and G-CSF has thus far not been reported. The combination treatment demonstrated significantly increased efficacy in comparison to either drug alone or to untreated controls, expressed by all evaluated criteria. The synergistic interaction between the polyene and the echinocandin against *Aspergillus* is apparently due to simultaneous effect of the AMB-IL on the fungal cell membrane and inhibition by CAS of synthesis of 1,3-β-D-glucan in the cell wall. Recombinant human G-CSF is a glycoprotein, which induces the bone marrow to reinforce the production of PMNs, and may lead to additive or synergistic effects when it is given with some antifungal drugs. We have reported previously that immunosuppressed mice with low PMN number receiving G-CSF presented a significant increase in neutrophil number. A synergistic effect of G-CSF with CAS was also observed in this study. The data demonstrated that combination of CAS with G-CSF was clearly more effective than CAS alone in treatment of experimental systemic aspergillosis (*P* < 0.001). The data are compatible with our previous study that revealed the synergistic effect of G-CSF in combination with polyenes. In contrast, G-CSF revealed no benefit in terms of survival of mice when it was given alone, as found by us previously and as reported also by Graybill et al. The results obtained from the experiments indicated that combination of all three agents (CAS+G-CSF+AMB-IL) markedly delayed the mortality of mice and
significantly increased their survival rates ($P < 0.001$). These therapies led also to a significant reduction in the fungal burden or even absence of *A. fumigatus* in tissues of organs, compared to animals treated with single therapy or no treatment. However, the experiments showed that the combined therapies consisting of three agents were not more effective than combinations involving only two components – antifungal drug and cytokine G-CSF.

In our model of systemic aspergillosis (i.e. via iv inoculation) the fungi disseminated to all internal organs investigated, e.g. kidneys, spleen (data not shown) and lungs. *Aspergillus* CFU were completely eliminated from the organs of the majority of mice at day 14 of infection. Histologically, no hyphal presence was observed in the lungs throughout infection, despite the massive bronchopneumonia found in untreated mice. It should be noted that also other research groups, that evaluated histologically *Aspergillus* infected mice,\textsuperscript{29,30} did not indicate presence of hyphae in the lungs of the animals with disseminated aspergillosis, while fungi were detected in other organs (e.g. kidneys, brain).

In summary, our study demonstrated that treatment of experimental aspergillosis by combinations of antifungal agents and immunotherapy is significantly more effective, in comparison to single therapy. Thus, further clinical studies are needed to investigate the benefit of combination therapy for invasive aspergillosis in humans.
References


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30. Wallace TL, Paetznick V, Cossum PA, Lopez-Berestein G, Rex JH, Anaissie E. Activity of liposomal nystatin against disseminated
A. Survival of *Aspergillus*-infected mice treated with single or combination therapy

Control – filled squares; AMB – filled triangles; AMB-IL – open triangles; CAS – filled diamonds; CAS+AMB – filled circles; CAS+AMB-IL – open squares.

B. Survival of *Aspergillus*-infected mice treated with antifungals and G-CSF

Mice were infected with $0.8 \times 10^6$ 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. (a) 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. (b) All combination treatments with G-CSF significantly increased survival rate of mice ($P < 0.01$) (Kaplan-Meier test). * The data referring to combinations of AMB+G-CSF and AMB-IL+G-CSF were published previously.$^{24}$
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).
Figure 3.

A. Galactomannan levels in *Aspergillus*-infected mice treated with single or combination therapy

Control – open squares; AMB – open triangles; AMB-IL – open circles; CAS – open diamonds; CAS+AMB – crosses; CAS+AMB-IL – filled squares.

B. Galactomannan levels in *Aspergillus*-infected mice treated with antifungals and G-CSF

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, CAS+G-CSF, CAS+AMB-IL+G-CSF-treated animals, compared with those that received single agents or untreated controls.
Figure 4. Histopathology of kidneys

Kidney from untreated control shows foci of acute pyelonephritis, within medulla (a). In one of these foci fungal hyphae are demonstrated (original magnification, x400). *A. fumigatus* hyphae in the kidney tissue of untreated control stained with GMS (b) (original magnification, x200). In kidney from mouse that received CAS one focus of chronic and acute inflammatory infiltrate is seen within the kidney pelvic mucosa (c) (original magnification, x200). No evidence of inflammation of kidneys from mice treated with AMB or with combinations of CAS+AMB and CAS+AMB+G-CSF (d, e, f) (original magnification, x100).
Figure 5. Histopathology of lungs

Lung tissue of untreated mouse shows diffuse areas of massive bronchopneumonia, involving also pleura (a) (original magnification, x100). Diffuse heavy bronchopneumonia in lung of mouse treated with CAS alone (b) (original magnification, x200). Small foci of very mild neutrophilic infiltrate within parenchyma in the lung of an AMB-treated mouse (c) (original magnification, x100).

In lungs of mice that received combination therapies of CAS+AMB or CAS+AMB+G-CSF few areas are involved by bronchopneumonia (d, e) (original magnification, x100).
### Table 1. MIC, MEC and FICI data of combinations of CAS+AMB, CAS+AMB-IL for *A.fumigatus* (ATCC 64026).

<table>
<thead>
<tr>
<th></th>
<th>CAS+AMB</th>
<th>CAS+AMB-IL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>End point</td>
<td>CAS (alone)</td>
</tr>
<tr>
<td>MIC (µg/ml)</td>
<td>125</td>
<td>15.6</td>
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<td>MEC (µg/ml)</td>
<td>0.78</td>
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### Table 2. Fungal burden in kidneys

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of CFU/organ (mean ± SD)&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>24h</td>
</tr>
<tr>
<td>Control</td>
<td>345±80</td>
</tr>
<tr>
<td>AMB</td>
<td>295±117</td>
</tr>
<tr>
<td>AMB-IL</td>
<td>200±39</td>
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<tr>
<td>Cas</td>
<td>337±105</td>
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<tr>
<td>Cas+AMB</td>
<td>293±116</td>
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<tr>
<td>Cas+AMB-IL</td>
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<tr>
<td>G-CSF</td>
<td>348±131</td>
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<tr>
<td>Cas+G-CSF</td>
<td>550±259</td>
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<tr>
<td>AMB+G-CSF</td>
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<tr>
<td>AMB-IL+G-CSF</td>
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<tr>
<td>Cas+AMB+G-CSF</td>
<td>287±83</td>
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<tr>
<td>Cas+AMB-IL+G-CSF</td>
<td>217±27</td>
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</tbody>
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<sup>a</sup> Determined as described in Materials and Methods. Mean value of two to three mice per group for each time point

<sup>b</sup> n.d. – not done, because the animals succumbed
Table 3. Fungal burden in spleen

<table>
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<tr>
<th>Group</th>
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<th>48h</th>
<th>72h</th>
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<th>14d</th>
<th>21d</th>
<th>28d</th>
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<td>718±237</td>
<td>467±208</td>
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<tr>
<td>AMB-IL</td>
<td>1327±304</td>
<td>518±146</td>
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<tr>
<td>Cas</td>
<td>1965±415</td>
<td>1727±276</td>
<td>637±55</td>
<td>128±31</td>
<td>45±23</td>
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<tr>
<td>Cas+AMB</td>
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<td>107±33</td>
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<tr>
<td>Cas+AMB-IL</td>
<td>1167±263</td>
<td>115±45</td>
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<td>G-CSF</td>
<td>1795±153</td>
<td>945±176</td>
<td>672±119</td>
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<td>768±339</td>
<td>260±77</td>
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<td>AMB-IL+G-CSF</td>
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<td>110±49</td>
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<td>338±170</td>
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*a* Determined as described in Materials and Methods. Mean value of two to three mice per group for each time point. *b* n.d. – not done, because the animals succumbed.

Table 4. Fungal burden in lungs

<table>
<thead>
<tr>
<th>Group</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
<th>5d</th>
<th>7d</th>
<th>14d</th>
<th>21d</th>
<th>28d</th>
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<tr>
<td>Control</td>
<td>898±194</td>
<td>525±155</td>
<td>102±86</td>
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<td>n.d.</td>
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<tr>
<td>AMB</td>
<td>495±82</td>
<td>65±34</td>
<td>20±18</td>
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<tr>
<td>AMB-IL</td>
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<td>42±12</td>
<td>22±22</td>
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<td>10±14</td>
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<tr>
<td>Cas</td>
<td>668±99</td>
<td>170±76</td>
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<tr>
<td>Cas+AMB</td>
<td>467±217</td>
<td>20±18</td>
<td>10±8</td>
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<td>730±213</td>
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<td>225±106</td>
<td>35±23</td>
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<td>670±208</td>
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<td>AMB+G-CSF</td>
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<td>425±91</td>
<td>33±28</td>
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<td>32±39</td>
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<tr>
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<td>50±28</td>
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</table>

*a* Determined as described in Materials and Methods. Mean value of two to three mice per group for each time point. *b* n.d. – not done, because the animals succumbed.
Antifungal effect and possible mode of activity of a compound from the marine sponge *Dysidea herbacea*

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**Abstract**

Objectives. Assessment of antifungal activity of a compound isolated from the marine sponge *Dysidea herbacea* against the fungal pathogens *Candida* (primarily *C. albicans*) and *Aspergillus* (primarily *A. fumigatus*) species, and investigations of the possible mode of activity of the compound.

Methods. Freeze dried sponges were extracted with EtOAc-MeOH. Bioassay guided separation was used to identify the active compound. Antifungal activity was assessed in vitro by a modified NCCLS technique. For determination of the possible mode of activity of the compound we tested the effect on fungal cellular morphology (light, scanning and transmission electron microscopy) and possible site of activity in the fungal cells, such as cell membrane (ion leakage kinetics) as well as toxicity (cytotoxicity tests).

Results and conclusions. The active compound was determined to be 3,5-dibromo-2-(3,5-dibromo-2-methoxyphenoxy) phenol. This compound exhibited in vitro activity against the tested fungal pathogens.

The experiments on the mode of activity revealed that there are significant changes in fungal cell morphology, as demonstrated by scanning and transmission electron microscopy. The compound, apparently, affects the fungal cell membrane, expressed primarily in leakage of potassium ions from the fungal cells. Two other bromo diphenyl ethers were also found to be active.

Further experiments in in vivo models are planned.

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Introduction

Fungal infections have increased in the last years and became an important medical problem, due to an increased number of immunocompromised patients with innate or acquired immunodeficiencies. Invasive mycoses caused by fungal organisms, even those with low virulence, can be life-threatening and are associated with high morbidity and mortality for patients such as cancer patients receiving intensive chemotherapy, bone marrow and solid organ transplant recipients with acute neutropenia and patients with AIDS. In addition, other risk factors, such as prolonged corticosteroid and antibiotic treatments may contribute to development of fungal infections.

The most frequently seen pathogens that cause fungal diseases in humans include Candida and Aspergillus spp. The prevention, diagnosis and treatment of fungal infections remain difficult. Although much progress has been made towards developing different agents for the treatment of systemic mycoses, there are in fact a limited number of efficacious antifungal drugs. Many of the drugs currently available have undesirable effects and may be toxic. Considering the fact that the available antifungal agents originate from a limited number of sources, and that most of them have similar modes of activity, it is very important to explore additional sources for substances with potential antifungal activity, which could possibly have different modes of activity or affect different sites in the fungal cell.

The oceans are the source of a large number of unique natural products that were mainly isolated from sponges, soft corals and tunicates. Many of these secondary metabolites possess interesting biological activities. The real source, however, of the natural compounds, i.e. the host marine organism or guest bacteria, cyanobacteria or other microorganisms is, in most cases, unknown. The marine sponge Dysidea herbacea, whose extract was found to be active, occurs, according to its secondary metabolites, in two chemotypes, one containing sesquiterpenes and polychlorinated amino acid derivatives and the other polybrominated diphenyl ethers. It was suggested by Unson et al. that the polybrominated diphenyl ethers are produced by cyanobacteria. These compounds may play a role in the chemical defense of the sponge against potential predators and bacterial invasion. Polybrominated diphenyl ethers were reported to inhibit enzymes, show antibacterial, antifungal and cytotoxic activities. Tetra to hexabromo-2-phenoxy phenyl ethers were isolated from D. herbacea.

Materials and methods

Marine sponge

Dysidea herbacea was collected by scuba diving (−6 m) near the coast of Zanzibar on March 1999. A voucher sample (# M0020) is deposited in the Department of Zoology, Tel-Aviv University.

Extraction and isolation

The fresh sponge was immediately frozen and kept at −20 °C until investigated chemically. The freeze-dried sponge (10 g) was extracted at room temperature in EtOAc-MeOH (1:1, 3×50 ml). The brown gum obtained after evaporation of the combined organic phase (1.2 g) was subjected to chromatography on Sephadex LH-20 (eluting with CHCl3-MeOH, 1:1) (compound 1, 200 mg, 2%). 1H NMR, 13C NMR and 2D NMR spectra were recorded on a Bruker ARX-500 spectrometer. Mass spectra (MS) data were recorded on a Fisons Autospec-Q spectrometer. The spectral data of compound 1 were found to be identical to the earlier reported compound isolated from D. herbacea collected from the Caroline Islands in the South Pacific Ocean.

Fungal strains

Aspergillus fumigatus (ATCC 64026) and Candida albicans (CBS 562) (type species) were used throughout the study. In addition, in some susceptibility tests also A. niger, A. flavus, C. tropicalis and C. glabrata (laboratory strains) were used. The strains of Aspergillus spp. were grown on Sabouraud dextrose agar slants (SDA) (Difco, USA) at 28 °C for 96 h. Conidia were collected in sterile saline and the conidial suspension was adjusted to the required concentration by counting in a hemacytometer.

Candida spp. were cultured on SDA plates at 37 °C for 24 h. The test strains were suspended in sterile saline and the inoculum was adjusted to the required concentration by counting in a hemacytometer. The inoculum of the test strains was verified.
by plating on SDA plates for determination of colony counts.

Antifungal activity testing

The in vitro activities of the natural compounds against *Aspergillus* and *Candida* spp. were determined by a modified technique based on the NCCLS protocols\(^{11,12}\) 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) of the compounds were determined after 48 h incubation. The MIC value was considered as the lowest compound concentration with no visible growth.

Following the MIC assay, samples from microplate wells with no visible growth were examined for the killing effect of the compounds, through inoculation on SDA plates and incubation for 48 h at 37\(^\circ\)C. The lowest concentration of compounds that killed the fungi was considered as the minimal fungicidal concentration (MFC) value.

Microscopy

For light microscopy *A. fumigatus* was treated with \(\frac{1}{2}\)-MIC of compound 1 for 24 h at 37\(^\circ\)C. The samples were examined under a Zeiss microscope.

For scanning electron microscopy (SEM) *A. fumigatus* or *C. albicans* at a concentration of 2.5-5 \(\times\) 10^5 cells/ml were suspended in YNB containing \(\frac{1}{2}-1\) MIC of compound 1 and incubated at 37\(^\circ\)C for 6 h and 48 h, respectively. The specimens were fixed in 1% glutaraldehyde overnight at 4\(^\circ\)C, post-fixed with 1% OsO\(_4\) solution for 2 h at room temperature and dehydrated in an alcohol series, air-dried, coated with gold (15 nm) and examined with a Joel 840 SEM at 20 kV.

For transmission electron microscopy (TEM) observations *C. albicans* was prepared as described above till the step of glutaraldehyde fixation. The samples were subjected to propylene-oxide, propylene-oxide with Epon 812 (1:1) and thereafter to Epon 812 for 48 h for polymerization. Thin sections were placed on copper grids and stained with uranyl-acetate and lead-citrate, and examined in a Joel TEM 100SX electron microscope.

Ion leakage kinetics

Ion leakage kinetics was performed according to the method of Polacheck et al.\(^{13}\) Briefly, *C. albicans* was suspended in YNB to a final concentration of 5 \(\times\) 10^5/ml. MIC concentrations of compound 1 and AMB—ampicillin B (as a positive control) were added to the test tubes. Samples of 0.5 ml and 10 ml were taken for determination of viable counts and ion concentration, respectively, immediately after adding compound 1 and drug control (AMB), and at 15 min intervals thereafter. Equal samples were taken from the control at the same times of incubation. The cells were washed twice with 15 ml of twice distilled water (TDW), were suspended in 3 ml of TDW and disrupted by incubation at 70\(^\circ\)C for 1 h. After centrifugation the supernatant was taken for ion determination in a Perkin Elmer 403 atomic absorbance spectrophotometer. The concentration of potassium ions was determined after calibration with KCl at 383 nm.

Cytotoxicity test

The XTT test\(^{14}\) is based on the fact that live cells reduce tetrazolium salts into colored formazan compounds. The biochemical procedure is based on the activity of mitochondria enzymes that are inactivated shortly after cell death.

**HEp2** cells were grown at 37\(^\circ\)C in Dulbecco’s modified Eagle’s medium, DMEM (Gibco, UK) containing 2 mM glutamine and 10% fetal calf serum supplemented with 200 \(\mu\)g of streptomycin and 200 IU of penicillin per ml. After 48 h the cells were harvested by trypsin treatment and resuspended in growth medium to a final concentration of 10^5 cells/ml. Hundred microlitre of the suspension was added to a microplate and incubated overnight in 37\(^\circ\)C.

One to three folds of MIC concentrations of compound 1 and AMB (as a positive control) were added to the wells, non-treated HEp-2 cells used as a negative control. The plate was incubated at 37\(^\circ\)C, and after 1 h 50 \(\mu\)l of XTT—cell proliferation
kit (Biological Industries)—was added into the wells. The increase in OD was proportional to the number of live cells after treatment, compared to non-treated cells. OD was measured with a microplate spectrophotometer Spectra MAX 340 at 450 nm.

Red blood cells (RBC) lysis

RBC were obtained from human blood of healthy donors with normal blood chemistry, after centrifugation at 3000 rpm for 10 min. The cells were washed and resuspended in phosphate buffered saline (PBS), containing 0.1% EDTA (Merck, Germany) to a final concentration of 1-5×10⁸/ml.15 Three millilitre of the suspended RBC were incubated for 1 h at 37°C with compound 1 at different concentrations, or with 1 µg/ml AMB (MIC concentration) and 5 µg/ml (as an indicator of complete hemolysis), and without treatment as control. The cells were centrifuged, and the supernatant was taken for estimation of hemolysis using a microplate reader at 540 nm.

Results and discussion

In vitro susceptibility testing

In vitro susceptibility of *A. fumigatus* (ATCC 64026) and *C. albicans* (CBS 562) to a series of sponge extracts was examined. The extract of *D. herbacea* was found to be active against both fungal species. We used amphotericin B (AMB) as a control antifungal agent. The MIC of compound 1 against *A. fumigatus* and *C. albicans* was 7.81 µg/ml (Table 1). The in vitro activity of compound 1 was assayed against additional pathogenic *Aspergillus* (*A. flavus, A. niger*) and *Candida* (*C. tropicalis, C. glabrata*) species. The MIC values against *A. flavus, A. niger* and *C. tropicalis* were 7.8, 1.95 and 7.81 µg/ml, respectively. *C. glabrata* revealed less susceptibility to compound 1 and the MIC of this species was 15.62 µg/ml (Table 1).

AMB is a most potent antifungal agent, as expressed in very low MIC values (1-2 µg/ml). Although the MIC values found in our study for compound 1 are higher than those of AMB, such data have also been reported for other antifungal agents, such as azoles, which are considered not in the range of resistance.

In addition, fungicidal activity of compound 1 was measured, and MFC values were determined towards *A. fumigatus* (15.62 µg/ml) and *C. albicans* (7.81 µg/ml).

Mode of activity of compound 1

The next step in our study was focused on attempts to determine the possible mode of activity of compound 1. Toward this end we examined the effect on fungal cellular

<table>
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<th>MIC (µg/ml)</th>
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<tr>
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<td><em>C. albicans</em></td>
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<tr>
<td><em>C. tropicalis</em></td>
<td>7.8</td>
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<tr>
<td><em>C. glabrata</em></td>
<td>15.62</td>
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Figure 1  *A. fumigatus* treated with compound 1 under a light microscope. (A) *A. fumigatus* without treatment (control); (B) *A. fumigatus* treated with 3.9 µg/ml (½ MIC) of compound 1 for 24 h (original magnification: ×200).
morphology and ultrastructure, possible sites of activity in the fungal cells, such as cell membrane. We also examined the toxicity towards mammalian cells.

Effect of compound 1 on morphology of C. albicans and A. fumigatus

To assess the effect of compound 1 on the fungal morphology we exposed fungi for different exposure times to MIC and sub-MIC concentrations and examined them under a light microscope, scanning and transmission electron microscopes.

Examination of A. fumigatus treated with $\frac{1}{2}$ MIC of compound 1 under a light microscope showed irregular branching of the hyphae (Fig. 1); C. albicans treated cells were markedly swollen as compared to the controls.

Further studies included SEM and TEM observations. Treated A. fumigatus spores and C. albicans yeasts were analysed by SEM in comparison to untreated fungi. In both fungi, treated with compound 1 at MIC concentration, the cells lost their symmetrical appearance, they were completely deformed, clumped together and deep wrinkles were observed. Cell wall invaginations were also noticed. Leakage of cellular content could be noticed. The changes were in correlation with the compound 1 concentration, and were already noticed at $\frac{1}{2}$ MIC; the higher the concentration of the compound, the greater the change of the cells (Figs. 2 and 3).

TEM observations of C. albicans treated with compound 1 showed shrinkage of the protoplast, disruption of the cytoplasmatic membrane in presence of the lower concentrations ($\frac{1}{2}$ MIC–3.9 $\mu$g/ml) (Fig. 4(B)), whereas incubation in higher concentrations led to a more significant damage; all the inner organelles are completely discomposed, no evidence of cytoplasmatic membrane, and even the cell wall is deeply affected and looks like undulant (Fig. 4(C)). The control cells are well preserved (Fig. 4(A)).
Effect of compound 1 on cell membrane

In order to examine the effect of the compound on the cell membrane, ion leakage assays were performed. The potassium ion leakage from *C. albicans* treated with compound 1, was assessed in comparison to untreated controls and to cells treated with AMB. AMB is known for its activity on

**Figure 3** Scanning electron microscopy of *C. albicans* treated with compound 1. (A) *C. albicans* without treatment (control); (B) *C. albicans* after treatment with 3.9 μg/ml of compound 1 for 48 h; (C) *C. albicans* after treatment with 7.8 μg/ml of compound 1 for 48 h (original magnification: ×10 000).

**Figure 4** Transmission electron microscopy of *C. albicans* treated with compound 1. (A) *C. albicans* without treatment (control) (original magnification: ×10 000); (B) *C. albicans* after treatment with 3.9 μg/ml of compound 1 for 48 h (original magnification: ×15 000); (C) *C. albicans* after treatment with 7.8 μg/ml of compound 1 for 48 h (original magnification: ×10 000).

Effect of compound 1 on cell membrane

In order to examine the effect of the compound on the cell membrane, ion leakage assays were performed. The potassium ion leakage from *C. albicans* treated with compound 1, was assessed in comparison to untreated controls and to cells treated with AMB. AMB is known for its activity on
the membrane, leading to increased permeability of monovalent cations. The experiments revealed that C. albicans exposed to compound 1 at the MIC concentration lost 50% of its potassium ion content already after 15 min of exposure, with no further changes during 1 h of treatment, while AMB treated cells lost 100% of potassium already after 15 min (Fig. 5).

**Cytotoxicity tests**

For evaluation of the toxicity of compound 1, XTT assays were performed. A human cell line—HEp2 was treated with MIC concentration of the compound and metabolic activity was assayed. A decrease of almost 25% in the metabolic activity of the cells was noted after 1 h of exposure. Treatment with MIC amounts of AMB caused a decrease of 27% after 1 h in the metabolic activity of the cells (Fig. 6).

In addition, we tested the effect of compound 1 on human RBC. The MIC concentration of compound 1 caused hemolysis in 10% of RBC following 1 h exposure, whereas exposure to AMB at the same conditions led to hemolysis in 30% of RBC. A five-fold increase of MIC value of compound 1 (39 μg/ml) increased the hemolysis of RBC to almost 70%, while AMB caused complete hemolysis at the five-fold MIC value (5 μg/ml) (Fig. 7).

In addition to compound 1, another Dysidea metabolite, 3,4,5-tribromo-2-(2,4-dibromophenoxy) phenol (2) as well as the synthetic 2,6-dibromo-4-phenoxyphephol (3) (see Scheme 1) were also found to be active against A. fumigatus and C. albicans at MIC concentrations of 7.81, 15.62 μg/ml and 1.95, 15.62 μg/ml, respectively.

In conclusion, the results obtained in this study revealed that compound 1 is active in vitro both against Candida and Aspergillus species and apparently affects, primarily, the fungal cell membrane. The effect on the membrane may be resulting from different mechanisms of activity: binding to sterols and thereby causing higher membrane permeability, inhibition of ergosterol synthesis or effect on membranal enzymes. Additional experiments, which could further elucidate the mode of activity and in vivo investigations will be planned.
Acknowledgements

We wish to thank Prof. Micha Ilan for collecting the marine samples and Mrs Neta Avni for her skillful technical assistance in preparing the compounds.

References

Discussion

The main goal of our study was to find novel antifungal formulations or combinations with efficacy against systemic aspergillosis in an experimental animal model. In the past two decades the incidence of aspergillosis, especially in immunocompromised persons, has continued to increase. The management of aspergillosis is particularly problematic, because diagnosis of this disease is difficult and comes late, and the efficacy of current antifungal therapy regimens in heavily immunosuppressed patients is poor. Despite the advances offered with current available antifungal drugs, the morbidity and mortality associated with *Aspergillus* infections remains unacceptable, especially for the most at-risk patients. For individuals with severe immunosuppression as a result of chemotherapy, graft-versus-host disease and its therapy, or transplantation, new antifungal preparations/formulations and strategies are greatly needed.

Hence, toward the main purpose we adapted an animal model of experimental systemic aspergillosis. Experimental aspergillosis was obtained by iv inoculation of immunocompromised mice (pretreated with CY), as a useful model. A number of investigators support this observation, indicating that this model is valuable and suitable for assessment of efficacy of novel antifungal drugs (12,69,70,75). Our experiments revealed that inoculating 4-5 weeks old female ICR mice with 0.8-1 x $10^6$ conidia/mouse of *A.fumigatus* (ATCC 64026) resulted in 100% of death within 3-13 days (134,135). Systemic aspergillosis was determined by fungal colonization of viscera (kidneys, spleen, lungs). Macroscopically examined organs (kidneys and lungs) of dead mice revealed presence of microabscesses; in histopathological and calcofluor stained samples of renal tissue homogenates, hyphal elements were detected microscopically.
Following the adaptation of a suitable experimental model the first step of our study focused on the investigation of efficacy of amphotericin B-intralipid and nystatin-intralipid admixtures in experimental systemic aspergillosis. The rationale of this approach was based on the information from literature (88,136) and our own studies on Candida (92,93) that AMB-fat emulsions were less toxic than conventional AMB and could be administered at higher doses, hence could be more effective. Indeed, Shadkhan & Segal described the efficacy of AMB-IL in experimental candidiasis. The data of this study show that AMB-IL was more effective than conventional AMB also in treatment of experimental systemic aspergillosis. AMB-IL at the higher dose of 2 mg/kg/day significantly increased survival rate and mean survival time (MST) of infected mice in comparison to AMB ($P < 0.05$). Previous studies involving systemic candidiasis (92,93) revealed that treatment with higher doses of AMB-IL led to approximately 100% survival of the treated animals. The results obtained in our experimental aspergillosis model seem to be compatible with clinical and epidemiological observations of Aspergillus infections, which are considered more severe complications in immunocompromised patients with higher levels of mortality and more difficult therapy (3,8,137) than candidiasis, hence the better outcome of treatment of candidiasis with AMB-IL in comparison to treatment of aspergillosis. Physicochemical characterization of the AMB-IL admixtures prepared by overnight vigorous agitation revealed that the preparations contained particles of similar sizes as before mixing with Fungizone (350.6 ± 6.1 nm) and about 90% of AMB remained in emulsion particles, as determined following ultracentrifugation (91). The characteristics of the AMB-IL admixtures remained unchanged for a month, pointing to their stability. This was different in preparations obtained by gentle mixing, which separated spontaneously into several phases. In addition, the AMB-IL admixture
caused significant lower hemolysis of RBC and reduced $K^+$ leakage in comparison to Fungizone.

Comparative pharmacokinetic studies of AMB, AMB-IL and liposomal AMB (Ambisome) have revealed that the blood and tissues (kidneys, liver, spleen, lungs) distribution of AMB-IL has a similar pattern to Ambisome (138). It was shown that AMB blood levels were higher in animals administered with AMB-IL than in those with AMB. In addition, it was also found that the level in liver was higher and in kidneys lower.

Nystatin is similar in structure to AMB. The mode of action of both drugs has been attributed to the binding to ergosterol in the fungal membrane, creating transmembrane channels resulting in membrane permeability and leakage of monovalent cations ($K^+$), sugars and metabolites (139). But, unlike AMB, nystatin is not suitable for intravenous administration. Therefore, development of alternative formulations of nystatin, that could enable intravenous therapy, was needed. Liposomal formulation of nystatin was developed and resulted in good efficacy against fungal infections (96,97,140), but this antifungal agent has not yet reached clinical use.

A novel admixture of nystatin with intralipid (Ny-IL), which we prepared similarly to the AMB-IL formulation, was examined in this study. The results obtained in the experiments indicate that Ny-IL at the higher concentration of 6 mg/kg/day improved the survival rate and prolonged the MST of infected mice, compared to untreated controls and to Ny-IL treated groups at lower doses, albeit the group treated with the higher dose of Ny-IL, included relatively a small number of mice.

Comparison of data of both formulations, AMB-IL and Ny-IL, revealed that survival rate and MST of animals treated by AMB-IL at the dose of 2 mg/kg/day was
significantly higher ($P < 0.05$) than those of animals treated by Ny-IL. It seems that in our system the Ny-IL is less potent than AMB-IL. It is of interest, that Denning and Warn (141) found that liposomal nystatin at a dose of 5 mg/kg/day was superior to liposomal AMB at the same dose. High doses of liposomal nystatin (10 mg/kg/day) were toxic. In our study the highest non-toxic dose of Ny-IL was 6 mg/kg/day.

The data in this work provide evidence, that AMB-IL admixture is very effective against experimental systemic aspergillosis; Ny-IL may also be a possible additional therapeutic option that should be considered.

In the next step of our study we investigated the activity of recombinant G-CSF, the cytokine that increases production of neutrophils by the bone marrow, in combination with AMB, AMB-IL or Ny-IL admixtures against experimental aspergillosis induced in immunocompromised mice. As stated above, a compromised state in mice was achieved by ip injection of the immunosuppressive agent cyclophosphamide. Administration of this agent at a dose of 200 mg/kg led to a neutropenic state in mice (neutrophils $< 500/\mu l$). Such state is analogous to clinical situations that are associated with high risk of development of aspergillosis. Reduction in the number of circulating neutrophils leads to decreased resistance to opportunistic pathogens, such as *Aspergillus* (54). Our data indicated that immunosuppressed mice receiving G-CSF presented increased numbers of neutrophils, already 24 h after administration of the cytokine, with the effect peaking after 3 days. This cytokine is known for its ability to restore the number of neutrophils, and apparently return their antifungal activity (43,53).

In our study we found a synergistic effect of G-CSF with AMB or the polyene-intralipid admixtures, AMB-IL or Ny-IL. The data revealed that combination of AMB or AMB-IL with G-CSF was clearly more effective than AMB or AMB-IL alone in
treatment of experimental systemic aspergillosis. G-CSF combined with AMB or AMB-IL significantly improved survival rates and MST-s of infected mice in comparison to AMB or AMB-IL alone ($P < 0.001$). Our data are compatible with other studies, pointing to the additive effect of G-CSF in combination with antifungal drugs (53,54,142,143). It should be pointed out that in those studies therapy with G-CSF began 3 days before infection, while in our study G-CSF-treatment began together with the antifungal therapy, after induction of infection.

The results obtained from our experiments indicated that G-CSF alone was ineffective in increasing significantly the survival or delaying mortality of infected animals, although a reduction in the CFU counts in organ tissues below that of the untreated mice was noted. It also was reported that G-CSF alone has no beneficial effect on survival of experimental cryptococcal meningitis (144).

We did not evaluate the effect of free nystatin (Ny) on aspergillosis, as in this form Ny is not suitable for systemic administration. However, the admixture of nystatin and intralipid is a formulation, which can be used systemically. Thus we assessed the efficacy of the combination of G-CSF with Ny-IL. Treatment of experimental aspergillosis with Ny-IL was less effective than AMB-IL, but nevertheless had a therapeutic effect in comparison to the untreated group. When G-CSF was administered with Ny-IL at a dose of 4 mg/kg/day the survival rate of the mice increased to 38.5% in comparison to 29% in Ny-IL treated animals. However, this therapy also was inferior to the combination treatment of G-CSF with AMB or G-CSF with AMB-IL.

In conclusion, the data of the present part of the study indicate that addition of the cytokine G-CSF to polyene or polyene-intralipid therapy has a synergistic effect in experimental aspergillosis and improves the outcome.
In addition, our study focused on combination therapy of the echinocandin CAS and the polyene AMB or AMB-IL against experimental murine aspergillosis. Moreover, we also explored the effect of supplementing the antifungal combination with the cytokine G-CSF. Combination antifungal therapy has become of interest in view of the poor outcomes in treatments with the available antifungal agents in immunocompromised patients. To the best of our knowledge the evaluation of efficacy of CAS and G-CSF as well as combination of two antifungals and G-CSF has thus far not been reported in the literature.

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, reduction of CFU in organs and reduced galactomannan antigenemia, a criterion of IA. The synergistic interaction between the polyene and the echinocandin against *Aspergillus* is apparently due to the 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. Interestingly, combinations involving polyene and azole in treatment of aspergillosis (145) or candidiasis (146) have been reported to be antagonistic.

Recombinant human G-CSF is a glycoprotein, which induces the bone marrow to reinforce the production of PMNs, and may lead to additive or synergistic effects when it is given with antifungal drugs (53,54,142-144,147). We have found (134) that immunosuppressed mice with low PMN numbers receiving G-CSF presented a significant increase in the number of neutrophils. Thus, a synergistic effect was observed against aspergillosis when G-CSF was administrated with CAS. The data demonstrated that combination of CAS with G-CSF was clearly more effective than CAS alone in treatment of experimental systemic aspergillosis. This combination
significantly increased survival rates and MST-s of infected mice, as compared to results obtained by treatment with CAS alone \( (P < 0.001) \), and led to a rapid decline of the galactomannan level in the serum of the animals. The data are compatible with our other results (134) that revealed the synergistic effect of G-CSF in combination with polyenes. In contrast, G-CSF revealed no benefit in terms of survival of mice when it was given alone (134) and as reported also by Graybill et al. (144).

The results obtained from these experiments indicated that combination of CAS, G-CSF and either AMB or AMB-IL markedly delayed the mortality of mice and significantly increased their survival rates \( (P < 0.001) \). These therapies led also to a significant reduction in the fungal burden or even absence of \textit{A. fumigatus} in tissues of organs, particularly in the kidneys and lungs, compared to animals treated with single therapy or no treatment. We therefore assumed that addition of G-CSF to the antifungal combination might increase efficacy of the treatment even more. However, the experiments showed that the combined therapies consisting of three agents were not more effective than combinations involving only two components – antifungal drug and cytokine G-CSF.

In our model of systemic aspergillosis (i.e. via iv inoculation) the fungi disseminated to all internal organs investigated, e.g. kidneys, spleen and lungs. \textit{Aspergillus} CFU were completely eliminated from the organs of the majority of treated mice at day 14 of infection (untreated controls succumbed to infection by day 10). Histologically, no hyphal presence was observed in the lungs throughout infection, despite the massive bronchopneumonia found in untreated mice. It should be noted that also other research groups, that evaluated histologically \textit{Aspergillus} infected mice (96,148), did not indicate presence of hyphae in the lungs of the animals with disseminated aspergillosis, while fungi were detected in other organs (e.g. kidneys, brain).
The significant rise in the incidence of fungal infections, coupled with awareness of the importance of these infections, has resulted in increased efforts to search for newer effective antifungals. It is of significance to look for new sources of potential substances with biological activity and, by enlarging the search for different sources in nature, it might be possible to find novel substances which would affect fungi by different mechanisms, and thereby result eventually in availability of effective antifungals with diminished toxicity for the host than the currently existing agents.

The oceans are a unique resource that provides a diverse array of natural products, primarily from invertebrates such as sponges, and from marine bacteria and cyanobacteria. As the etiological agents of various infectious diseases develop resistance to existing medicaments, the marine environment provides novel leads against fungal, bacterial and viral diseases (149). Recently, our laboratory took part in a project that focused on high throughput screening (HTS) for antifungal activity of compounds, which have been isolated from various natural sources. One of the extracts, isolated from the marine sponge *Dysidea herbacea*, revealed antifungal activity in vitro. Therefore, it was of importance for us to further investigate the antifungal effect and the possible mode of activity of the compound, that was determined to be 3,5-dibromo-2-(3,5-dibromo-2-methoxyphenoxy) phenol. This compound exhibited in vitro activity against various *Candida* and *Aspergillus* spp.

The experiments on the mode of activity revealed that exposure to the compound leads to significant changes in fungal cell morphology, as demonstrated by scanning and transmission electron microscopy. The compound, apparently, affects the fungal cell membrane, which is expressed primarily in leakage of potassium ions from the fungal cells. The effect on the membrane may be resulting from different mechanisms.
of activity: binding to sterols and thereby causing higher membrane permeability, inhibition of ergosterol synthesis or effect on membranal enzymes.

Our study provides strong evidence that treatment of experimental aspergillosis by combinations of antifungal agents or combination of immunotherapy with antifungals is significantly more effective, in comparison to single therapy. Thus, further clinical studies are needed to investigate the benefit of combination therapy for invasive aspergillosis in humans.

**Summary and conclusions**

The data presented in this study show that:

1. AMB-IL was more effective than conventional AMB in treatment of experimental systemic aspergillosis, especially when high doses of AMB-IL were used
2. Ny-IL may also be a possible additional therapeutic option against aspergillosis that should be considered
3. Combination of polyenes and G-CSF, particularly G-CSF with AMB or AMB-IL, was synergistic in experimental aspergillosis and significantly increased the survival rate and prolonged the survival time of the animals in comparison to treatment with either polyene alone
4. The polyene-echinocandin combination (CAS + AMB or CAS + AMB-IL) demonstrated significantly increased efficacy in comparison to either drug alone or to untreated controls, as expressed in increased or prolonged survival of infected mice, reduction of CFU in organs and reduced galactomannan antigenemia
5. Combination of CAS with G-CSF was clearly more effective than CAS alone in treatment of experimental systemic aspergillosis
6. Combined therapies consisting of three agents (CAS, G-CSF and either AMB or AMB-IL) markedly increased the survival of animals compared to those treated with single therapy or no treatment; however, these protocols were not more effective than combinations involving only two components – antifungal drug and cytokine G-CSF.

7. The compound isolated from the marine sponge *Dysidea herbacea* exhibited in vitro activity against *Aspergillus* and *Candida* species and apparently affects the fungal cell membrane.

In conclusion, our promising data pointing to the efficacy of lipid formulations of polyenes, polyene-echinocandin combinations and adjunct immunotherapy in the treatment of disseminated experimental aspergillosis warrant further clinical investigations.
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טיפולי תרופתיים נסוניים
Aspergillosis
מנגד
מозвращת במודלים ניסיוניים
בבליל חיים

히ובר לשב קבלת התואר “דוקטור לפילוסופיה”
מאיה

אדוארד סירוב

הוגה לסנטא של אוניברסיטת תל-אביב
יולי 2005
עבדה זו נעשתה בהדרכון

פרופסור אסתר סגל
ה rekl הovidוטיים

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Aspergillosis: Clinical Aspects and Treatment Options

Edward Sekunda

ABSTRACT

Aspergillosis remains a major public health concern, particularly in compromised host settings. The disease is caused by Aspergillus species, which have become increasingly resistant to conventional antifungal agents. The most common clinical manifestations of aspergillosis include systemic infections and allergic fungal diseases.

INTRODUCTION

Aspergillus species are ubiquitous in the environment, and they are opportunistic pathogens that can cause serious infections in immunocompromised individuals. The prevalence of aspergillosis has increased due to the rise in the number of individuals with underlying medical conditions, such as AIDS and hematopoietic stem cell transplantation.

METHODS

Aim: To evaluate the effectiveness of antifungal therapy in the treatment of aspergillosis.

Materials and Methods: A retrospective review of medical records was conducted to identify patients diagnosed with aspergillosis and treated with antifungal agents.

Results: The study found that antifungal therapy was effective in controlling the infection in most cases. However, there was a high rate of relapse and drug resistance.

Conclusion: Antifungal therapy remains the mainstay of treatment for aspergillosis, but the development of drug resistance and relapse rates highlight the need for continued research and development of new therapeutic strategies.

ACKNOWLEDGMENTS

This research was supported by a grant from the National Institutes of Health.

REFERENCES


Aspergillosis studies and interventions in experimental model Prof. Asaf Yaffe, AMB-IL+G-CSF and AMB+G-CSF

1. AMB-IL+G-CSF preparations, which showed higher efficacy compared to the standard AMB treatment in experimental Aspergillosis, were adopted by
2. Ny-IL as an alternative to AMB treatment in Aspergillosis cases
3. AMB-IL+G-CSF and AMB+G-CSF, their combination, and G-CSF preparations showed promising results in experimental Aspergillosis.
Aspergillosis in mice as a model of milder treatment

4. Shikou et al.-acinetomycins (AMB-IL+CAS, AMB+CAS) and the outcome of CFU bacteria and fungal cells in the blood, liver, and respiratory system of the mouse, which improved by treatment with G-CSF

5. The combination of CAS with G-CSF significantly improved survival, with a decrease in the number of CFU in blood and lungs, and in galactomannan in the blood, compared to treatment with either drug alone.

6. Combining AMB-IL+CAS with G-CSF was more effective in vitro than the combination of AMB-IL alone. In addition, the combination of AMB-IL+CAS with G-CSF increased survival in vivo, compared to treatment with either drug alone.

7. The aqueous extract of D. herbacea showed activity in vitro against Aspergillus and Candida, and appears to disrupt the membrane of the cell. This suggests that the results of this work are encouraging, and that the combination of polyunsaturated fatty acids and G-CSF may be effective in the treatment of Aspergillosis in humans.
Aspergillus-1 Candida

Invasive Aspergillosis (IA) –

In the treatment of Aspergillus infections, itaconazole – (AMB) B and voriconazole – (itraconazole) are the first-choice drugs, as well as liposomal formulations of itaconazole and voriconazole.

The hypothesis is that the addition of antifungal agents to the treatment of Aspergillosis can improve the outcome of the treatment.

The research was conducted in a model of experimental Aspergillus infection.
ב (א)
בדיקת פעילות של פוליאנים ואנמוללות של פוליאנים-אינטראליפיד (אינטראליפיד)

(AMB-IL)אנפיטריצין-B (Ny-IL)אינטראקציה עם תערובות של פעילות והערכת בדיקת
בושת שרוף. כמות-כם,伧 Fernandez רדה פטרייה
בנד AMB-IL

G-CSF ובדיקת פעילות של פוליאנים ואנמוללות של פוליאנים-אינטראליפיד بشילוב עם ציהוק
בנד אספרטנילויס נסייה

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בדיקת פעילות של פוליאנים ואנמוללות של פוליאנים-אינטראליפיד (אינטראליפיד)

G-CSF מופחתה נסייה

ב (ג)
בדיקת פעילות של אפריגניד (CAS) בשילוב עם ציהוק
בנד אספרטנילויס נסייה

ב (ד)
בדיקת פעילות של פוליאנים-אינטראליפיד ואמבולコーヒ
בנד AMB

(AMB/AMB-IL CAS) שיטות

ב (ה)
בדיקת פעילות של פוליאנים-אינטראליפיד بشילוב עם ציהוק
בנד מילוי

Dysidea herbacea

(1) בודקה מתקרר פטרייה

שינית

מודל גולימ מכנון פטרייה

מות העצומית של פוליאנים-אינטראליפיד

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ונפרדו 30 ימים לאחר הזרקה. בنظمי אינטראווסטריכרה וכספית ספיקת תוכן (לקיבוץ מטרות
(lysate: מספג ל-105 במילון).

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שיפור "ע"הנוגעבשפלחונינעשplementsגנואספריגוליזיספסיפיתבישית

שעתימ lặngההדבקקההחותשקופה"ירכוזיםשניתשלNy-ILאוAMB-ILבבאמש5ימים.מעקב
בוחרפרצופצויותסטנאריתיסחפשמיתשלהפוליאנים.המוצרהואשהחקבלהריאהAMB-IL-שהעלכה0%
לאחתה AccessToken הרוחמה(המשווהלת% לשילוח)
אורעעוריההשידרהשלNy-ILרבעכבריםש المتوسط$"(P<0.001) לש浼יתה ב16.2-ל0% (P<0.001) Ny-IL
17.3-AMB-IL$"טרופיקותוהנוגעבשלבריכודיםוהבריכה самомמצプリンוגтанופחת$6.13(7ימים)ואלאלםופלת
8.61-16.8: Ny-IL: 23.07(7ימים)גנוזילשלופחטוע najwyżיםAMB,AMB-IL,AMB-IL+G-CSF.
אנסיילוידם,אנסיילוידעםNy-IL交流合作ה,וייהיויליךנגנואספיגוליזיס

םופשתניסית

שיפורבאספיגוליזיסטופיובבamatנהבפיליאניםיעוצק

בנשתהלהבוביריציעותשלהתרופהאטנירפסיפיתשהżąמלפיופחילופשלשהבטוריפםפוליאנים
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הbsites: $"P<0.001
(97.40,51.48,51.48
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15.35-126.2528.838.47(7ימיםבהbsitesל coisa)
$"(Ny-IL+G-CSF-1AMB-IL+G-CSF,AMB+G-CSF,G-CSF

תוראהוהנוגעבשלמרידוהםוהנוגעב-securit enactment לש
AMB-ILואAMBG-CSF

םלוכוספוריב

 carnagy, - 100%תמותהבקבריחתותוך-30ימים.ידוהוםטסנוםעבוןייטיידמצאותהאלנטים.
The research that follows in the next stage of the combination of the efficiency tested CAS with AMB or AMB-IL against Aspergillus and immunotherapy in mice.

Yes, in addition, with the addition of antifungal combination effects, we studied the cytokine G-CSF. In the combination showed that was received the survival and extended life of 69.2% of the treated mice compared to 44.4%, 40.7%, and 50% (hence forth).

The combination of G-CSF with the combination of AMB+CAS or AMB-IL+CAS — does not contribute to the帅气, and the survival did not extend or extend the life of the animals.

The combinations led to colonization of the host in the portal and in the antigenic galactomannan. In the receiving all the tests, which showed that the possibilities exist for the effective use of antifungal future, especially, and also that there are opportunities for people to use them.

Antifungal activity in vitro is described in the extract of Dysidea herbacea new products, which contain antifungal and antiviral, with natural sources, especially, which constitutes the problems and consequent immunizations of people with the infections of parasites and fungi.

For the first time we tested the activity of Dysidea herbacea — 3,5 dibromo 2-(3,5 dibromo 2 methoxyphenoxy) phenol on fungi and parasites. Tested with the help of electronic and light microscopes, the effects of the morphology of the tested components, and also with human cell lines on the extract of Dysidea herbacea, which indicated that the mixture of the extract is effective in in vitro conditions.
ה>--יתnia שיגניה מעשהית של עצות התא. עובדה, הוצאתו של העצם והูטריתו על הממברנה של התא

**מסקנה חמשית**

1. סטטיפל מראות הנוכחיים במחקר שהוצגושם תערובת באמצעות AMB-IL, בריכוז בשיעור בגבוה, מאשרת עיקל יותר מניסויית מפושטת אספרגילים בנגד AMB-IL+G-CSF,AMB+G-CSF,AMB-IL+G-CSF יספורל נו"יNy-IL,AMB+IL- CAS או AMB+CAS

2. ישוב פולי-ל-/signature סע פולי-ב,במעורבות עם G-CSF,ับ"ל הלוחמל התערובת שחל החוזור ממספר CFL מחלת החוזה בוירגניהית התא בברחת גלוקטומנת המקבילה


4. בטיפול溢价 גלקטומן בברחת היו צפופים,בנובא המקבילה CAS של פולי-ל-/signature

5. מעובדת שובדד במעורבות של התערובת של יונת לווה המקבילה in vitro הדבורת D. herbeca.ivors, לפיכך,Canon-1 Aspergillus}

6. ישוב מעולב נו"יNy-IL מתגלה תערובת עם G-CSF,בנובא המקבילה CAS של פולי-ל-/signature

7. בטיפול溢价 גלקטומן בברחת היו צפופים,בנובא המקבילה CAS של פולי-ל-/signature

לטוס, והתואמת המנהרות חשישות שקולות בברחת והรับผิด ולצארית על עיולה של תמרונית של פולי-ל-/signature עם אנטרפלייד, שולובים של פולי-ל-/signature,אינטראקציה זכויות וסינת CONNECTIONS מעורבות עם פולי-ל-/signature בברחת pewp. כ- נושאם לטופגי כללי, ראש зрמונג להווה לשונמה והיו לעיל פוליאגנולית בברחת.

אэм