Eur J Cancer Clin Oncol, Vol. 24, No. 3, pp. 527–538, 1988
Printed in Great Britain

0277–5379/88$3.00+0.00

Pilot Study of Amphotericin B Entrapped in Sonicated Liposomes in Cancer Patients with Fungal Infections

JEAN-PAUL SCULIER, ANDRÉ COUNE, FRANÇOISE MEUNIER, CHRISTIANE BRASSINNE, CHANTAL LADURON, CATHY HOLLAERT, NADIA COLLETTE, CORINNE HEYMANS and JEAN KLASTERSKY

Service de Médecine et Laboratoire d'Investigation Clinique H.J. Tagnon, Institut J. Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, 1 rue Héger-Bordet, 1000 Bruxelles, Belgium.

Abstract—*A pilot study with amphotericin B incorporated in sonicated liposomes (ampholiposomes) made of egg phosphatidylcholine, cholesterol and stearylamine in a molar ratio* 4:3:1 *was performed in cancer patients with fungal infections. Fifteen patients received a total of* 117 *intravenous infusions of ampholiposomes. The total dose of amphotericin B administered per patient ranged from* 20 *to* 1004 mg (*mean* 472 mg). *The number of infusions per patient varied from* 1 *to* 20 (*mean* 8) *and the duration of treatment from* 1 *to* 29 *days* (*mean* 10 *days*). *Infusion of doses up to* 1.8 mg/kg *was well tolerated. None of the common side-effects of Fungizone®, the colloidal suspension of amphotericin B, occurred; it was noteworthy that patients had no renal function impairment.*

Serum amphotericin B concentrations given as ampholiposomes were much higher than those obtained with Fungizone®. With a daily treatment schedule, peak and trough serum amphotericin B concentrations, as measured by HPLC, were 10 *to* 20 μg/ml *and* 5 *to* 10 μg/ml *respectively; while they did not exceed* 2 μg/ml *and* 1 μg/ml *with Fungizone®. Amphotericin B given as ampholiposomes had a prolonged serum* β *half-life* (25.3 ± 16.0 h). *Higher serum antifungal activity was observed with ampholiposomes as compared to Fungizone®. We concluded that ampholiposomes have a better therapeutic index than Fungizone.®*

INTRODUCTION

Opportunistic fungal complications are increasingly frequent in patients with neoplastic diseases and have become an important cause of death [1]. *Candida* spp. and *Aspergillus* spp. are the most common pathogens. Invasive fungal infections are often difficult to diagnose and the treatment which essentially consists of the parenteral administration of amphotericin B [2] is associated with a high rate of failure [3–5].

Amphotericin B is a poorly water-soluble drug and for intravenous administration,it is supplied as a colloidal suspension (Fungizone®) using sodium deoxycholate as a dispersing agent [6]. This preparation has many serious side-effects such as fever, chills, bronchospastic reactions or nephrotoxicity [7]. Despite the development of new classes of antifungal agents, amphotericin B remains the drug of choice for most offending pathogens. However, there is a need to investigate the potential value of other galenic preparations of this drug, with the aim of avoiding the side-effects as well as achieving better antifungal therapy. Amphotericin B methylester represents such an attempt. It has, however, been a failure due to the neurotoxicity of the preparation. Another alternative could be the encapsulation of the drug in liposomes. Liposomes are lipid vesicles that can be used as carriers for the administration of hydrophilic, hydrophobic or amphiphilic compounds. We have used sonicated liposomes made of egg yolk lecithin, cholesterol and stearylamine to administer to cancer patients a water-insoluble antimitotic compound, NSC-251635, by

Accepted 2 October 1987.
This work was presented in part at the 22nd meeting of the American Society of Clinical Oncology (Los Angeles, 1986).
Address requests for reprints to: J.P. Sculier, Service de Médicine, Institut J. Bordet, 1 rue Héger Bordet, 1000 Bruxelles, Belgium.
This work was supported in part by the 'Fondation Lefèvre' (Belgium), the Squibb Company (U.S.A.) and the 'Fonds National de la Recherche Scientifique' (Belgium).

the intravenous route [8, 9]. Tolerance by the patients was excellent, even when large volumes of liposomes were given. As amphotericin B, an amphiphilic drug, was successfully entrapped in this type of liposomes, we performed a pilot (phase I) study in cancer patients with suspected fungal complications in order to evaluate the toxicity and the pharmacology of that preparation (ampholiposomes).

METHODS

Patient selection

Patients were eligible for the study if they had a microscopically proven solid or hematological malignancy and suspected or documented invasive fungal infection.

The diagnosis of microbiologically documented fungal infections required the presence of clinical signs of infection plus positive cultures from the suspected site of a fungemia. In case of lung aspgillosis, a radiological infiltrate and a positive culture of the sputum, obtained by tracheal aspiration or bronchoalveolar lavage (BAL), were necessary. Fungemia had to be demonstrated by several positive blood cultures. The disease was considered as a 'suspected' fungal infection in the case of an obvious site of a likely infection, clinically progressive and consistent with a mycotic disease, in spite of the lack of a microbiological proof of a fungal agent.

Poor tolerance to prior treatment of the infection with Fungizone® was the main criterion for inclusion in this pilot study.

Adequate cardiac, renal (serum creatinine <3 mg/dl) and hepatic (serum bilirubin <3 mg/dl) functions were required. Patients had to give informed consent prior to starting treatment with ampholiposomes. The investigation was conducted according to a protocol that had been accepted by the human research committee of the hospital.

Preparation of liposomes containing amphotericin B (ampholiposomes)

Deoxycholate-free amphotericin B (AmB) was provided by Squibb Co. (U.S.A.). Egg phosphatidylcholine (PC) was purchased from Sigma Chemical Co. (St Louis, MO); cholesterol (CH) from BDH Chemicals, Ltd (Poole, U.K.); stearylamine (SA) from Aldrich Europe (Beerse, Belgium) and Limulus amebocyte lysate, from M.A. Bioproducts (Walkersville, MD). All other chemicals were reagent grade.

Liposomes were freshly and pyrogen-free prepared in a sterile fashion on the day of the experiment. For the first two patients, the following procedure was used: egg PC, CH and SA at molar ratio of 4:3:1 were dissolved in chloroform:methanol at 2:1 (vol:vol) and after mixing 0.52 mM of AmB dissolved in methanol, the lipids were dried by evaporation under vacuum. One milliliter of 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl was added for each 20 mg of dried lipids. The mixture was shaken vigorously until suspension of the lipid film was complete and then the suspension was sonicated for 15 mm at 140 W, under an N_2 atmosphere, with a Branson model B-30 sonifier (Branson Sonic Power Co, Danbury, CT).

Undispersed lipids, free AmB and titanium particles were removed by centrifugation at 2000 ***g*** for 30 minutes. Liposomes were recovered in the supernatant. AmB concentration in liposomes was measured at 405 nm by optical density or by HPLC determination as a methanol extraction of the liposomes [10]. For the other patients, empty liposomes of the same lipid composition were sonicated on powdered AmB (0.52 mM per ml of liposomal preparation) during 30 min at 140 W, under an N_2 atmosphere.

Concentration of amphotericin B entrapped in liposomes ranged from 163 to 396 μg/ml of liposomes with a mean value of 297 μg/ml. Hydrodynamic diameters of the liposomes were measured by dynamic laser light scattering (Nicomp Model 370, Pacific Scientific, Silver Spring, MD); whatever the type of preparation, the majority of the vesicles had a diameter of about 60 nm and there was a small proportion of larger size liposomes around 250 nm. The sterility of the preparations was verified by a culture on blood agar and Sabouraud's dextrose agar; the absence of endotoxin was confirmed by the Limulus assay [11].

Administration of ampholiposomes

Initial work up consisted of clinical examination, blood tests (blood cell counts, serum glucose, electrolytes, magnesium, urea, creatinine, uric acid, bilirubin, hepatic and pancreatic enzymes, lipids, creatine phosphokinase; blood coagulation tests including fibrinogen degradation products and antithrombin III), urine analysis, ECG, fungal serology and chest X-ray. Blood, urine and sputum cultures as well as nose, throat, rectal and vaginal swabs were performed before starting therapy. If clinically indicated, arterial blood gas and pH were monitored. Other usually investigations such as CT scan or echography were performed as clinically indicated.

Patients were hospitalized in the medical intensive care unit; the ECG was monitored and if indicated, a central venous line was inserted. The initial dose of AmB given to the first patient was 0.4 mg/kg. The subsequent daily doses and the intervals between two infusions of ampholiposomes were decided according to the level of AmB obtained in the serum of the patient. The liposomal preparation was administered intravenously by an

Isoflux perfusion system (Van Leer Medical, Isigny, France) at a rate of 100 drops per min (about 7 ml per min).

Blood tests, urine analysis, ECG were repeated daily during therapy. Chest X-rays and cultures were performed at least twice weekly. After the end of treatment, patients were periodically assessed for possible late side-effects and relapse of fungal disease.

Treatment evaluation

Although this was not the purpose of this phase I study, the response to therapy was assessed for a few patients whenever possible. Improvement was defined as fungal cultures became negative, fever disappeared as well as all clinical and radiological signs of active infection. Failure was defined as the persistence of infection or death from fungal infection. Patients were classified as not evaluable when they died during liposomal therapy, from complications unrelated to the fungal infection and its treatment. Side-effects were related to the liposomal treatment if there was no other clear explanation to their cause.

Pharmacokinetic studies

Serum concentrations of phospholipids and amphotericin B were determined in all patients, except for the first one, at least just before (trough) and at the end (peak) of the ampholiposome infusion. In the majority of the patients blood was obtained at various intervals during the 24–48 h following the start of the infusion. Time 0 was defined as the time when the infusion started. Half-lifes were graphically measured; other pharmacokinetic parameters, i.e. the area under the plasma concentration–time curve from zero to infinity (AUC_0^∞), the total body clearance and the distribution volume, were calculated by standard methods [12].

Amphotericin B concentration was determined by high-performance liquid chromatography at 405 nm after precipitation of the serum proteins with methanol. Serum phospholipids were measured as inorganic phosphorus by the method of Fiske and Subbarow [13], after extraction of the blood samples in chloroform:methanol and subsequent mineralization.

Microbiological investigations

Serum amphotericin B concentration was also measured by a bioassay using *Paecilomyces variotti* [14]. Serum fungicidal activity was determined as previously described [15] against three test organisms: *Candida albicans* (ATCC 28516), *Torulopsis glabrata* (clinical isolate from patient 4) and *Aspergillus fumigatus* (clinical isolate from patient 7). Isolations and identification of fungi in patients were performed according to standard routine laboratory methods.

Statistical methods

Association between antifungal activities and HPLC serum level of amphotericin B was performed by one way ANOVA (our purpose is to test main effects and not to take into account any interaction). The assumptions of the ANOVA (homogeneity of variance and normality of data) were successively tested with Bartlett's test and chi-square goodness of fit tests. If homogeneity of variance could not be met, a Box–Cox transformation of data was done (square roots of data in our case). For analysis of the ampholiposome fungistatic activities against *Candida albicans*, a Box–Cox or other transformation could not approach the equal variance assumption. Thus for this particular set of data the assumptions of ANOVA could not reasonably be met and a Kruskal–Wallis non-parametric test was used. *A posteriori* tests were performed on the main effect by using the extended Tukey test (Duncan's test gave the same results in our case): Table 6 displays where significance was *not* present. The methodology of this analysis was based on Godfrey [16] and Sokal and Rolhf [17].

RESULTS

Patient characteristics

Fifteen patients were included in the present phase I pilot study. All had malignant disease: acute leukemia (9), lymphoma (1) or solid tumors (5). Sex, age, weight, body surface, antineoplastic treatment, other associated therapies, granulocytes count and serum creatinine level on day 1 of ampholiposome therapy are described in Table 1. Six patients were severely neutropenic (<500 granulocytes/mm^3) and four presented with moderate renal failure (serum creatinine between 1.6 and 2 mg/100 ml).

Documented fungal diseases (Table 2) were lung aspergillosis (7) and fungemia (5), of which four were related to a central venous catheter. Three patients had suspected fungal diseases; two were considered to be caused by *Candida albicans*. Patient 8 presented with a relapsing brain abscess that was previously shown at surgery to be caused by *Aspergillus* spp. Twelve of the patients had received, prior to ampholiposomes, Fungizone®, all with severe side-effects such as fever, chills and/or dyspnea which precluded further administration of this antifungal therapy.

Ampholiposomes treatment: description, tolerance and outcome

The first patient to whom ampholiposomes were administered had a disseminated candidiasis, severe neutropenia and respiratory failure requiring arti-

Table 1. Patient characteristics at start of ampholiposomes

Patient No.	Sex	Age (years)	Weight (kg)	Body surface (m^2)	Neoplastic disease	Antineoplastic treatment	Associated treatment	Granulocyte count (cells/mm^3)	Serum creatinine (mg/100 ml)
1	F	28	50	1.6	CML in blastic crisis, furosemide, AV	Ara C	Vancomycin, erythromycin ceftazidime, cotrimoxazole	<100	0.9
2	F	30	55	1.66	Hodgkin's disease	Ara C	Cotrimoxazole, dopamine, methylprednisolone, AV	5600	1.7
3	F	25	65	1.84	AML	Ara C + CPA + autologous BMT	Ceftazidime, amikacin	<100	1.2
4	F	71	54	1.67	Ovary cancer	Carboplatin	Cimetidine	5000	1.8
5	F	57	55	1.72	AML	Ara C + DNR + VCR	—	56,000	0.9
6	M	52	53	1.60	Larynx cancer	irradiation	—	10,100	1.0
7	M	50	65	1.86	AML	AMSA + Ara C	Heparin, ceftazidime	350	0.9
8	F	23	61	1.72	ALL	Ara C + CPA + TBI + allogeneic BMT	Methylprednisolone, isoniazid, cyclosporine A	2700	2.0
9	M	66	55	1.67	Head and neck cancer	—	Theophyllin	15,000	1.5
10	F	75	56	1.55	Cervix cancer	—	Teicoplanin, AV	20,000	1.5
11	M	39	65	1.78	CML in blastic crisis	Ara C + CPA + TBI + autologous BMT	Ceftazidime, amikacin	<100	1.1
12	F	32	53	1.52	AML allogeneic BMT	Ara C + CPA + TBI	Methylprednisolone, furosemide, timentin, ceftazidime, acyclovir, ranitidine, cyclosporine A, AV	700	1.0
13	M	62	36	1.48	NSCLC	6-aminochrysene	Ceftazidime, cotrimoxazole, rifampicin, isoniazide, ranitidine, AV	30,000	1.9
14	F	25	67	1.78	AML	Ara C + CPA + TBI + allogeneic BMT	Cotrimoxazole, metronidazole, methylprednisolone, ranitidine digoxine, timentin	<100	1.5
15	M	60	75	1.82	AML	Ara C + DNR + VCR	Ceftazidime, vancomycin, cimetidine	200	1.5

F = female; M = male; TBI = total body irradiation; NSCLC = non small cell lung cancer; CML = chronic myeloid leukemia; AML = acute myeloblastic leukemia; ALL = acute lymphoblastic leukemia; CPA = cyclophosphamide; BMT = bone marrow transplantation; DNR = daunorubicin; VCR = vincristine; AV = artificial ventilation.

ficial ventilation. She had a severe intolerance to Fungizone® and accepted to receive a dose of 0.4 mg/kg (20 mg) of amphotericin B incorporated in sonicated liposomes. No side-effects were observed; the patient died 5 days later from bacteremia. In the following patients the serum level of amphotericin B was monitored and the dosage as well as the interval between two consecutive administrations were decided according to the clinical tolerance and the serum concentration of amphotericin B. As described in Table 3, the total dose of ampholiposomes ranged from 20 to 1004 mg (mean: 472 mg). The number of infusions per patient ranged from 1 to 20 (mean: 8) and the duration of treatment from 1 to 29 days (mean: 10 days). A total of 117 infusions were given. The daily volume of liposomes administered ranged from 90 to 260 ml according to the dose of amphotericin B prescribed. Figures 1a, b and c show the profile of amphotericin B serum concentration in patients 5, 10, 12. In the last patients, a serum concentration between 5 and 20 μg/ml could be maintained by a daily dose of 0.5–1.8 mg/kg of ampholiposomes.

Tolerance to ampholiposomes was excellent. Patients who had presented severe side-effects with Fungizone® did not develop any with ampholiposomes. The most common complaint of the patients was a slight somnolence lasting a few hours after the end of the infusion. Hyperkalemia (1 case), mild hypokalemia (2), nausea and vomiting (3) and arthralgia (1) were observed after some infusions (Table 4) but there were always several other possible explanations for these manifestations since all these patients had an advanced neoplastic disease and multiple concurrent therapies. In patient 5 who received 60 mg of ampholiposomes over 10 min, acute lumbar pain with concomitant elevation of serum amylase and lipase levels was observed; spontaneous resolution occurred within 48 h. No sign of hemolysis was seen in the patients. We did not observe hypomagnesemia or renal insufficiency due to ampholiposomes; a rapid correction of an elevated serum creatinine or hypokalemia, which was the consequence of prior treatment with Fungi-

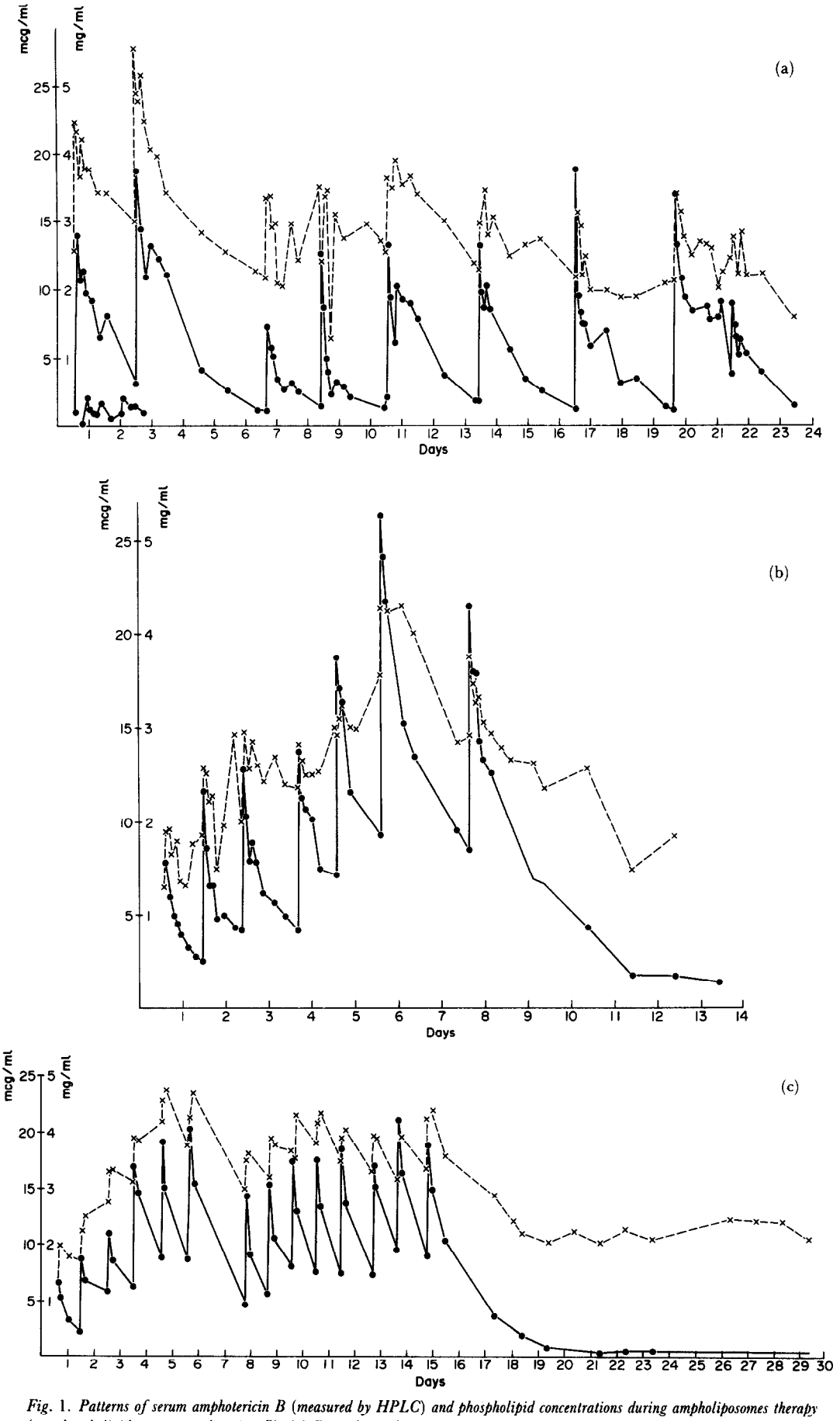

Fig. 1. *Patterns of serum amphotericin B (measured by HPLC) and phospholipid concentrations during ampholiposomes therapy (--- phospholipids; —— amphotericin B). (a) Data obtained in patient* 5, *who received the following total doses of ampholiposomes*: 80 mg (*days* 1 *and* 2), 40 mg (*days* 7 *and* 9), 50 mg (*days* 11, 14, 17 *and* 20), 25 mg (*day* 22). *The lower curve, near the basal level, represents the blood level obtained with* 1.2 mg/kg *of Fungizone® prior to onset of ampholiposomes. (b) Data in patient* 10, *who received the following total doses of ampholiposomes*: 60 mg (*days* 1, 2, 3 *and* 5), 100 mg (*day* 6) *and* 60 mg (*day* 8). *(c) Data in patient* 12, *who received a daily dose of* 60 mg *of ampholiposomes from day* 1 *to day* 15 *except on day* 7.

Table 2. Type of infections and clinical outcome

Patient No.	Type of infection	Pathogen isolated	Site of positive culture	Response of fungal diseases	Survival	Cause of death	Autopsy data
1	Fungemia	*Candida albicans*	Blood (5)†	Failure	5 days	Infection	Disseminated candidiasis
2	Suspected candidiasis	*Candida albicans*	Sputum	Not evaluable	10 days	Lung tumor	No fungal disease
3	Lung aspergillosis	*Aspergillus fumigatus*	BAL	Failure	11 days	Infection	CNS and lung aspergillosis
4	Fungemia*	*Torulopsis glabrata*	Blood (4)†	Improvement	4 weeks	Unknown	NA
5	Lung aspergillosis	*Aspergillus fumigatus*	Sputum	Improvement	10 months‡	—	—
6	Fungemia*	*Candida albicans*	Blood (6)†	Improvement	2 months‡	—	—
7	Lung aspergillosis	*Aspergillus fumigatus*	Sputum post BAL	Improvement	5 months	Leukemia	No fungal disease
8	Suspected CNS aspergillosis	—	—	Improvement	4½ months	Leukemia	NA
9	Fungemia*	*Candida albicans*	Blood (17)†	Not evaluable	3 days	Cardiac arythmias	No fungal disease
10	Lung aspergillosis	*Aspergillus fumigatus*	BAL	Not evaluable	13 days	ARDS	No fungal disease
11	Suspected esophagitis	*Candida albicans*	Mouth, stool	Improvement	2½ months	Leukemia	Lung aspergillosis
12	Lung aspergillosis	*Aspergillus fumigatus*	BAL	Improvement	2 months	Leukemia	NA
13	Lung aspergillosis	*Aspergillus fumigatus*	Sputum	Failure	3 days	Respiratory failure	Lung aspergillosis
14	Fungemia*	*Candida albicans*	Blood (13)†	Failure	9 days	CNS bleeding	Disseminated candidiasis and lung aspergillosis
15	Lung aspergillosis	*Aspergillus fumigatus*	BAL	Failure	8 days	Septic shock	Lung aspergillosis

CNS: central nervous system; BAL: bronchoalveolar lavage; ARDS: adult respiratory distress syndrome; NA: no autopsy.
*Central catheter related.
†Number of positive blood culture.
‡Still alive.

zone®, was seen as soon as ampholiposomes were started in two and four patients respectively. No cardiac, lung or CNS toxicity was documented during treatment and in the post-treatment follow-up.

Table 2 shows the response of fungal diseases to ampholiposomes and the outcome of the patients; three patients were not evaluable for response because of early death due to non-infectious causes. In the group of seven evaluable patients with lung aspergillosis, three improved; the patient with a suspected *Aspergillus* spp. brain abscess improved as well as another with fungemia. There were five failures: two fungemia and three lung aspergillosis. Autopsy was obtained in 10 patients. No fungal disease was seen in the three inevaluable cases and in patient 7, who died 5 months after treatment from a lung aspergillosis. One patient had disseminated candidiasis, four had aspergillosis, and the last one had both infections.

Pharmacokinetic analysis

All patients but one (patient 1) had, at least, a determination of peak and trough levels of amphotericin B in the serum. In some patients the assay was also performed after the administration of Fungizone® and prior to the outset of ampholiposomes therapy. As shown in Fig. 1, the serum peak level of amphotericin B after Fungizone® never exceeded 2.5 μg/ml and trough levels were lower than 1 μg/ml. On the other hand, with ampholiposomes, a peak concentration of more than 5 μg/ml was uniformly obtained; after a few infusions it was higher than 10 μg/ml. A single dose of ampholiposomes between 0.5 and 1.5 mg/kg was able to maintain serum levels of amphotericin B higher than 2 μg/ml during 3–4 days. There was a concomitant increase in blood phospholipid levels.

Figure 2 shows the semi-logarithmic profile of serum amphotericin B concentration after a single dose of ampholiposomes (1.2 mg/kg on day 1 in

Table 3. Treatment characteristics

Patient No.	Fungizone therapy prior to ampholiposomes Total dose (mg)	Ampholiposome treatment Total dose (mg)	Number of infusions	Duration of treatment (days)	Dose per infusion (number of infusions)
1	350	20	1	1	20 mg (1)
2	—	350	6	8	20 mg (1), 40 mg (1), 60 mg (1), 70 mg (1), 80 mg (2)
3	415	660	8	8	80 mg (7), 100 mg (1)
4	120	380	6	7	60 mg (5), 80 mg (1)
5	330	465	9	22	25 mg (1), 40 mg (2), 50 mg (4), 80 mg (2)
6	95	480	9	12	35 mg (3), 50 mg (2), 60 mg (1), 70 mg (2), 75 mg (1)
7	1080	555	10	12	25 mg (1), 40 mg (1), 50 mg (3), 60 mg (3), 80 mg (2)
8	1135	1004	20	29	24 mg (1), 30 mg (1), 40 mg (3), 50 mg (8), 60 mg (6), 70 mg (1)
9	231	226	3	3	66 mg (1), 80 mg (2)
10	—	480	7	8	60 mg (5), 80 mg (1), 100 mg (1)
11	88	616	7	7	88 mg (7)
12	—	840	14	15	60 mg (14)
13	40	80	2	2	40 mg (2)
14	280	380	8	8	40 mg (5), 50 mg (2), 80 mg (1)
15	150	540	7	8	60 mg (3), 90 mg (4)

Table 4. Side-effects of ampholiposomes

Type of side-effects	Number of patients	Comments
Slight somnolence	6	Patients 3, 4, 5, 6, 7, 15
Hyperkalemia	1	Patient 2 on days 1 and 2
Hypokalemia	2	Patients 12 and 15 during all the treatment (serum K did not fall below 2.5 mmol/l)
Nausea and vomiting	3	Patients 2, 5 and 12, respectively during 3, 2, and 1 days
Arthralgia	1	Patient 5 after infusions 1, 2 and 4
Lumbar pain (pancreatitis)	1	Patient 4 during infusion 1

Table 5. Pharmacokinetic parameters calculated for the first infusion of ampholiposomes

Patient No.	Amphotericin dose given (mg/kg)	$T_{\frac{1}{2}}\beta$ (h)	β (/h)	AUC_0^∞ (μg./h/ml)	Total clearance (ml/h/kg)	Distribution volume (ml/kg)
4	1.1	34	0.02	160	6.9	345
5	1.45	67	0.01	1120	1.3	130
6	1.3	11.5	0.06	150	8.6	144
7	1.2	20.5	0.033	307	3.9	130
8	1.2	23.5	0.03	287	4.2	140
9	1.2	15	0.046	126	9.5	207
10	1.1	19	0.036	153	7.2	200
11	1.4	24.5	0.028	182	7.6	272
15	1.2	13.5	0.05	186	6.5	126
Mean ± σ	1.2 ± 0.1	25.3 ± 16	0.044 ± 0.022	297 ± 296	6.2 ± 2.4	188 ± 73

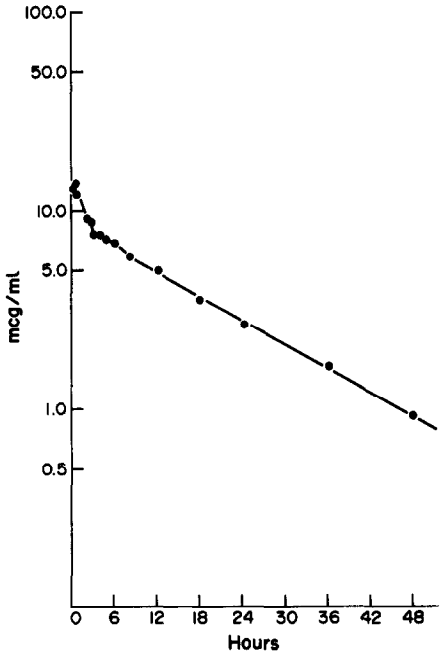

Fig. 2. Semi-logarithmic plot of the blood amphotericin B concentration after a single dose of ampholiposomes (90 mg *on day* 1 *in patient* 15).

patient 15); it has a bi-exponential pattern: the first phase (α) has a short half-life (42 min) while the second (β) has a much longer half-life (810 min). Calculated AUC_0^∞, total clearance and distribution volume were respectively 186 μg, h/ml, 6.5 ml/h/kg and 126 ml/kg body wt. In practice the first phase is negligible. We have been able to calculate the pharmacokinetic parameters for the first infusion of ampholiposomes in nine patients (Table 5). For a mean given dose of amphotericin B of 1.2 mg/kg of body wt, the mean β half-life was 25.3 h, the calculated AUC_0^∞ 217 μg. h/ml, the mean total clearance 6.2 ml/h/kg and the mean distribution volume 188 ml/kg. In patient 12 (Fig. 1c), a third half-life (γ) of 15 days was observed after treatment completion and followed a β half-life of 32 h obtained with the last infusion of ampholiposomes.

Microbiological investigations

Serum amphotericin B concentration was measured in eight patients by a bioassay using *Paecilomyces variotti*. As shown in Fig. 3 for patient 8 (177 samples), there was a good linear relationship between the values obtained with the HPLC method and those obtained with the bioassay as shown by linear regression ($r^2 = 0.897$).

Serum fungicidal and fungistatic activities were determined on 771 samples against three test organisms (Table 6). Increased antifungal activity was observed when the serum level of amphotericin B measured by HPLC was high. This observation was statistically significant as evaluated by ANOVA or the Kruskal–Wallis non-parametric test and confirmed by the Scheffe, least square difference modi-

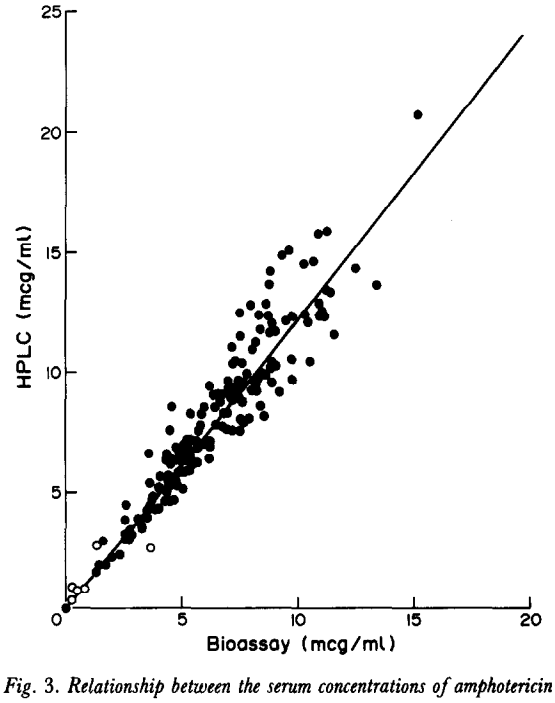

Fig. 3. Relationship between the serum concentrations of amphotericin B as measured by HPLC and those obtained by a bioassay in patient 8. (●) *Ampholiposomes*; *O Fungizone*®).

Table 6. *Relation between amphotericin B serum level and serum fungicidal and fungistatic activities*

		Sample No.	Fungizone® <1/2	1/2	1/4	1/8	*P** value (ANOVA)
Fungistatic activity	$X \pm \sigma$	53	0.51 ± 0.63	0.41 ± 0.14	1.16 ± 0.45	1.88 ± 0.48	<0.0001
Candida albicans	*n*		16	4	25	8	
Torulopsis glabrata	$X \pm \sigma$	51	0.59 ± 0.45	1.24 ± 0.34	1.90 ± 0.43		<0.0001
	n		30	10	12		
Aspergillus fumigatus	$X \pm \sigma$	52	0.65 ± 0.51	1.44 ± 0.53	1.90 ± 0.41		<0.0001
	n		32	13	7		
Fungicidal activity	$X \pm \sigma$	53	≤1/2		1.94 ± 0.51		<0.0001
Candida albicans	*n*		0.90 ± 0.62		6		
			47				
Torulopsis glabrata	$X \pm \sigma$	52	1.02 ± 0.69				NA
	n		52				
Aspergillus fumigatus	$X \pm \sigma$	52	1.02 ± 0.69				NA
	n		52				

		Sample No.	Ampholiposomes <1/2	1/2	1/4	1/8	1/16	1/32	1/64	*P** value (Kruskal–Wallis test)
Fungistatic activity	$X \pm \sigma$	463	1.84 ± 2.45	0.81 ± 0.62	3.27 ± 2.22	4.76 ± 2.54	7.94 ± 3.03	11.09 ± 3.33	18.43 ± 5.76	<0.0001
Candida albicans	*n*		25	16	70	114	138	95	5	
Torulopsis glabrata	$X \pm \sigma$	462	2.93 ± 3.29	2.27 ± 1.58	5.11 ± 3.30	7.63 ±3.13	10.83 ± 3.46	15.45 ± 3.38		<0.0001
	n		69	33	121	139	93	7		
Aspergillus fumigatus	$X \pm \sigma$	464	2.55 ± 2.72	4.06 ± 3.19	5.93 ± 3.54	9.04 ± 3.32	12.42 ± 3.18	18.72 ± 2.91		<0.0001
	n		66	70	155	131	40	2		
Fungicidal activity	$X \pm \sigma$	459	≤1/2		6.79 ± 3.62	9.24 ± 3.03	12.86 ± 3.31	20.78		<0.0001
Candida albicans	*n*		4.41 ± 4.04		131	108	19	1		
			200							
Torulopsis glabrata	$X \pm \sigma$	458	5.34 ± 4.09		9.44 ± 3.24	14.24 ± 3.26				<0.0001
	n		325		125	8				
Aspergillus fumigatus	$X \pm \sigma$	459	6.31 ± 4.34		9.89 ± 2.91	12.91 ± 6.95				<0.0001
	n		422		34	3				

n = number of samples; $X \pm \sigma$ = mean serum concentration ± standard deviation (μg/ml); NA = not applicable because all tested samples had a fungicidal activity <½.

fied or Wilcoxon tests. Only *P* values obtained with ANOVA (Fungizone®) or the Kruskal–Wallis test (ampholiposomes) are shown in Table 6. By increasing the serum level of amphotericin B, ampholiposome administration resulted in higher serum antifungal activities.

DISCUSSION

Previous attempts to decrease the toxicity of Fungizone®, the colloidal preparation of amphotericin B, have been unsuccessful. The clinical investigation of a semi-synthetic amphotericin B methylester, with promising antifungal activity, had to be stopped, because of the delayed occurrence in some treated patients of a lethal leukoencephalopathy [18]. In the present report, intravenous administration of amphotericin B entrapped in sonicated liposomes made of egg phosphatidylcholine, cholesterol and stearylamine in a molar ratio 4:3:1 was found to be outstandingly well tolerated by otherwise severely ill and often neutropenic patients. Although many of the patients had developed severe side-effects with Fungizone®, none of those reactions such as fever, chills or renal function impairment [2, 6] were observed during ampholiposomes treatment. No limiting toxicity was reached with the dosages used in this study.

The most frequent side-effect was transient and slight somnolence; this might have been related to the liposomes because we have also observed dizziness when we administered an hydrophobic

cytostatic agent incorporated in liposomes of the same composition [8, 9]. No late neurological toxicity was observed and there was no delayed impairment of renal function. One patient, who received a very rapid infusion, had symptoms and transient activity of serum enzymes consistent with an acute pancreatitis. However in each patient, amphotericin B given as ampholiposomes could be given much more rapidly than when administered as Fungizone® (30 min to 1 h compared to 4–6 h). We observed one case of hyperkalemia, a complication reported with Fungizone® given as a rapid infusion [19]. Other possible side-effects were difficult to interpret because our patients had many medical problems and were receiving multiple drugs. Lopez-Berestein *et al.* [20], using the intravenous administration of amphotericin B incorporated in multilamellar liposomes made of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol in a 7:3 molar ratio, also reported a good tolerance to their treatments. In a study on peritoneal transport performed in rabbits, it has been shown that amphotericin B tended to raise ultrafiltration while deoxycholate induced peritoneal irritation and raised clearances of urea, potassium, phosphate and dextrose without affecting ultrafiltration [21]. These observations suggest that some of the effects of Fungizone® might not be due to amphotericin B itself but rather to the galenic preparation. It should be emphasized here that the type of liposomal vector could have a critical impact on the properties of the drug. Chemical composition, charge, structure and mode of preparation are all variables that can modify the physico-chemical, biological and pharmacological properties of the liposomes and thus of the carried drug [22, 23]. Administration of amphotericin B entrapped in liposomes of another composition might therefore result in values of pharmacokinetic and tissue distribution parameters different from those obtained in the present study.

Although difficult to assess in some patients, significant clinical improvement was observed in several patients, particularly those with aspergillosis; moreover all fungemias were eradicated by the ampholiposomes. However, these observations do not prove that ampholiposomes are superior therapeutically to Fungizone®, at least, they suggest clinical effectiveness. Other types of uni- or multilamellar liposomes incorporating amphotericin B [24–29] have been shown to be superior to Fungizone® in experimental models of cryptococcosis, histoplasmosis or candidiasis in non-neutropenic or neutropenic mice. A preliminary clinical study with multilamellar liposomes by Lopez-Berestein *et al.* has also suggested superior antifungal activity [20].

Compared to Fungizone®, the ampholiposomes used in this study are characterized by pharmacokinetic properties suggesting a higher therapeutic activity in some clinical conditions. Their administration resulted in prolonged and higher amphotericin B serum level; the peak and trough concentrations, obtained with daily ampholiposomes infusions (1.0–1.5 mg/kg of amphotericin B), were respectively 10–20 μg/ml and 5–10 μg/ml while with Fungizone® (0.6 mg/kg/day or 1.2 mg/kg/2 day) serum peak levels of amphotericin B did not exceed 2 μg/ml and trough levels were lower than 1μg/ml [14]. It is difficult to extensively compare the pharmacokinetic parameters of amphotericin B given as ampholiposomes to those of Fungizone® because information about the pharmacokinetics of Fungizone® has not been so far extensively studied. In the best available study where two patients have been investigated after receiving their last dose of Fungizone® [30], the distribution of the drug was described by a three-compartment model. Elimination half-life of amphotericin B for both patients were 14 and 16.5 days. This value is similar to the half-life observed for patient 12 with ampholiposomes (15 days). This observation suggests that the elimination of amphotericin B could occur at a similar rate for both types of preparation.

The potential biological value of the high amphotericin B levels obtained after the administration of ampholiposomes is suggested by the good linear regression between the HPLC determination and the bioassay dosage. It is also supported by the demonstration of a measurable serum antifungal activity after the administration of ampholiposomes and in relationship with the elevated levels of amphotericin B. However, it remains to be shown whether the latter test [15] has a clinically significant predictive value for the response to a treatment with antifungal agents, as it is the case for the bactericidal serum titration [31].

In conclusion, ampholiposomes have been shown to have a better therapeutic index than Fungizone®, because of their better tolerance and possibly increased antifungal activity which seems to be related to higher serum concentrations. However, the clinical effectiveness of ampholiposomes in the management of fungal diseases remains to be established in controlled studies. As no limiting toxicity has yet been demonstrated, increasing the daily dose of this type of ampholiposomes and/or reducing interval between the courses should also be further investigated. The role of ampholiposomes in the treatment of other conditions such as macrophage disorders [32], parasitic diseases [33] or cancer [34] remains also to be investigated.

Acknowledgements—We are pleased to acknowledge Drs. D. Bron and L. Debusscher for providing patient management and D. Daneau, P. Mommen, A. Devenyn, L. Themelin, N. Verboven and all the staff of the medical ICU for their assistance.

REFERENCES

1. Gold JWM. Opportunistic fungal infections in patients with neoplastic disease. In: Brown AE, Armstrong D, eds. *Infectious Complications of Neoplastic Disease. Controversies in Management.* New York, Yorke Medical Books, 1985, 111–121.
2. Medoff G, Kobayashi GS. Strategies in the treatment of systemic fungal infections. *N Engl J Med* 1980, **302**, 145–155.
3. De Gregorio MW, Lee WMF, Linker CA, Jacobs RA, Ries CA. Fungal infections in patients with acute leukemia. *Am J Med* 1982, **73**, 543–548.
4. Aisner J, Schimpff SC, Wiernik PH. Treatment of invasive aspergillosis: relation of early diagnosis and treatment to response. *Ann Intern Med* 1977, **86**, 539–543.
5. Meunier-Carpentier F, Kiehn TE, Armstrong D. Fungemia in the immunocompromised host. Changing patterns, antigenemia, high mortality. *Am J Med* 1981, **71**, 363–370.
6. Kucers A, McBennett N. The use of antibiotics. In: *A Comprehensive Review with Clinical Emphasis.* London, Heinemann, 1979, 865–884.
7. Maddux MS, Barriere SL. A review of complications of amphotericin B therapy: recommendations for prevention and management. *Drug Intel Clin Pharm* 1980, **14**, 177–181.
8. Coune A, Sculier JP, Frühling J *et al.* Iv administration of a water-insoluble antimitotic compound entrapped in liposomes. Preliminary report on infusion of large volumes of liposomes to man. *Cancer Treat Rep* 1983, **67**, 1031–1033.
9. Sculier JP, Coune A, Brassinne C *et al.* Intravenous infusion of high doses of liposomes containing NSC 251635, a water-insoluble cytostatic agent. A pilot study with pharmacokinetic data. *J. Clin Oncl* 1986, **4**, 789–797.
10. Brassinne C, Laduron C, Coune A *et al.* High-performance liquid chromatographic determination of amphotericin B in human serum. *J Chromatogr* 1987, **419**, 401–407.
11. Cooper JF, Hochstein HD, Seligmann EB Jr. The limulus test for endotoxin (pyrogen) in radiopharmaceutical biological. *Bull Parent Drugs Assoc* 1972, **26**, 153–162.
12. Labaume JP. *Pharmacocinétique, Principes Fondamentaux.* Paris, Masson, 1984.
13. Fiske CH, Subbarow Y. Colorimetric determination of phosphorus. *J Biol Chem* 1925, **66**, 375–400.
14. Bindschalder DD, Bennett JE. A pharmacologic guide to the clinical use of amphotericin B. *J Infect Dis* 1969, **120**, 427–436.
15. Meunier F. Serum fungistatic and fungicidal activity in volunteers receiving antifungal agents. *Eur J Clin Microbiol* 1986, **5**, 103–109.
16. Godfrey K. Comparing the mean of several groups. *N Engl J Med* 1985, **313**, 1450–1456.
17. Sokal RR, Rohlf FJ. *Biometry.* San Francisco, W.H. Freeman, 1981.
18. Ellis WG, Sobel RA, Nielsen SL. Leukoencephalopathy in patients treated with amphotericin B methylester. *J Infect Dis* 1982, **146**, 125–137.
19. Craven PC, Gremillion DH. Risk factors of ventricular fibrillation during rapid amphotericin B infusion. *Antimicrob Agents Chemother* 1985, **27**, 868–871.
20. Lopez-Berestein G, Fainstein V, Hopfer R *et al.* Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer, a preliminary study. *J Infect Dis* 1985, **151**, 704–710.
21. Maher JF, Hirzel P, Chakrabarti E, Bennett RR. Contrasting effects of amphotericin B and the solvent sodium deoxycholate on peritoneal transport. *Nephron* 1986, **43**, 38–42.
22. Witzke NM, Bittman R. Dissociation kinetics and equilibrium binding properties of polyene antibiotic complexes with phosphatidylcholine/sterol vesicles. *Biochemistry* 1984, **23**, 1668–1674.
23. Hopfer RL, Mills K, Mehta R, Lopez-Berestein G, Fainstein V, Juliano RL. *In vitro* antifungal activities of amphotericin B and liposome-encapsulated amphotericin B. *Antimicrob Agents Chemother* 1984, **25**, 387–389.
24. Tremblay C, Barza M, Fiore C, Szoka F. Efficacy of liposome intercalated amphotericin B in the treatment of systemic candidiasis in mice. *Antimicrob Agents Chemother* 1984, **26**, 170–173.
25. Graybill JR, Craven PC, Taylor RL, Williams DM, Magee WE. Treatment of murine cryptococcosis with liposome-associated amphotericin B. *J Infect Dis* 1982, **145**, 748–752.
26. Taylor RL, Williams DM, Craven PC, Graybill JR, Drutz DJ, Magee WE. Amphotericin B in liposomes, a nasal therapy for histoplasmosis. *Am Rev Resp Dis* 1982, **125**, 610–611.
27. Ahrens J, Graybill JR, Craven PC, Taylor RL. Treatment of experimental murine candidiasis with liposomes-associated amphotericin B. *Sabouradia* 1984, **22**, 163–166.
28. Lopez-Berestein G, McQueen T, Mehta K. Protective effect of liposomal-amphotericin B against *C. albicans* infection in mice. *Cancer Drug Delivery* 1985, **2**, 183–189.
29. Lopez-Berestein G, Hopfer RL, Mehta R, Mehta K, Hersh EM, Juliano RL. Liposome-encapsulated amphotericin B for treatment of disseminated candidiasis in neutropenic mice. *J Infect Dis* 1984, **150**, 278–283.
30. Atkinson AJ Jr, Bennett JE. Amphotericin B pharmacokinetics in humans. *Antimicrob Agents Chemother* 1978, **13**, 271–276.
31. Sculier JP, Klastersky J. Significance of the serum bactericidal test in Gram-negative bacillary bacteremia in patients with and without granulocytopenia. *Am J Med* 1984, **76**, 429–435.

32. Lin HS, Medoff G, Kobayashi GS. Effects of amphotericin B on macrophages and their precursor cells. *Antimicrob Agents Chemother* 1977, **11**, 154–160.
33. New RRC, Chance ML, Heath S. Antileishmanial activity of amphotericin and other antifungal agents entrapped in liposomes. *J Antimicrob Chemother* 1981, **8**, 371–381.
34. Schiffman FJ, Klein I. Rapid induction of amphotericin B sensitivity in L1210 leukaemia cells by liposomes containing ergosterol. *Nature* 1977, **269**, 65–66.