COMMUNICATION

Assessing the Antifungal Activity and Toxicity Profile of Amphotericin B Lipid Complex (ABLC; Abelcet®) in Combination with Caspofungin in Experimental Systemic Aspergillosis

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ABSTRACT: The purpose of this study was to assess the antifungal activity and renal and hepatic toxicity of amphotericin B lipid complex (ABLC; Abelcet®) following co-administration of Caspofungin to rats infected with *Aspergillus fumigatus*. *Aspergillus fumigatus* inoculum ($1.3–2.3 \times 10^7$ colony forming units [CFU]) was injected via the jugular vein; 48 h later male albino Sprague–Dawley rats (350–400 g) were administered either a single intravenous (IV) dose of Fungizone® (1 mg AmpB/kg), ABLC (1 or 5 mg AmpB/kg), or an equivalent volume of normal saline (NS) (vehicle control) once daily for 4 days. Rats were further randomized into groups to receive 3 mg/kg Caspofungin or physiologic saline IV once daily for 4 days. To assess antifungal activity, brain, lung, heart, liver, spleen, and kidney sections were homogenized with NS (2 mL; 1 g of each tissue/mL) and a 0.1-mL aliquot was spread plated onto a Sabouraud dextrose agar plate. The plates were incubated for 48 h at 37°C, at which time the numbers of CFU were determined and corrected for tissue weight. To assess renal and hepatic toxicity, serum creatinine and aspartate aminotransferase levels were determined. Fungizone and ABLC at a dosing regimen of 1 mg/kg i.v. once daily for four consecutive days and Caspofungin at a dosing regimen of 3 mg/kg i.v. once daily for four consecutive days had similar effectiveness in decreasing the total number of *Aspergillus fumigatus* CFUs found in all organs analyzed compared to non-treated controls. A combination of ABLC (1 mg/kg i.v. × 4 days) and Caspofungin (3 mg/kg i.v. × 4 days) significantly decreased the total number of *Aspergillus fumigatus* CFUs found in all organs analyzed compared to Caspofungin alone and non-treated controls. ABLC at a dosing regiment of 5 mg/kg i.v. once daily for four consecutive days was more effective in decreasing the total number of

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Aspergillus fumigatus CFUs found in all organs analyzed compared to Fungizone or ABLC alone at 1 mg/kg and Caspofungin alone at 3 mg/kg. However, a combination of ABLC (5 mg/kg i.v. × 4 days) and Caspofungin (3 mg/kg i.v. × 4 days) was not more effective than ABLC at 5 mg/kg or the combination of ABLC at 1 mg/kg and Caspofungin 3 mg/kg in reducing the total number of *Aspergillus fumigatus* CFUs compared to controls. Except for non-treated infected control rats, none of the treatment groups tested displayed a greater than 50% increase in serum creatinine concentrations from baseline. In addition, only ABLC at a dosing regimen of 1 mg/kg i.v. once daily for four consecutive days displayed a greater than 50% increase in AST concentration from baseline. Taken together, these findings suggest that ABLC at 5 mg/kg once daily × 4 days appears to be the best therapeutic choice in this animal model. J Pharm Sci 93:1382–1389, 2004

Keywords: amphotericin B lipid complex; caspofungin; *Aspergillus fumigatus*; rats

INTRODUCTION

Disseminated fungal infections such as candidiasis, histoplasmosis, and aspergillosis are on the rise in organ transplant recipients, diabetics and patients with cancer or AIDS.[3,7,10–12,14,18–20,26,29] In these patients, invasive fungal infections may account for as many as 30% of deaths.[7,19,31,41] Despite the development of a number of new antifungal agents,[11,20,22,27] amphotericin B (AmpB) formulated as a micelle suspension, remains an effective agent in the treatment of systemic fungal infections.[21,27,30,37] However, AmpB use is often limited by the development of kidney toxicity manifested by renal vasoconstriction with a significant decrease in glomerular filtration rate and renal plasma flow and by renal potassium and magnesium wasting.[8,14,21,27,30,39]

Incorporation of many drugs, including chemotherapeutic and antifungal agents, into liposomes minimizes toxicity without loss in pharmacological effect.[1,4,16,23,24,34,38] In addition, when AmpB was complexed with lipid to form amphotericin B lipid complex (ABLC) it was selectively taken up by mononuclear phagocytes and delivered principally to the liver and the lung.[17,22,23,33] Survival of mice infected with *Histoplasma capsulatum* was greater with ABLC than with AmpB treatment, in part due to higher concentrations of AmpB in liver and lung tissue.[33] Moreover, these animals were less toxic than infected mice administered equivalent amounts of AmpB. Recent studies by Bhamra et al. have suggested the very low levels of circulating protein-bound AmpB that they observed after administration of ABLC to rats was a result of rapid tissue uptake leading to reduced toxicity.[4]

Amphotericin B's mechanism of action has been postulated as interacting with the sterol component of fungal cells, ergosterol, which results in pores and channels within the fungal membrane. This results in leaky fungal cell membranes that can cause cell lysis and death. Caspofungin is a new echinocandin in the antifungal group of compounds that inhibits the biosynthesis of beta-(1,3)-D-glucan an essential component of fungal cell walls.[10,15,25,35] One study has reported that Caspofungin has been utilized as salvage therapy for refractory invasive aspergillosis with much success.[15] The use of Caspofungin with other antifungal agents is appealing due to the drug's distinct mechanism of action. Since both Amphotericin B and Caspofungin distinctly disrupt fungal cell membrane integrity and formation by different mechanisms, it has been hypothesized that a combination of these compounds may enhance their antifungal activity in an additive or synergistic manner without associated toxicity. Although some *in vitro*[3,10] and clinical[2,9,15,35] studies have already reported additive and/or synergistic antifungal activity, to date, few studies have investigated the effectiveness and renal and hepatic toxicity profile of liposomal amphotericin B (i.e., AmBisome) when co-administered with Caspofungin in a rat model of systemic *Aspergillus fumigatus*. In addition, no studies investigating the efficacy and toxicity of Caspofungin in combination with ABLC has been reported and only one case study has been cited.[18] Therefore, the purpose of this study was to assess the antifungal activity of ABLC; Abelcet® following co-administration of Caspofungin in experimental systemic aspergillosis. We hypothesize that co-administration of ABLC and Caspofungin will have an additive antifungal effect without observable toxicity.

MATERIALS AND METHODS

Aspergillus fumigatus isolated from a patient with disseminated aspergillosis (provided by the BC Centre for Disease Control, F1048) was used

to infect the rats. The inoculum was grown for 48 h at 37°C on Sabouraud dextrose agar. Spores were harvested from the agar using glass beads and suspended in pyrogen-free saline. Spore suspensions were standardized to 1% transmission at 540 nm (LKB Ultraspec II). The *Aspergillus fumigatus* inoculum ($1.3–2.3 \times 10^7$ colony-forming units [CFU]) was injected through the jugular vein of male albino Sprague Dawley rats (350–400 g). The jugular vein of the rat was cannulated by a similar method used for rabbits.[36] After 48-h post-aspergillus injection a single IV dose of either Fungizone® (1-mg AmpB/kg), ABLC (1 or 5 mg AmpB/kg), or an equivalent volume of normal saline (NS) (vehicle control) was administered once daily for 4 days. Rats were further randomized into groups to receive 3 mg/kg Caspofungin [dosage based on published manuscript by Petraitiene et al.[25]] or physiologic saline by IV once daily for 4 days (Table 1).

Fungizone® (purchased from Vancouver General Hospital Department of Pharmacy contains 50 mg of AmpB for every 41 mg of sodium deoxycholate) was reconstituted with sterile water. ABLC; Abelcet® was donated by Élan Pharmaceuticals. Serum creatinine and aspartate aminotransferase (AST) kits were purchased from Sigma Chemical Co. (St. Louis, MO) and Termo DNA Co. (Arlington, TX) respectively. Caspofungin was purchased from Merck Research Laboratories (NJ).

All of the animals used in the present study were cared for in accordance with the principles promulgated by the Canadian Council on Animal Care and the University of British Columbia. A total of 48 male albino Sprague–Dawley rats (weight range, 350–400 g; Charles River Canada, Montreal, Quebec, Canada) were housed in an animal facility with a 12-h dark–light cycle and controlled temperature and humidity. The availability of water and food (Purina rat chow) was unrestricted throughout the duration of the study. Renal function was measured by determining serum creatinine (SCr) concentrations prior to, 48 and 144 h after administration of the drugs or NS. For the purposes of this study, the criteria for measurable kidney toxicity was set as a 50% increase in serum creatinine concentration from baseline. Liver function was measured by determining serum AST concentrations prior to, 48 and 144 h after administration of the drugs or NS. For the purposes of this study the criteria for measurable liver toxicity was set as a 50% increase in serum AST concentration from baseline. To assess antifungal activity, brain, lung, heart, liver, spleen, and kidney sections (1 g of each tissue) were homogenized with NS (2 mL; concentration of 0.5 gram tissue/1 mL) (Heidolph diax 900) for 5 min on ice. Ten-fold serial dilutions of 0.1 mL homogenate were spread plated onto Saboraud dextrose agar plates and incubated for 48 h at 37°C. Surviving colonies of *A. fumigatus* were counted (CFU/mL homogenate, corrected for tissue weight).

The numbers of CFUs in tissues, serum creatinine concentration, and serum AST concentration prior to and following administration were compared between each treatment group by analysis of variance (INSTAT2; GraphPad Inc.). Critical differences were assessed by Tukey post hoc tests.[6] A difference was considered significant if the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$). All data were expressed as a mean ± standard error of the mean (SEM).

Table 1. Treatment Groups in the Study

Treatment Groups	N	Dosage
Normal saline + vehicle control	12	1 mL + 1 mL
Fungizone® + vehicle control	6	1 mg/kg + 1 mL
ABLC + vehicle control	6	1 mg/kg + 1 mL
ABLC + vehicle control	6	5 mg/kg + 1 mL
Normal saline + caspofungin	6	1 mL + 3 mg/kg
ABLC + caspofungin	6	1 mg/kg + 3 mg/kg
ABLC + caspofungin	6	5 mg/kg + 3 mg/kg

Prior to, 24, 48, and 144 h following ABLC or NS administration, serum samples were obtained for serum creatinine measurements as an indirect evaluation of renal function and for serum AST measurements as an indirect evaluation of hepatic function. Following the 144 h blood collection, each rat was sacrificed by injecting a single intraperitoneal dose of sodium pentobarbital (300 mg/kg); the kidneys, spleen, lung, liver, heart, and brain were removed, blotted dry, and weighed. The degree of antifungal activity was determined by measuring the number of colony forming units (CFU) of *Aspergillus fumigatus* in whole blood and all tissues.

RESULTS

Fungizone and ABLC at a dosing regimen of 1 mg/kg i.v. once daily for four consecutive days and Caspofungin at a dosing regiment of 3 mg/kg i.v. once daily for four consecutive days had similar effectiveness in decreasing the total number of *Aspergillus fumigatus* CFUs found in all organs analyzed compared to non-treated controls (Table 2). A combination of ABLC (1 mg/kg i.v. $\times$ 4 days) and Caspofungin (3 mg/kg i.v. $\times$ 4 days) significantly decreased the total number of *Aspergillus fumigatus* CFUs found in all organs analyzed compared to Caspofungin alone and non-treated controls (Table 2). ABLC at a dosing regimen of 5 mg/kg i.v. once daily for four consecutive days was more effective in decreasing the total number of *Aspergillus fumigatus* CFUs found in all organs analyzed compared to Fungizone and ABLC at 1 mg/kg and Caspofungin at 3 mg/kg. However, a combination of ABLC (5 mg/kg i.v. $\times$ 4 days) and Caspofungin (3 mg/kg i.v. $\times$ 4 days) was not more effective than ABLC at 5 mg/kg or the combination of ABLC at 1 mg/kg and Caspofungin 3 mg/kg in reducing the total number of *Aspergillus fumigatus* CFUs compared to controls (Table 2). Except for non-treated infected control rats, none of the treatment groups tested displayed a greater than 50% increase in serum creatinine concentrations from baseline (Table 3). In addition, only ABLC at a dosing regimen of 1 mg/kg i.v. once daily for four consecutive days displayed a greater than 50% increase in AST concentration from baseline (Table 4).

DISCUSSION

The use of Caspofungin as salvage therapy for invasive aspergillosis has resulted in a response rate of 40–50% among a variety of patient populations. The use of Caspofungin with other antifungal agents is appealing due to the drug's distinct mechanism of action. Since both Amphotericin B and Caspofungin distinctly disrupt fungal cell membrane integrity and formation by different mechanisms, it has been hypothesized that a combination of these compounds may enhance their antifungal activity in an additive or synergistic manner without associated toxicity. The purpose of this study was to assess the antifungal activity of ABLC; Abelcet® following co-administration of Caspofungin within rats infected with experimental Aspergillosis.

Previous studies, primarily within patients, have reported the benefits of caspofungin in combination with liposomal amphotericin (and not ABLC) as primary or salvage treatment of invasive aspergillosis in cancer [2,15] and pediatric patients.[9] However, few studies have investigated the effectiveness and the possibility of reduced toxicity of ABLC when co-administered with Caspofungin in a rat model of systemic *Aspergillus fumigatus*. Our investigation reports that a combination of ABLC at 1 mg/kg $\times$ 4 days and Caspofungin at 3 mg/kg $\times$ 4 days to have significantly greater antifungal activity than either Fungizone (1 mg/kg $\times$ 4 days), ABLC (1 mg/kg $\times$ 4 days), or Caspofungin (3 mg/kg $\times$ 4 days) alone. Based on our selected criteria for kidney toxicity as a 50% increase in SCR from baseline none of the treatment groups displayed measurable kidney toxicity (Table 3). We further report that ABLC at 5 mg/kg $\times$ 4 days appears to be the most effective antifungal single drug therapy group (Table 2) with no measurable renal toxicity (as determined by changes in serum creatinine levels; Table 3) or hepatic toxicity (as determined by changes in serum AST levels; Table 4). To our surprise, the combination of ABLC at 5 mg/kg $\times$ 4 days and Caspofungin at 3 mg/kg $\times$ 4 days only displayed greater antifungal activity than Caspofungin treatment alone and did not exhibit significantly greater antifungal activity than ABLC 5 mg/kg treatment alone. This combination had a greater increase in SCr from baseline (without altering hepatic toxicity) than ABLC 5 mg/kg or Caspofungin 3 mg/kg therapy alone. This result may be due to Caspofungin's ability to increase amphotericin B-induced renal toxicity. Ripeau et al. have recently reported that Caspofungin can increase the expression of *Candida albicans* secretory proteinase 5.[28] Secretory proteinases may selectively release active amphotericin B from the ABLC at sites of fungal infection in a similar way as fungal cell-derived phospholipases[32] resulting in more active amphotericin B available to not only cause damage to fungal cells but also to surrounding renal cells. Since a substantial percentage of Caspofungin following IV administration is recovered in the kidney[13] this explanation maybe possible. Further studies to test this explanation are required.

Our results further showed that ABLC at the higher dose (5 mg/kg) and in combination with Caspofungin had lower hepatic toxicity than ABLC at the lower dose (1 mg/kg) (Table 4). This

Table 2. Fungi Analysis of *Aspergillus fumigatus* Infected Male Sprague Dawley Rats Treated with Single Intravenous (IV) Doses of Normal Saline (NS) (Non-Treated Control), Fungizone (1 mg/kg × 4 days), ABLC (1 mg/kg × 4 days), ABLC (5 mg/kg × 4 days), Caspofungin (3 mg/kg × 4 days), ABLC (1 mg/kg × 4 days) + Caspofungin (3 mg/kg × 4 days), and ABLC (5 mg/kg × 4 days) + Caspofungin (3 mg/kg × 4 days)

	Infected Tissues (CFU/0.5 g of Homogenized Tissue)						
Treatment Groups	Brain	Lungs	Heart	Liver	Spleen	Kidney	All Organs
Non-treated controls (n = 12)	2595 ± 1050	811 ± 458	164 ± 59	682 ± 418	936 ± 292	156 ± 40	5343 ± 1515
Single drug therapy							
Fungizone (n = 6)	113 ± 56^{a}	30 ± 9^{a}	14 ± 4^{a}	149 ± 68^{a}	667 ± 510	20 ± 6^{a}	993 ± 632^{a}
ABLC 1 (n = 6)	468 ± 334^{a}	90 ± 65^{a}	24 ± 12^{a}	52 ± 14^{a}	232 ± 115^{a}	$70 \pm 17^{a,b}$	936 ± 346^{a}
ABLC 5 (n = 6)	364 ± 123^{a}	$8 \pm 2^{a,b,c}$	14 ± 4^{a}	$8 \pm 2^{a,b,c}$	$20 \pm 5^{a,b,c}$	$16 \pm 3^{a,c}$	$430 \pm 126^{a,c}$
Caspofungin 3 (n = 6)	$403 \pm 86^{a,b}$	$73 \pm 37^{a,d}$	$30 \pm 10^{a,b,d}$	$73 \pm 50^{a,d}$	$328 \pm 53^{a,d}$	$98 \pm 45^{b,d}$	$1003 \pm 55^{a,d}$
Combination therapy							
ABLC 1 + Caspofungin 3 (n = 6)	$342 \pm 162^{a,b}$	$10 \pm 6^{a,b,c,e}$	32 ± 15^{a}	$56 \pm 25^{a,b}$	$124 \pm 39^{a,d,e}$	34 ± 22^{a}	$598 \pm 140^{a,e}$
ABLC 5 + Caspofungin 3 (n = 6)	$504 \pm 151^{a,b}$	$8 \pm 2^{a,b,c,e}$	26 ± 11^{a}	$22 \pm 6^{a,b,c,d}$	$18 \pm 6^{a,b,c,e,f}$	$8 \pm 2^{a,b,c,d,e,f}$	$586 \pm 149^{a,e}$

[a] $p < 0.05$ vs. non-treated controls.
[b] $p < 0.05$ vs. fungizone alone.
[c] $p < 0.05$ vs. ABLC 1 mg/kg alone.
[d] $p < 0.05$ vs. ABLC 5 mg/kg alone.
[e] $p < 0.05$ vs. caspofungin alone.
[f] $p < 0.05$ vs. ABLC 1 mg/kg + caspofungin using PCANOVA.
All data are presented as mean ± SEM. All rats were infected with $1.1–2.3 \times 10^{7}$ colony forming units (CFU)/0.3 mL/rat of *Aspergillus fumigatus* prior to initiation of treatment.

Table 3. Serum Creatinine (SCr) Analysis of *Aspergillus fumigatus* Infected Male Sprague Dawley Rats Treated with Single IV Doses of NS (Non-Treated Control), Fungizone (1 mg/kg × 4 days), ABLC (1 mg/kg × 4 days), ABLC (5 mg/kg × 4 days), Caspofungin (3 mg/kg × 4 days), ABLC (1 mg/kg × 4 days) + Caspofungin (3 mg/kg × 4 days), and ABLC (5 mg/kg × 4 days) + Caspofungin (3 mg/kg × 4 days)

	SCr Concentration (mg/dL)			
Treatment Groups	Baseline[b]	48 h[c]	144 h[d]	% Change from Baseline[e] (%)
Non-treated controls (n = 6)	0.45 ± 0.12	0.40 ± 0.06	0.86 ± 0.40[a]	+91.1
Single drug therapy				
Fungizone (n = 6)	0.61 ± 0.20	0.94 ± 0.25[a]	0.62 ± 0.30	+1.6
ABLC 1 (n = 6)	0.65 ± 0.25	0.62 ± 0.15	0.41 ± 0.15	−36.9
ABLC 5 (n = 6)	0.74 ± 0.15	0.92 ± 0.20	0.64 ± 0.15	−13.5
Caspofungin 3 (n = 6)	0.60 ± 0.17	0.69 ± 0.12	0.43 ± 0.29	−28.3
Combination therapy				
ABLC 1 + Caspofungin 3 (n = 6)	0.79 ± 0.05	0.79 ± 0.10	1.05 ± 0.10[a]	+32.9
ABLC 5 + Caspofungin 3 (n = 6)	0.71 ± 0.18	0.48 ± 0.18	0.72 ± 0.09	+1.4

[a] $p < 0.05$ vs. Baseline using PCANOVA.
[b] Baseline-SCr levels prior to the rats being infected with *Aspergillus fumigatus*.
[c] 48 h-SCr levels 2 days after the rats were infected with *Aspergillus fumigatus* and prior to treatment.
[d] 144 h-SCr levels 2 days after the rats were infected with *Aspergillus fumigatus* following 4-days of treatment.
[e] % change in SCr levels between baseline and 144 h after the initiation of the infection.
All rats were infected with 1.1–2.3 × 10^7 colony forming units (CFU)/0.3 mL/rat of *Aspergillus fumigatus* prior to initiation of treatment. All data are presented as mean ± SEM.

may be due to several factors including: (a) the ability of ABLC at the higher dose to kill the fungal infection to a greater extent within the liver, thus reducing the associated hepatic toxicity and/or (b) the ability of Caspofungin to modify the liver concentration of ABLC. The presence of Caspofungin may reduce ABLC tissue concentration resulting in less ABLC-induced hepatic toxicity. Studies to confirm these explanations are on going.

In conclusion, this study assesses the antifungal activity and renal and hepatic toxicity of ABLC in combination with Caspofungin in Experimental

Table 4. Aspartate Aminotransferase (AST) Analysis of *Aspergillus fumigatus* Infected Male Sprague Dawley Rats Treated with Single IV Doses of Normal Daline (Non-Treated Control), Fungizone (1 mg/kg × 4 days), ABLC (1 mg/kg × 4 days), ABLC (5 mg/kg × 4 days), Caspofungin (3 mg/kg × 4 days), ABLC (1 mg/kg × 4 days) + Caspofungin (3 mg/kg × 4 days), and ABLC (5 mg/kg × 4 days) + Caspofungin (3 mg/kg × 4 days)

	AST Concentration (U/L)			
Treatment Groups	Baseline[b]	48 h[c]	144 h[d]	% Change from Baseline[e] (%)
Non-treated controls (n = 6)	61.6 ± 2.8	62.3 ± 6.6	40.7 ± 12.4[a]	−34
Single drug therapy				
Fungizone (n = 6)	77.3 ± 5.0	98.4 ± 5.5	72.9 ± 11.0	−32
ABLC 1 (n = 6)	36.3 ± 7.9	65.5 ± 8.7[a]	69.6 ± 30.1	+92
ABLC 5 (n = 6)	54.0 ± 5.9	103.2 ± 25.8[a]	55.4 ± 5.9	+3
Caspofungin 3 (n = 6)	59.3 ± 9.4	92.6 ± 17.7[a]	61.1 ± 28.5	+3
Combination therapy				
ABLC 1 + Caspofungin 3 (n = 6)	48.0 ± 12.0	151.3 ± 26.3[a]	58.6 ± 5.8	+22
ABLC 5 + Caspofungin 3 (n = 6)	42.3 ± 2.2	124.5 ± 52.1[a]	58.0 ± 7.0	+37

[a] $p < 0.05$ vs. Baseline using PCANOVA.
[b] Baseline-AST levels prior to the rats being infected with *Aspergillus fumigatus*.
[c] 48 h-AST levels 2 days after the rats were infected with *Aspergillus fumigatus* and prior to treatment.
[d] 144 h-AST levels 2 days after the rats were infected with *Aspergillus fumigatus* following 4-days of treatment.
[e] % change in AST levels between baseline and 144 h after the initiation of the infection.
All rats were infected with 1.1–2.3 × 10^7 colony forming units (CFU)/0.3 mL/rat of *Aspergillus fumigatus* prior to initiation of treatment. All data are presented as mean ± SEM.

Systemic aspergillosis. Our findings suggest that ABLC at 5 mg/kg once daily × 4 days appears to be the best therapeutic choice in this animal model exhibiting the greatest antifungal activity compared to the other treatment groups tested with no apparent renal and hepatic toxicity. It appears that the presence of Caspofungin may increase Amphotericin B-induced renal toxicity of ABLC without providing additional antifungal activity.

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