Caspofungin benefit on phagocytes from patients with renal dysfunction infected with multidrug-resistant Candida glabrata. [*V.Allizond is the corresponding author]

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Caspofungin benefit on phagocytes from patients with renal dysfunction towards multidrug-resistant *Candida glabrata*

Aims: We evaluated the potential impact of caspofungin (CAS) on the functional activities of polymorphonuclear leukocytes (PMNs) from haemodialysed patients (HDs) and renal transplant recipients (RTRs) towards a multidrugï resistant (MDR) clinical strain of *Candida glabrata* compared with those of PMNs from healthy subjects (HSs).

Materials & methods: CAS effects on PMN phagocytosis and intracellular killing towards MDR *C. glabrata* were evaluated in 66 HDs, 54 RTRs and 30 HSs in absence and presence of CAS at MIC and sub-MIC concentrations.

Results: The obtained results underline the role of CAS in restoration of the impaired PMN functions in HD and RTR patients: when HD-PMNs and RTR-PMNs were exposed to both MIC and sub-MIC concentrations of CAS, their fungicidal activity against the MDR *C. glabrata* strain was significantly higher than that of drug-free controls, with Survival Index values that overlapped with those achieved by HS-PMNs.

Conclusion: Caspofungin might still constitute an effective therapeutic option for the treatment of IFIs caused by MDR *C. glabrata* in patients with altered phagocyte-dependent innate immunity.

Keywords*:* phagocytes, haemodialysed patients, renal transplant recipients, caspofungin, *Candida glabrata*

Introduction

Non-*albicans Candida* species are emerging as key opportunistic pathogens, especially among immunocompromised patients such as those with renal dysfunction [1, 2]. Currently, epidemiological trends indicate a worldwide shift towards infections by *Candida glabrata*, that has become the second most common cause of invasive candidiasis after *C. albicans* [3]. Invasive fungal infections (IFIs) caused by *C. glabrata* are associated with a high infection-related mortality rate and often prove to be refractory to treatment due to both reduced susceptibility and resistance to available antifungal drugs, especially to azoles [3]. Consequently, based on recent published guidelines, the echinocandins have replaced azole drugs as first-line therapy against these yeast infections [4, 5]. Among the echinocandins, caspofungin (CAS) is safe and effective with a favorable toxicity profile [3]. Documentation of acquired resistance to CAS remains sporadic; however recent reports of bloodstream infections due to *C.glabrata* strains resistant to both fluconazole and CAS have focused further concern on this species [5-7]. During the last decade, a large body of preclinical data has shown that antifungal drugs and immune effector cells can work synergistically to oppose fungal infection [8, 9]. Host defense against invasive candidiasis requires recruitment and activation of phagocytes, mediated through the induction and sensing of cytokines, chemokines, and the development of Th1-type adaptive immunity [10]. The addition of an antifungal drug in these processes may impact directly and indirectly on phagocytes, leading to a potential improvement of host responses to infectious agents [8, 11-13]. Since the whole efficacy of antifungal drugs may not be fully captured by conventional methods such as minimum inhibitory concentration (MIC) determination and fungal species may differ in the immune reactions that they evoke mainly in high-risk patients, this work aimed at evaluating the effects exerted by CAS on the functional activities of polymorphonuclear leukocytes (PMNs) from haemodialysed patients (HDs) and renal transplant recipients (RTRs) towards a clinical strain of *C. glabrata* compared with those of PMNs from healthy subjects (HSs).

Materials & methods

Study populations

Sixty-six HDs, comprising 42 males and 24 females (mean age 69.3 years), and 54 RTRs, comprising 31 males and 23 females (mean age 53.9 years), followed at the Ivrea Hospital (Turin, Italy), were recruited for the study. Patients with acute or chronic infections, active immunological diseases, history of malignancy, haematological and liver disease, antibiotic therapy, were

excluded. The control population was made up of 30 age-matched HSs. Venous blood samples from HDs, RTRs and HSs were obtained after receipt of written informed consent.

HD patients had been on haemodialysis maintenance for between 1 and 359 months (mean 53 ± 39 months). The mean Kt/V (single pool) was 1.44 and the protein catabolic rate (PCR = 1.15) indicated an adequate dialysis prescription and nutrition. All patients were dialysed with biocompatible modified cellulose dialyser membrane. Underlying diseases of the HD patients were nephroangiosclerosis (18), chronic renal failure (18), diabetic nephropathy (12), chronic glomerulonephritis (8), polycystic kidney disease (2), kidney myeloma (2), interstitial nephritis (1 patients), and other causes (5).

The mean time since RTR patients underwent first-time kidney transplantation was 83.94 months (range 7 - 248 months), and the main causes of renal disease were nephroangiosclerosis in 16, chronic renal failure in 10, chronic glomerulonephritis in 6, interstitial nephritis in 5, polycystic kidney disease in 4, diabetic nephropathy in 4, and other causes in 9. The mean value of serum creatinine concentration in RTR patients was 1.7 ± 0.8 mg/dl. Maintenance immunosuppressive therapy was based on the use of tacrolimus (FK), mycophenolate mofetil (MMF), and prednisone (P) in 32 out of 54 patients (59%); cyclosporine (CyA) and MMF in 2 patients (4%); CyA, P, and sirolimus in 7 patients (13%); P and FK in 3 patients (6%); FK and MMF in 4 patients (7%); FK alone in 5 patients (9%); and CyA alone in 1 patient (2%).

Yeasts

A *C. glabrata* bloodstream isolate was used in this study. Once identified by biochemical methods, it was plated onto Sabouraud dextrose (SAB) agar (Oxoid S.p.A., Milan, Italy) to ensure viability and purity.

Antifungal susceptibility testing

Reference powders of fluconazole (FLC) (Sigma-Aldrich, Milan, Italy), voriconazole (VCZ) (Sigma-Aldrich, Milan, Italy) and CAS acetate (Merck Sharp & Dohme Ltd., Hoddesdon, UK) were reconstituted according to the manufacturersô instructions. The *C. glabrata* isolate was tested for *in vitro* susceptibility to FLC, VCZ and CAS using Clinical and Laboratory Standards Institute (CLSI) broth microdilution method and interpretative criteria [14] with an inoculum of 10^3 colony-forming units (CFU)/ml for MIC determination. The recently revised CLSI clinical breakpoints were used for *C. glabrata* [5]: CAS MIC value of Ó0.5 µg/ml, FLC MIC value of Ó64 µg/ml, and VCZ MIC value of > 0.5 µg/ml were considered resistant.

To perform phagocytosis and intracellular killing assays (PMNs/yeasts in a ratio of $10^6/10^6$), the *C. glabrata* isolate was also tested for susceptibility to CAS with an inoculum of 10^6 CFU/ml.

PMN isolation

PMNs were isolated from the patientsô and controlsô whole venous blood samples after dextran gravity sedimentation , followed by Ficollï Paque™ (Pharmacia S.p.A., Milan, Italy) centrifugation and hypotonic lysis of the residual erythrocytes, as described previously [11, 15]. Next, purified PMNs were suspended in RPMI 1640 medium (Sigma-Aldrich, Milan, Italy) and adjusted to 10^6 cells/ml. PMN viability was assessed by trypan blue exclusion and cell viability of > 95% was kept as the cutoff; the test was performed before and after each experiment.

Yeast labelling

For *C. glabrata* labelling, aliquots of 200 µl of frozen yeast cells were inoculated into fresh SAB broth containing 150 µl of ^{3}H-uracil (specific activity 1270 GBq/mmol) (NEN Life Science Products, Milan, Italy) and incubated at 37ºC for 4 h. Radiolabelled yeast cells were washed by centrifugation with SAB broth, resuspended in fresh medium and adjusted to yield 10^6 CFU/ml as confirmed by colony counts in triplicate.

Effect of caspofungin on PMN functions

Phagocytosis assay. The effect of CAS on PMN phagocytosis of radiolabelled yeast cells was investigated by incubating yeasts and PMNs at a 1:1 ratio for different incubation times (30, 60 and 90 min), under constant shaking (150 rpm) at 37°C with CAS at 1 and 0.5 x MIC concentrations. Controls without CAS were included. After each incubation time point to remove free radiolabelled yeast cells, PMNs were washed twice by centrifugation (200 x *g* for 5 min) and suspended in sterile water for 5 min. Sample analysis was performed by transferring 100 µl into scintillation fluid (Atomlight; NEN Life Science Products), counting by spectrophotometry, and expressing data as counts per minute (cpm). The % phagocytosis was calculated as [(cpm in PMN pellet)/(cpm in total yeast pellet)]×100 [13, 16, 17].

IntraPMN killing assay. Intracellular killing was investigated by incubating yeast cells and PMNs (1:1 ratio) for 30 min to allow phagocytosis to proceed. To remove extracellular yeasts the PMN-yeast cells mixtures were washed by centrifugation (200 x *g* for 5 min). After PMN lysis with sterile water, at time zero (t_0) the intracellular viability of yeast cells was determined by colony counting method. After 10-fold serial dilutions of the lysate PMNs, aliquots of 100 µl were plated onto SAB

agar and incubated at 37°C for 24 h and the CFUs grown were counted. Further CAS at 1 and 0.5 x MIC was added and at times *x* (tx) viable counts were measured in the same way. The survival index (SI) that defines the PMN intracellular killing activity was calculated as follows: SI = surviving yeast cells at t_0 added to survivors at t_x / survivors at t_0. The SI value is 1, when PMN fungal killing is 100% effective [13, 16, 17].

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) for 10 separate experiments assessed in quadruplicate. Statistical significance of differences between experimental and control results was determined by using Studentôs unpaired *t*-test. Statistical analysis was carried out with GraphPad Prism v. 6 for Windows (GraphPad Software, San Diego, CA). A *P*-value of < 0.05 was considered statistically significant.

Results

MIC/Susceptibility results. The clinical *C. glabrata* isolate was a multidrugï resistant (MDR) strain being resistant to FLC (MIC Ó64 µg/ml), to VCZ (MIC > 0.5 µg/ml), and to CAS (MIC = 2 µg/ml), respectively. With a yeast cell inoculum of 10^6 CFU/ml, necessary to perform tests with PMNs, MIC of CAS was 4 µg/ml.

Phagocytosis assay. In all experiments, the viability of PMNs remained unchanged throughout. In drug-free samples, no difference in the phagocytic activity by HD-PMNs and RTR-PMNs against *C. glabrata* was observed compared with HS-PMNs (Table 1): within 30 min of incubation, more than 70% of yeast cells were phagocytosed by HD, RTR and HS-PMNs, resulting in relatively stable phagocytic percentages between 30 and 90 min.

IntraPMN killing assay. A slight decrease in the fungicidal activity of RTR-PMNs was observed over time compared with HS controls (Table1). In HD-PMNs the intracellular fungicidal activity was significantly lower than that observed in healthy controls, and it was totally absent at 90 min due to intracellular yeast cell replication (SI > 2 *vs* 1.58, Table 1).

Influence of 1 x MIC CAS on PMN functions. CAS at 1 x MIC (4 µg/ml) had no significant effect on the phagocytic activity of PMNs from HDs, RTRs or HSs, since yeast cells were phagocytosed at the same rates as untreated controls ($P > 0.05$, Table 1).

In contrast, the addition of CAS to HS-PMNs resulted in a marked effect *(P* < 0.01) on intracellular fungicidal activity against *C. glabrata* compared with that detected in drug-free controls, leading to a significant decrease in SI values during the 90 min of incubation (Table 1). Likewise, when HD-PMNs and RTR-PMNs were exposed to 1 x MIC of CAS, their fungicidal activity was significantly higher than that of drug-free controls (*P* < 0.01, Table 1), with SI values that overlapped with those achieved by HS-PMNs.

Influence of 0.5 x MIC CAS on PMN functions. A similar pattern was also detected when HD-PMNs and RTR-PMNs were exposed to 0.5 x MIC of CAS (2 μg/ml). *C. glabrata* cells were engulfed by HD-PMNs and RTR-PMNs at rates similar to those of HD-PMNs and RTR-PMNs exposed to the MIC. In fact, the mean phagocytosis rates were 83.9%, 85.7% and 76.5% for HD-PMNs and 72.0%, 74.1% and 74.3% for RTR-PMNs, after 30, 60 and 90 min incubation time. Analogously, after 30, 60 and 90 min incubation time the SI values overlapped with those of HD-PMNs and RTR-PMNs exposed to the MIC. In details, SI values were 1.36, 1.33 and 1.28 for HD-PMNs and 1.37, 1.32 and 1.28 for RTR-PMNs, respectively.

Discussion

Outcome of IFIs in immunocompromised patients depends on a complex interplay between host and pathogen factors, as well as treatment modalities. The sporadic emergence of MDR strains recently detected [5-7] and recognized as an emerging threat could be related to the selection pressure for resistant organisms due to the wide use of echinocandins coupled with broadening azole resistance for treatment of infections due to *C. glabrata*. In our study the clinical isolate of *C. glabrata*, used to examine the interactions between CAS and PMNs from RTR and HD patients compared with HS-PMN controls, was a MDR strain being resistant to FLC, VCZ, and CAS.
In agreement with our previous works on *C. albicans* [11, 16, 17], we found that the intracellular uptake of MDR *C. glabrata* by HD-PMNs and RTR-PMNs is not impaired by either uremia or immunosuppressive regimens, as we detected phagocytosis rates similar to those of HS drug-free controls (Table 1). In contrast, following yeast cell uptake, the intracellular fungicidal activity of PMNs was slightly reduced in RTR patients, and it was significantly impaired in HD patients compared with that detected in HS controls (P < 0.05, Table 1): these results may contribute to explain the greater risk of IFIs in these immunocompromised patients [1, 2]. No synergistic effect between CAS and PMNs on phagocytosis towards MDR *C. glabrata* in HD, RTR and HS-PMNs (Table 1) throughout the observation period occurred, indicating that CAS does not adversely interfere with PMN yeast cell uptake. On the other hand, the addition of CAS at 1 x MIC after phagocytosis had occurred, resulted in a significant increase in yeast cell death, as significant

differences in intracellular killing rates between CAS-exposed HD, RTR-PMNs and drug-free controls were detected ($P < 0.01$, Table 1). Approximately 60% of MDR *C. glabrata* was efficiently killed by PMNs from patients with renal dysfunction within the first 30 min of incubation, with a further increase in killing rates during the entire incubation time. Taken together, these data underline the role of CAS in restoration of the impaired PMN functions in these immunocompromised patients. Similar enhancement of the PMN fungicidal activity was also detected with CAS at 0.5 x MIC level, indicating the absence of a drug-dose dependent effect. Moreover, these findings are in line with recent studies reporting that CAS, following intracellular drug accumulation, significantly influences oxidative burst metabolism and improves intracellular killing activity of PMNs against *C. albicans*, but has no effect on phagocytosis [12, 13]. In support of this, our recently published data further indicate that CAS, besides exerting a direct antifungal effect, may also modulate phagocyte activity through an indirect synergistic antifungal effect even on PMNs from patients with renal dysfunction against *C. albicans*, being able to strength the depressed intracellular fungicidal phagocytic activity of either HD or RTR patients [16, 17].

Future perspectives

In recent years, therapeutic failures strongly related to multidrug resistant fungal strains have been increasingly reported and greater efforts are needed to prevent or reduce the spread of fungal resistance. Therefore, new approaches aimed at discovering the unknown immunomodulating properties of established antifungals may have the potential to provide us with a decisive advantage in our asymmetric war with microbial pathogens. Importantly, caspofungin displayed a significant additive *in vitro* effect with PMNs against the clinical MDR *C. glabrata* strain not only in healthy subjects but overall in patients with renal dysfunction, where such synergy may enhance fungal killing restoring the compromised ability to clear invading yeast pathogens. As a consequence, our findings provide evidence that caspofungin might still play a key role for treatment of IFIs even caused by MDR *C. glabrata*, especially in patients with altered phagocyte dependent innate immunity. Within the limitations of this study, to fully elucidate the clinical relevance of these results further studies are needed, including *ex vivo* experiments on PMNs from patients receiving CAS for therapeutic purposes.

Executive summary

Background

- Outcome of invasive fungal infections in immunocompromised patients remains difficult and depends upon a complex interaction of antifungal drugs and their activity on both pathogen and host cells. Resistance to echinocandins in clinical *C. glabrata* isolates has begun to occur, representing an emerging threat to their clinical use.

Objective

- To assess the caspofungin effects on the activities of PMNs from haemodialysed patients and renal transplant recipients towards a clinical MDR strain of *C. glabrata* compared with those of PMNs from healthy subjects.

Results

- Caspofungin plays a key role for treatment of MDR *C. glabrata* being capable of restoring the impaired functions of PMNs from patients with renal dysfunction, through an indirect synergistic effect.

Conclusion

- Caspofungin might still constitute an effective therapeutic option for the treatment of IFIs caused by MDR *C. glabrata* in patients with altered phagocyte-dependent innate immunity.

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Ethical conduct of research

Informed consent has been obtained from the participants involved in this study.

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Table 1. Effect of caspofungin (CAS) at 1 x MIC (4 µg/ml) on phagocytosis and intracellular killing of polymorphonuclear cells (PMNs) from haemodialysed patients (HDs), renal transplant recipients (RTRs) and healthy subjects (HSs) against multidrugï resistant *C. glabrata*

	Mean % phagocytosis ± S.E.M.		Survival Index (SI) ± S.E.M. (%)[a]	
Time (min)	Controls	CAS	Controls	CAS
HSs				
30	72.0 ± 0.22	79.1 ± 0.78	1.62 ± 0.02 (38%)	1.39*** ± 0.05
60	74.9 ± 0.14	86.8 ± 0.54	1.61 ± 0.07 (39%)	1.33*** ± 0.05
90	75.2 ± 0.44	68.2 ± 0.29	1.58 ± 0.05 (42%)	1.31*** ± 0.03
HDs				
30	81.9 ± 0.09	83.4 ± 0.02	1.86* ± 0.12 (14%)	1.37*** ± 0.03
60	85.9 ± 0.10	86.1 ± 0.10	1.86* ± 0.07 (14%)	1.32*** ± 0.02
90	82.7 ± 0.23	77.6 ± 0.23	>2**	1.26*** ± 0.03
RTRs				
30	70.9 ± 0.13	72.3 ± 0.20	1.70 ± 0.04 (30%)	1.39*** ± 0.04
60	75.9 ± 0.14	74.9 ± 0.18	1.68 ± 0.03 (32%)	1.33*** ± 0.03
90	69.7 ± 0.40	75.0 ± 0.40	1.67 ± 0.03 (33%)	1.27*** ± 0.03

S.EM., standard error of the mean.

[a] Percentage of yeast cells killed by PMNs in absence and presence of the antifungal drug.

* Significantly different from healthy subject controls ($P < 0.05$).

** Significantly different from healthy subject controls ($P < 0.01$).

*** Significantly different from controls without drug ($P < 0.01$).