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A synthetic peptide as a novel anticryptococcal agent

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Summary

An engineered, killer decapeptide (KP) has been synthesized based on the sequence of a recombinant, single-chain anti-idiotypic antibody (KT-scFv) acting as a functional internal image of a yeast killer toxin. Killer decapeptide exerted a strong fungicidal activity against *Candida albicans*, which was attributed to peptide interaction with β-glucan. As this polysaccharide is also a critical component of the cryptococcal cell wall, we wondered whether KP was also active against *Cryptococcus neoformans*, a human pathogen of increasing medical importance. We found that KP was able to kill both capsular and acapsular *C. neoformans* cells *in vitro*. Furthermore, KP impaired the production of specific *C. neoformans* virulence factors including protease and urease activity and capsule formation, rendering the fungus more susceptible to natural effector cells. *In vivo* treatment with KP significantly reduced fungal burden in mice with cryptococcosis and, importantly, protected the majority of immunosuppressed animals from an otherwise lethal infection. Given the relevance of cryptococcosis in immunocompromised individuals and the inability of conventional drugs to completely resolve the infection, the results of the present study indicate KP as an ideal candidate for further studies on novel anticryptococcal agents.

Introduction

The antimicrobial effects of a killer toxin (KT) produced by the yeast *Pichia anomala* (*Pa*-KT) have been demonstrated against prokaryotic and eukaryotic pathogens, including a variety of important microorganisms such as *Mycobacterium tuberculosis, Pneumocystis carinii* and *Candida albicans* (Magliani *et al*., 1997a; Séguy *et al*., 1998).

To overcome the intrinsic toxicity and the chemical instability of *Pa*-KT, taking advantage of its immunogenicity, a *Pa*-KT-neutralizing monoclonal antibody (mAb KT4) has been produced (Polonelli and Morace, 1987), which, when used as an immunogen in different animals, was able to induce production of anti-idiotypic antibodies sharing structural and/or functional similarities with the active toxic site of *Pa*-KT (Polonelli *et al*., 1991; 1993; 1994; Polonelli and Morace, 1988). The production of *Pa*-KT-like antibodies was also shown to occur in the antibody repertoire following either experimental or natural infections by *Pa*-KT-sensitive microorganisms (Polonelli *et al*., 1996). On the basis of these observations, the anti-idiotypic monoclonal antibody (IdmAb), representing the internal image of *Pa*-KT (KT-IdmAb), has been generated by conventional fusion from spleen lymphocytes of rats idiotypically immunized with mAb KT4 (Polonelli *et al*., 1997).

The wide spectrum of activity of KT-IdmAb against bacteria, fungi and protozoa has been demonstrated in a variety of *in vitro* studies (Magliani *et al*., 1997b; Conti *et al*., 1998; 2000; Séguy *et al*., 1997; Savoia *et al*., 2002). The microbicidal capacity observed *in vitro* was confirmed in experimental models *in vivo*, demonstrating the therapeutic effect of this mAb in vaginal candidiasis (Magliana *et al*., 1997b), pulmonary aspergillosis (Cenci *et al*., 2002) and pneumocystosis (Séguy *et al*., 1997). A recombinant KT-IdmAb in the single-chain format (KT-scFv) has also been generated in a recombinant phage antibody system (Magliani *et al*., 1997b), and experimental data indicated that KT-IdmAb as well as KT-scFv showed comparable antimicrobial activity *in vitro* and remarkable therapeutic efficacy in an experimental model of candidiasis (Magliani *et al*., 2001).

The KT-scFv gene was cloned and expressed in the human commensal *Streptococcus gordonii* (Oggioni *et al*., 2001). Engineered *S. gordonii* strains stably colonized mucosal surfaces and exerted a strong therapeutic effect in experimental models of vaginal candidiasis, with efficacy equal to a full therapeutic course of fluconazole (Beninati *et al*., 2000).

To fulfill its microbicidal capacity, *Pa*-KT and its immunological derivatives must bind to a receptorial component, shared by a number of pathogens, recently characterized as cell wall β1,3-glucan (Cassone *et al*., 1997; Guyard *et al*., 2002a,b). In fact, blockade of β1,3-

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glucan abrogates or strongly reduces the microbicidal capacity of *Pa*-KT (Guyard *et al*., 2002a). Although the cellular target has been identified, the killing mechanisms remain undefined.

The exquisite range of activity of KT-IdmAb and KT-scFv, and the remarkable therapeutic effect observed in several experimental models of fungal infections led to the synthesis of a decapeptide killer mimotope based on the known sequence of the KT-scFv gene. A detailed description of this engineered synthetic killer anti-idiotypic Ab fragment (KP) has been reported recently, together with the demonstration of its strong candidacidal activity *in vitro* and *in vivo* (Polonelli *et al*., 2003). Namely, KP was able to cure rat vaginal infections caused by fluconazole-susceptible and -resistant *C. albicans* strains, and to protect against systemic candidiasis in murine models set up both in immunocompetent and severely immunocompromised (SCID) mice (Polonelli *et al*., 2003). Of particular relevance was the demonstration that the peptide anticandidal effect could be abolished by laminarin (Polonelli *et al*., 2003), a soluble β1,3-glucan, thus showing that a β1,3-glucan molecule was possibly the target of the peptide, as previously demonstrated with the KT-IdmAb (Cassone *et al*., 1997).

Cryptococcus neoformans causes serious infections in immunocompromised individuals such as AIDS or transplant patients and, occasionally, in immunocompetent hosts (Casadevall and Perfect, 1998). In immunocompromised individuals, this fungus is not eradicated with conventional therapeutic approaches, and survivors need lifelong suppressive therapy (Powderly, 1996). Moreover, the immunosuppressive effects of some cryptococcal capsular compounds, such as glucuronoxylomannan, released during infection, could be an additional deleterious, life-threatening factor (Vecchiarelli, 2000). It has been reported that the *C. neoformans* cell wall is composed of 86% glucose and 7.3% N-acetylglucosamine (James *et al*., 1990). The presence of both α- and β-glucans in the cell wall, following chemical extraction, has been documented in detail (James *et al*., 1990). Moreover the β1,3-glucan synthase encoding gene has been cloned in *C. neoformans* and proved essential for fungal cells (Thompson *et al*., 1999). Given (i) the importance of cryptococcosis and the difficulties in curing this insidious infection; (ii) the critical role of β-glucan, the supposed receptor of the peptide, in the cell wall of *C. neoformans*, we examined whether KP could also exert anticryptococcal activity.

We found that KP killed *C. neoformans in vitro* in a dose-dependent, laminarin-sensitive manner. When this peptide was used at concentrations that did not affect fungal growth it impaired the production of specific *C. neoformans* virulence factors including protease and urease activity and capsule formation, rendering the fungus more susceptible to natural effector cells. *In vivo* treatment with KP significantly reduced fungal burden in mice with cryptococcosis and dramatically improved survival of immunosuppressed *C. neoformans* infected mice.

Results

KP killing activity of C. neoformans in vitro

To assess whether KP could affect *C. neoformans* growth *in vitro*, encapsulated (6995) or acapsular (7698) strains were incubated with different concentrations (20, 10 and 5 μg ml^{-1}) of KP or its own scramble peptide (SP) and both the optical density (OD) and the number of CFU were evaluated after a 48 h incubation. The results show that KP, at a concentration of 20 μg ml^{-1}, exerted a strong fungicidal activity. No significant effect was observed at lower KP concentrations (10 and 5 μg ml^{-1}) whereas SP had no effect at all, at any concentration, thus confirming the specificity of KP activity (Fig. 1).

Laminarin abrogates KP activity

Because β1,3-glucan has been indicated as the putative receptor, or part of it, for *Pa*-KT and KT-IdmAb (Cassone *et al*., 1997; Guyard *et al*., 2002a,b) and, indeed, addition of laminarin (soluble β1,3-glucan) completely inhibited the effects of KP on *C. albicans* (Polonelli *et al*., 2003), different concentrations of laminarin and pustulan (soluble β1,6-glucan) were added to *C. neoformans* in the presence of KP. The results indicate that laminarin, but not pustulan, completely abrogated the KP-induced anticryptococcal effect in a dose-dependent manner (Fig. 2). These results suggest the involvement of β1,3-glucan in specific binding of KP to *C. neoformans* cells.

Effect of KP on C. neoformans *virulence factors*

Cryptococcus neoformans is endowed with many virulence factors protecting the fungus from host immune defence, thus facilitating tissue invasion. Among them, the most important is the polysaccharide capsule, responsible for potent antiphagocytic and immunomodulating activities (Vecchiarelli, 2000). Production of melanin is considered another important factor protecting the fungus during infection (Gomez and Nosanchuk, 2003). Moreover, a number of secreted proteins with enzymatic activity appear to facilitate survival within the host (Perfect and Casadevall, 2002). To assess the activity of KP on cryptococcal virulence factors the following effects were evaluated.

Effect of KP on capsule size of unmelanized and melanized C. neoformans

Initial experiments performed to evaluate the effect of KP on melanin production indicated that the addition of differ-

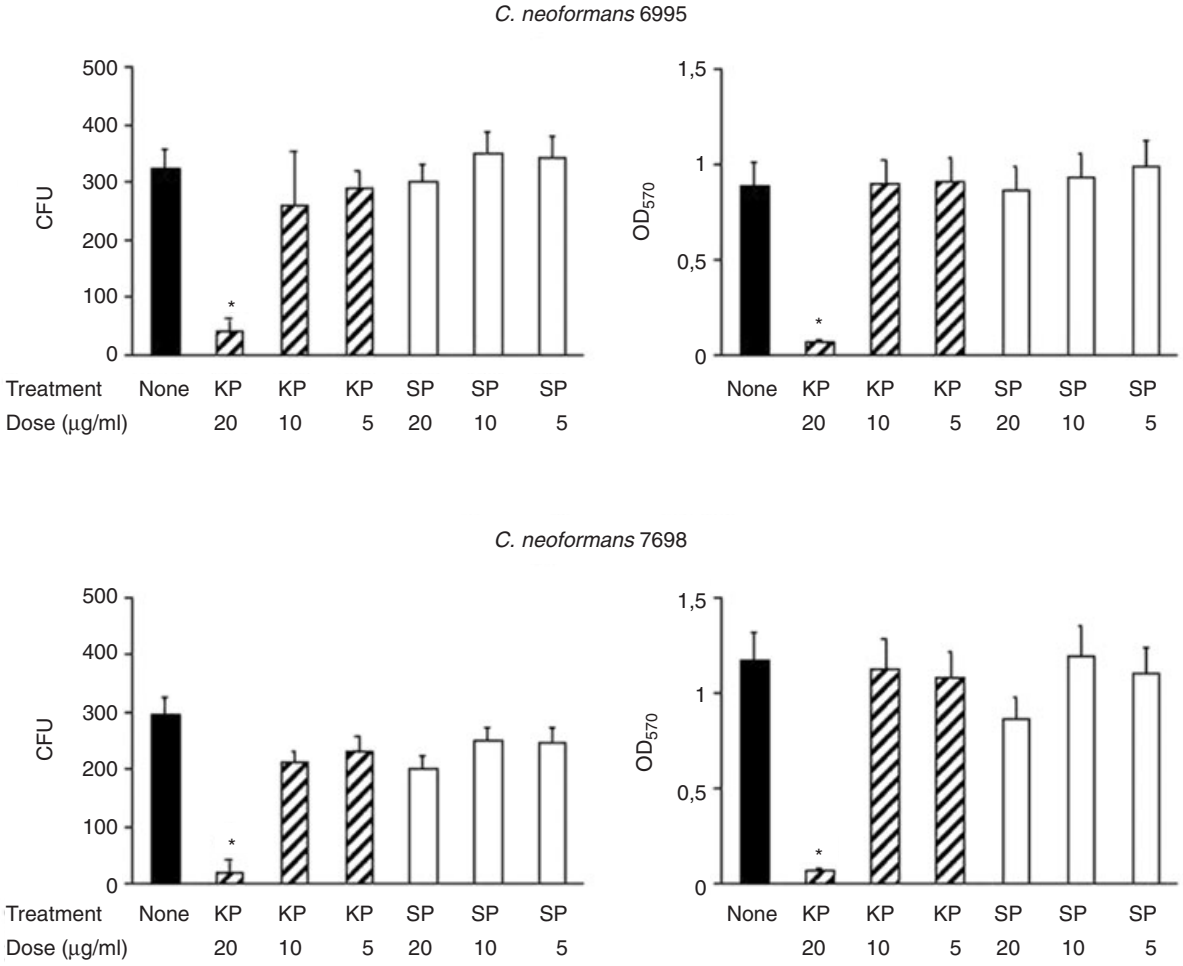

Fig. 1. Antifungal effect of KP on *C. neoformans in vitro*. For CFU determination, 10^4 *C. neoformans* 6995 or 7698 were incubated at 37°C in 1 ml of RPMI medium, alone or in the presence of 20, 10 and 5 μg ml^{-1} KP or 20 and 10 μg ml^{-1} SP. After 24 h, 100 μl of suspension were recovered, plated onto Sabouraud agar, and CFU (mean ± SE) were calculated. For the colorimetric assay, the same concentrations of KP or SP were added to 10^3 *C. neoformans* in a final volume of 200 μl, in 96-well flat-bottomed microtitre plates. After 24 h incubation at 37°C, MTT was added to the cultures for an additional 3 h and, after solubilization of formazan precipitate, the OD was recorded by an ELISA plate reader, at a wavelength of 570 nm. *$P < 0.01$, KP-treated versus controls, according to Student's *t*-test.

ent concentrations of KP or SP to *C. neoformans*, cultured in medium containing L-DOPA, did not affect pigment production (data not shown). The effect of KP on fungal capsule thickness was subsequently evaluated in unmelanized and melanized *C. neoformans* cells, treated with different doses of KP. The results show that KP inhibited both fungal growth (Fig. 3A) and capsule production (Fig. 3B) in unmelanized and melanized cells, confirming the above results. Importantly, a dramatic reduction of capsule formation was evident also at a KP concentration (5 μg ml^{-1}) clearly unable to cause a fungicidal effect (see Fig. 1).

Effect of KP on C. neoformans *enzyme activity*

Protease, phospholipase and urease activities were tested after addition of different, non-microbicidal concentrations of KP (10 and 5 μg ml^{-1}) to the cell suspension before inoculation on agar plates. The results show that addition of 10 μg ml^{-1} KP caused an impairment of the extracellular protease activity, as indicated by an increased Pz value, and a slower urea hydrolysis (Table 1). In contrast, phospholipase activity of *C. neoformans* was unaffected by KP treatment (Table 1).

Susceptibility of KP-treated C. neoformans *to killing activity of natural effector cells*

Because KP impairs some cryptococcal virulence factors including capsule size, the capacity of KP to affect the susceptibility of *C. neoformans* to murine effector cells was evaluated. To this end, KP-treated or untreated *C.*

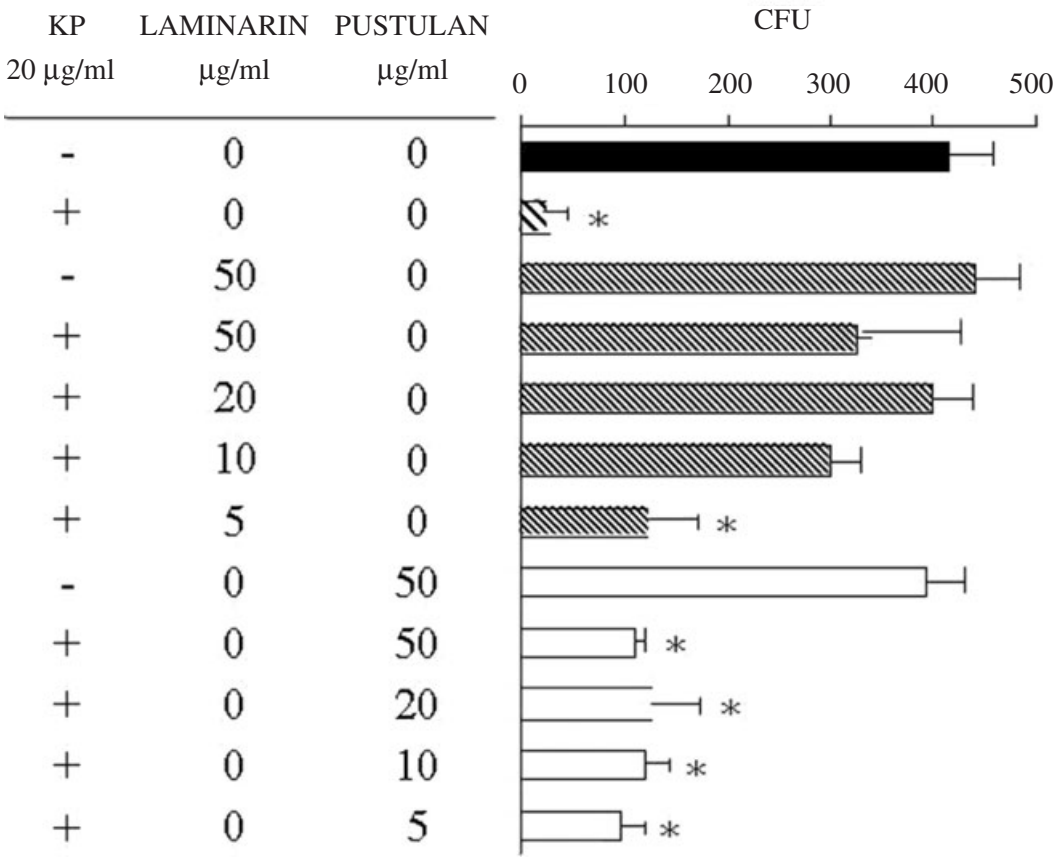

Fig. 2. Laminarin abrogates KP activity *in vitro*. *C. neoformans* (10^3 cells/0.1 ml RPMI medium) were cultured alone or in the presence of 20 μg ml^{-1} KP and different doses of laminarin (soluble β1,3 glucan) or pustulan (soluble β1,6 glucan). After 24 h incubation at 37°C, 100 μl of suspension were recovered, plated onto Sabouraud agar, and CFU (mean ± SE) were calculated. * $P < 0.01$, treated versus untreated controls, according to Student's *t*-test.

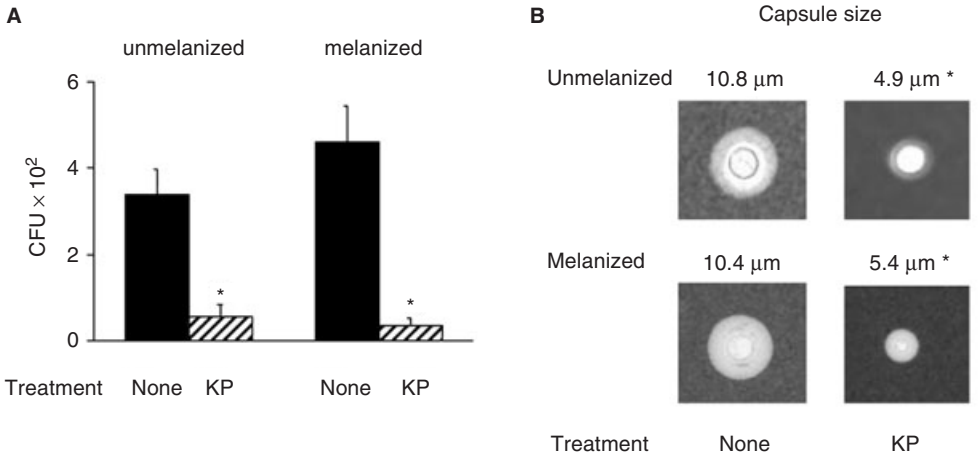

Fig. 3. KP impairs CFU recovery and capsule production of unmelanized and melanized *C. neoformans in vitro*. Synthesis of melanin was induced by culturing *C. neoformans* on agar plates of defined minimal medium containing 1.0 mM L-DOPA as described in *Experimental procedures*.
A. CFU were determined as in Fig. 1.
B. Reports the values of capsule size, measured with the help of a grid, in India-ink preparations of untreated *C. neoformans* or after addition of 5 μg ml^{-1} KP. Micrographs (original magnification ×40) were also taken (reported as inserts in panel B). The data reported are from one experiment representative of four with similar results. SE (<10%) has been omitted in B. * $P < 0.01$, KP-treated versus untreated controls, according to Student's *t*-test.

Table 1. Effect of KP or SP on *C. neoformans* enzyme activity.

Treatment		Enzyme activity			
Molecule	Dose (μg ml^{-1})	Protease[a]	Phospholipase[a]	Urease[b] 12 h	24 h
None	–	0.478 ± 0.098	0.580 ± 0.077	+	+
KP	10	0.675 ± 0.060*	0.565 ± 0.112	–	+
KP	5	0.463 ± 0.124	0.591 ± 0.102	+	+
SP	10	0.494 ± 0.056	0.587 ± 0.099	+	+
SP	5	0.486 ± 0.095	0.577 ± 0.132	+	+

a. Determination of protease and phospholipase production was performed on Sabouraud-egg yolk or bovine serum albumin-containing agar plates respectively. The plates were inoculated with 10 μl of a suspension of 10^5 ml^{-1} *C. neoformans* cells, alone or in the presence of 10 and 5 μg ml^{-1} KP or SP, and incubated at 37°C for 7 days. Protease and phospholipase activity was expressed by the Pz value, as described in *Experimental procedures*. The values range between 0 and 1, with the highest level of enzymatic activity being the nearest to 0.
b. Urease activity was determined 12 and 24 h after inoculation of *C. neoformans*, alone or in the presence of 10 and 5 μg ml^{-1} KP or SP, on urea agar slants. The signs + or – indicate the presence or absence, respectively, of urease activity; *, $P < 0.01$, treated versus untreated, according to Student's *t*-test.

neoformans cells were incubated with neutrophils or peritoneal macrophages for 2 and 4 h respectively. Killing activity was determined as the percentage of CFU inhibition. The results show that treatment with KP rendered *C. neoformans* significantly more susceptible to the killing activity of natural effector cells (Table 2). The maximum effect was observed at the 20 μg ml^{-1} dose (Table 2).

Therapeutic efficacy of KP on C. neoformans *infection*

To assess whether the *in vitro* activity of KP on *C. neoformans* paralleled a therapeutic effect *in vivo*, an experimental model of systemic cryptococcosis was used. Immunocompetent or cyclophosphamide-immunosuppressed BALB/c mice were intravenously infected with *C. neoformans* yeast cells and subsequently treated with KP intraperitoneally, 1 h after infection, and 1 and 2 days later. The course of infection was evaluated in terms of organ clearance, histological examination of brain, and survival. The results showed that KP dramatically reduced fungal burden and capsule formation in brains of infected mice (Fig. 4A and B) and, importantly, protected the majority of immunosuppresed animals from an otherwise lethal infection (Fig. 4C).

Table 2. Effect of KP or SP on susceptibility of *C. neoformans* to killing by natural effector cells.

Treatment		Killing activity (%)[a]	
Molecule	Dose (μg ml^{-1})	Neutrophils	Macrophages
None	–	18.0 ± 1.3	11.4 ± 1.4
KP	20	35.6 ± 2.1*	27.0 ± 2.5*
KP	5	23.6 ± 2.0	14.6 ± 2.9
SP	20	19.5 ± 1.3	10.2 ± 1.1
SP	5	18.7 ± 1.8	9.5 ± 1.0

a. 10^5 *C. neoformans* ml^{-1} RPMI were incubated at 37°C for 18 h, alone or in the presence of KP or SP. After washing, cells were mixed with thioglycollate-induced murine peritoneal neutrophils or resident macrophages (effector to target ratio 10 : 1) in the presence of 10% heat-inactivated FCS. Killing activity was determined as the percentage of CFU inhibition, according to the formula 100 – (CFU experimental group/CFU control) × 100. *, $P < 0.01$, treated versus untreated, according to Student's *t*-test.

Discussion

Cryptococcosis is a life-threatening infection in immunocompromised and, occasionally, immunocompetent hosts (Casadevall and Perfect, 1998). Even though HAART therapy has reduced the impact of cryptococcosis in AIDS patients, new groups at risk for this infection have emerged, including transplant recipients (John *et al.*, 1994; Gallis *et al.*, 1975). Therapeutic options are still quite unsatisfactory because of the toxicity of available drugs, their inability to eradicate the fungus, and the potential emergence of drug resistance (Friese *et al.*, 2001). In patients with AIDS, cryptococcosis is usually incurable so that the survivors of an acute infection must receive lifelong therapy (Powderly, 1996). Therefore, there is increasing interest in discovering novel molecules active against *Cryptococcus* that could become useful tools in clinical management of the infection.

It has recently been demonstrated that KP exerts a strong anticandidal activity both *in vitro* and *in vivo*, an activity correlated with the binding to yeast β1,3-glucan (Polonelli *et al.*, 2003). As glucans are critical structural constituents also of *C. neoformans*, we felt encouraged in assessing whether KP could affect cryptococcal cells. Fulfilling this rationale, here we demonstrate that KP possesses a strong, dose-dependent anticryptococcal activity both *in vitro* and *in vivo*. Of particular relevance is our observation that non-cytocidal concentrations of KP markedly reduced the production of cryptococcal virulence factors, including the capsule. Moreover, KP exerted a remarkable therapeutic activity in experimental cryptococcosis.

The fact that laminarin completely abrogates the activity

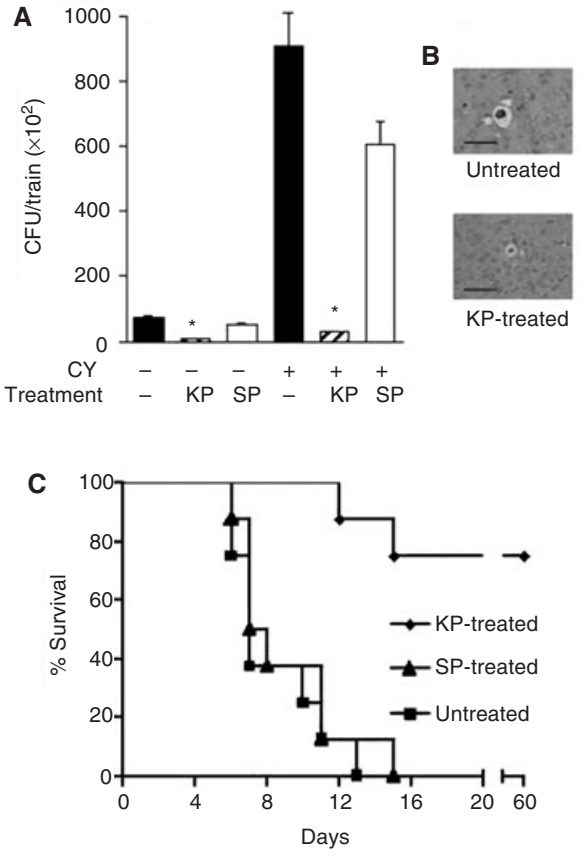

Fig. 4. Therapeutic activity of KP in murine cryptococcosis. Immunocompetent or cyclophosphamide (CY)-immunosuppressed BALB/c mice were intravenously infected with *C. neoformans* yeast cells and treated with 50 μg KP or SP intraperitoneally, 1 h after infection, and 1 and 2 days later.
A. For the quantification of fungal burden in brains, mice were sacrificed 5 days after infection, serial dilutions of organ homogenates were plated onto Sabouraud agar, and CFU were counted after a 48-h incubation. $^*P < 0.01$, KP treated versus untreated controls, according to Student's *t*-test.
B. For histological analysis, periodic acid-Schiff stained sections were prepared 5 days after infection from brains of mice untreated or treated with KP. Note the smaller yeast and capsule size in treated animals. Bar = 50 μm.
C. Per cent survival of infected mice. $P < 0.01$, KP treated versus untreated controls, according to Mantel-Cox Logrank test.

of KP against *C. neoformans* suggests that, as for anticandidal activity, linkage of KP to β1,3-glucan on the yeast surface is a crucial step in the antifungal effect of this molecule. This is particularly relevant because glucan is a major structural component of the cell wall, whose integrity is crucial for the fungal cell. Indeed, the β1,3-glucan synthase encoding gene is essential for yeast viability and growth (Thompson *et al.*, 1999) thus, it is conceivable that KP could interfere with the regular formation of the *Cryptococcus* cell wall.

Molecules able to selectively interact with fungal cell wall components, which are not present in mammalian cells (Selitrennikoff and Nakata, 2003), should be rationally considered as putative antifungal drugs. Indeed, the present paper demonstrates that administration of KP to *C. neoformans*-infected mice reduced fungal load in target organs, and dramatically improved survival in immunosuppressed mice. In fact, 80% of treated animals survived the infection for more than 60 days while all untreated animals died within 7 days.

Cryptococcus neoformans is unique among fungal pathogens for its major virulence factor, a complex polysaccharide capsule. It has been shown that highly virulent isolates produce more capsule than weakly virulent isolates, both *in vitro* under tissue culture conditions and *in vivo* (Blackstock *et al.*, 1999). To what extent KP inhibits the synthesis of the capsule and/or its attachment to the cell wall is yet to be determined. It has been recently shown that the capsule is anchored to the *C. neoformans* cell through α1,3-glucan (Reese and Doering, 2003), but it is not known whether β1,3-glucan could play the same role. Furthermore, binding of KP to α1,3-glucan has not yet been reported. Here we demonstrate that KP impairs capsule thickness *in vitro* and, consistently increases susceptibility of *C. neoformans* to the killing activity of natural effector cells. Moreover capsule thickness was reduced during infection in KP-treated mice, as evidenced by histological examination of brain sections. Thus, the beneficial effect of KP against *C. neoformans* infection is due not only to direct, β1,3-glucan-mediated, fungicidal activity, but also to enhanced susceptibility of the fungus to the host immune response.

It is possible, although not demonstrated here, that even lower, non-microbicidal concentrations of KP could affect glucan synthesis, thus indirectly hampering capsule organization, which probably needs the integrity of the underlying cell wall. This hypothesis finds support in the observation that the outermost capsular-like mannoprotein component requires previous synthesis of the underlying β-glucan layer during protoplast regeneration in *Saccharomyces cerevisiae* and *Candida* (Sentandreu *et al.*, 1983).

Beside the capsule, urease, extracellular proteases, phospholipase and melanin production represent other important cryptococcal virulence factors (Perfect and Casadevall, 2002). Here we demonstrate that KP impairs the activity of some of these factors, even at concentrations unable to affect fungal growth. First, we observed a delay in urease production. This is particularly relevant, and suggests that KP could exert *in vivo* a sort of beneficial effect on the antifungal activity of phagocytes, as urease has been associated with the capacity of *C. neoformans* to survive in phagolysosomes (Cox *et al.*, 2000).

The role of extracellular proteases in the pathogenesis of fungal infections has been well established (Ghannoum, 2000). Secretion of aspartyl proteinases is essential for mucosal infections by *C. albicans* (De Bernardis

et al., 2001; Naglik *et al*., 2003). It is possible that the therapeutic effect of KP observed in *C. albicans* infection (Polonelli *et al*., 2003) could also be related to the inhibition of aspartyl proteinase activity. Here we observed that KP significantly reduces cryptococcal proteases *in vitro*. This could be an additional mechanism through which KP reduces colonization and tissue invasion *in vivo*, given that a role for secreted cryptococcal proteinases in cleaving host proteins involved in immunity has been suggested (Chen *et al*., 1996).

Phospholipase activity, responsible for destabilization of membranes and cell lysis (Schmiel and Miller, 1999; Ghannoum, 2000), has been correlated with virulence of *C. neoformans* (Chen *et al*., 1997). Finally, melanin is considered an important factor in protecting the microorganism against multiple toxic insults during infection (Gomez and Nosanchuk, 2003). The finding that KP, able to inhibit urease and protease production, does not affect phospholipase activity and melanin synthesis, suggests a selective effect on the inhibition of specific extracellular cryptococcal proteins. Such an effect could be somehow dependent on the loss of cell wall integrity caused by KP-induced binding to β-glucan.

In our experimental system a thinly encapsulated strain of serotype A *C. neoformans* was used. This strain, endowed with low virulence, closely resembles the infectious particles that give rise to infection in humans (Retini *et al*., 1998). The results of our present study therefore become an interesting working hypothesis for further investigations with more virulent strains of *C. neoformans*.

In conclusion, KP shows a remarkable antifungal activity on *C. neoformans in vitro* and a strong therapeutic activity *in vivo* in immunosuppressed hosts. This is particularly relevant, considering that cryptococcosis is still incurable in immunocompromised patients. Furthermore, KP can selectively impair or retard the synthesis/release of fungal virulence factors at doses unable to kill yeast cells *in vitro*. These data provide evidence that therapeutic activity of KP is dependent not only on its direct fungicidal activity, but also on its capacity to render *C. neoformans* less aggressive and invasive, thus facilitating the control of infection by the host immune system. The beneficial effects of KP observed in animal model studies could be useful to predict outcomes in the treatment of human infections, as observed for other antimicrobial drugs, suggesting a reasonable basis for a novel therapeutic option to cure fungal infections.

Experimental procedures

Microorganisms

Two strains of *C. neoformans* were used, *C. neoformans* var. *neoformans* 6995, a thinly capsulated isolate of serotype A, and the acapsular mutant *C. neoformans* var. *neoformans* 7698. The origin of the two strains has been described in detail elsewhere (Monari *et al*., 2002). The cultures were maintained by serial passages on Sabouraud agar (BioMerieux, France). The cells were harvested by suspending a single colony in saline, washed twice, counted in a hemocytometer and adjusted to the desired concentration. Unless otherwise stated, experimental data refer to *C. neoformans* strain 6995.

Killer peptide

An engineered KP fragment was used in this study. An SP was included as a negative control. The synthesis of KP on the basis of the sequence of a single-chain recombinant anti-idiotypic mAb, acting as a functional internal image of a microbicidal yeast killer toxin, and its optimization through alanine scanning have been described in detail elsewhere (Polonelli *et al*., 2003).

In vitro *evaluation of KP activity on* C. neoformans

The antifungal activity of KP against *C. neoformans in vitro* was evaluated as the number of CFU recovered after plating on Sabouraud agar and by a colorimetric assay (Levitz and Diamond, 1985). Briefly, for determination of CFU, 10^4 *C. neoformans* were incubated at 37°C in 1 ml of RPMI medium, alone or in the presence of 20, 10 and 5 μg ml^{-1} KP or SP. After 24 h, 100 μl of suspension were plated onto Sabouraud agar and the CFU evaluated after a 48 h incubation at 37°C. In selected experiments, different concentrations of laminarin (β1,3-glucan; Sigma Chemical) and pustulan (β1,6-glucan; Calbiochem) were also added, as described elsewhere (Polonelli *et al*., 2003). For the colorimetric assay 20, 10 or 5 μg ml^{-1} KP or SP were added to 10^3 *C. neoformans* in a final volume of 200 μl, in 96-well flat-bottomed microtitre plates. After incubation for 24 h at 37°C, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) was added to the cultures for an additional 3 h, formazan precipitate was solubilized with acid isopropanol, and *C. neoformans* viability was determined by measuring the resulting stain with an ELISA plate reader at a wavelength of 570 nm.

Production of melanin by C. neoformans

In vitro synthesis of melanin was induced by culturing *C. neoformans* on agar plates of defined minimal medium containing 1.0 mM L-DOPA (Sigma), as described elsewhere (Franzot *et al*., 1998).

C. neoformans *capsule measurement*

The thickness of the polysaccharide capsule of *C. neoformans*, untreated or treated with KP or SP, was measured in India-ink preparations under 400× magnification as described elsewhere (Franzot *et al*., 1998). The distance from the edge of the capsule to the cell was measured with the help of a grid. Measurement was done on at least 20 cells, and a mean value was determined.

C. neoformans *enzyme activity*

Determination of phospholipase production was performed by a plate method as described elsewhere (Echevarria *et al*., 2002).

Briefly, Sabouraud-egg yolk plates were inoculated with 10 μl of a suspension of 10^5 ml^{-1} *C. neoformans* cells, alone or in the presence of 10 and 5 μg ml^{-1} KP or SP, and incubated at 37°C. After 7 days of incubation, the diameter of the colony (a) and that of the colony plus the precipitation zone (b) was recorded. Phospholipase activity was expressed as Pz = a/b. The values range between 0 and 1, with the highest level of enzymatic activity being nearest to 0. Determination of protease activity was performed in bovine serum albumin-containing agar plates (Aoki *et al.*, 1994). After inoculation with 10 μl of a suspension of *C. neoformans* cells, as above, the plates were incubated at 37°C for 7 days, and the protease activity was expressed as the Pz value, as for phospholipase. Urease activity was determined 12 and 24 h after inoculation of *C. neoformans*, alone or in the presence of 10 and 5 μg ml^{-1} KP or SP, on BBL urea agar slants (Becton-Dickinson, Franklin Lakes, NJ).

Mice, infection and KP treatment

Female, 8–10 weeks old, inbred BALB/c mice were obtained from Harlan Nossan (Milan, Italy) and housed at the Animal Facilities of the University of Perugia, Perugia, Italy. Procedures involving animals and their care were conducted in conformity with national and international laws and policies. To induce systemic infection the mice, untreated or treated with 150 mg kg^{-1} cyclophosphamide three days before the challenge, were injected intravenously with 5×10^7 *C. neoformans* cells. KP (50 μg/dose) was administered intraperitoneally 1 h after the infection, and 1 and 2 days later. Infected animals were monitored for organ clearance and survival. Quantification of fungal growth, 5 days after infection, was assessed by plating serial dilutions of brain homogenates onto Sabouraud agar.

For histology, 5 days after infection brains were excised and immediately fixed in formalin. Sections (3–4 μm) of paraffin-embedded tissues were stained using the periodic acid-Schiff procedure.

Evaluation of antifungal activity of neutrophils and macrophages against KP-treated C. neoformans

Neutrophils were collected by lavaging the peritoneal cavity of mice 18 h after intraperitoneal injection of 1 ml of aged, endotoxin-free 10% thioglycollate solution (Difco). Resident macrophages were collected by peritoneal lavage of untreated animals. *C. neoformans* (10^6 ml^{-1} RPMI) were incubated at 37°C for 18 h, alone or in the presence of different doses of KP. *Cryptococcus neoformans* cells (10^5), treated or not with KP, were mixed with 10^6 neuthrophils or macrophages in the presence of 10% heat-inactivated FCS. After 2 or 4 h of incubation, the killing activity of neutrophils or macrophages, respectively, was determined as the percentage of CFU inhibition (mean ± standard error), calculated as follows: 100 − (CFU experimental group/CFU control cultures) × 100.

Statistical analysis

Student's *t*-test was used to determine the statistical significance of differences in organ clearance and *in vitro* assays. Survival data were analyzed by the Mantel-Cox Logrank test. Significance was defined as $P < 0.01$. *In vivo* groups consisted of 6–8 animals. Unless otherwise stated, the data reported were pooled from three to five experiments.

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