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In vitro activity of a monoclonal killer anti-idiotypic antibody and a synthetic killer peptide against oral isolates of *Candida* spp. differently susceptible to conventional antifungals

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Background/aims: A monoclonal killer anti-idiotypic antibody (mAbK10) and a synthetic killer peptide, acting as internal images of a microbicidal, wide-spectrum yeast killer toxin (KT) have been recently shown to express candidacidal *in vitro* and an *in vivo* therapeutic activity against experimental mucosal and systemic candidosis models caused by a reference strain of *Candida albicans* (10S).
Material and methods: The *in vitro* candidacidal activity of mAbK10 and synthetic killer peptide was compared using a colony forming unit assay against a large number of isolates of different *Candida* spp., obtained from oral saliva of adult diabetic (type 1 and 2) and nondiabetic subjects from Parma (Italy) and London (UK).
Results: Both the KT-mimics exerted a strong dose-dependent candidacidal activity, probably mediated by the interaction with β-glucan KT receptors on target yeast cells, against all the tested strains, regardless of their species and pattern of resistance to conventional antifungal agents.
Conclusions: These observations open new perspectives in the design and production of candidacidal compounds whose mechanism reflects that exerted in nature by killer yeasts.

Key words: antifungal resistance; *Candida* spp.; monoclonal antibodies; synthetic peptides

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Yeast killer toxins (KTs) are proteins or glycoproteins secreted for ecological competition by a number of yeasts, capable of killing susceptible cells, belonging to the same or congeneric species, that present specific cell wall receptors (KTR) (20). Interest in the yeast killer phenomenon has increased particularly with respect to the surprising susceptibility of eukaryotic and prokaryotic microorganisms of clinical interest to killer toxins from species of the genera *Pichia and Williopsis* (28). In particular, a toxin produced by the killer strain *Pichia anomala* ATCC 96603 (PaKT) has been studied for its biological activity against *Candida albicans*, characterised by a KTR mainly comprising β-glucans (17). Regretfully, PaKT proved to be toxic and very labile at physiological pH and temperatures, as well as strongly antigenic, so is not useful for systemic antifungal therapy (8, 25).

In order to obtain, for therapeutic purposes, large amounts of standardised reagents exploiting PaKT's antimicrobial activity without undesired effects, anti-idiotypic antibodies representing the internal image of PaKT and able to mimic its biological activity have been produced in the monoclonal (KTmAb) and recombinant

format (KTscFv) by using a PaKT-neutralising monoclonal antibody (mAbKT4) as immunogen (idiotypic vaccination) (19, 29, 30). KTmAb (mAbK10) and KTscFv (scFvH6) displayed a microbicidal activity *in vitro* and a therapeutic effect *in vivo* against KTR-bearing eukaryotic and prokaryotic pathogenic microorganisms (7, 9–11, 19, 31, 32). The KTscFv encoding gene was cloned and expressed in the human commensal *Streptococcus gordonii*. Engineered strains were able to stably colonise the mucosal surfaces and to exert a therapeutic effect. They were also as efficacious as a full course of fluconazole, when used to treat experimental vaginal candidiasis in rats (5).

In an attempt to identify biologically active fragments for potential therapeutic use, the scFvH6 encoding gene was sequenced and a series of overlapping decapeptides, each displaced by two residues, with special focus on those pertaining to complementary-determining regions (CDR) domains, were synthesised and tested for their candidacidal activity. The most active decapeptide (P6), containing part of the CDR-L1 region, was selected for large scale synthesis and analysed through alanine scanning to evaluate the critical relevance of each residue. In comparison with its own scramble peptide (SP), a decapeptide (KP) proved to exert an implemented candidacidal activity *in vitro* that was inhibited by laminarin (a soluble β-1,3-glucan preparation) but not by pustulan (a soluble β-1,6-glucan preparation). It also had a potent therapeutic effect *in vivo* in immunocompetent and immunosuppressed mice against vaginal and systemic candidiasis caused by fluconazole-sensitive as well as -resistant strains (27).

In recent years, the epidemiological relevance of *C. albicans* as a major human opportunistic pathogen, the growing importance of other non-*albicans* species often less susceptible to antifungal agents, and the frequent isolation of *Candida* species from the oral cavities of patients with diabetes mellitus, have attracted the interest of many researchers (1, 4, 15, 16, 23, 26, 33). In this study we report the *in vitro* fungicidal activity of mAbK10 and KP against a large number of oral isolates of different *Candida* spp. In particular, we comparatively tested yeast strains isolated in Parma and London from oral secretions of adult diabetic and nondiabetic subjects, previously identified by conventional and molecular methods (3, 21), showing different patterns of susceptibility to conventional antifungal drugs.

Material and methods

Candida isolates

A total of 229 oral yeasts (177 *C. albicans* and 52 non-*albicans* spp.) were isolated in Parma (DPR group, 71 strains) and London (DL group, 83 strains) from adult diabetic patients (both type 1 and type 2) and nondiabetic subjects (ND group, 75 strains), and identified by conventional and molecular methods (3, 21). All the isolates were tested for their antifungal susceptibility *in vitro* to six antifungal agents with a commercial kit following the methodology outlined by the manufacturer (Fungitest®, Biorad, Marnes-la-Coquette, France). The antifungal agents were assessed at two different concentrations (fluconazole: 8 and 64 μg, miconazole: 0.5 and 8 μg, ketoconazole: 0.5 and 4 μg, itraconazole: 0.5 and 4 μg, amphotericin B: 2 and 8 μg, 5-fluorocytosine: 2 and 32 μg) with both standard positive and negative controls. After incubation at 37°C for 48 h, growth was assessed by colorimetric means. Results are expressed as resistant (R), intermediate resistant (I) or sensitive (S) to each antifungal agent. A total of 71 representative strains (*C. albicans* and non-*albicans* spp.) were randomly selected for this study. Of these, 35 showed complete *in vitro* susceptibility to the tested antifungal agents (11 DPR, 12 DL, 12 ND), while 36 (12 from each group) were characterised by different patterns of susceptibility.

Monoclonal killer anti-idiotypic antibody (mAbK10)

The production and characterization of mAbK10, a rat IgM functionally mimicking PaKT, have been described elsewhere (30). Hybridoma cells secreting mAbK10 were grown in supplemented RPMI 1640 medium (Sigma-Aldrich Co., Irvine, UK) with 15% foetal calf serum (Sigma). MAbK10 was obtained from culture supernatants by precipitation with ammonium sulphate and dialysis against phosphate-buffered saline (PBS) and filtered through a 22 μm filter (Millex-GP, Millipore, Bedford, MA). Antibody concentration was determined by capture ELISA by means of a pair of mouse mAbs against μ heavy chain of rat Ig (LO-IMEX, Brussels, Belgium). A previously heat-inactivated mAbK10 acted as a negative control.

Synthetic killer peptide (KP)

An engineered synthetic killer decapeptide (KP) acting as a functional internal image of PaKT, obtained on the basis of the sequence of a single-chain recombinant anti-idiotypic mAb (27), was synthesised (Neosystem, Strasbourg, France) for use in this study. A scramble peptide (SP) containing the same amino acids of KP in a different sequence was also included as a negative control.

In vitro evaluation of candidacidal activity

The *in vitro* candidacidal activity of mAbK10 and KP was evaluated by a colony forming unit assay as previously reported (27, 30). Briefly, *Candida* cells from a single colony on Sabouraud Dextrose agar (SDA) were suspended in 199 Medium (Sigma-Aldrich) and incubated with shaking at 37°C for 2 h. After counting in a Burker haemocytometer, 10 μl of yeast cell suspension ($2–3 \times 10^2$ cells) was added to 90 μl of H_2O containing mAbK10 (final concentration 20 μg/ml) or KP (final concentration, 20 or 100 μg/ml). Heat-inactivated mAbK10 or SP at the same concentrations was used as control. A reference strain of *C. albicans* (UP10), known to be sensitive to mAbK10 and KP (27, 30), was used as a positive control. After incubation for 18 h at 37°C, the fungal cells were dispensed onto SDA plates that were then incubated at 30°C for 48 h before CFU enumeration. Each experiment was performed in triplicate and the results were expressed as the percentage inhibition of each isolate in comparison with the respective control.

To establish the minimal fungicidal concentration, corresponding to 100% killing of yeast cells, the above mentioned CFU assay was also carried out in the presence of decreasing concentrations of KP (15, 10, and 5 μg/ml) on four of the *Candida* strains (two *C. albicans*, one susceptible and one resistant to itraconazole; two *Candida* non-*albicans*, one susceptible and one of intermediate resistance to azoles) against which KP demonstrated a 100% killing activity at higher concentrations. Each experiment was performed in triplicate.

Colonies of *Candida* strains grown in SDA plates after treatment were re-grown on SDA for 24 h, re-suspended in 199 Medium and re-incubated in the presence of mAbK10 or KP, as described above, in order to establish if they were intrinsically resistant to KP or if they were occasionally not sensitive to KP, under the experimental conditions adopted.

Statistical analysis

Statistical analysis was performed to establish possible differences in the candidacidal activity of mAbK10 and KP on the 71 *Candida* isolates evaluated in this study. In particular, we compared the mAbK10 and KP fungicidal activity on *C. albicans* isolates vs. non-*albicans* ones (Table 1). In addition, a comparison was made between *Candida* isolates previously showing a resistance to one or more antifungal agents vs. completely susceptible isolates to the same agents tested (Tables 2 and 4).

Finally, we evaluated differences in mAbK10 and KP activity on *Candida* isolates from each group of patients (Diabetic patients from London, from Parma and nondiabetic subjects) (Table 3).

Statistical analysis of data was performed with Mann–Whitney (when two groups of data were analysed) or Kruskal–Wallis (when more than two groups of data were analysed) nonparametric tests and differences within or between groups were assumed to be significant when the probability (P) was less than or equal to 0.05.

Results

Antifungal susceptibility of *Candida* isolates

Data concerning the *Candida* strains isolated from diabetics and nondiabetics in Parma and London and their susceptibility patterns against six different antifungal agents are summarised in Table 4. Most of the isolates (77.2%) were *C. albicans*, especially genotype A (81.9%). A greater percentage of strains resistant and intermediately resistant to azoles and amphotericin B was observed among the isolates from London. On the basis of these observations, 71 randomly selected strains from the three different groups, species (both *C. albicans* and non-*albicans*), and susceptibility profiles have been chosen for further investigation and testing against mAbK10 and KP (Tables 1–4).

Table 1. mAbK10 and KP candidacidal activity on *C. albicans* isolates vs. non-*albicans* isolates

C. albicans isolates	K10[a] (%)	KP[a] (%)	*Candida* isolates	K10[a] (%)	KP[a] (%)
C. albicans A DL 2	99	100	*C. dubliniensis* DL 63	90	91.7
C. albicans A DL 4	97.1	97.7	*C. dubliniensis* DL 41	97	89.7
C. albicans A DL 7	77.4	98.6	*C. dubliniensis* DL 92	75.1	92
C. albicans A DL 1	83.4	97.9	*C. dubliniensis* DL 72	70	68.7
C. albicans A DL 18	71	93.1	*C. dubliniensis* DPR 47	73.4	99
C. albicans A DL 24	70.5	89.8	*C. dubliniensis* CZ 69	65.2	75.3
C. albicans A DL 66	84.1	92.2	*C. dubliniensis* CM 24	93.1	88.2
C. albicans A DL 121	78.2	58.2	*C. dubliniensis* CM 31	96.1	95.6
C. albicans A DPR 59	65	99.7	*C. dubliniensis* CM 35	70	98.1
C. albicans A DPR 67	50.1	100	*C. dubliniensis* CM 72	60.3	92.5
C. albicans A DPR 96	74.3	97.4	*C. glabrata* DL 48	73.4	96.3
C. albicans A DPR 98	65.9	98.4	*C. glabrata* DL 102	65	83.3
C. albicans A DPR 100	90.4	100	*C. glabrata* DL 107	51.6	99.5
C. albicans A DPR 38a	52.7	82.9	*C. glabrata* DL 125	93	95.9
C. albicans A DPR 39	62.4	98	*C. glabrata* DPR 28	99.5	84.4
C. albicans A DPR 95	80	97.1	*C. glabrata* DPR 33	82	96.9
C. albicans A DPR 107	97	100	*C. glabrata* DPR 24	62.1	90.5
C. albicans A CZ 58	99	92.4	*C. glabrata* CZ 96a	45.4	95.4
C. albicans A CM 75	70.5	76.9	*C. glabrata* CZ 20	80	87.2
C. albicans A CZ 22	74.4	97.1	*C. glabrata* CZ 98	54.1	96.3
C. albicans A CM 76	92.2	93.4	C. *guilliermondii* DL 73	78.4	95.2
C. albicans A CM 95	60	100	*C. guilliermondii* CZ 29	94.4	92.2
C. albicans B DL 67	78.3	61	*C. krusei* DL 70	45	94.2
C. albicans B DL 13	77	92.9	*C. krusei* CZ 30b	56.1	95.1
C. albicans B DPR 89	76.2	98	*C. krusei* CZ 96b	87	79.4
C. albicans B DPR 111	55.3	99	*C. krusei* CM 20	60	94
C. albicans B CM 104	80.2	99.3	*C. parapsilosis* DL 98	72.1	93
C. albicans B CM 23	85.3	84.7	*C. parapsilosis* DPR 66	30	99.3
C. albicans C DL 54	80.2	90.5	*C. parapsilosis* DPR 23	93.4	94.1
C. albicans C DL 84	75	87.2	*C. parapsilosis* DPR 68	88	99.2
C. albicans C DPR74	87.4	97.8	*C. tropicalis* DL 86	78.4	100
C. albicans C DPR 9	91.4	99.8	*C. tropicalis* DPR 51	50	84
C. albicans C CM 29	77	90.1	*C. lusitaniae* DPR 31	91.1	98.8
C. albicans C CM 110	60	96.7	*C. lusitaniae* DPR 65	56.4	98.7
			C. lusitaniae CZ 30a	86.2	93.7

[a]mAbK10 and KP were used at a concentration of 20 μg/ml; their activity is expressed as the percentage of killing evaluated by the CFU assay in comparison with the proper controls. Statistical analysis was performed with the Mann–Whitney nonparametric test.

In vitro candidacidal activity of mAbK10

As shown in Tables 1–3, mAbK10 at a concentration of 20 μg/ml demonstrated significant antifungal activity on all of the 71 *Candida* strains evaluated in this study, with a percentage of killing ranging from 30 to 99.5%. A second CFU assay, performed in the same experimental conditions on cells from colonies of *Candida* spp. which survived the killer activity of mAbK10 showed a similar percentage of killing, proving that the re-tested isolates were not resistant mutant clones.

No statistically significant differences ($P = 0.45$) were observed in the candidacidal activity of mAbK10 between *C. albicans* (50–99% killing, mean = 76.9, sd = 13.1) and non-*albicans* isolates (30–99.5% killing, mean = 73.2, sd = 17.8).

No statistically significant differences ($P = 0.76$) were observed in the candidacidal activity of mAbK10 against the isolates that were either resistant to or of intermediate resistance to the antifungals (30–99.5% killing activity, mean = 74, sd = 17) and those that were susceptible to commercially available antifungal agents (50–97% killing activity, mean = 76.1, sd = 13.8).

No statistically significant differences ($P = 0.71$) were observed in the candidacidal activity of mAbK10 against the isolates from diabetic patients in London (45–99% killing activity, mean = 77.5, sd = 12.8), Parma (30–99.5% killing activity, mean = 72.7, sd = 18.4), and nondiabetic controls (45–99% killing activity, mean = 74.9, sd = 15).

In vitro candidacidal activity of KP

Synthetic peptide KP showed a significant candidacidal activity on all of the *Candida* isolates examined in this study, with 100% killing when a concentration of 100 μg/ml was employed. Although the killing rate was reduced when a concentration of 20 μg/ml was employed, in most isolates the killing was still above 90% (Table 1–3). A second CFU assay performed on cells from colonies of *Candida* spp. that survived the killer activity of KP confirmed that those colonies were not resistant mutant clones.

No statistically significant differences ($P = 0.14$) were observed in the candidacidal activity of KP between *C. albicans* isolates (58–100% killing, mean = 92.8, sd = 10.1) and non-*albicans* isolates (68.7–100% killing, mean = 92.2, sd = 7.1).

Table 2. mAbK10 and KP candidacidal activity on *Candida* isolates that previously showing a resistance to one or more antifungal agents vs. completely susceptible *Candida* isolates

Candida isolates	K10[a] (%)	KP[a] (%)	AS[b]	*Candida* isolates	K10[a] (%)	KP[a] (%)	AS[b]
C. albicans A DL 2	99	100	itr^R	*C. albicans* A DL 1	83.4	97.9	S
C. albicans A DL 4	97.1	97.7	mcz^I	*C. albicans* A DL 18	71	93.1	S
C. albicans A DL 7	77.4	98.6	itr^R ket^R 5-FC^R	*C. albicans* A DL 24	70.5	89.8	S
C. albicans B DL 67	78.3	61	ket^I mcz^I flu^R itr^R	*C. albicans* A DL 66	84.1	92.2	S
C. albicans C DL 54	80.2	90.5	mcz^I	*C. albicans* A DL 121	78.2	58.2	S
C. dubliniensis DL 63	90.0	91.7	$flu^I mcz^I$	*C. albicans* B DL 13	77	92.9	S
C. glabrata DL 48	73.4	96.3	$ab^I flu^I$ itr^I mcz^I	*C. albicans* C DL 84	75	87.2	S
C. glabrata DL 102	65.0	83.3	$mcz^I flu^R$ ket^R itr^R	*C. dubliniensis* DL 41	97.0	89.7	S
C. glabrata DL 107	51.6	99.5	flu^I ket^I itr^I mcz^I	*C. dubliniensis* DL 92	75.1	92.0	S
C. guilliermondii DL 73	78.4	95.2	$ket^I itr^I mcz^I$	*C. dubliniensis* DL 72	70.0	68.7	S
C. krusei DL 70	45.0	94.2	$itr^I mcz^I flu^R$	*C. glabrata* DL 125	93.0	95.9	S
C. parapsilosis DL 98	72.1	93	flu^I mcz^I 5-FC^I	*C. tropicalis* DL 86	78.4	100	S
C. albicans A DPR 59	65	99.7	itr^I	*C. albicans* A DPR 38a	52.7	82.9	S
C. albicans A DPR 67	50.1	100	5-FC^R	*C. albicans* A DPR 39	62.4	98	S
C. albicans A DPR 96	74.3	97.4	itr^I	*C. albicans* A DPR 95	80	97.1	S
C. albicans A DPR 98	65.9	98.4	ket^I flu^R itr^R	*C. albicans* A DPR 107	97	100	S
C. albicans A DPR 100	90.4	100	itr^I	*C. albicans* B DPR 111	55.3	99	S
C. albicans B DPR 89	76.2	98	ket^I	C: *albicans* C DPR 9	91.4	99.8	S
C. albicans C DPR74	87.4	97.8	itr^I	*C. dubliniensis* DPR 47	73.4	99.0	S
C. glabrata DPR 28	99.5	84.4	flu^I itr^I mcz^I	*C. glabrata* DPR 24	62.1	90.5	S
C. glabrata DPR 33	82.0	96.9	itr^I	*C. parapsilosis* DPR 23	93.4	94.1	S
C. lusitaniae DPR 31	91.1	98.8	flu^I ket^I itr^I mcz^I5-FC^I	*C. parapsilosis* DPR 68	88.0	99.2	S
C. lusitaniae DPR 65	56.4	98.7	flu^I ket^I $itr^I mcz^I$ 5-FC^I	*C. tropicalis* DPR 51	50.0	84.0	S
C. parapsilosis DPR 66	30.0	99.3	ket^I	–	–	–	–
C. albicans A CZ 58	99	92.4	itr^R	*C. albicans* A CZ 22	74.4	97.1	S
C. albicans A CM 75	70.5	76.9	$ab^I mcz^I$ flu^R itr^R ket^R	*C. albicans* A CM 76	92.2	93.4	S
C. albicans B CM 71	70.2	94.4	ket^I itr^R	*C. albicans* A CM 95	60	100	S
C. albicans B CM 104	80.2	99.3	mcz^I	*C. albicans* B CM 23	85.3	84.7	S
C. albicans C CM 29	77	90.1	5-FC^I	*C. albicans* A CZ 11	81.4	94.4	S
C. dubliniensis CZ 69	65.2	75.3	mcz^I	*C. albicans* C CM 110	60	96.7	S
C. glabrata CZ 96a	45.4	95.4	mcz^I	*C. dubliniensis* CM 24	93.1	88.2	S
C. guilliermondii CZ 29	94.4	92.2	itr^I	*C. dubliniensis* CM 31	96.1	95.6	S
C. krusei CZ 30b	56.1	95.1	flu^I ket^I mcz^I 5-FC^I	*C. dubliniensis* CM 35	70.0	98.1	S
C. krusei CZ 96b	87.0	79.4	flu^I ket^I itr^I mcz^I5-FC^I	*C. dubliniensis* CM 72	60.3	92.5	S
C. krusei CM 20	60.0	94.0	$ab^I flu^I$ ket^I itr^I mcz^I 5-FC^I	*C. glabrata* CZ 20	80.0	87.2	S
C. lusitaniae CZ 30a	86.2	93.7	5-FC^I	*C. glabrata* CZ 98	54.1	96.3	S

[a]mAbK10 and KP were used at a concentration of 20 μg/ml; their activity is expressed as the percentage of killing evaluated by the CFU assay in comparison with the proper controls. KP, decapeptide.
[b]AS, antifungal susceptibility: flu, fluconazole; ke, ketoconazole; itra, itraconazole; mic, miconazole; am, amphotericin B; FC, 5 fluorocytosine; I, intermediate resistant; R, resistant; S, sensible to all the antifungals tested.
Statistical analysis was performed with the Mann–Whitney nonparametric test.

No statistically significant differences ($P > 0.05$) were observed in the candidacidal activity of KP against the isolates that were either resistant or of intermediate resistance to these commercial antifungal agents (61–100% of killing, mean = 93, sd = 8.5) and those that were susceptible to commercially available antifungal agents (58–100% of killing, mean = 92.1, sd = 8.7).

Statistically significant differences ($P < 0.01$) were observed in the antifungal activity of KP against the isolates from diabetic patients in London (58–100% inhibition, mean = 89.9, sd = 11.4) and from Parma (84–100% inhibition, mean = 96.2, sd = 5.3). Statistically significant differences ($P < 0.01$) were also observed in the antifungal activity of KP against the isolates from diabetic patients from Parma (84–100% inhibition, mean = 96.2, sd = 5.3) and from controls (75–100% inhibition, mean = 91.7, sd = 6.7).

The fungicidal activity of KP was dose-dependent as assessed by testing the peptide at different concentrations against a small group of isolates of different antifungal susceptibilities (Table 5). Almost 100% fungicidal activity was obtained at 20 μg/ml of KP, but still good candidacidal activity was also observed at 15 (90–99% killing) and 10 μg/ml (48–96% killing). At a concentration of 5 μg/ml, KP showed considerably variable candidacidal activity, and against one isolate of *C. albicans,* which expressed a resistance to itraconazole, it had no activity at all.

Discussion

The therapeutic activity of a monoclonal killer anti-idiotypic antibody (mAbK10) and a synthetic killer peptide (KP) (19, 27) against experimental mucosal and systemic candidosis, caused by a reference strain (10S) of *C. albicans* has been recently reported. In the present study, the candidacidal activity of both the monoclonal anti-idiotypic antibody (mAbK10) and the synthetic killer peptide (KP) have been evaluated against a large number (71) of isolates belonging to different *Candida* species. (Fig. 1 and 2).

mAbK10 and KP have been found to have a significant candidacidal activity on all the oral *Candida* isolates, irrespective of the different species or the different susceptibility to conventional antifungal drugs. Recent data indicate that the antifungal susceptibility test (Fungitest) used in the present study can be useful and reliable for testing clinical *Candida* isolates even though it only measures a limited number of drug concentrations (22).

Table 3. mAbK10 and KP candidacidal activity on *Candida* isolates from diabetic patients from London, from Parma, and from nondiabetic control subjects

D/L *Candida* isolates	K10[a] (%)	KP[a] (%)	D/PR *Candida* isolates	K10[a] (%)	KP[a] (%)	ND *Candida* isolates	K10[a] (%)	KP[a] (%)
C. albicans A DL 2	99	100	*C. albicans* A DPR 59	65	99.7	*C. albicans* A CZ 58	99	92.4
C. albicans A DL 4	97.1	97.7	*C. albicans* A DPR 67	50.1	100	*C. albicans* A CM 75	70.5	76.9
C. albicans A DL 7	77.4	98.6	*C. albicans* A DPR 96	74.3	97.4	*C. albicans* B CM 71	70.2	94.4
C. albicans B DL 67	78.3	61	*C. albicans* A DPR 98	65.9	98.4	*C. albicans* B CM 104	80.2	99.3
C. albicans C DL 54	80.2	90.5	*C. albicans* A DPR 100	90.4	100	*C. albicans* C CM 29	77	90.1
C. dubliniensis DL 63	90.0	91.7	*C. albicans* B DPR 89	76.2	98	*C. dubliniensis* CZ 69	65.2	75.3
C. glabrata DL 48	73.4	96.3	*C. albicans* C DPR74	87.4	97.8	*C. glabrata* CZ 96a	45.4	95.4
C. glabrata DL 102	65.0	83.3	*C. glabrata* DPR 28	99.5	84.4	*C. guilliermondii* CZ 29	94.4	92.2
C. glabrata DL 107	51.6	99.5	*C. glabrata* DPR 33	82.0	96.9	*C. krusei* CZ 30b	56.1	95.1
C. guilliermondii DL 73	78.4	95.2	*C. lusitaniae* DPR 31	91.1	98.8	*C. krusei* CZ 96b	87.0	79.4
C. krusei DL 70	45.0	94.2	*C. lusitaniae* DPR 65	56.4	98.7	*C. krusei* CM 20	60.0	94.0
C. parapsilosis DL 98	72.1	93	*C. parapsilosis* DPR 66	30.0	99.3	*C. lusitaniae* CZ 30a	86.2	93.7
C. albicans A DL 1	83.4	97.9	*C. albicans* A DPR 38a	52.7	82.9	*C. albicans* A CZ 22	74.4	97.1
C. albicans A DL 18	71	93.1	*C. albicans* A DPR 39	62.4	98	*C. albicans* A CM 76	92.2	93.4
C. albicans A DL 24	70.5	89.8	*C. albicans* A DPR 95	80	97.1	*C. albicans* A CM 95	60	100
C. albicans A DL 66	84.1	92.2	*C. albicans* A DPR 107	97	100	*C. albicans* B CM 23	85.3	84.7
C. albicans A DL 121	78.2	58.2	*C. albicans* B DPR 111	55.3	99	*C. albicans* A CZ 11	81.4	94.4
C. albicans B DL 13	77	92.9	*C. albicans* C DPR 9	91.4	99.8	*C. albicans* C CM 110	60	96.7
C. albicans C DL 84	75	87.2	*C. dubliniensis* DPR 47	73.4	99.0	*C. dubliniensis* CM 24	93.1	88.2
C. dubliniensis DL 41	97.0	89.7	*C. glabrata* DPR 24	62.1	90.5	*C. dubliniensis* CM 31	96.1	95.6
C. dubliniensis DL 92	75.1	92.0	*C. parapsilosis* DPR 23	93.4	94.1	*C. dubliniensis* CM 35	70.0	98.1
C. dubliniensis DL 72	70.0	68.7	*C. parapsilosis* DPR 68	88.0	99.2	*C. dubliniensis* CM 72	60.3	92.5
C. glabrata DL 125	93.0	95.9	*C. tropicalis* DPR 51	50.0	84.0	*C. glabrata* CZ 20	80.0	87.2
C. tropicalis DL 86	78.4	100				*C. glabrata* CZ 98	54.1	96.3

[a]mAbK10 and KP were used at a concentration of 20 μg/ml; their activity is expressed as the percentage of killing evaluated by the CFU assay in comparison with the proper controls.
Statistical analysis was performed with the Kruskal–Wallis nonparametric test.

Table 4. Antifungal susceptibility of *Candida* spp. isolates from different groups of subjects

	Diabetics Parma No. of isolates/total (%)	Diabetics London No. of isolates/total (%)	Nondiabetic subjects No. of isolates/total (%)	Total subjects No. of isolates/total (%)
C. albicans	52/71(73.2),	69/83 (83.1)	56/75 (74.6)	177/229 (77.2)
C. albicans A[a]	40/52 (76.9)	59/69 (85.5),	46/56 (82.1)	145/177 (81.9)
C. albicans B[a]	10/52 (19.2)	7/69 (10.1)	8/56 (14.2)	25/177 (14.1)
C. albicans C[a]	2/52 (3.8)	3/69 (4.3)	2/56 (3.5)	7/177 (3.9)
Other *Candida* spp.	19/71 (29.7)	14/83 (16.8)	19/75 (25.3)	52/229 (22.7)
Fluconazole				
S	66/71 (92.9)	66/83 (79.5)	66/75 (88)	198/229 (86.4)
I	4/71 (5.6)	12/83 (14.4)	8/75 (10.6)	24/229 (10.4)
R	1/71 (1.4)	5/83 (6.0)	1/75 (1.3)	7/229 (3.06)
Miconazole				
S	67/71 (94.3)	54/83 (65.8)	59/75 (78.6)	180/229 (78.6)
I	4/71 (5.6)	29/83 (34.9)	16/75 (21.3)	49/229 (21.4)
R	0/71	0/83	0/75	0/229
Ketoconazole				
S	63/71 (88.7)	60/83 (72.2)	67/75 (89.3)	190/229 (82.9)
I	8/71 (11.2)	12/83 (14.4)	5/75 (6.6)	25/229 (10.9)
R	0/71	11/83 (13.2)	3/75 (4)	14/229 (6.1)
Itraconazole				
S	60/71 (84.5)	60/83 (72.2)	64/75 (85.3)	184/229 (80.3)
I	9/71 (12.6)	11/83 (13.2)	7/75 (9.3)	27/229 (11.8)
R	2/71 (2.8)	12/83 (14.4)	4/75 (5.3)	18/229 (7.8)
Amphotericin B				
S	70/71 (98.5)	77/83 (92.7)	67/75 (89.3)	214/229 (93.4)
I	1/71 (1.4)	6/83 (7.2)	8/75 (10.6)	15/229 (6.5)
5-Fluorocytosine				
S	68/71(95.7),	78/83 (93.9)	67/75 (89.3)	213/229 (93)
I	2/71 (2.8)	4/83 (4.8)	8/75 (10.6)	14/229 (6.1)
R	1/71 (1.4)	1/83 (1.2)	0/75	2/229 (0.8)

[a]*C. albicans* isolates were characterised by molecular methods into genotypes A, B, and C.
S: susceptible; I: intermediate resistant; R: resistant. (Ref. 21.)

Interestingly, KP activity on the *Candida* isolates from diabetic patients from Parma was greater than that on isolates from the diabetic patients from London ($P < 0.01$) and from the nondiabetic control subjects ($P < 0.01$). This observed variation is not readily explicable, but may reflect the different susceptibility of the *Candida* isolates from the three groups of patients to all antifungal agents (Table 4), or perhaps the natural variability of expression of the KTR throughout all fungi.

Table 5. KP activity at different concentrations on four selected *Candida* spp. isolates

Candida isolates	AS[a]	KP 20[b] (%)	KP 15[b] (%)	KP 10[b] (%)	KP 5[b] (%)
C. albicans A DPR107	S	100	91.1	47.7	25.5
C. tropicalis DL86	S	100	99.8	98.2	68.4
C. albicans A DL2	itr^R	100	90.4	80	0
C. glabrata DL107	flu^I ket^I itr^I mcz^I	99.5	98.5	95.9	72.8

[a]AS, antifungal susceptibility: S, sensible to all the antifungal tested; flu, fluconazole; ket, ketoconazole; itr, itraconazole; mcz, miconazole; am, amphotericin B; FC, 5 fluorocytosine; I, intermediate resistant; R, resistant.
[b]KP was tested at 20, 15, 10, and 5 μg/ml; its activity is expressed as the percentage of killing evaluated by the CFU assay in comparison with the proper control. KP, decapeptide.

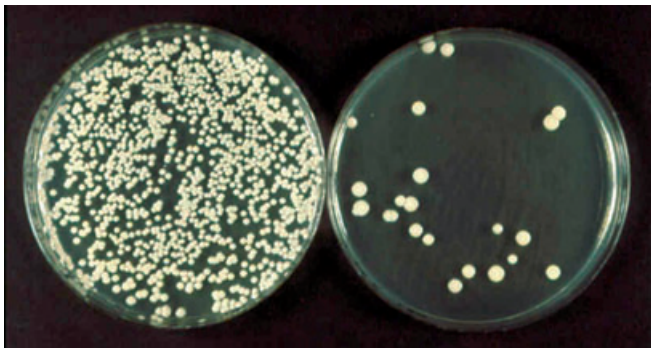

Fig. 1. Effect of mAbK10 on one of the *Candida* isolate used in the study. Left: Yeast cells treated with heat-inactivated mAbK10. Right: Yeast cells treated with mAbK10.

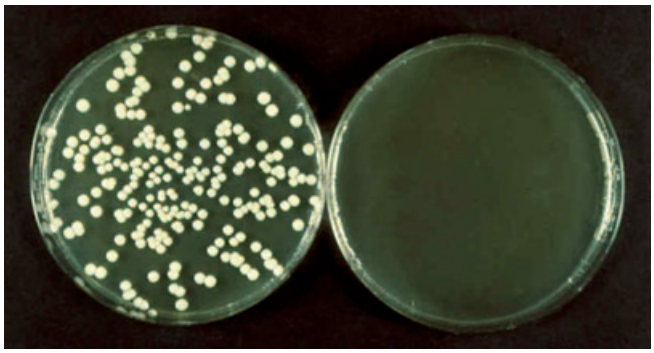

Fig. 2. Effect of KP on two different *Candida* isolates used in this study. Left: Yeast cells treated with the scramble peptide SP. Right: Yeast cells treated with KP. KP, decapeptide.

Nevertheless, the minimum inhibition of candidal growth by KP was over 58% even for the isolates that were completely resistant to the conventional antifungal agents (Table 2). It could be therefore postulated that, although the KTR is variably expressed in nature, it is ubiquitous and may therefore be exploited for a novel antifungal agent. The observed variability of susceptibility to KP and mAbK10 among strain of *C. albicans* would appear not to be caused by genetic mutations in a small number of cells. This is evidenced by the persistence of the same sensitivity upon re-testing.

It has been previously shown that germinating cells are especially susceptible to KT and its immunological derivatives, such as mAbK10 and KP (27, 30). This observation is of particular interest, given the relevance of germination for tissue invasion in *C. albicans* pathogenicity (12). It may well be that the variability observed in the present study was due to the different phases of growth of *Candida* cells during the experimental assay used in the present study.

The exquisite susceptibility to mAbK10 and KP of strains of *Candida* spp. resistant to conventional antifungal agents is of particular interest in view of the increased clinical concern about antimicrobial resistance in these and allied fungi (2). The mechanisms of resistance to conventional antifungal agents found in the *Candida* spp. isolates investigated in the present study do not affect the susceptibility of these isolates to mAbK10 and KP. Even though the mechanism of action of these antibody derivatives is still undetermined, their evident interaction with a presumptive KTR localised in the cell wall, such as β glucan, suggests the occurrence of a fungal target that has therapeutic potential. This may be similar to β-1,3-glucan synthesis inhibitors, such as pneumocandin and echinocandin, that display a fungicidal activity on different species of yeasts and moulds (13, 24). Resistance to these antifungal agents has been reported in spontaneous mutants of *Saccharomyces cerevisiae* and *C. albicans*; however, this resistance may not be of clinical relevance as the mutants exhibit attenuated virulence in animal models of experimental infections (14, 18).

The wide spectrum of candidacidal activity of mAbK10 and KP probably reflects their ability to interact with a β-glucan KTR. As glucan and glucan-like molecules are widely present in microbes, where they exert critical structural and/or virulence properties, their targeting may provide a novel and extensive therapeutic approach to microbial infections. These molecules can display a wide spectrum of antimicrobial activity, inclusive of yeasts and filamentous fungi, other than *Candida* spp., and this activity has been shown in diverse species such as *Aspergillus fumigatus* (7), *Cryptococcus neoformans* (6), *Paracoccidioides brasiliensis*, *Pneumocystis carinii* (32), as well as protozoa, such as *Leishmania* spp. (31) and bacteria (9–11).

KP had a higher candidacidal activity upon all the isolates tested in the study ($P < 0.001$) than did mAbK10. This may reflect the different structure of the two tested molecules compared at the same concentration (20 μg/ml) but characterised by a different structure, K10 being a rat monoclonal Ig M antibody (30) and KP a synthetic decapeptide containing the first three amino acids of the light chain CDR1, representing an active fragment of the KTscFv. Interestingly, just the deletion of the COOH-terminal serine caused a remarkable fall (about three logs) in KP activity, as recently reported by Polonelli *et al*. (27). Nevertheless, the engineered synthetic anti-idiotype fragment KP represents the evolution of KT-IdAbs, through the monoclonal (K10) and recombinant format (19, 30).

Although both mAbK10 and KP expressed a good candidacidal activity in this *in vitro* study, KP in particular, as a synthetic peptide, may represent the compound for the generation of a new class of broad-spectrum antimicrobial molecules for the prevention and treatment of superficial and systemic infections caused by KT-sensitive microorganisms. The mechanism of action of this new class of drugs reflects a naturally occurring phenomenon, that of killer yeasts, and as such may be able to overcome the currently recognised mechanisms of resistance to conventional antimicrobial drugs in epidemiologically relevant prokaryotic and eukaryotic pathogenic microorganisms.

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