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Inhibition of *Candida albicans* secreted aspartic protease by a novel series of peptidomimetics, also active on the HIV-1 protease

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Abstract

Nineteen reduced amide, monohydroxy- or dihydroxyethylene-based transition-state peptidomimetics, known to be good inhibitors of the aspartic protease of HIV-1, were tested against a secreted aspartic protease (Sap2), purified from the culture medium of a virulent strain of *Candida albicans*. Ten of these compounds exhibited IC_{50}s against Sap2 lower than 15 μM; the best inhibitor, Kyn–Val–Phe–Ψ[OH–OH]–Phe–Val–Kyn, when added to the *C. albicans* culture, repressed the hydrolysis of bovine serum albumin (BSA), contained in the culture medium, and inhibited the growth of the fungus.

Keywords: Peptidomimetic; Aspartic protease; Sap; *Candida albicans*

Candida albicans, a pathogen responsible for cutaneous, mucocutaneous, and systemic infections, possesses at least ten distinct genes (*SAP*) encoding secretory aspartic proteases (Sap), which are involved in nutrition, invasion, adherence, dissemination, and colonization by the fungus [1–6]. Individual members of the *SAP* gene family might be expressed at various stages of the infection process, during which different Saps might play specific roles. Saps1–3 appear to be produced during mucosal adherence and cause tissue damage, whereas the proteases encoded by the hyphal-associated genes, *SAP4–6*, may be more important for systemic infections [4,5,7–9]. Saps can thus be considered virulence factors.

In log-phase *Candida* cells, cultured in artificial media containing bovine serum albumin (BSA), *SAP2* mRNA is the main *SAP* transcript and Sap2 is thus the major secretory aspartic protease [10,11]. Other *SAP* genes may be expressed at significantly lower levels and later in the culture, depending on the *C. albicans* strain and environmental factors such as temperature of growth or absence of BSA as nitrogen source in the culture medium [10–12].

The *Candida* Saps belong to a category of enzymes, which are found in several prokaryotes and eukaryotes, as well as in viruses such as HIV [13]. The three-dimensional structure of Sap2, complexed with either a tight-binding or a general aspartic protease inhibitor, has revealed variations on the classical aspartic protease theme, mainly due to several sequence insertions and deletions [14,15]. Despite the difference in structure between Sap2 and HIV protease, several inhibitors of the latter are also active on the fungal enzyme [6,16–19]. This has led to the conclusion that the beneficial effect on *Candida* infections observed in AIDS patients treated with HIV protease inhibitors might be due to a direct inhibition of the fungal protease(s) [16,18].

Since several years we have contributed to the design and production of efficient, transition-state peptidomimetic inhibitors of the HIV-1 aspartic protease [20–24]. Here we present the results obtained from an evaluation of the inhibitory effects of a series of these compounds on Sap2, purified from a virulent strain of *C. albicans*.

Materials and methods

Fungal strain and culture. Candida albicans H12, a highly vaginopathic strain commonly used to study disseminated candidosis in

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animal models [7,25], was purchased from the American Type Culture Collection (ATCC56879). The yeast was grown overnight at 30 °C in Sabouraud's dextrose (Difco, Detroit, MI) as a starter culture, before inoculation (~10^3 cells/ml) into YCB–BSA medium (1.2% [wt/vol] yeast carbon base, 0.2% bovine serum albumin), pH 4 or 5. Cultures were shaken in an orbital incubator at 30 °C, generally for 48 h; media were collected by centrifugation, followed by passage through a 0.2 μm filter (Sarstedt, Nümbrecht, D) to yield sterile supernatants. Culture samples were utilized for enumerating colony forming units (CFU) on Sabouraud's dextrose agar.

Purification of Sap2. A 48 h-culture supernatant (40 ml) was lyophilized; the powder was dissolved in about 2.5 ml of 100 mM Na-phosphate buffer, pH 6.5, passed in two aliquots through a column (1 × 48 cm) of Sephadex G-75 (Amersham–Biosciences, Uppsala, Sweden), equilibrated, and eluted with 10 mM Na-phosphate buffer, pH 6.5. Sap activity was present in the fractions immediately following the void volume. These fractions were vacuum-dried to 1 ml.

Protein concentration and enzyme assays. Protein concentration was measured by the bicinchoninic acid method (Pierce, Rockford, IL). Fluorometric assays of Sap activity were carried out in 200 μl of 50 mM Na-citrate–HCl buffer, pH 3.2, with Arg–Glu–[5-(aminoethyl)aminonaphthalene sulfonate]–Ile–His–Pro–Phe–His–Leu–Val–Ile–His–Thr–Lys–[4′-dimethylaminoazobenzene-4-carboxylate]–Arg (20 μM; Molecular Probes Europe, Leiden, NL), as substrate [26]. Enzyme kinetic measurements were initiated by the addition of the protease (0.3 μg protein) and the inhibitory effects were evaluated by addition of each peptidomimetic at various concentrations about 1 min thereafter. The slopes of the fluorometric curves were utilized for calculating arbitrary 'protease units.' The HIV-1 protease activity was measured as described in [24]. Inhibitory data were converted into 50% inhibitory concentrations (IC_{50}) from a Dixon plot.

SDS–PAGE. Acrylamide (12.5%) gel electrophoresis was run in a standard fashion to monitor either the hydrolysis of BSA in the yeast culture medium or the purification of Sap2.

Peptidomimetic inhibitors. Reduced amide monohydroxyethylene and diaminodiol-based transition-state peptidomimetics were synthesized as reported [20,24]. Peptidomimetics with the mono- or di-hydroxyethylene in the central core were synthesized under stereochemical control by a regioselective reduction of amino acid-derived epoxyalcohols [27], followed by addition of the flanking residues [24]. The correct structure of the peptidomimetics was confirmed by ES-MS, the compounds were purified by semipreparative C18 RP-HPLC (Waters Radial-Pak), and concentrated stock solutions were prepared by dissolving in DMSO (which did not exceed the concentration of 1% in either of the enzyme assays or the yeast cultures).

Results and discussion

Purification of Sap2

Candida albicans H12, a strain which expresses *SAP2* at high levels [7], was cultured for 48 h in YCB medium in the presence of BSA, at pH 4. BSA hydrolysis products act as nutrient and BSA-derived peptides are also known to induce *SAP2* expression [8]. SDS–PAGE of the culture medium shows a major component with an apparent molecular weight of 44 kDa, equivalent to that of Sap2 [9,28], and also that the serum albumin is extensively hydrolyzed to relatively small peptides (Fig. 1, right lane). By gel permeation through Sephadex G-75, we succeeded in extensively purifying this component (Fig. 1, left lane), which exhibits aspartic protease activity. The one-step purification procedure adopted led to a 16-fold increase in protease specific activity and to a 60% recovery of total enzyme activity.

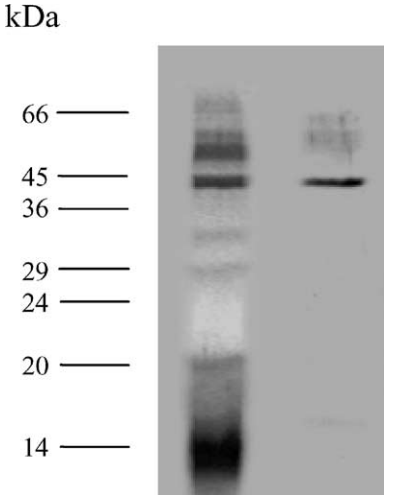

Fig. 1. SDS–PAGE of the supernatant of a 48 h-culture (YCB-BSA, pH 4) of C. *albicans* H12 (left) and of the purified Sap2 (right).

Peptidomimetic inhibitors of Sap2

Several peptidomimetics, that are known to be efficient inhibitors of the aspartic protease of HIV-1, were tested on the purified Sap2 (Table 1). All of them were less efficient in inhibiting Sap2 than the aspartic protease of HIV-1, against which they were actually designed, independently of the type of uncleavable central core and of flanking residues. However, ten out of nineteen had IC_{50}s lower than 15 μM, a value which compares well with the anti-Sap properties of those inhibitors of HIV-1 aspartic protease that are currently in use as anti-AIDS drugs [15–18], and which are likely also inhibiting *Candida* Saps in vivo [17,18]. Among the peptidomimetic inhibitors tested, TS-70, which can be synthesized by a rather simple route [24], appears to be the most efficient with an IC_{50} of 0.2 μM.

Effects of a peptidomimetic inhibitor on Sap2 secretion and C. albicans growth

The inhibition of aspartic protease(s) secreted from *C. albicans* should lead to a decreased cleavage of peptides from BSA, with a concomitant loss in the induction of the *SAP* genes and in the production of Sap [17]. We have thus cultured the *Candida* yeast for 48 h, in the absence and in the presence of 50 μM TS-70, with BSA as sole nitrogen source. As shown in Fig. 2, while BSA is almost fully hydrolyzed in untreated *Candida* cultures, the presence of TS-70 prevents to a variable degree the complete hydrolysis of BSA. The variance in hydrolysis patterns may derive from the complexity of *SAP*

Table 1
Inhibition of *C. albicans* Sap2 by transition-state peptidomimetics

		IC_{50}	
		Sap2	HIV-AP
TS-2	Arg–Ile–Phe–Ψ[CH_2–NH]–Phe–Gln–Arg	>100 μM	2 μM
TS-10	Trp–Ile–Phe–Ψ[CH_2–NH]–Phe–Glu–Trp	15 μM	2 nM
TS-23	Lys–Ile–Phe–Ψ[CH_2–NH]–Phe–Gln–Arg	>100 μM	60 μM
TS-41	Ac–Phe–Ile–Phe–Ψ[OH–OH]–Phe–Glu–Phe–Ac	7 μM	12 nM
TS-42	Ac–Trp–Val–Phe–Ψ[OH–OH]–Phe–Val–Trp–Ac	2 μM	6 nM
TS-43	Ac–Trp–Val–Phe–Ψ[OH–OH]–Phe–Glu–Phe–Ac	5 μM	9 nM
TS-49	Ac–Trp–Phe–Ψ[OH–OH]–Phe–Trp–Ac	37 μM	25 μM
TS-53	Boc–Phe–Ψ[OH–OH]–Phe–Glu–Phe–Ac	40 μM	0.6 μM
TS-54	Boc–Phe–Ψ[OH–OH]–Phe–Dtg–Poa	5 μM	0.2 μM
TS-57	Ac–Trp–Ser–Phe–Ψ[OH–OH]–Phe–Kyn	70 μM	4 μM
TS-59	Kyn–Thr–Phe–Ψ[OH–OH]–Phe–Kyn	13 μM	2 μM
TS-63	Kyn–Dtg–Phe–Ψ[OH–OH]–Phe–Poa	1.4 μM	9 nM
TS-70	Kyn–Val–Phe–Ψ[OH–OH]–Phe–Val–Kyn	0.2 μM	15 nM
TS-75	Ac–Trp–Val–Phe–Ψ[OH–OH]–Phe–Val–Ac	9 μM	5 nM
TS-90	Xan–Dtg–Val–Ψ(S,R,S) [OH]–Val–Dtg–Xan	30 μM	3 μM
TS-91	Kyn–Val–Phe–Ψ(S,R,S) [OH]–Phe–Val–Kyn	12 μM	4 nM
TS-92	Kyn–Dtg–Phe–Ψ(S,R,S) [OH]–Phe–dmPoa	83 μM	3 nM
TS-93	Kyn–Thr–Phe–Ψ(S,R,S) [OH]–Phe–dmPoa	120 μM	4 nM
TS-94	Kyn–Val–Phe–Ψ(S,R,S) [OH]–Phe–dmPoa	140 μM	3 nM

AP, aspartic protease; Ac, acetyl; Boc, *t*-butyloxycarbonyl; Dtg, D-α-(2-thienyl)glycine; Poa, phenoxyacetic acid; Kyn, kynurenic acid; Xan, xanturenic acid; dmPoa, dimethyl phenoxyacetic acid.

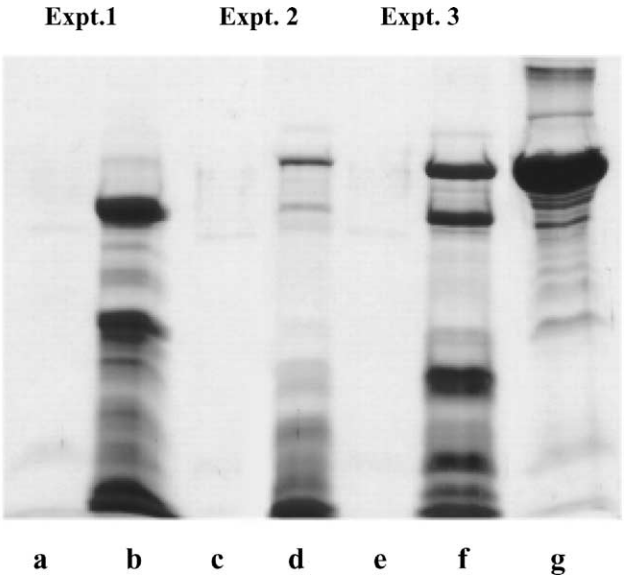

Fig. 2. SDS–PAGE of the supernatant of a 48 h-culture (YCB-BSA, pH 5) of *C. albicans* H12, in the abscence (lanes a, c, e) and in the presence (lanes b, d, f) of 50 μM TS-70. YCB-BSA, pH 5, corresponding to a t_0 culture, was run in lane g.

expression in *Candida*. Despite this, CFU counts carried out in parallel reproducibly showed that, while the control cultures reached $4.4 \pm 1.7 \times 10^8$ CFU/ml, the presence of 50 μM TS-70 caused a decrease in cell density to $4.7 \pm 0.7 \times 10^7$ CFU/ml (means ± SEM of three experiments).

In conclusion, we have found that several transition-state peptidomimetics, that are potent inhibitors of the HIV-1 aspartic protease, are also inhibiting Sap2. When one of these inhibitors was added to a culture of *C. albicans* at acidic pH, it partly inhibited the hydrolysis of BSA and also the growth of *C. albicans*. Growth inhibition did not exceed one logCFU, as residual activity of Sap2, or presence of other Saps, may contribute to yeast nutrition and growth. However, it is worth noticing that other inhibitors of Sap2, such as indinavir and ritonavir, although also causing a comparable incomplete inhibition of *C. albicans* growth in vitro, are still effective in inducing a rapid clearance of the fungus from the vagina of experimentally infected rats [18].

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