PEPTIDOMIMETIC INHIBITORS OF EXTRACELLULAR ASPARTIC PROTEINASES OF *Candida albicans* AND *Candida tropicalis*

Kateřina BROŽKOVÁ[a], Ivana KŘÍŽOVÁ[a], Libuše PAVLÍČKOVÁ[a1], Martin HRADÍLEK[a2], Martin FUSEK[a3,+], Tomáš RUML[b], Milan SOUČEK[a4] and Iva PICHOVÁ[a5,*]

[a] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, Czech Republic; e-mail: [1] pavlick@uochb.cas.cz, [2] hradilek@uochb.cas.cz, [3] sigmaldr@czn.cz, [4] soucek@uochb.cas.cz, [5] pich@uochb.cas.cz*

[b] *Department of Biochemistry and Microbiology, Prague Institute of Chemical Technology, 166 28 Prague 6, Czech Republic; e-mail: tomas.ruml@vscht.cz*

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In an attempt to develop effective inhibitors of *Candida* secreted aspartic proteinases, we have prepared a series of *N*-protected peptides varying in the type of scissile bond replacement, in the P and P' side chains as well as in the N- and C-terminal modifications. The compounds were tested *in vitro* with the chromogenic peptide substrate using purified secreted proteinases of *C. albicans* and *C. tropicalis*. Our results have confirmed that the binding of inhibitors and their effectiveness is influenced by a number of enzyme–inhibitor interactions. Moreover, factors like solvation/desolvation contribute to the optimal binding energy of the inhibitors.

Key words: Peptidomimetics; Peptides; Peptide bond isosteres; Secreted aspartic proteinases; *Candida albicans*; *Candida tropicalis*; Peptide inhibitors; Subsite specificity.

Candida infections cause diseases ranging from superficial disorders to invasive, rapidly fatal infections in the host with abrogated immunity. This yeast is a major agent of vaginitis, affecting a substantial group of the female population. *Candida* spp. have become also a serious pathogen of mucous membranes in persons infected with HIV (refs[1–3]).

The ability of *Candida* spp. to cause diseases is likely a complex process involving multiple interactions bewtween *Candida* and the host. Different factors, like secretion of extracellular proteinases (SAP), adherence, persistence, dimorphism, and phenotypic variability, contribute to candidal viru-

+ Present address: Sigma-Aldrich Ltd., Pobřežní 46, 186 21 Prague 8, Czech republic

lence[3–5]. The role of *Candida* SAP in the pathogenesis of candidosis has been experimentally demonstrated. Specific antibodies against SAP were detected in patients[6] and the level of proteinase expression correlates with the virulence of *Candida* sp.[7]. Treatment of mice with pepstatin A, selective inhibitor of aspartic proteinases, caused a protective effect against dissemination of *C. albicans*[2,8].

Initially, *Candida* strains were believed to express a single SAP. Later it was shown that *C. albicans* genome contains at least seven distinct genes encoding extracellular proteinases (SAP1–SAP 7) and at least one intracellular proteinase[9–11]. These enzymes are aspartic proteinases and they form two subfamilies represented by SAP 1–SAP 3, and SAP 4–SAP 7. Most studies have been addressed to SAP 2. This proteinase is composed of 342 residues. pH optimum of the proteinase is about 3. However, the proteinase is active on some substrates at neutral pH (ref.[12]). Recently, the 3D structure of *C. albicans* (SAP 2) in complexes with pepstatin A and the renin inhibitor A 70450, containing a hydroxyethylene isostere, and the 3D structure of CTAP have been determined[13–15]. Substrate specificity studies[16,17] and the structural data have revealed that the broad flap extending towards the active site and the large S3 subsite play a key role in determining specificity in SAP. These features differentiate *Candida* proteinases from mammalian aspartic proteinases such as renin, pepsin, gastricin, and cathepsin D.

To define the structural features of the inhibitor that influence the inhibition of secreted aspartic proteinases of *C. albicans* and *C. tropicalis*, we have synthesized a series of pseudopeptide inhibitors varied in the peptide bond isostere, in the neighboring residues, and in the N- and C-terminal modifications and analyzed their activities against both proteinases.

EXPERIMENTAL

Abbreviations Used

Boc, *tert*-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate; CAAP, *C. albicans* aspartic proteinase; CTAP, *C. tropicalis* aspartic proteinase; DCC, *N*,*N*'-dicyclohexylcarbodiimide; DEAE, diethylaminoethyl; DMSO, dimethyl sulfoxide; HOBt, 1-hydroxybenzotriazole; IC_{50}, concentration of compound that inhibited a reaction by 50%; Nph, 4-nitrophenylalanine; P3, P2, P1, P1', P2', S3, S2, *etc*., designation of amino acid residues of a substrate or inhibitor and corresponding regions of the enzyme active site involved in a complex according to Schehter and Berger[29]; Piv, pivaloyl; Pst, (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid; RP HPLC, reverse-phase high-performance liquid chromatography; SAP, secreted aspartic proteinase; SDS, sodium dodecylsulfate; TBDMS, *tert*-butyldimethylsilyl; TFA, trifluoroacetic acid; YCB, yeast carbon base.

Yeast Strains and Their Growth

C. albicans (strain C-74) and *C. tropicalis* (strain ATCC-75) were grown in 1.2% YCB medium, containing 0.4% bovine serum albumin, adjusted to pH 4.0. The cultures were shaken (200 min^{-1}) at a temperature 30 °C for three days. The cells were harvested and then pelletized by centrifugation at 13 000 g for 1 h. The resulting supernatant containing proteinases was used for isolation of CAAP and CTAP.

Isolation of Proteinases

Proteinases were isolated according to a modified method of Fusek[17]. The supernatant with SAP of *C. albicans* was dialyzed against 15 mM sodium citrate buffer pH 5.6 (buffer A) and then applied onto a column of DEAE-Sephadex A-25 equilibrated with buffer A. The proteinase was eluted with 100 mM sodium citrate buffer (pH 5.6). The fractions were assayed for proteolytic activity with chromogenic substrate Lys-Pro-Ala-Glu-Phe-Nph-Ala-Leu. The purity of the proteinase was screened by SDS electrophoresis on 10% polyacrylamide gels. To obtain a pure proteinase, the fractions containing enzyme were dialyzed against buffer A and rechromatographed on DEAE-Sephadex. The proteinase was eluted by a linear gradient of 15 to 100 mM sodium citrate buffer.

Proteinase secreted by *C. tropicalis* was isolated from the supernatant by ammonium sulfate precipitation at 60% of saturation. The precipitate was pelletized by centrifugation at 13 000 g for 1 h, redissolved in buffer A and dialyzed against the same buffer. The following purification of CTAP was similar to that of CAAP (see above).

Synthesis of Inhibitors

All peptides were synthesized by an established solution method[18] using orthogonal Boc/benzyl protection and DCC–HOBt couplings. Inhibitors **1**–**5** (Table I) were prepared by reductive amination[19] of *N*-Boc-phenylalaninal[20] in the presence of H-Phe-Glu-Phe-OMe (**1**), H-Phe-Gln-Phe-NH_2 (**2**), H-Phe-Cys-Phe-NH_2 (**3**), H-Phe-Asp-Phe-NH_2 (**4**) and H-Phe-Val-Phe-OMe (**5**). Inhibitor **6** was prepared by the same method from Piv-phenylalaninal and H-Phe-Asp-Phe-NH_2. Inhibitor **7**, with hydroxyethylene isostere, was prepared from *N*-Boc, *O*-TBDMS protected (2*R*,4*S*,5*S*)-5-amino-2-benzyl-4-hydroxy-6-phenylhexanoic acid[21–23], and dipeptide amide, H-Glu-Phe-NH_2, by BOP mediated couplings. Inhibitors **8** and **9**, with hydroxyethylamine isostere, were prepared by alkylation of tripeptide amides, H-Phe-Glu-Phe-NH_2 and H-Phe-Ile-Phe-NH_2, with *N*-protected (1*S*,2*S*)-(1-amino-2-phenylethyl)oxirane[24]. Inhibitors **10**–**14** with phenylstatine group were prepared by coupling of *N*-Boc protected (2S,3*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (Pst) with corresponding peptides. Crude inhibitors were purified by RP HPLC on a semipreparative Vydac 218TP510 column using a linear elution gradient from 0.05% TFA in 50% aqueous MeOH to 0.05% TFA in MeOH. Pooled fractions were mixed with dilute acetic acid and lyophilized. The purity of the inhibitors was confirmed by isocratic RP HPLC on a Vydac 218TP54 column in 0.05% TFA in aqueous MeOH, by amino acid analyses and by high-resolution fast-atom-bombardment mass spectrometry. Stereochemical purity of inhibitor intermediates was unequivocally proved by ^{1}H NMR spectroscopy (FT-NMR spectrometer Varian UNITY 500).

Activity Assays

The activities of both proteinases were monitored using a synthetic peptide containing the chromogenic 4-nitrophenylalanine residue, Lys-Pro-Ala-Glu-Phe-Nph-Ala-Leu. The assay was performed in 100 mM sodium acetate buffer pH 3.3 (buffer B). Typically, 1.5 nmol of proteinase was incubated with 28 nmol of the substrate in 1 ml of buffer B at 37 °C. The reaction rate was determined from a change in absorbance at 300 nm with an Aminco DW 2000 spectrophotometer. Proteinase concentrations were determined by the Bradford method[25].

Inhibition Studies

IC_{50} values of inhibitors were carried out by a spectrophotometric assay with the chromogenic substrate mentioned above. In a typical experiment, 1.5 nmol of SAP was added to 1 ml of buffer B containing 28 nmol of the substrate and an inhibitor. The final concentration of the inhibitors ranged from 0.6 $mmol^{-1}$ to 8 $nmol^{-1}$. The inhibitors were dissolved in DMSO, the final concentration of DMSO in the assay being lower than 2.5%. The IC_{50} values were determined from the Dixon plot using Enzfitter program.

TABLE I
IC_{50} values of inhibitors for secreted aspartic proteinases of *C. albicans* and *C. tropicalis*

No.	Inhibitor	IC_{50}, μmol l^{-1}	
		C. albicans SAP	*C. tropicalis* SAP
1	Boc-Phe[CH_2NH]Phe-Glu-Phe-OMe	28	32
2	Boc-Phe[CH_2NH]Phe-Gln-Phe-NH_2	>1 000	>1 000
3	Boc-Phe[CH_2NH]Phe-Cys-Phe-NH_2	23	23
4	Boc-Phe[CH_2NH]Phe-Asp-Phe-NH_2	238	389
5	Boc-Phe[CH_2NH]Phe-Val-OMe	130	>1 000
6	Piv-Phe[CH_2NH]Phe-Asp-Phe-NH_2	112	230
7	Boc-Phe[CH(OH)CH_2]Phe-Glu-Phe-NH_2	14	15
8	Boc-Phe[CH(OH)CH_2NH]Phe-Glu-Phe-NH_2	37	35
9	Boc-Phe[CH(OH)CH_2NH]Phe-Ile-Phe-NH_2	39	43
10	Boc-Pst-Ile-Phe-OMe	98	110
11	Boc-Pst-Glu-Phe-OMe	3	6
12	Boc-Pst-Glu-Phe-OH	160	230
13	Boc-Pst-Glu-Phe-NH(3-quinolyl)	65	85
14	Boc-Pst-Glu-Phe-NH-$(CH_2)_2$-NH-Boc	35	70

N-Terminal Sequence Analysis

N-Terminal sequences were determined from isolated material using an Applied Biosystems 470A Sequencer. The phenylthiohydatoins were identified by HPLC on an Ultrasphere ODS column.

RESULTS AND DISCUSSION

Most of the efforts of medicinal chemistry to design inhibitors of aspartic proteinases playing a key role in serious diseases, like AIDS, leukemia, neoplasia, muscle diseases, breast cancer or high blood pressure, have been based on classical substrates or transition-state analogs. Secreted aspartic proteinases of *Candida* species were shown to hydrolyze preferentially substrates containing the sequence Phe-Phe, Phe-Tyr, His-Thr and Lys-Thr (refs[17,26]). For our study, we have isolated secreted aspartic proteinases of *C. albicans* and *C. tropicalis*. N-Terminal sequencing of CAAP has revealed the sequence Gln-Ala-Val-Pro-Val-Thr-Leu- that represents the N-terminus of SAP 2 (ref.[14]). CTAP had the N-terminal sequence Ser-Asp-Val-Pro-Thr-Thr-Leu-Ile- which is identical to that published by Symersky *et al.*[15].

To study the inhibition of both SAPs, we have chosen a lead structure of inhibitors containing the motif Phe-isostere-Phe. To study the compatibility of various isosteric groups with various P2' side chains for fine tuning of inhibitors into the CAAP and CTAP, we have synthesized *N*-protected tetrapeptide inhibitors containing reduced amide [CH_2NH], hydroxyethylamine [$CH(OH)CH_2NH$], hydroxyethylene [$CH(OH)CH_2$], phenylstatine [(3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid] isosteres and as well as different amino acid residues in the P2' position.

The results are summarized in Table I. Inhibitor **1** with the reduced amide bond and glutamic acid in the P2' position exhibits similar potency for both proteinases. An unexpected change in the inhibition activity was observed for compound **2** with Gln at P2', which lacks inhibition activity for both CTAP and CAAP. Cysteine residue in the P2' position (inhibitor **3**) interacts with residues from S2' subsites of CAAP and CTAP similarly to Glu of inhibitor **1**. The IC_{50} values of inhibitor **3** are almost identical with those for inhibitor **1**. The low potency of Asp-containing inhibitor **4** is observed with both proteinases. The side chain of Asp in inhibitor **4** is probably too short to form enzyme–inhibitor interactions similar to Glu. Inhibitor **5** with a hydrophobic isopropyl side chain of Val exhibits a low inhibition activity for CAAP and CTAP. A slight improvement of the inhibition activity of inhibitor **6** was achieved by the replacing the Boc-protective group by the pivaloyl group [$(CH_3)_3$–C–CO] which lacks oxygen atom.

The replacement of the reduced bond in inhibitor **1** by the hydroxyethylene isostere, mimicking a transition state of peptide bond proteolysis, has not significantly improved the inhibition potency of inhibitor **7**. Likewise, the introduction of hydroxyethylamine isostere (inhibitor **8**) exhibited only a slight effect on the effectiveness of this compound for both proteinases. Almost no difference in inhibition activities was found between inhibitors **8** and **9** containing Glu and Ile residues in P2', respectively.

The introduction of phenylstatine residue, an analog of efficient statine residue from pepstatine, into the inhibitor containing Ile at the P2' position (inhibitor **10**) decreased the inhibition activity 2.5 times for both proteinases. However, replacement of Ile by Glu in the P2' position caused an improvement of the inhibition activity of inhibitor **11** by one order of magnitude. An unexpectedly high increase in IC_{50} values of two orders of magnitude was observed for inhibitor **12** in which an OH group is present at the C-terminus. The increase in IC_{50} values for both proteinases was likely caused by solvation/desolvation of the inhibitor. Recently, it was shown that this effect plays a significant role in the binding of HIV-1 proteinase inhibitors. A major contribution to the Gibbs energy of binding comes from the favourable entropy term resulting from the release of water molecules associated with the desolvation of inhibitors[27]. Inhibitor **12** containing the hydroxyl group offers more acceptors for formation of hydrogen bonds than the ester group of inhibitor **11**. The solvation of the OH group is thus higher, however, the desolvation step for this molecule is energetically more demanding. The energy for desolvation of inhibitor **12** decreases the Gibbs energy of binding and thus can increase the IC_{50} of this compound.

Modification of the parent compound **11** by the (3-quinolyl) amino residue at the C-terminus (inhibitor **13**) caused an increase in inhibition potency by one order of magnitude for both proteinases when compared with compound **12**. The extension of the Boc-Pst-Glu-Phe-NH_2 motif by -CH_2-CH_2-NH-CH_2-Boc has decreased the effectiveness of inhibitor **14** by one order of magnitude in comparison with inhibitor **11**.

Our strategy for the design of inhibitors of secreted aspartic proteinases of *C. albicans* and *C. tropicalis* is based on the transition-state mimetic concept, an approach that has been used successfully in the design of potent inhibitors of other aspartic proteinases[28]. Our results show that good inhibitors might have Glu or Cys residues in the P2' position. Introduction of Val into this position abolishes the activity. Both proteinases prefer hydrophobic residues in the P1 and P1' positions. Inhibitors containing Ala in the P1 position show higher activity that those with Phe in this position.

The X-ray structure and molecular modeling of the active site of SAP of *C. albicans*[13,14] have shown that the binding cleft is rather restricted toward the primed site (S1'–S4'). The non-primed site (S1–S4) forms a broad cleft that can accommodate large side chain residues. The data collected here show that the primed site of CAAP and CTAP is sensitive to the character of the side chains in the individual compounds. The results also show that fine-tuning of the inhibitor structure into the binding clefts of the enzymes involves a number of interactions throughout the active site. A single interaction is buffered by the adaptability of the plastic active cleft as a whole and multiple contacts of varying importance result in an acceptable fit for an inhibitor. Moreover, other factors, like solvation/desolvation of inhibitors significantly influence the binding of inhibitors to the active sites of proteinases. The results of this study will be used as a background for future design of potent inhibitors of CAAP and CTAP.

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REFERENCES

1. Odds F. C., Smidt J., Soll D. R.: *Mycoses in AIDS Patients*, p. 67. Plenum Press, London 1990.
2. Ruchel R. in: *Oral Candidosis* (L. P. Samaranayake and T. W. MacFarlane, Eds), p. 47. Wright, London 1990.
3. Cutler J. E.: *Annu. Rev. Microbiol.* **1991**, *45*, 187.
4. Ruchel R.: *Biochim. Biophys. Acta* **1981**, *659*, 99.
5. Macdonald F., Odds F. C.: *J. Med. Microbiol.* **1980**, *13*, 423.
6. Macdonald F., Odds F. C.: *J. Gen. Microbiol.* **1983**, *129*, 431.
7. Louie A., Dixon B. M., El-Maghrabi E. A., Burnett J. W., Baltch A. L., Smith R. P.: *J. Med. Vet. Mycol.* **1994**, *32*, 59.
8. Fallon K., Bausch K., Noonan J., Huguenel E., Tamburini P.: *Infect. Immun.* **1997**, *65*, 551.
9. Ray T. L., Payne C. D.: *Infect. Immun.* **1990**, *58*, 508.
10. White T. C., Miysaki S. H., Agabian N.: *J. Bacteriol.* **1993**, *175*, 6126.
11. Monod M., Togni G., Hube B., Sanglard D.: *Mol. Microbiol.* **1994**, *13*, 357.
12. Wagner T., Borg V., Zepelin M., Ruchel R.: *J. Med. Vet. Mycol.* **1995**, *284*, 618.
13. Cutfield S. M., Dodson E. J., Anderson B. F., Moody P. C. E., Marshall C. J., Sullivan P. A., Cutfield J.: *Structure* **1995**, *3*, 1261.
14. Abad-Zapatero C., Goldman R., Muchmore S. W., Hutchins Ch., Stewart K., Navaza J., Payne C. D., Ray T. L.: *Protein Sci.* **1996**, *5*, 640.
15. Symersky J., Monod M., Foundling I. S.: *Biochemistry* **1997, *36***, 12700.
16. Fusek M., Smith E. A., Monod M., Foundling S. I.: *FEBS Lett.* **1993**, *327*, 108.
17. Fusek M., Smith E. A., Monod M., Dunn B. M., Foundling S. I.: *Biochemistry* **1994**, *33*, 9791.

18. Bodanszky M., Bodanszky A.: *The Practice of Peptide Synthesis*. Springer Verlag, Berlin 1984.
19. Urban J., Konvalinka J., Stehlíková J., Gregorová E., Majer P., Souček M., Andreánsky M., Fábry M., Štrop P.: *FEBS Lett.* **1992**, *298*, 9.
20. Souček M., Urban J.: *Collect. Czech. Chem. Commun.* **1995**, *60*, 693.
21. Litera J., Buděšínský M., Souček M.: *Collect. Czech. Chem. Commun.* **1998**, *63*, 541.
22. Evans B. E., Rittle K. E., Hommick C. F., Springer J. P., Hirshfeld J., Veber D. E.: *J. Org. Chem.* **1985**, *50*, 461.
23. Chakravarty P. K., de Laszlo S. E., Sarnella C. S., Springer J. P., Schuda P. F.: *Tetrahedron Lett.* **1989**, *30*, 415.
24. Rich D. H., Sun C.-Q., Vara Prasad J. V. N., Pathiasseril A., Toth M. V., Marshall G. R., Clare M., Muller R. A., Houseman K.: *J. Med. Chem.* **1992**, *34*, 1225.
25. Bradford M. M.: *Anal. Biochem.* **1976**, *72*, 248.
26. Capobianco J. O., Lerner C. G., Goldman R. C.: *Anal. Biochem.* **1992**, *204*, 96.
27. Bardi J. S., Luque I., Freire E.: *Biochemistry* **1997**, *36*, 6588.
28. Vaca J. P.: *Methods Enzymol.* **1994**, *241*, 311.