Introduction

Pathogenic yeasts of the *Candida* species are among the leading causative agents of hospital-acquired yeast infections as they are often spread on the hands of health-care personnel. They prevail in specific hosts and environments such as low-birth-weight infants in neonatal intensive-care units. Three classes of antifungal drugs (azoles, polyenes and echinocandins) are recommended for the treatment of candidiasis, mainly caused by *C. albicans*. However, their effectiveness is hampered by the growth of other species, most notably *C. parapsilosis*, as well as the development of resistance due to biofilm formation. It is thus necessary to develop new types of antifungal drugs against new molecular targets.

Secreted aspartic proteases (Saps) of *Candida* species are extracellular enzymes with broad substrate specificity that enable the yeasts to overcome barriers used by the human host to prevent invasive infection. Moreover, Saps enhance the yeast virulence by degrading the host proteins involved in defence. They are also required for proper yeast adhesion during biofilm development. Saps are differentially transcribed depending on ambient conditions or disease progression. Their inhibition, albeit by aspartic protease inhibitors developed against other enzymes, e.g. HIV-1 protease, in combination with administration of other antifungal drugs, has reduced the formation of *C. albicans* biofilms and disrupted their mature form. The effect of these inhibitors in the reduction of oropharyngeal candidiasis and other surface mycoses has been reported in several studies.

The structure-based discovery and optimisation of indolone derivatives as Sap2 inhibitors is promising for the treatment of resistant *Candida albicans* infections. *C. parapsilosis*, as a major non-albicans *Candida* pathogen, possesses at least three Sap isoenzymes, of which Sap1p appears to contribute most significantly to the host-cell damage and the survival of *C. parapsilosis* in the host. Therefore, the development of specific Sap inhibitors is an important pharmaceutical goal.

Understanding the three-dimensional structure of aspartic proteases is a key to successful inhibitor design, as was exemplified in the 1990s for HIV protease in search for anti-AIDS drugs. Yeast Saps are similar in structure to pepsin-like aspartic proteases. They are arranged as two domains, consisting mostly of β-sheets, with a spacious substrate-binding cleft between them. Each domain contributes one DT(S)G triad to the active site, with the aspartate residue indispensable for catalysis. The central part of the extended active site is covered by an anti-parallel β-sheet, called flap. Peptidic substrates and peptidomimetic inhibitors span the active-site cavity in an extended conformation, placing their amino-acid side chains P₄–P₁ and P₁–P₄ in their respective S₄–S₅ and S₁–S₃ pockets. Sap1p is an enzyme with wide substrate specificity and relatively open substrate-binding cleft. The entrance to the substrate-binding site is lined with four entrance loops (N-ent loop 1, N-ent loop 2, C-ent loop 1 and C-ent loop 2) which modulate inhibitor affinities. Two disulphide bridges (Cys 47 – Cys 53 and Cys 258 – Cys 292) contribute to the stability of the structure.

Peptidomimetic aspartic protease inhibitors combine an uncleavable isostere of the peptide bond, bound in the S₁′/S₁′-S₄/S₅′-S₂′ pockets (Figure 1(B)).
inhibition was achieved using the nonpeptidic HIV PR inhibitor parapsilosis inhibitors were tested with Sap1 from cathepsin D26 which is structurally similar to Saps. A series of pepcavity were found to efficiently inhibit human aspartic proteases. Recently, novel types of macrocyclic mimetics designed thoroughly studied inhibitor of this type is pepstatin A (Figure 1(B))5,24. Pepstatin A inhibits most of aspartic proteases in subnanomolar concentrations (K_i of 0.3 nM for Sapp1p)25. It has inspired the design and synthesis of numerous analogues (Figure 1(B))24. Pepstatin A inhibits most of aspartic proteases in subnanomolar concentrations (K_i of 0.3 nM for Sapp1p)25. It has inspired the design and synthesis of numerous inhibitors targeted towards pharmacologically important proteases. Recently, novel types of macrocyclic mimetics designed using the knowledge of pepstatin A conformation in the binding cavity were found to efficiently inhibit human aspartic protease cathepsin D26 which is structurally similar to Saps. A series of pepstatin A based inhibitors varied at the P3, P2 and P2' positions were targeted against Sap1, Sap3, Sap5, and Sap6 from C. albicans27. Other phenylstatine (Pst)-containing pepstatin A inspired inhibitors were tested with Sap1 from C. albicans, Sapp1p from C. parapsilosis, and Sapt1p from C. tropicalis25. The K_i values of the Pst-containing inhibitors of this series (designated as KB32, KB70, KB74 and KB75) ranged from 0.1 nM to 14.6 nM for Sapp1p25. As alluded to above, Saps from different pathogenic Candida spp. are also inhibited by clinically used HIV-1 protease (PR) inhibitors, but in micromolar concentrations.6,25,28,–30. The best in vitro Sap1p inhibition was achieved using the nonpeptidic HIV PR inhibitor ritonavir (K_i 1.9 µM)25. In our previous work, we determined the structures of Sapp1p and the related Sapp2p in complex with pepstatin A23,31 and Sapp1p with ritonavir.12. These structural studies shed light into the binding modes but did not quantify the molecular determinants for differential affinity.

This work presents four crystal structures of Sapp1p complexes with four Pst-containing pepstatin-based inhibitors. The protein–ligand hydrogen bonding and nonpolar interactions in these complexes have been analysed in detail and compared with Sapp1p/pepstatin A23 and Sapp1p/ritonavir32 complexes. This analysis has made it possible to pinpoint the structural determinants responsible for the subnanomolar inhibition of this enzyme.

**Materials and methods**

**Sapp1p purification**

Sapp1p, used in this study, was an authentic enzyme purified from C. parapsilosis culture supernatant, as described previously. C. parapsilosis strain P69 from the mycological collection of the Faculty of Medicine, Palacky University, Olomouc, Czech Republic was cultivated in the YCB-BSA medium consisting of 1.2% (w/v) Yeast Carbon Base (Difco), 0.2% (w/v) BSA and 15 mM sodium citrate, pH 4. The culture supernatant obtained after approximately 70 h of cultivation and the removal of the cells by centrifugation (5000 g, 15 min) was subjected to ion-exchange chromatography, followed by gel filtration. Finally, Sapp1p and Sapp2p proteases were separated using chromatofocusing. Enzyme-activity assays were performed using the fluorogenic substrate Dabcyl-Glu-His-Val-Lys-Leu-Val-Glu-EDANS, which is cleaved at the Leu-Val bond by Sapp1p33.

**Crystallisation and data collection**

The complexes of Sapp1p with inhibitors were prepared and crystallised as follows. Purified Sapp1p (15 mg mL^{-1}) was incubated for 30 min at room temperature with a twofold molar excess of the inhibitor (applied as a DMSO solution) in 15 mM sodium citrate, pH 3.75 (the final DMSO concentration did not exceed 2%). The mixture was centrifuged for 5 min at 16,000 g. The initial crystallisation trials were performed with the help of a Gryphon crystallisation workstation (Art Robbins Instruments) by the sitting-drop vapour-diffusion method at 18 °C in 96-well plates; 0.2 µl of protein solution were mixed with 0.2 µl of well solution, and the mixture was equilibrated over a 200 µl reservoir solution. PEGs Suite I and JCSG Core I Suite (Qiagen) were used for the crystallisation condition screen. The initial microcrystals of Sapp1p with inhibitors appeared in several days. The subsequent optimisation of crystallisation conditions involved a change to the hanging-drop method, which was performed in NeXtal plates (Qiagen). The ratio of the protein to the precipitant solution was 2:1 for Sapp1p with KB32 and KB74 complexes and 1:1 for Sapp1p with KB70 and KB75 complexes. The final crystals were obtained under the following conditions. For the complexes of Sapp1p with KB32 and KB70, they were: 0.1 M MES pH 6.5, 40% PEG 200; for the complexes of Sapp1p with KB74 and KB75: 0.1 M MES pH 6.5, 30% PEG 400. The crystals were flash-cooled in liquid nitrogen with the precipitant solution as a self-cryoprotectant.

Diffraction data were collected at 100 K on MX 14.1 operated by the Joint Berlin MX-Laboratory at the BESSY II electron-storage ring in Berlin-Adlershof, Germany.34

Diffraction data for Sapp1_KB74 were processed, integrated, and reduced using XDS and scaled by XSCALE from the XDS suite, whereas the data for the other complexes were integrated by mosflm and scaled by the CCP4 package SCALA.35 The crystals belonged to the P212121 or P6222 space group and contained
Table 1. Crystal data and diffraction data collection and refinement statistics

|                  | Sapp1p–KB70 | Sapp1p–KB75 | Sapp1p–KB32 | Sapp1p–KB74 |
|------------------|-------------|-------------|-------------|-------------|
| Crystal data     |             |             |             |             |
| Space group      | P6_22       | P6_22       | P2_12,2_1   | P2_12,2_1   |
| a, b, c (Å)      | 172.62, 172.62, 253.25 | 172.62, 172.62, 253.25 | 87.26, 87.29, 157.95 | 87.25, 87.35, 157.68 |
| α, β, γ (°)      | 90.00, 90.00, 120.00 | 90.00, 90.00, 120.00 | 90.00, 90.00, 90.00 | 90.00, 90.00, 90.00 |
| Molecules per asymmetric unit | 4 | 4 | 4 | 4 |
| Max. resolution (Å) | 1.70 | 1.80 | 1.30 | 1.35 |
| Data collection and processing |             |             |             |             |
| Wavelength (Å)   | 0.9184      | 0.9184      | 0.9184      | 0.9184      |
| Resolution limits (Å) | 50.00–1.70 (1.75–1.70) | 50.00–1.80 (1.85–1.80) | 50.0–1.25 (1.28–1.25) | 50.0–1.35 (1.43–1.35) |
| No. of observed reflections | 3494125 (214086) | 1961990 (87469) | 1554178 (74826) | 838724 (319425) |
| No. of unique reflections | 234657 (15854) | 202267 (13667) | 323787 (18707) | 253539 (31857) |
| Multiplicity     | 14.5 (13.5) | 9.7 (6.4)   | 4.8 (4.0)   | 3.3 (3.0)   |
| Rmerge (Å)       | 0.098 (1.67) | 0.13 (1.10) | 0.073 (0.708) | 0.064 (0.537) |
| Rcrys (Å)        | 0.094 (1.61) | 0.113 (0.92) | 0.060 (0.548) | 0.054 (0.443) |
| CC1/2            | 100.0 (68.6) | 99.7 (52.3) | 99.9 (65.3) | 99.9 (78.0) |
| Completeness (%) | 98.3 (90.9) | 99.3 (78.2) | 84.3 (76.5) | 96.2 (90.3) |
| No. of reflections in working set | 221538 (14326) | 192014 (12888) | 274147 (19750) | 240826 (14664) |
| No. of reflections in test set | 11694 (759) | 10179 (656) | 14594 (1050) | 12767 (777) |
| No. of observations | 200267 (13667) | 1961990 (87469) | 274147 (19750) | 240826 (14664) |
| No. of unique observations | 10406/223/1649 | 10393/160/1101 |
| RMSD (Å)         | 0.013 | 0.012 | 0.013 | 0.012 |
| RMSD (°)         | 1.70 | 1.70 | 1.70 | 1.70 |
| Number of molecules in AU (protein/inhibitor/solvent molecules) | 10246/220/985 | 10198/212/1073 | 10406/223/1649 | 10393/160/1101 |

Structure solution

All structures were solved by molecular replacement with CCP4 Molrep, using available structures of identical proteins (3FV3)23. The initial models were refined through several cycles of manual building using Coot and automated refinement with CCP4 REFMACS. Structural data were visualised in PyMOL. Atomic coordinates and structure factors were deposited in the Protein Data Bank under the codes 7AGB, 7AGC, 7AGD and 7AGE (Table 1).

The analysis of enzyme–inhibitor interactions

Four crystal structures of Sapp1p in complex with inhibitors (KB32, KB70, KB74 and KB75) and Sapp1p/pepstatin A complex (PDB: 3FV3)23 were analysed for protein–ligand hydrogen bonds and nonpolar interactions using Protein–Ligand Interaction Profiler PLIP for non-peptidic parts of the ligands and UCSF CHIMERA for peptidic parts of the ligands. Prior to the analyses, the structures were protonated using CHIMERA. For the catalytic Asp dyad, a probable protonation state was assigned based on our previous quantum chemical calculations in Sapp2p/inhibitor complexes (Figure 2). The cut-off atom–atom distances were set to 4.1 Å for hydrogen bonding, the minimum D–H–A angle was set to 100°. The analyses were carried out for chains A of the structures. PyMol version 1.7.6.3 (Molecular Graphics System, Version 2.0 Schrödinger, LLC) was used for visualisation.

Results

The structures and binding modes in Sapp1p-inhibitor complexes

The crystal structures of Sapp1p complexes with four pepstatin-based inhibitors, KB70 (tert-butyl-oxycarbonyl-valine-valine-phenylstatine-alanine-phenylstatine-NH₂), KB32 (tert-butyl-oxycarbonyl-valine-valine-phenylstatine-alanine-phenylstatine-O), KB75 (tert-butyl-oxycarbonyl-valine-valine-phenylstatine-alanine-3R, 4R)-phenylstatine-OME) and KB74 (tert-butyl-oxycarbonyl-valine-valine-phenylstatine-alanine-OMe), were obtained at the resolution of 1.25–1.80 Å. Four nearly identical protein–ligand chains were present in the asymmetric unit cell, differing only in the conformations of the C-terminal phenylstatine (Pst) backbone (except for KB74, which contains only the central Pst residue). The overall structures of all the complexes were similar to each other (with the RMSD being 0.191–0.293 Å) and also to the Sapp1p complex with pepstatin A (the PDB entry 3FV3).33

The binding modes of all pepstatin-based inhibitors towards Sapp1p were nearly identical on the non-prime side (P4–P1) and in the P2′ position. The main differences appeared in the P1′ and P3′ regions (Figure 2(A)); they were caused by the different moieties and their stereochemistry. Ritonavir utilises a similar backbone binding mode to pepstatin A, including its isosteric hydroxyl and P2, P1 side chains, but it differs in the binding of the remaining moieties: on the one hand, it lacks the P4 and P3′ side chains;
on the other hand, its P3 and P2′ moieties extend further than those of pepstatin A (Figure 2(B)).

The interaction of the inhibitors in the Sapp1p-binding subsites

In all the crystal structures, we analysed hydrogen bonds and nonpolar interactions. Pepstatin A and derived inhibitors form a total of 11–12 H-bonds with an average distance of 2.8–2.9 Å, whereas ritonavir make only eight H-bonds with an average distance of 3.0 Å. There are 11–12 nonpolar contacts for all the inhibitors except for the weakest pepstatin-based inhibitor KB74 (8) and ritonavir (9), with an average distance of 3.7–3.8 Å (Table 2). This smaller number of both hydrogen-bonding and nonpolar interactions probably contributes to the low affinity of ritonavir to Sapp1p.

Protein-ligand hydrogen-bonding interactions

As far as hydrogen-bonding interactions in the individual Sapp1p subsites are concerned, pepstatin A and four derived inhibitors exhibit strictly conserved hydrogen-bonding patterns in the S3–S1′ subsites, including the catalytic aspartate dyad Asp32/Asp220, with heavy-atom distances differing at most by 0.3 Å (Table 1, Figure 3). This is contrasted to ritonavir, which lacks one hydrogen bond in the S3 pocket with Thr:OG1 due to the methylation of N74 in ritonavir and one in the S2 pocket with Asp80:OD2 due to its side-chain rotation (Figure 3). Furthermore, its hydrogen bond in the S1′ pocket (Gly79) is substantially weakened because of slight adjustments of the backbones of both the protein and the ligand.

In the S2′ pocket, pepstatin A and three pepstatin-based inhibitors (KB32, KB70 and KB74) form two hydrogen bonds to Sapp1p (Gly34:O and Asn125:ND2; Table 2(A)). KB75 lacks the latter one because of the different stereochemistry of the C-terminal Pst moiety and thus its inverted placement (Figure 2(A)). Ritonavir lacks the former one because of a slight repositioning of both the protein and ligand backbones.

The most variable region was the S3′ pocket as a result of different P3′ substituents – (3-R-4R)Pst for KB75, Pst for KB32 and KB70, missing moiety for KB74 and ritonavir, and Sta for pepstatin A. Only KB75 was able to form hydrogen bonds with Sapp1p (Arg77) thanks to its C-terminal Pst-moiety rotation (Table 2(A)).

Protein-ligand nonpolar interactions

The nonpolar contacts between Sapp1p and pepstatin-based inhibitors were mostly conserved with differences in distances up to 0.5 Å (Table 1(B)). In the S4 subsite, one nonpolar contact (Thr:224) was missing for pepstatin A due to the different identity of the P4 substituent (isovaleric acid vs. tert-butyl-oxy-carbonyl in other pepstatin-based inhibitors). Both S4 nonpolar contacts (Thr:224, Tyr:285) were missing for ritonavir, which does not possess the P4 residues and forms different contacts in the S3 and S2 pockets (Pro:112, Tyr:227). In contrast to pepstatin-based inhibitors, ritonavir had different contacts in S3 and S2 pockets (Pro:112, Tyr:227). Larger changes were observed in the S1′/S3′ pockets. Five contacts were present for pepstatin A, KB70, and KB32 (Ile:303, Leu:218, Asp:301 and two from Gly:79; Table 2(B)). KB75 lacked three of them but formed another two from Ala:127 because of its different stereochemistry of the C-terminal Pst. KB74 does not possess the P3′ substituent and thus did not have any S1′/S3′ nonpolar contacts. Ritonavir formed only one nonpolar interaction in S1′ (Ile:303); one (Leu:218) was missing as a result of the atom-atom distances slightly exceeding the threshold of 4.1 Å.

Discussion

The analysis of protein–inhibitor hydrogen-bonding and nonpolar contacts in crystal structures can be a useful tool for the interpretation of the molecular basis of differences in inhibitor affinities. In this work, we searched for structure–activity relationships (SAR) and found two molecular determinants of the inhibitor structures which defined their nanomolar affinity for Sapp1p. The first one was the conformation and orientation of the unprimed (P4-P1) and P2′ side chains as well as the backbones of the KB70, KB75 and KB32 inhibitors in the Sapp1p binding cleft, which was nearly indistinguishable from the binding mode of pepstatin A (the black dashed lasso in Figure 2(A)). The presence of this trait is likely to have been, at least in part, responsible for the low nanomolar K of values of these four inhibitors (from 0.1 to 6.6 nM; Figure 3). The absence of this characteristic feature (the missing P4 residue and the different orientation and contacts of the P3 residue of ritonavir; the black dashed lasso in Figure 2(B)) probably led to a significant affinity decrease (K; 1900 nM).

The second molecular determinant for the low nanomolar affinity of pepstatin A as well as three other Pst-containing inhibitors (KB70, KB75, and KB32) was the presence of the C-terminal extension beyond P2′ (the cyan dashed lasso in Figure 3), forming nonpolar contacts in the S1′ and S3′ subsites (as they are adjacent...
Table 2. (A) The hydrogen bonding of X-H···Y type, where X and Y are oxygen and nitrogen and (B) Nonpolar contacts of C···C type between Sapp1p and the four inhibitors studied here, pepstatin A and ritonavir. The atom···atom distances are in Å. The inhibitor names, composition and $K_i$ values (nM) are indicated.

| SUBSITE | PROTEIN | LIGAND | $A, K_i = 0.3$ nM | $K_i = 0.1$ nM | $K_i = 0.4$ nM | $K_i = 6.6$ nM | $K_i = 14.6$ nM | $K_i = 1900$ nM |
|---------|---------|--------|-------------------|----------------|----------------|----------------|----------------|----------------|
| S3      | Thr     | 224    | Val2             | N              | 3.0            | 2.9            | 2.9            | 2.9            | n.d.*         | n.d.*         |
| S2      | Asp     | 80     | Val3             | N              | 2.9            | 3.1            | 3.1            | 3.2            | n.d.*         | n.d.          |
| S1      | Gly     | 222    | Sta/Pst          | N              | 3.0            | 2.9            | 2.9            | 2.9            | 3.0           | 3.2           |
| S1'     | Gly     | 79     | Sta/Pst          | O              | 2.8            | 2.8            | 2.8            | 2.8            | 2.8           | 3.5           |
| S2'     | Gly     | 34     | Ala              | N              | 2.8            | 2.8            | 2.8            | 2.9            | n.d.          | n.d.          |
| S2'     | Asn     | 125    | Ala              | N, O           | 3.0            | 3.1            | n.d.           | 3.3           | 3.1           | 3.3           |
| S3'     | Arg     | 77     | (3R-4R) Pst      | N              | n.d.           | n.d.           | 2.8            | n.d.           | n.d.          | n.d.          |
| S4      | Thr     | 224    | Iva/Boc          | CG2/C2         | n.d.*          | 4.0            | 4.0            | 3.9            | 3.9           | n.d.          |
| S3      | Pro     | 12     | CG2/C2           | n.d.           | 3.5            | 3.6            | 3.5            | 3.9            | 3.8           | n.d.          |
| S2      | Ile     | 303    | Val3             | CG1            | 3.7            | 3.8            | 3.8            | 3.8            | 3.7           | 3.7           |
| S1      | Ile     | 30     | CD1              | Sta/Pst CD1/C1 | 3.8            | 3.6            | 3.5            | 3.3            | 3.6           | 3.6/4.0       |
| S1'/S3' | Ile     | 303    | CD1              | Sta/Pst CD1/CZ | 3.9            | 3.6            | n.d.           | 3.5           | n.d.          | 3.7           |
| S5      | Thr     | 224    | Iva/Boc          | CG2/C2         | n.d.*          | 4.0            | 4.0            | 3.9            | 3.9           | n.d.          |
| S5      | Tyr     | 285    | CE1/CE2          | n.d.           | 3.5            | 3.6            | 3.5            | 3.9            | 3.8           | n.d.          |
| S3      | Pro     | 12     | CG2/C2           | n.d.           | 3.5            | 3.6            | 3.5            | 3.9            | 3.8           | n.d.          |
| S2      | Ile     | 303    | Val3             | CG1            | 3.7            | 3.8            | 3.8            | 3.8            | 3.7           | 3.7           |
| S1      | Ile     | 30     | CD1              | Sta/Pst CD1/C1 | 3.8            | 3.6            | 3.5            | 3.3            | 3.6           | 3.6/4.0       |
| S1'/S3' | Ile     | 303    | CD1              | Sta/Pst CD1/CZ | 3.9            | 3.6            | n.d.           | 3.5           | n.d.          | 3.7           |

* n.d. not determined.
in the structure, they are collectively denoted as S1'/S3'). The absence of this extension in KB74 resulted in smaller numbers of hydrogen bonds as well as nonpolar contacts in the S1'/S3' sub-sites (cf. Table 2) and thus presumably in the lower affinity (Ki: 14.6 nM).

Admittedly, it is beyond the capabilities of the used approach to pinpoint the fine structural determinants of potency between pepstatin A and the pepstatin-based inhibitors. One reason is the disorder in the crystal structures, which has prevented unequivocal localisation of the C-terminal parts of the pepstatin-based inhibitors as well as water molecules at the protein-inhibitor interface that may stabilise the complexes. Another factor is the dynamics of the inhibitor and the surrounding protein amino acids, which may also influence the binding affinities. Last but not least, quantum effects such as proton transfer may occur between the active-site aspartates. Various computational approaches could be used to address these phenomena. Molecular dynamics (MD) can provide insights into the flexibility of the complexes and solvation patterns. MD-based energy analyses, such as the popular MM-GB/PBSA, are useful for obtaining interaction energies and their components and residue contributions. Quantum mechanics (QM) is a method of choice for cases where quantum effects play a role in protein–ligand binding.

Conclusions
We have determined four new crystal structures of nanomolar pepstatin-based inhibitors complexed to Sapp1p, an aspartic protease from the pathogenic yeast *Candida parapsilosis*. A
comparison of hydrogen bonding and nonpolar contacts with Sapp1p/pepsatin A and Sapp1p/ritonavir complexes has defined the molecular determinants responsible for high vs. low affinities. Most importantly, sub/low nanomolar inhibition occurred when the ligand filled the whole Sapp1p binding cavity from S4 to S3' pocket and formed numerous hydrogen bonds and nonpolar contacts. Such a structure-based insight can thus provide the basis for the development of novel medically useful compounds.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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