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Design, synthesis, inhibition studies, and molecular modeling of pepstatin analogues addressing different secreted aspartic proteinases of *Candida albicans*

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This paper is dedicated to the memory of Professor Hans C. Korting.

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ABSTRACT

The family of secreted aspartic proteinases is known as an important virulence factor of yeast infections by *Candida albicans* in particular, which is the most common fungal pathogen for humans with respect to systemic disease. Due to the continuing increase of drug resistant strains, these proteinases are currently considered as promising drug target candidates. Based on the known Sap2-substrate specificity data and X-ray analyses of Sap/inhibitor complexes, three libraries of inhibitors were designed and synthesized by modifying the structure of pepstatin A, a common non-selective aspartic proteinase inhibitor, at the P3, P2, or P2′ position. These novel inhibitors showed high inhibitory potencies for the isoenzymes Sap1, Sap3, Sap5 and Sap6. Then, the affinity and selectivity of the peptide ligands were investigated by molecular modeling, highlighting new key structural information for the design of potent and selective anti-virulence agents targeting *Candida albicans*.

1. Introduction

Infections caused by fungal pathogens are widespread and currently increasing, constituting a major threat in hospitals [1]. Most systemic fungal infections are caused by *Candida* species, with *Candida albicans* (*C. albicans*) being the most common representative. *C. albicans* is widespread as a commensal organism and can be found on mucosal surfaces in about 50% of the population. However, if the balance of the normal flora is compromised, this yeast can become virulent [2,3]. The most frequent forms of manifest candidosis affect mucous membranes like in the mouth and vagina, but systemic infections are also common today and can be life-threatening. The risk of serious candidosis increases after use of immunosuppressive agents [1].

Multiple factors are believed to be conducive to fungal pathogenicity. Hyphal formation, surface recognition molecules, phenotypic switching, and extracellular hydrolytic enzyme production have been the most widely studied factors in recent years [4]. The importance of extracellular hydrolytic enzymes in the context of virulence is well known in bacteria, protozoa, and yeasts. In terms of *Candida* species, the role of secreted aspartic proteinases (Sap) in candidosis has been experimentally demonstrated [5–8]. These enzymes are involved in the adherence and invasion of mucosal surfaces and subsequent dissemination by the *Candida* organism [9–12]. Therefore, the inhibition of Saps represents a promising strategy for the design of anti-virulence drugs, especially in consideration of the alarming rise in life-threatening systemic fungal infections paralleled by increased

Abbreviations: Abu, amino-butyric acid; ACN, acetonitrile; Boc, *tert*-butyloxycarbonyl; CI, 95% Confidence Interval; Dab, diaminobutyric acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; DIC, *N*,*N*-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; ESI, electrospray ionization; Fmoc, 9-fluorenylmethoxycarbonyl; HOAt, 1-hydroxy-7-azabenzotriazole; HPLC, high performance liquid chromatography; Nle, *nor*-leucine; *O*-MeTyr, *O*-methyl-tyrosine; Orn, ornithine; Pbf, 2,2,4,6,7-pentamethyl-dihydro-benzo-furan-5-sulfonyl; *p*-MePhe, para-methyl-phenylalanine; TBS, *tert*-butyldimethylsilyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Sap, secreted aspartic proteinases; Sta, statine; SPPS, solid phase peptide synthesis.

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drug resistance of the *Candida* species [13,14]. A combined therapy using a Saps inhibitor together with anti-fungal drugs would then increase the efficiency of the treatment against a prospective *Candida* species.

The Sap proteins belong to the aspartic proteinase family, a class of proteolytic enzymes which includes human and non-human proteins that are associated with widespread pathological conditions like hypertension (renin), AIDS (HIV proteinase), cancer (cathepsin D), and peptic ulcer disease (pepsin) [15]. Aspartic proteinases also play a major role in amyloid disease and malaria [15]. The Sap family of *C. albicans* includes 10 isoenzymes with molecular weights ranging from 35 to 50 kDa [2]. They are encoded by a family of ten *Sap* genes as preproenzymes, which have additionally 60–200 amino acid residues compared to the mature enzymes [2,3]. Distinct differences in pH optima of activity, ranging from 2 to 7, are evident among these isoenzymes. This property may be crucial to the survival of *C. albicans* and may also be associated with the different roles of Sap proteins during the colonization of the host. The Sap1–3 isoenzymes (pH optimum 3–5) seem to fulfill a function in adherence and tissue damage during localized infections [9], whereas Sap4–6 (pH optimum 5–7) appear to be important in systemic diseases [16–18]. The role of the Sap9 and Sap10 isoenzymes in cell surface integrity, cell separation, and adhesion has been characterized recently, but almost nothing is known about Sap7 and Sap8 [19,20].

The Sap2 isoenzyme is the most abundantly secreted protein in vitro when cells are grown in the presence of protein as the sole source of nitrogen [2]. Therefore, at present, most of the information available about Sap substrate specificity is based on the in vitro Sap2 activity. This isoenzyme is able to degrade many human proteins including albumin, hemoglobin, collagen, immunoglobulins and others. This broad spectrum of activity likely accounts for the ability of pathogenic *C. albicans* to efficiently overcome host cell barriers [2].

The structure of Sap2 was also the first of the Sap family to be solved crystallographically in complex with the inhibitor A-70450 [21,22], initially designed as a renin inhibitor at Abbott Laboratories, and with pepstatin A [22], which is a common non-selective inhibitor of aspartic proteinases. Recently, Borelli et al. [23,24] have also reported the crystal structures of Sap1 and Sap3 apoenzymes, and Sap3 and Sap5 in complex with pepstatin A. The availability of three-dimensional structures of several inhibited complexes of *C. albicans* Saps represents one of the major preconditions for structure-based inhibitor design.

Pepstatin A (Fig. 1A), a microbial pentapeptide produced by Actinomycetes [25], is the archetypal competitive inhibitor of aspartic proteinases. This molecule binds in an extended conformation inside the active site cleft of the enzyme (Fig. 1B). Its central Sta residue, which is a γ-amino acid containing the nonscissile bond –(CHOH–CH2)–, is responsible for the inhibitory activity as it acts as a tetrahedral transition-state mimic of amide bond hydrolysis [22]. Pepstatin A has been widely used to carry out studies in vitro, which provided the first direct evidence of the contribution of Sap enzymes to *C. albicans* virulence. Murine and rat mucosal model studies have shown the ability of pepstatin A to reduce *C. albicans* colonization and invasion of host tissues [26,27]. However, pepstatin A was virtually ineffective in systemic infections when administered intravenously [10], probably due to its unfavourable pharmacokinetic properties. In addition, pepstatin A exhibits low selectivity as it is also able to inhibit the mammalian aspartic proteinases. For the aforementioned reasons, this inhibitor is not to be considered as a drug candidate. Nevertheless, the discovery and characterization of pepstatin A is of paramount importance as modifications of its structure represent a starting point for the design of novel potent and selective aspartic proteinase inhibitors.

In the present work, the rational design and the biological evaluation of novel peptide inhibitors of Saps was undertaken. Three series of pepstatin analogues varied at the P3, P2, or P2′ positions were synthesized. Their inhibition activities were measured against different isoenzymes: Sap1, Sap3, Sap6 and few of them against Sap5 as well [28]. The aim of this study was to identify new key amino acids for the inhibition of each Sap subgroup, and to report a precise interaction map relevant for the design of new potent anti-virulence agents targeting the *C. albicans* proteinases. Similarly, this work also aimed at highlighting novel chemical features involved into the ligand selectivity among the studied isoenzymes. We envisage that this work is of great value for the future design of selective inhibitors that cross-react with Sap isoenzymes responsible for the virulence of *C. albicans*.

2. Material and methods

All reagents of synthetic grade were used as supplied. 2-chlorotritylchloride resin (1.55 mmol/g) was purchased from Iris Biotech GmbH (Marktredwitz, Germany). HOAt and all amino acids with the exception of statine (*N*-Fmoc-*O*-TBS-statine), which was synthetically prepared [29], were purchased from Gerhardt (Wolfhagen, Germany). Amino acid side chain protection was as follows: Lys(Boc), Dab(Boc), Orn(Boc), Arg(Pbf), Sta(TBS). DMF, ACN (HPLC grade), DBU, DIPEA, DIC, TFA, TIS, piperidine (99% extra pure), were purchased from Acros Organics (Geel, Belgium). HPLC

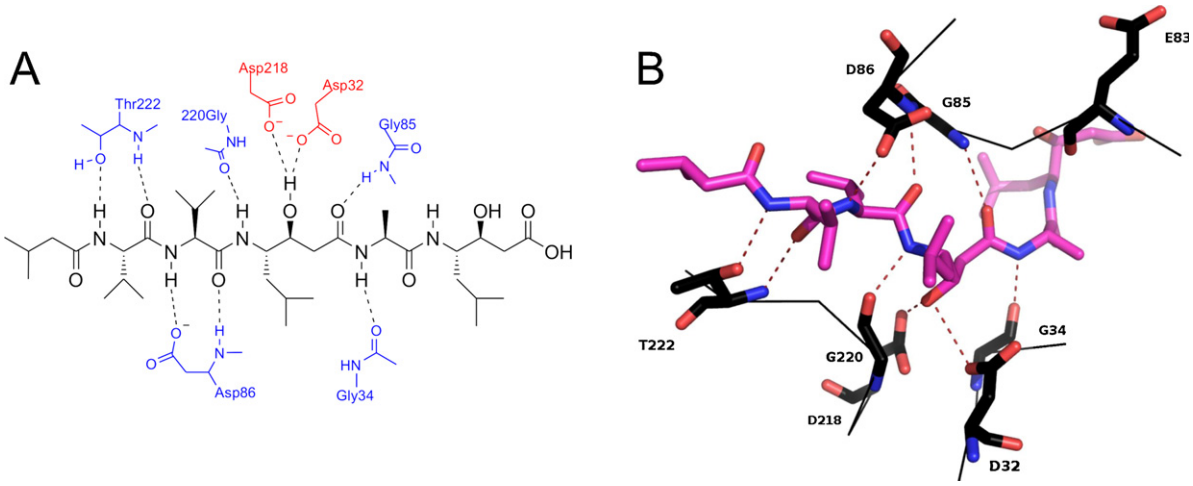

Fig. 1. 2D (A) and 3D (B) representation of the 9 H-bonds stabilizing the pepstatin A in the Sap binding clefts (based on X-ray crystallographic data).