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Research paper

Macrocyclization of a potent PACE4 inhibitor: Benefits and limitations

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ABSTRACT

PACE4, one of the seven members of the proprotein convertase family, plays an important role in the progression of prostate cancer. Therefore, its inhibition has become an attractive target to develop new therapies against this disease. Recently, we have developed a highly potent and selective PACE4 inhibitor, known as the multi-Leu peptide with the following sequence Ac-LLLLRVKR-NH_2. Herein, with the aim of improving the stability profile of this inhibitor for potential *in vivo* application, we investigated the impact of different cyclization strategies. The inhibitory activity of new peptides was tested and compared to their linear counterparts. The potent analogues were further selected for stability evaluation. Our results showed that the cyclization involving a *C*-terminal carboxylic acid (head-to-tail or side chain-to-tail) led to compounds with significantly diminished inhibitory potency towards PACE4, indicating that an appropriate balance between rigidity and flexibility of the structure is necessary to allow the optimal binding with the enzyme. On the other hand, the modification within a multi-Leu core in combination with the incorporation of a *C*-terminal 4-amidinobenzylamide (Amba) residue yielded potent cyclic analogues. The best compound derived from this group, (&)[Mpa]LLLC(&)RVK[Amba] (where & indicates cyclization, Mpa – 3-mercaptopropionic acid), exhibited promising overall profile comprising of potent inhibitory effect against PACE4 and prostate cancer cell lines as well as improved stability. We believe that this cyclic framework could be further used to design even more potent and stable PACE4 inhibitors.

1. Introduction

Prostate cancer (PCa) is one of the most frequently diagnosed and deadly malignancies, which occurs among 17% of the world's men population (Torre et al., 2015). Several therapeutic strategies have been approved for PCa, including surgery, radiotherapy, hormone therapy, and chemotherapy (Lavery and Cooperberg, 2016). Despite the notably prolonged survival in patients with PCa diagnosed in its early stages, for patients with advanced disease, the disease eventually progresses and yield poor prognostic for the majority of patients (Wu et al., 2014). Thus, there is an urgent need for the development of more effective therapeutic strategies for advanced PCa (molecularly targeted with greater efficacy and fewer side effects) that could improve disease survival. This targeted therapy might be used alone or in combination with one or more traditional treatments. In the past few years, numerous studies have been conducted to identify alternative pathways for an effective treatment for PCa including tyrosine kinase inhibitors (Qureshi et al., 2004), antiangiogenic agents (Feldman and Feldman, 2001), endothelin receptor antagonists (Qiao et al., 2015), anti-apoptotic

Abbreviations: γAbu, 4-aminobutyric acid; Amba, 4-amidinobenzylamide; AMC, 7-amino-4-methylcoumarin; ATCC, American Type Culture Collection; 6-Cl-HOBt, 1-hydroxy-6-chloro-benzotriazole; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; D6R, hexa-D-arginine; DMSO, dimethyl sulfoxide; EDT, 1,2-ethanedithiol; Et_2O, diethyl ether; FBS, foetal bovine serum; HFIP, hexafluoro-2-propanol; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; IC_{50}, the half maximal inhibitory concentration; K_i, the inhibition constant; ML, multi-Leu peptide inhibitor; Mpa, 3-mercaptopropionic acid; MS, mass spectrometry; Mtt, 4-methyltrityl; PCa, prostate cancer; PCs, proprotein convertases; PEG, polyethylene glycol; PyBOP, benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)-phosphonium hexafluorophosphate; RP-HPLC, reversed-phase high-performance liquid chromatography; RT, room temperature; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane; UPLC, ultra performance liquid chromatography; UV–vis, ultraviolet–visible.

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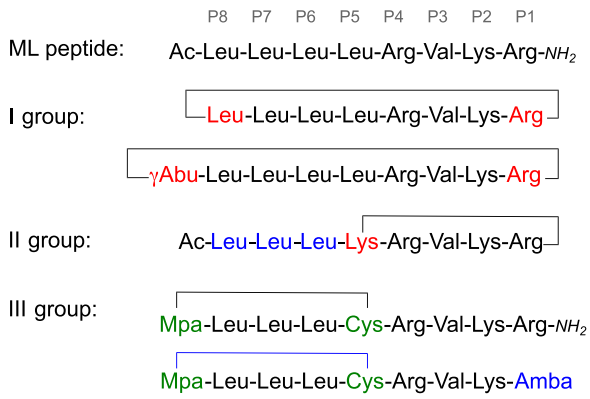

Fig. 1. General strategy for the ML peptide cyclization. New analogues have been classified into three groups based on the cyclization method. Group I comprises of peptides obtained by head-to-tail cyclization. Group II consists of compounds synthesized by side chain-to-tail cyclization between the side-chain of ε-amino group of a Lys residue at various positions of the multi-Leu core (which are indicated in blue) and a *C*-terminal carboxylic acid. Group III is composed of compounds with a Cys residue and its deaminated counterpart, mercaptopropionic acid (Mpa), both residues are indicated in green. Two analogues from this series have additional modifications such as the substitution of an Arg^{P1} residue with an Amba moiety or the replacement of the disulfide bond by *trans*-2-butene bridge (both modifications are indicated in blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein inhibitors and proteasome inhibitors (Lee et al., 2016), many of these pathways being dependent on proteins requiring a proteolytic step for biological activation.

Proprotein convertases (PCs) are serine proteases responsible for the processing of a wide variety of proproteins (including proforms of receptors, pro-hormones, growth factors, zymogens or cell membrane proteins) at a consensual R-X-X-R motif in various cell compartments, mainly in *trans*-Golgi network, endosomes, secretory granules and at the surface of cells (Seidah and Prat, 2012). Our work has shown that a single member of the PC family is crucial for the progression of PCa, namely PACE4 (Couture et al., 2012). In cancer cells, PCs are often deregulated and used to support tumour progression by sustaining tumour cells self-stimulation with mitogenic factors (Couture et al., 2011; Khatib et al., 2002). In PCa cells, we demonstrated that downregulation of PACE4, which is strongly overexpressed compared to normal prostate tissues (D'Anjou et al., 2011; Kang et al., 2014), reduces tumour progression, whereas no similar effect was observed for other cancer-related PCs, such as PC7 or furin (Couture et al., 2012). For this reason, the study of PACE4 holds promise as a potential druggable target for PCa.

Despite a relatively high degree of sequence and structural homology between the catalytic domains of PCs (Henrich et al., 2005), we were able to develop a highly potent (K_i = 22 nM) PACE4 inhibitor, known as a multi-Leu peptide (ML) with the following sequence: Ac-LLLLRVKR-NH_2 (Levesque et al., 2012) (Fig. 1). This inhibitor displayed antiproliferative effects on PCa cell lines (IC_{50} for DU145: 100 ± 10 μM; LNCaP: 180 ± 60 μM). Generally, peptides might be considered as good drug candidates because of their high biological activity and specificity to biological targets as well as low toxicity and minimal drug–drug interactions. However, their major drawback is poor *in vivo* stability, which can result in a short half-life ($t_{1/2}$) and low bioavailability (Adamska et al., 2015; Chen et al., 2015; Craik et al., 2013). In our case, stability studies revealed exoproteases hydrolysis susceptibility at both ends, which was marginalized by the substitution of both *N*- and *C*-termini by an unnatural amino acid (D-Leu) and an arginine mimetic (4-amidinobenzylamide, Amba), respectively (Kwiatkowska et al., 2014). The peptidomimetic variant (Ac-[DLeu]LLLRVK-Amba, known as compound C23) displayed significantly improved activity and stability profile and even recapitulated the effect observed with PACE4-silencing on PCa xenografts as an intravenously administered compound (Levesque et al., 2015). Nevertheless, this improved PACE4 inhibitor still is prone to proteolytic degradation; therefore, the incorporation of the additional modifications is necessary to transform it into a drug-like compound.

Various approaches have been developed to improve resistance of peptides to proteolysis, including *N*- and *C*-terminal modifications (Brinckerhoff et al., 1999; Dasgupta et al., 2002; Green et al., 2004), replacement of labile amino acids (Ritzel et al., 1998; Werle and Bernkop-Schnurch, 2006), increasing molecular mass (for instance: oligomerization and PEGylation) (Lee et al., 2005), cyclization (Osapay et al., 1997; Rubin and Qvit, 2016) and grafting the biologically active sequences into a cyclic framework (Chan et al., 2016). While some of these strategies have been already applied for the ML peptide, they have not always provided improved cellular and *in vivo* results. For example, to increase the size of the ML inhibitor, we have synthesized its *N*-terminally modified analogues containing a PEG or a fatty acid (octanoic acid) group (Kwiatkowska et al., 2016). In this case, PEGylation led to a loss of the antiproliferative effect, whereas lipidation resulted in analogues with similar or improved activity profile against PCa cell lines, but with significantly enhanced toxicity.

In the present study, we investigated whether the cyclization of the ML peptide can improve its stability profile. To design the first generation of cyclic PACE4 inhibitors, we took into account that the PCs recognition motif (RVKR↓) and the multi-leucine core responsible for selectivity towards PACE4 of the ML peptide should be preserved (Levesque et al., 2012). The general strategy used to obtain cyclic ML analogues is presented in Fig. 1. The first group consist of compounds synthesized by head-to-tail (*C*-terminus to *N*-terminus) cyclization of a ML peptide and its analogue *N*-terminally modified with a 4-aminobutyric acid (γAbu). The second series was based on the lactam cyclization between *C*-terminus and ε-amino group of the Lys residue in the P5, P6, P7 or P8 position, respectively. The third group of compounds contains peptides with thiol groups linked by the disulfide (analogue **8**, **9**) or alkene bridge (peptide **10**). Herein, we present the synthesis and biological evaluation (inhibitory activity, antiproliferative properties and plasma stability) of new cyclic compounds as potent PACE4 inhibitors.

2. Material and methods

All reagents and solvents were purchased from commercial suppliers and were used without additional purification. All coupling reagents, amino acid derivatives, *S*-trityl-3-mercaptopropionic acid, Boc-L-4-aminobutyric acid were obtained from Chem-Impex International (Wood Dale, IL, USA) and ChemPep (Miami, FL, USA). 2-Chlorotrityl-chloride resin and TentaGel S RAM resin were purchased from Rapp Polymere (Tübingen, Germany). Hexa-D-arginine inhibitor was obtained from Sigma–Aldrich (St. Louis, MA, USA). 4-Amidinobenzylamine·2HCl was synthesized from 4-(aminomethyl)benzonitrile hydrochloride according to the previously described procedure (Kwiatkowska et al., 2014) with the slight modifications concerning the formation of the amidine moiety, which was obtained by the reduction of acylated amidoximes with potassium formate in the presence of 10%Pd/C (Nadrah and Dolenc, 2007).

Reversed-phase high-performance liquid chromatography (RP-HPLC) was carried out on an Agilent Technologies 1100 system (analytical and semi-preparative) equipped with a diode array detector (λ = 210, 230, and 254 nm) and a VARIAN ProStar preparative system equipped with an UV–vis detector (λ = 214). The purity of the peptides was determined by HPLC with a Phenomenex Jupiter (5 μm, 4.6 mm × 250 mm, 300 Å) or an Agilent