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Original article

C-terminal constrained phenylalanine as a pharmacophoric unit in peptide-based proteasome inhibitors

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Abstract

Here we report the synthesis and biological properties of peptide-based molecules bearing constrained analogues of phenylalanine at the C-terminal. Compounds were tested as proteasome subunits' inhibitors. Dehydro-peptides showed good inhibition, in particular against trypsin-like (T-L) proteasome activity while some C-terminal Tic-derivatives inhibit only caspase-like activity in enzymatic β1 subunits with a certain degree of efficacy. The best analogues of the series demonstrated good resistance to proteolysis and a capacity to permeate the cell membrane.

Keywords: Constrained phenylalanine analogues; Synthesis; Pseudopeptides; Proteasome inhibition

1. Introduction

The ubiquitin–proteasome pathway is considered to be the best means of extralysosomal cytosolic and nuclear protein degradation in cells [1–5]. The 26S proteasome expressed in eukaryotic cells is a large multicatalytic intracellular protease complex and represents the central proteolytic machinery of the system. The proteasome regulates basic cellular processes and is responsible for the degradation and proteolytic processing of proteins essential for the regulation of development, differentiation, proliferation, cell cycling, senescence, apoptosis, gene transcription, signal transduction, antigen presentation, immune activation and the inflammatory and stress responses [6]. The proteasomal pathway represents a new approach in the treatment of a range of pathologies such as cancer, inflammation, immune diseases and others [7–12].

Cell proteins must be targeted for recognition and subsequent degradation by covalent attachments of a ubiquitin polypeptide [13]. The proteolytic activities of the 26S proteasome are carried out in its barrel-shaped 20S catalytic core, which is flanked by two 19S regulatory caps. Ubiquitinated substrates are recognized and bound to the complex, then unfolded and deubiquitinated, and subsequently transferred to the catalytic chamber, where they are degraded. Proteasome 20S is composed of four axially-stacked rings; the outer ring consists of seven different non-proteolytic α subunits, which allow substrate translocation into the central cavity formed by two inner rings formed by seven β subunits [14–16]. Only the β1, β2, and β5 subunits retain proteolytic activity by means of N-terminal threonine residues which face the central cavity. Based on their specificity towards peptidyl substrates, the β1, β2, and β5 subunits have been assigned caspase-like (PGPH), trypsin-like (T-L) and chymotrypsin-like (ChT-L) peptidase activities, respectively [17,18].

Several classes of synthetic and biological compounds which inhibit the proteolytic activities of the multicatalytic-complex have been developed [19–23], and have contributed greatly to the identification of essential functions of the 26S proteasome in various processes and pathways in eukaryotic

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cells [6]. In particular, proteasome inhibitors could act as therapeutic agents in the prevention of tumoral cell proliferation and as modulators of antigen presentation [8,12,24]. The elucidation of the 3D structure of proteasomal inhibitors provides interesting informations required for improving existing inhibitors and in the design of new compounds [25].

The major family of multicatalytic-complex inhibitors has a peptide-based structure with a C-terminal functional group able to interact with proteasomal catalytic threonine. Short peptide inhibitors include synthetic and natural molecules with a pharmacophoric function, such as vinyl sulfone, boronic acid, aldehyde and epoxyketone. Other classes are comprised of peptide macrocycles and non-peptidic inhibitors with a wide structural variety [26–39].

Involvement of the different proteasomal subunits in the process of protein degradation is evaluated by the employment of selective inhibitors of the individual active sites [40]. We have previously developed new oligopeptidic proteasome inhibitors bearing different pharmacophoric units at the C-terminal [41–46]. In particular we have identified and characterized a new class of inhibitors selective for trypsin-like activity and specific for the multicatalytic 20S complex with a vinyl ester function. This class of inhibitors is able to interact with enzymatic threonine in the same way that has been suggested for the well-known vinyl sulfone peptide. The best of these derivatives inhibit the β2 subunit in a nM range, are non-toxic, do not affect cell proliferation and are able to modulate the generation of antigenic peptides linked by MHC class I molecules. Moreover, vinyl ester inhibitors have demonstrated good resistance to proteolysis in plasma, as well as an ability to permeate the cell membrane [42].

On the basis of our previous results we prepared and tested a new series of compounds presented in Fig. 1 with reference vinyl ester inhibitors HMB-AA_1-AA_2-Leu-VE. New oligopeptide-based molecules contain selected amino-acidic sequences derived from the most representative inhibitors of the previous series. All pseudotri- and pseudotetrapeptides are functionalized at the N-terminal position with a 2-methyl-3-hydroxybenzoyl (HMB) group, while the C-terminal pharmacophoric unit consists of constrained phenylalanine analogues. Compounds **5–8** bear an α,β-dehydro-phenylalanine (ΔPhe), meaning that the substrate for Michael addition for catalytic threonine is directly inserted into the peptidic backbone. 1,2,3,4-Tetrahydro-isoquinoline-3-ethyl acrylate (Tic-VE) in an L or D configuration is the pharmacophore in the pseudopeptides **12–19**, analogous to reference leucine-vinyl ester (Leu-VE) inhibitors.

2. Chemistry

Pseudopeptides **5–8** contain pharmacophoric α,β-dehydrophenylalanine at the C-terminal position, as reported in Scheme 1. The ΔPhe moiety was obtained through azlactonization and dehydratation of Boc-Ser-(β-OH)Phe-OH (**1**) or Boc-Leu-(β-OH)Phe-OH (**2**), using sodium acetate in acetic anhydride [47,48]. Treatment of the azlactones with sodium ethylate resulted in the formation of N_α-protected dehydro-dipeptides **3** or **4** with ΔPhe in a *Z*-configuration [49]. Deprotection with trifluoroacetic acid and coupling steps utilizing water soluble carbodiimide and 1-hydroxybenzotriazole (WSC–HOBt) yielded the desired products.

Compounds **12–19** bearing a tetrahydro-isoquinoline vinyl ester (Tic) at the C-terminal in an L or D configuration were then synthesized stepwise by solution methods (Scheme 2). L or D N_α-protected Tic-VEs were prepared from the corresponding aldehyde, which is obtained via the Fehrentz method [50] by reaction with [(ethoxycarbonyl)methylidene]triphenylphosphorane [51]. HATU were employed for acylation of the H-Tic-VEs, the other coupling steps were accomplished using WSC/HOBt; Bocs were removed by TFA treatment.

All products were purified by preparative RP-HPLC, and structural verification was achieved by mass spectrometry and NMR spectroscopy. HPLC capacity factors (K') and other physicochemical properties of compounds **5–8** and **12–19** are reported in Table 1.

3. Biological activity

The capacity of the ΔPhe peptides **5–8** and tetrahydro-isoquinoline vinyl ester derivatives **12–18** to inhibit the three catalytic activities of isolated 20S proteasome was tested. All compounds were assayed at different concentrations (from 0.001 to 10 μM) for their capacity to inhibit the in vitro trypsin-like (T-L), chymotrypsin-like (ChT-L) and caspase-like (PGPH) activities of proteasome purified from lymphoblastoid cell lines using a fluorogenic substrate specific for the three major proteolytic activities of the enzymatic complex [52]. IC_{50} values against proteasome subsite activities obtained after 30 min of incubation are reported in Table 2, and compared to prototype vinyl ester inhibitor HMB-Val-Ser-Leu-VE and the two known inhibitors Epoxomicin and MG132.

The cell membrane permeation ability of dehydro-peptides and the most active Tic-VE analogues was tested against the β1 subsite (**13**, **15**) in live cells. After cell treatment, the proteasomes were purified and assayed for proteolytic activity as described above.

The same compounds tested in vivo were evaluated for their susceptibility to enzymatic hydrolysis by incubation at 37 °C in human plasma [53]. The degradation half-lives of the pseudopeptides, reported in Table 2, were determined as described in Section 6.

4. Results and discussion

The data obtained from enzyme inhibition tests against the catalytic subunits highlighted that the new peptide-based inhibitors generally show different potency and selectivity depending on their molecular structure.

The P1 position favours α,β-dehydro-phenylalanine (ΔPhe) to the bicyclic tetrahydro-isoquinolinic vinyl ester (Tic-VE) system. Compounds **4–8** are more active against the β2 and β5 subunits, while compounds **12–19** showed moderate inhibition of the β1 subunit of the 20S proteasome in certain cases. In terms of potency, the unsaturated residue was found to be