Bioorganic & Medicinal Chemistry 17 (2009) 3824–3828

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Cyclobutane-containing peptides: Evaluation as novel metallocarboxypeptidase inhibitors and modelling of their mode of action

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ARTICLE INFO

Article history:
Received 13 February 2009
Revised 14 April 2009
Accepted 17 April 2009
Available online 23 April 2009

Keywords:
Metalloprotease
Inhibitor screening
Ligand docking
Human carboxypeptidase B
Cyclobutane β-peptide
Non-natural amino acid

ABSTRACT

Different types of cyclobutane-containing peptides (CBPs) were screened for the first time as ligands of metallocarboxypeptidases (MCPs). CBPs are conformationally constrained, low molecular-weight compounds which showed moderate yet selective inhibitory activity against mammalian MCPs. The most potent compound was a carboxypeptidase B inhibitor. Docked protein–ligand complexes indicated that CBPs may bind to the target proteases via electrostatic interactions and aromatic stacking to catalytically crucial residues and that the placement of functional groups seems to be assisted by the rigid CBP backbone. The easily obtainable CBPs may offer a valuable alternative in the design of novel inhibitors to disease-linked metallocarboxypeptidases like human plasma carboxypeptidase B.

1. Introduction

Since their discovery, carboxypeptidase A (CPA) and carboxypeptidase B (CPB), members of the M14 family of metalloproteases, have been intensively investigated as a mechanistic model of metal-dependent proteases.[1–4] The assessment of the potential roles that MCPs play in different pathologies, like acute pancreatitis[5–7] inflammation,[8,9] diabetes,[10] and cancer[11–14] is a highly explored field today. Investigations on gastrointestinal proteolytic enzymes—such as pancreatic CPA and CPB—are currently underway to discern potential ways to overcome the problem of poor oral absorption of peptide and protein drugs.[15–17] In another context, CPA and CPB are considered to be good biomarkers for the early detection of acute pancreatitis and cancer.[18,19] These findings, together with the recent discovery of a complete new subfamily,[20] may warrant further developments of chemotherapeutic or bioimaging agents targeted to M14 proteases.[21]

Peptidomimetics containing non hydrolyzable bonds have been widely exploited in the design of extremely potent protease inhibitors.[22,23] Compound BX528 (a phosphinate-containing mimic of the tripeptide Phe-Val-Lys), an inhibitor to human plasma CPB, appears as a recent example of application of this strategy.[24] An alternative approach may involve the use of β- and γ-peptides. Such compounds proved to be resistant to hydrolytic cleavage by benchmark proteases, including CPA.[25] β- and γ-peptides share some common properties such as natural origin, stability, and propensity to form folding structures.[26]

Among the broad structural diversity known for natural and designed oligomers, CBPs are compounds scarcely explored to account for their biological properties. The CBP backbone contains the conformationally rigid cyclobutyl group instead of the freely rotatable (–CH_2–) units found in β- and γ-peptides. Moreover, CBPs constitute an attractive starting point for screening because of they bear some resemblance to the α-peptide substrates of MCPs: a low molecular weight, and an easy synthetic preparation.[27,28] We, therefore, decided to test the ability of CBPs to block the enzymic reaction carried out by M14 proteases and to investigate whether their rigid backbone might impose a demanding constraint on binding as shown by our recent determination of an α-peptide-bound MCP three-dimensional structure.[29] We obtained a series of CBPs and screened them against two prototypical MCPs with different specificities. Some CBPs were found to perform an inhibitory activity in vitro against bovine CPA and human CPB. Selected CBPs were computationally docked to the enzymes and their binding properties analysed in depth. As a result of this work, the identification and characterisation of a novel class of MCP inhibitors, the cyclobutane-containing peptides is described herein.

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doi:10.1016/j.bmc.2009.04.035

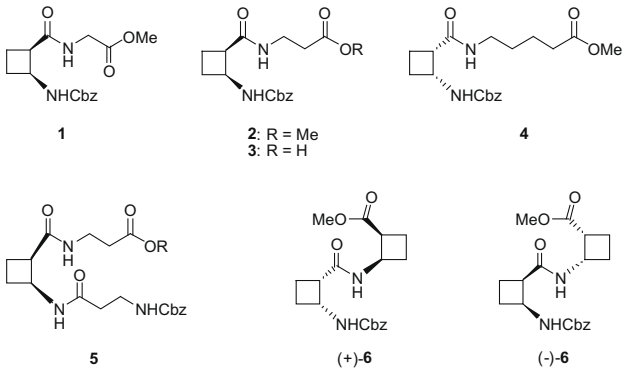

Chart 1. Structures of the CBPs analysed in this study.

2. Results and discussion

The structures of the cyclobutane containing peptides studied in this work are shown in Chart 1. Compounds **1–4** differ on the length of the linear residue. Thus, compound **1** is an α,β-dipeptide, **2** and **3** are β-dipeptides differing in the protected or unprotected terminal carboxyl group, **4** is a mixed β,δ-dipeptide, **5** is a β-tripeptide containing two β-alanine segments joined by a cyclobutane residue, and (+)- and (−)-**6** are enantiomeric bis(cyclobutane) β-dipeptides.

These compounds range in MW from 320 to 405 Da and their structures display a central cyclobutane ring flanked by a Cbz moiety and a carboxylate group at both ends. According to previous work, the backbone of the CBPs is bent around the cyclobutane ring and their functionalities may engage in a number of intermolecular interactions with neighbour molecules.[27,28]

2.1. Inhibition of carboxypeptidases

The results from seven CBPs acting against the two prototypical metallocarboxypeptidases, CPA and CPB, are collected in Table 1.

CBPs were active against CPA and CPB in the micromolar range. Dipeptide **1**, containing a glycine residue and synthesized to bear resemblance to the natural MCP substrates, showed the weakest inhibitory effect of the set. On the contrary, the β-dipeptide **2**, which is a $-CH_2-$ unit longer than **1**, behaved as a more effective inhibitor, especially against CPA. The short alkyl branch and the blocked carboxylate in compounds **1** and **2**, may weaken binding strength. Analogue **3**, with a free carboxylate, showed the lowest K_i value for both enzyme forms, with a preference for CPB. Compound **4**, with an alkyl branch which only differs from **2** in being two $-CH_2-$ units longer, performed as a twofold more potent inhibitor towards CPA, while its effect on CPB remained similar to that of compound **2**. These observations highlight the preference of CPA to bind and/or process aliphatic compounds. However, the fact that compound **4** displays a methyl ester instead of a free carboxylate may also explain its poor K_i against CPB and further investigations are necessary to clarify this issue. Compound **5**, the largest structure of the set with two alkyl branches, is similar to the latter in binding CPA, suggesting that the extra branch might have a minimal contribution to binding. Enantiomeric compounds (+)- and (−)-**6**, which contain a second cyclobutyl unit, showed also moderate inhibitory potency against both target proteins. Nevertheless, while (+)-**6** did not show any selectivity towards CPA and CPB, the enantiomer (−)-**6** was slightly CPA selective. A close inspection of CPA and CPB **6**-docked structures suggested a common mode of binding, with the Cbz moiety buried into the active site cleft while the blocked carboxylate is located at the protein's surface (not shown), in clear contrast with the mode of binding of compounds displaying a single cyclobutyl unit (see below). The settlement of the Cbz moiety is similar in the chiral active site cleft which imposes a symmetrical binding fashion of the solvent exposed groups in both enantiomers. Although the fitting in the active site is analogous in both structures, some minor offset was observed to occur, which may possibly be related to the inhibitory activity.

In summary, cyclobutane containing peptides were identified as a new kind of ligands for metallocarboxypeptidases from the M14 family. The most potent compound was **3**, which showed some preference to interact to CPB as compared to the other members of the series. Other slightly less potent CBPs acted with a high selectivity ratio against CPA, as is the case of compound **4**. Thus, both compounds **3** and **4** were chosen for further analysis of their binding mode.

Table 1
Biological activity of CBPs

Compound	K_i[a] (μM)	
	CPA	CPB
1	550 (100)	500 (95)
2	180 (30)	400 (70)
3	70 (12)	43.0 (7.5)
4	72.5 (9.5)	410 (80)
5	70 (15)	165 (55)
(+)-**6**	67 (10)	70 (10)
(−)-**6**	95 (18)	275 (45)

[a] Values inside parentheses indicate the standard error of the mean (SEM).

2.2. Analysis of β-dipeptide 3 binding

β-Dipeptide **3** occupies three subsites within CPB's active site cleft: S1′ (around Asp255), S1 (defined by Arg145 and Glu270, a subsite for substrate anchoring and processing) and S2 (shaped by Arg71, Ser197, Tyr198 and Ser199) (Fig. 1).

The inhibitor backbone is bent at the cyclobutyl ring. This might result from the rigid conformation dictated by the four-membered ring itself and from the protein–ligand intermolecular interactions. The free carboxylate binds to the substrate-anchoring residues Asn144 and Arg145 via one of its oxygen atoms (at distances 3.0 and 2.9 Å, respectively). This functionality locates more than 5 Å apart from Asp255, the specificity-determinant residue in CPB.

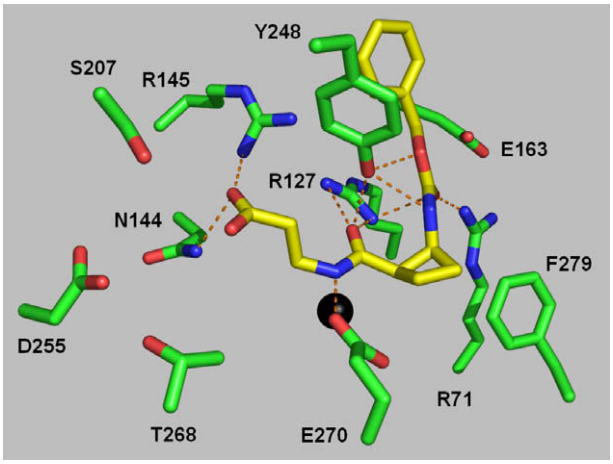

Figure 1. Predicted binding of compound **3** to human CPB structure (1zli). The ligand (yellow carbons) and selected CPB side-chains (green carbons) are shown in sticks model. Other atoms are coloured blue (nitrogen) and red (oxygen). Intermolecular interactions are as dashed orange lines. The catalytic zinc ion is shown as a black sphere.

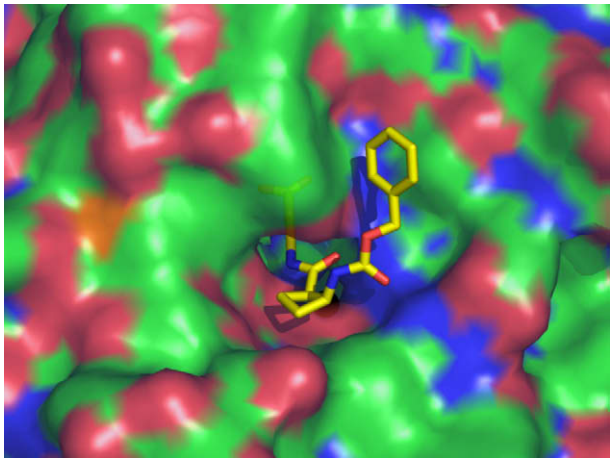

Figure 2. Placement of **3** over the CPB surface. The view shows the active site cleft pointing towards the reader, in approximately the same orientation as in Figure 1 (Tyr248 at about 12 o'clock). The ligand (yellow carbons) is shown in sticks model. Notice the bend of the ligand at the cyclobutyl unit. The figure is rendered as a translucent surface coloured as the atoms types beneath (see Fig. 1 legend for atom codes). The catalytic zinc ion is shown as a black sphere.

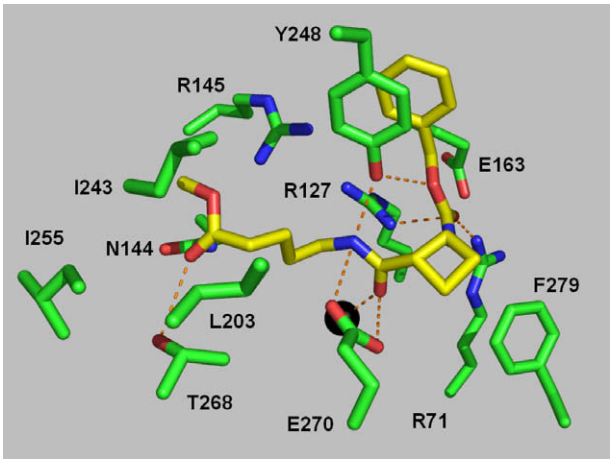

Figure 3. Predicted **4** binding to the CPA structure (2ctc). The view is in approximately the same orientation as in Figure 1. Other details as in the legend to Figure 1.

The amide bond of the carboxylate branch of the inhibitor makes a number of interactions to several residues at the active site: the N-atom is located in the second Zn^{2+} coordination sphere (at 3.1 Å) and hydrogen bonded to Glu270, the presumed proton donor during catalysis; the carbonyl oxygen interacts with the Arg127 side-chain. The cyclobutyl ring is close to two aromatic residues, Tyr198 and Phe279, and to the Ser199 side-chain. A further short interaction takes place between the carbonyl oxygen of the amide bond at the Cbz branch and Arg71 (separated by 2.7 Å). Most of the intermolecular interactions in the Cbz branch involve Tyr248 phenolic ring. This catalytically essential residue appears to be wrapped by **3,** with the Cbz aromatic ring facing the protein surface where it is accommodated in a hydrophobic depression (see Fig. 2). The Cbz ring sustains an edge-to-face interaction with Tyr248 phenolic ring on one face, while the side-chain carbon atoms from Thr164 and Glu163 are located near it at the solvent side. The binding of **3** to CPA proceeds much like as in CPB (not shown). The ligand is easily superimposable in both structures, and most interactions occur far from the specificity pocket of the enzyme. This can explain the almost similar K_i value for inhibition of CPA and CPB.

2.3. Analysis of β,δ-dipeptide 4 binding

Dipeptides **3** and **4** differ in the extension of the alkyl branch ending with either a free carboxylate in **3** or a methyl ester in **4**. Dockings predicted an opposite mode of binding for **4** into CPA and CPB. In CPA, the phenolic Tyr248 aromatic ring plays an important role in binding due to the number of interactions this residue makes to the ligand. These involve hydrogen bonds to the functionalities adjacent to the cyclobutane moiety as well as an face-to-face stacking of aromatic rings (Fig. 3).

One of the oxygen atoms of the methyl ester is hydrogen bonded to Thr268 hydroxyl (3.4 Å) and to one side chain amide nitrogen from Asn144 (3.2 Å). The long alkyl branch is within a hydrophobic environment lined by Leu203, Ile243, Ile247, and Ala250. The blocked carboxylate moiety is at the S1′ subsite, in the proximity of Ile255, the specificity determinant residue of CPA. The inner environment has a different nature in CPB (Table 2) and might provide an unsatisfactory fit of the alkyl branch. This probably caused the reversed orientation predicted for the CPB–**4** complex (not shown), where the Cbz branch binds into the S1′ pocket.

Table 2
MCP ligand-binding residues

Position	203	207	243	251	255
CPA	Leu	Gly	Ile	Ser	Ile
CPB	Ile	Ser	Gly	Ala	Asp

Residues at distance <5 Å from the ligand are tabulated.

Beyond the cyclobutyl ring and heading to the active site, the amide bond engages the catalytically crucial Glu270 (via interactions with the carbonyl O and N atoms) and the zinc ion with the carbonyl O-atom (at a distance of 2.6 Å), suggesting that the ligand may be coordinating the metal ion. The cyclobutyl ring faces two aromatic residues, Tyr198 and Phe279, while the carbonyl O atom from the Cbz moiety interacts with Arg71 and Arg127 (at distances 2.7 and 2.6 Å, respectively). Finally, the Cbz aromatic ring is in an face-to-face arrangement with the Tyr248 phenolic ring, and is surrounded by other carbon atoms from Glu163 and Thr164 side-chains. As shown in Figure 4, the β,δ-dipeptide **4** backbone is bent halfway between the Cbz and the blocked carboxylate group, i.e., at the position of the cyclobutyl ring. This suggests that conformationally constrained ligands are able to bind to into a narrow active site cavity such as that found in M14 proteases.

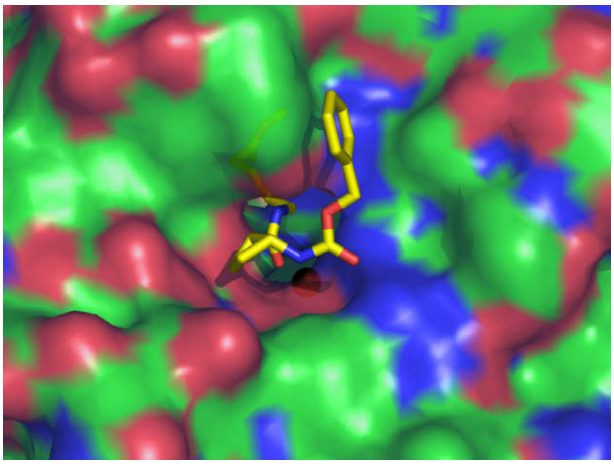

Figure 4. A view of the interactions between **4** and CPA. Same orientation as in Figure 2. Other details as in the legend to Figure 1.

3. Conclusion

We have recently identified a novel type of small-molecule ligands of MCPs from a collection of oxiranes.[30] As a continuation of the search for novel molecules, we have show herein that cyclobutane-containing peptides (CBPs) exert an inhibitory action against two prototypical M14 family proteases, CPA and CPB, in the micromolar range. Compound **3** was the most potent compound and behaved better as a CPB inhibitor, while compound **4**, provided with a longer alkyl chain with a blocked carboxylate, showed a preference to inhibit CPA. Based on the analysis of enzyme–inhibitor docked structures, further design of CBP-based inhibitors must consider, among other issues, the length of the alkyl branch and the chemical functionality displayed at its end, since both appear to interplay to define selectivity and binding strength towards one of the two types of MCPs. Docked structures also showed, as an unprecedented feature, that CBPs wrap the catalytically essential Tyr248 side chain, thus blocking the enzymic reaction. This would be a result from the constrained CBP conformation and the intermolecular interactions to the partner molecule. The most attractive inhibitors are ~350 Da compounds which may provide a convenient scaffold for chemical modification. CBPs might thus represent an alternative design to currently available MCP inhibitors, and might be considered interesting leads for further development in terms of potency and selectivity.

4. Experimental

4.1. Synthesis of cyclobutane containing peptides

Compounds **2–6** were prepared according to the methods previously described.[27,28] Compound **1** is a new dipeptide that was synthesized as follows:

To a solution of (1*R*,2*S*)-2-*N*-Bezyloxycarbonylaminocyclobutane-1-carboxylic acid (85 mg, 0.34 mmol) in anhydrous DMF (25 mL) dry Et_3N (0.2 mL), Gly–OMe (50.5 mg, 0.4 mmol), EDAC (200 mg, 1.04 mmol) and HOBt (70 mg, 0.52 mmol) were successively added. The mixture was stirred at room temperature under nitrogen atmosphere and the reaction was monitored by TLC. After 20 h, EtOAc (20 mL) was added and the combined organic layers were washed with saturated aqueous $NaHCO_3$ (3 × 15 mL). The organic phase was dried over $MgSO_4$ and solvents were removed under reduced pressure. The residue was chromatographed through Baker Silica gel using EtOAc as eluent to afford dipeptide **1** (40 mg, 44% yield) as a white solid crystals, mp 109–111 °C (AcOEt/pentane). $[\alpha]_D$ − 55.7 (*c* 1.43, CH_2Cl_2). IR (solid)**:** 3318 (NH), 2952 (CH), 1732(C═O), 1690 (C═O), 1542. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.93 (m, 1H), 2.10 (m, 1H), 2.32 (complex absorption, 2H), 3.32 (m, 1H, H_{14}), 3.74 (s, 3H, OMe), 3.89–4.02 (complex absorption, 2H, $H_{17a,b}$), 4.53 (t, J = 6.82 Hz, 1H, H_{11}), 5.10 (s, 2H, $H_{7a,b}$), 5.88 (d, J = 5.67 Hz, 1H, NH_{10}), 6.18 (broad s, 1H, NH_{16}), 7.37 (m, 5H, H_{arom}). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 18.2, 29.6, 41.0, 46.0, 46.5, 52.2, 66.4, 128.0, 128.4, 136.7, 155.7, 170.5, 173.3. Anal Calcd for $C_{16}H_{20}N_2O_5$: C, 59.99; H, 6.29; N, 8.74. Found: C, 59.93; H, 6.56; N, 8.77.

4.2. Biology

Recombinantly expressed human carboxypeptidase B was obtained as a zymogen, converted to the active form and purified as reported.[31] Bovine pancreatic carboxypeptidase A (CPA) was from Sigma. Enzyme concentrations for the kinetic studies were kept fixed at typically 5–50 nM. The chromogenic substrates, *N*-(4-methoxyphenylazoformyl)-L-phenylalanine and *N*-(4-methoxyphenylazoformyl)-L-arginine were from Bachem (Bubendorf, Switzerland). The experimental assays were performed at room temperature in 50 mM Tris (Tris = 2-amino-2-(hydroxymethyl)propane-1,3-diol), 0.5 M NaCl, pH 7.5, and 20 mM Tris, 0.1 M NaCl, pH 7.5, for CPA and CPB, respectively. Typically, the enzymic activity was measured using a fixed concentration of substrate and variable concentrations of inhibitor (five concentrations in the range from 0.15 μM to 750 μM). The initial velocity measurements were performed per triplicate in a 96-well microtiter plate or in 1 ml cuvettes. The changes in absorbance due to substrate breakdown were followed continuously at 340 nm. The GRAPHPAD Version 5.0 (www.graphpad.com) program was used to process the kinetic data. A non-linear global fit to a competitive one-site inhibition model gave the best fitting of the experimental observations. The enzyme inhibition K_i values were obtained from IC_{50} following the Cheng and Prussoff's equation.[32]

4.3. Bioinformatics: docking procedures

The binding properties of the two selected CBPs, **3** and **4**, were analysed by docking procedures with the program AUTODOCK Version 4.0 (The Scripps Research Institute, La Jolla, CA, USA). The binding of both **6** enantiomers was also investigated. The compounds were constructed, converted to 3D structures, and energy minimised with CHEM3D Version 9.0 (http://www.cambridgesoft.com). Then, the ligands and the target macromolecules were prepared with AUTODOCK. To validate the approach, two separate experiments were performed with the known 3D structures of bovine CPA bound to the ester substrate L-phenyllactate (2ctc) and porcine CPB bound to a small-molecule inhibitor (2jew). The results from this dockings were in excellent agreement with the crystallographic determinations. The PDB coordinates of CPA and human CPB (2ctc and 1zli, respectively) were used for our ligands. The protein–ligand interactions of the predicted complexes were inspected with PyMol (www.pymol.org) graphical user interface. All the final figures for publication were prepared with PyMol.

Acknowledgements

Financial support from the Ministerio de Ciencia e Innovación, Spain (Grants CTQ2007-61704/BQU and BIO2007-68046), Generalitat de Catalunya (Grants 2005SGR-103 and 2005SGR-1037) and CAMP project 108830 (VI EU Framework Programme) is gratefully acknowledged.

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