Reversible inhibition of cathepsin L-like proteases by 4-mer pseudopeptides

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Abstract A library of 121 pseudopeptides was designed to develop reversible inhibitors of trypanosomal enzymes (cruzan from \textit{Trypanosoma cruzi} and congopain from \textit{Trypanosoma congolense}). The peptides share the framework: Cha-X1-X2-Pro (Cha = cyclohexyl-alanine, X1 and X2 were phenylalanyl analogs), based on a previous report [Lecaille, F., Authié, E., Moreau, T., Serveau, C., Gauthier, F. and Lalmanach, G. (2001) Eur. J. Biochem. 268, 2733–2741]. Five peptides containing a nitro-substituted aromatic residue (Tyr/Phe) and one a 4-chloro-phenylalanine at the X1 position, and 3-(2-naphthyl)-alanine, homocyclohexylalanine or 3-nitro-tyrosine (3-NO\textsubscript{2}-Tyr) at the X2 position, were selected. They inhibited congopain more effectively than cruzain, except Cha-4-NO\textsubscript{2}-Phe-3-NO\textsubscript{2}-Tyr-Pro which bind the two parasitic enzymes similarly. Among this series, Cha-3-NO\textsubscript{2}-Tyr-HoCha-Pro and Cha-4-NO\textsubscript{2}-Phe-3-NO\textsubscript{2}-Tyr-Pro are the most selective for congopain relative to host cathepsins. No hydrolysis occurred upon prolonged incubation time with purified enzymes. In addition introduction of non-proteogenic residues in the peptidyl backbone greatly enhanced resistance to proteolysis by mammalian sera. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Cysteine protease; Cathepsin; Phe analog; Pseudopeptide; Trypanosome

1. Introduction

Lysosomal cathepsins B, L, S or K, which are implicated in the regulation of a broad range of biological functions, are attractive chemotherapeutic targets for cure of pathological processes, including arthritis, osteoporosis, or cancer invasion [1–3]. Related protozoan proteases also play crucial roles in the life cycle of parasites which are the etiological agents of American trypanosomiasis and sleeping sickness in Africa [4–6]. Cruzain from \textit{Trypanosoma cruzi}, the causative agent of Chagas disease in South America, and congopain, its homologous enzyme in \textit{Trypanosoma congolense}, which is responsible of nagana in African bovine domestic livestock, share a high sequence identity and an enzymatic specificity closely related to that of mammalian cathepsins B and L [7–11]. Inactivation of trypanosomal cysteine proteases (CPs) by peptide inhibitors offers a promising therapeutic pathway as recently demonstrated in a mouse model [12]. The substrate specificity of trypanosomal CPs, as that of their mammalian counterparts, is primarily determined by P2/S2 interactions, with a marked preference for aromatic residues such as Phe at P2. However we have recently found that trypanosomal enzymes can accommodate non-aromatic, unencoded cyclic amino acids such as 3-cyclohexyl-alanine (Cha) at P2 [13]. We also found that congopain and cruzain, but not cathepsins B and L, accommodate Pro at P2′ [8].

Reversible, tight-binding CP inhibitors would be the ideal drug target leads and considerable effort has been expended in optimizing interactions within the active site of CPs with natural amino acids. Some gave promising results when assayed in vitro but they most often displayed only a poor activity in tissue cultures, due to transport failure across cell membrane, susceptibility to endogenous proteolysis, and low water solubility [14,15]. Interactions with unencoded amino acids have not been studied in so great details until now, though their incorporation in the peptidyl backbone of small reversible inhibitors could improve their half-life and stability [16]. To reach this goal, a library of pseudopeptideylamides were prepared by solid phase synthesis and their ability to inhibit CPs was evaluated.

2. Materials and methods

Z-Phe-Arg-AMC (7-amino-4-methyl-coumarin hydrochloride) and Z-Ala-Ala-Pro-Phe-AMC were purchased from Bachem Biochimie (Voisin-le-Bretonneux, France). 1,3-carboxy-trans,2,3-epoxypropionyl-yl-leucylamido(4-guanido)butane (E-64), N,N-diethiothreitol (DTT) and Igepal CA-630 were from Sigma-Aldrich. All other reagents were of analytical grade. Fetal calf serum was purchased from Eurobio (Les Ulis, France). Bradykinin was obtained from Bachem Biochimie. Reversible inhibition of cathepsin L-like proteases by 4-mer pseudopeptides

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2.1. Enzymes

Cruzain (recombinant catalytic domain of cruzipain) was purified as previously described [17]. Congopain from T. congolense was prepared by affinity chromatography from trypomastigote lysates [18]. Human recombinant cathepsin L was a generous gift from Dr. John S. Mort (Shriners Hospitals for Children, Montreal, QC, Canada). Cathepsin B was purified from rat liver [19]. Trypsin and chymotrypsin from bovine pancreas were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Prior to kinetic measurements, enzymes were activated in their respective assay buffer for 5 min at 37°C: 0.1 M phosphate buffer pH 6.0, containing 6 mM DTT, 2 mM EDTA, 0.01% Igepal CA-630 for cruzain and 0.1 M phosphate buffer pH 6.0, containing 2 mM DTT, 1 mM EDTA, 0.01% Igepal CA-630 for cathepsins L and B. The enzyme active site was titrated by E-64 using Z-Phe-Arg-AMC as substrate [20].

The buffers for trypsin and chymotrypsin assays were 0.4 M Tris-HCl, pH 8.0 and 50 mM HEPES, 50 mM NaCl, pH 7.4 respectively. Trypsin was titrated as previously described [21], while chymotrypsin was titrated by ω1-antichymotrypsin using Z-Ala-Ala-Pro-Phe-AMC as substrate [22].

2.2. Peptides synthesis

Unless otherwise stated, all Fmoc-protected amino acids were of the L-configuration, and were purchased from Neosystem (Strasbourg, France) or Advanced ChemTech Europe (Bruxelles, Belgium). N-α-(3,4-dichlorophenyl)-D-isopropylglycine (DIPEPA), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Novabiochem (FranceBiochem, France). 121 pseudopeptidyl tetramers (for nomenclature and numbering see Table 1) were synthesized as peptidylamides. Peptides were constructed on the basis of a previous report [13].

2.3. Kinetic measurement

Cruzain (0.65 nM) was incubated with each of the 121 pseudopeptides (10 and 100 μM) in the activating buffer at 37°C for 10 min (final volume=30 μl). Two aliquots were removed, one for measuring the residual enzymatic activity in the presence of Z-Phe-Arg-AMC (5 μM) and the other was mixed with ethanol (300 μl) to inactivate the enzyme. After removal of the precipitate, the supernatant containing the native peptide and/or its proteolytic fragments was evaporated, redissolved in 0.1% TFA, and chromatographed by RP-HPLC on a C18 OD 300 Brownlee column, using a 30-min linear (0-60%) gradient of acetonitrile in 0.1% TFA at a flow rate of 0.5 ml/min. The elution profiles were analyzed by the software Spectacle. This experimental procedure was repeated with congopain, cathepsin L, cathepsin B, trypsin, and chymotrypsin in their respective activity buffer. Similar experiments were also carried out with peptides 72, 90, 111, 115 and 116. The resistance of Cha-4-nitro-phenylalanine (4-NO2-Phe)-3-nitro-tyrosine (3-NO2-Tyr)-Pro (100 μM) to endogenous proteolysis was checked after incubation at 37°C for 1 h in rat plasma and fetal calf serum. Peptides PB 8 and PB 11 derived from the proregion of procathepsin B [23], or bradykinin (final concentration: 100 μM), were used as control. The same experiments were done with peptides 72, 90, 111, 115 and 116.

3. Results and discussion

Based on previous reports [8,13], we have synthesized 121 tetrapeptides sharing the common structure Cha-X1-X2-Pro (Table 1), where Cha and Pro occupied the putative P2 and P2’ positions respectively, X1 and X2 being Phe derivatives (Fig. 1). Despite the presence of bulky hydrophobic residues, all peptides of this series are water soluble at a concentration of 10 mM. The ability of peptides to inhibit cruzain was screened by monitoring the residual enzymatic activity towards Z-Phe-Arg-AMC. Under our experimental conditions, the fluorescence of the 7-amino-4-methyl-coumaryl group

Table 1

<table>
<thead>
<tr>
<th>X1</th>
<th>X2</th>
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<tbody>
<tr>
<td>d-Phe</td>
<td>Phg</td>
</tr>
<tr>
<td>n-Phe</td>
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<tr>
<td>Phg</td>
<td>7</td>
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<tr>
<td>Chg</td>
<td>13</td>
</tr>
<tr>
<td>Cha</td>
<td>19</td>
</tr>
<tr>
<td>Hof</td>
<td>25</td>
</tr>
<tr>
<td>Ho-Cha</td>
<td>31</td>
</tr>
<tr>
<td>4-Cl-Phe</td>
<td>67</td>
</tr>
<tr>
<td>3,4-Cl2-Phe</td>
<td>73</td>
</tr>
<tr>
<td>3-NO2-Tyr</td>
<td>79</td>
</tr>
<tr>
<td>Nal2</td>
<td>91</td>
</tr>
</tbody>
</table>

The 121 tetramers were synthesized as peptidylamides. Peptides were constructed on the basis of a previous report [13].

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$\lambda_{ex} = 350$ nm and $\lambda_{em} = 430$ nm) was not affected by peptide concentration.

Six peptides of the series, i.e.

- Cha-4-chloro-phenylalanine (4-Cl-Phe)-homocyclohexyl-alanine (HoCha)-Pro (#72),
- Cha-3-NO$_2$-Tyr-HoCha-Pro (#90),
- Cha-4-NO$_2$-Phe-3-NO$_2$-Tyr-Pro (#110),
- Cha-4-NO$_2$-Phe-3-(2-naphthyl)-alanine (Nal$_2$)-Pro (#111),
- Cha-3-NO$_2$-Tyr-3-NO$_2$-Tyr-Pro (#115), and
- Cha-3-NO$_2$-Tyr-Nal$_2$-Pro (#116) inhibited cruzain over 50% (Fig. 2).

Compared to the fluorogenic substrate dansyl-Cha-Arg-Ala-Pro-Trp which is hydrolyzed at the Arg-Ala bond [13], no proteolytic fragment of the 4-mer peptides was generated upon incubation with cruzain as observed by RP-HPLC (not shown), demonstrating that peptides interacted as CP inhibitors, and not as substrates. At variance with peptidyl vinyl sulfones, where the most potent has a homophenylalanine (Hof) at P1 [26], peptides 25–30 and 57–61 that also have a Hof residue at P1 poorly inhibited cruzain. This agrees with recent structural data reporting that Hof is not essential for interactions between cruzain and vinyl sulfones [27]. Introduction of a saturated carbon cycle (cyclohexyl-glycine (Chg), Cha, or HoCha) led to a loss of inhibition, emphasizing the critical importance of aromaticity of the X1 side chain. Cruzain preferentially accommodated nitro-substituted Phe or Tyr residues at P1, which suggests that substitution of the benzyl ring by an electronegative group favored binding to the putative S1 subsite. In a previous report, Alves et al. [28] observed that the presence of a positively charged derivative of Cha (4-aminomethyl-cyclohexyl-alanine) at P1 in the series ortho-amino-benzoyl-Phe-X-Ser-Arg-Gln-N-(2,4-dinitro-phenyl)-ethylenediamine led to an efficient competitive inhibitor of cruzain and cathepsin L. This apparent discrepancy
is probably due to the broad specificity of the S1 subsite [11,27]. Our data also indicated that HoCha, Nal2, and 3-NO2-Tyr bound quite well to the S1’ subsite of cruzain (peptides 72, 90, 110, 111, 115, 116), while (o)-Phe, phenylglycine (Phg), and Chg are poorly accepted.

The \( K_i \) of the six selected peptides was determined according to [25] (Table 2). Peptides were slightly more potent competitive inhibitors of congopain than of cruzain, except Cha-4-NO2-Phe-3-NO2-Tyr-Pro (compound 110) which bound the two parasitic enzymes similarly. Cha-3-NO2-Tyr-HoCha-Pro and Cha-4-NO2-Phe-3-NO2-Tyr-Pro are respectively the most selective for congopain relative to cathepsin B and cathepsin L. Peptide 110 was the weakest inhibitor of human cathepsins L and B (\( K_i = 0.2 \) mM) due to the presence of a 3-NO2-Tyr at X2, a 3-(2-naphthyl)-alanyl residue being preferred at that position (peptide 111). Despite the inhibitory potential of small competitive inhibitors of trypanosomal CPs remains moderate [29], their \( K_i \) values compare to numerous low molecular weight competitive reversible inhibitors of thiol proteases (see for review [14]). Compared to reversible cystatin-derived inhibitors or propeptide-derived peptides [30,31], no hydrolysis of these peptides was detected by RP-HPLC upon prolonged incubation time (5 h) with cathepsins B and L, congopain, and cruzain, or unrelated proteins used as control (trypsin and chymotrypsin). This suggests that resistance of competitive pseudopeptidyl inhibitors towards their cognate CPs could be partly achieved by use of the structural analogs. At this stage, stability of the selected peptides was estimated in rat plasma and fetal calf serum. Approximately 25% of Cha-4-NO2-Phe-3-NO2-Tyr-Pro was recovered after 1 h incubation (not shown), while control peptides including PB 11 (a competitive procathepsin B-derived inhibitor) [23] were rapidly and fully metabolized by serum proteases. Similar increase of half-life was observed with peptides 72, 90, 110, 115 and 116, as reported for irreversible inhibitors [16]. Even though the affinity of such reversible low molecular weight inhibitors, containing unencoded amino acids in their peptide framework, remains weak in part due to their short length, their increased properties in terms of stability and resistance to proteolysis are promising vis a vis development of new inhibitory compounds of biological interest to control unwanted activity of CPs.

Acknowledgements: We thank Dr. John S. Mort (Shriners Hospital for Children, Montreal, QC, Canada) for his generous gift of human recombinant cathepsin L. Congopain was kindly provided by Dr. Edith Authié and Dr. Alain Boulangé (CIRAD-Emvt, Montpellier, France/ILRI, Nairobi, Kenya). We thank Karen Mercier and Elizabeth Hansell for technical assistance. This work was supported by a PRFMMP grant (Programme de Recherche Fondamentale en Microbiologie et Maladies Infectieuses et Parasitaires) from MENRT (Ministère de l’Education Nationale, de la Recherche et de la Technologie, France), by CEVA Santé Animale (Libourne, France), and by AI 35707 and the Sandler Family Foundation. F.L. holds a doctoral fellowship from MENRT.

References


Table 2

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Kᵢ (μM)</th>
<th>CZ 16</th>
<th>CG 18</th>
<th>CTB 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 Cha-4-C3-Phe-HoCha-Pro</td>
<td>39 ± 2</td>
<td>16 ± 4</td>
<td>74 ± 2</td>
<td>100 ± 11</td>
</tr>
<tr>
<td>90 Cha-3-NO2-Tyr-HoCha-Pro</td>
<td>47 ± 5</td>
<td>8 ± 4</td>
<td>49 ± 2</td>
<td>255 ± 35</td>
</tr>
<tr>
<td>110 Cha-4-NO2-Phe-3-NO2-Tyr-Pro</td>
<td>11 ± 1</td>
<td>17 ± 6</td>
<td>200 ± 8</td>
<td>282 ± 8</td>
</tr>
<tr>
<td>111 Cha-4-NO2-Phe-Nal2-Pro</td>
<td>38 ± 6</td>
<td>9 ± 4</td>
<td>11 ± 1</td>
<td>94 ± 10</td>
</tr>
<tr>
<td>115 Cha-3-NO2-Tyr-3-NO2-Tyr-Pro</td>
<td>31 ± 2</td>
<td>7 ± 2</td>
<td>58 ± 15</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>116 Cha-3-NO2-Tyr-Nal2-Pro</td>
<td>57 ± 13</td>
<td>11 ± 5</td>
<td>29 ± 4</td>
<td>93 ± 27</td>
</tr>
</tbody>
</table>

Enzymes were incubated with increasing amounts of peptides (0–100 μM final) for 10 min at 37°C, before triggering the enzymatic reaction by adding benzoyloxycarbonyl-Phe-Arg-AMC (1–20 μM), as described in Section 2. The inhibition constants were calculated by plotting 1/v against [I]. \( K_i \) values were expressed as the mean ± S.E.M. (triplicate experiments).