Crystal Structure of Novel Metallo carboxypeptidase Inhibitor from Marine Mollusk *Nerita versicolor* in Complex with Human Carboxypeptidase A4*

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Background: Only a few proteinaceous inhibitors of metallo carboxypeptidases have been characterized structurally in depth.

Results: The structure of human carboxypeptidase A4 in complex with a *Nerita versicolor* inhibitor (NvCI) was derived at 1.7 Å.

Conclusion: NvCI displays a different protein fold that inhibits carboxypeptidases in a substrate-like manner.

Significance: We deciphered the structural determinants for picomolar inhibition constants for A-type carboxypeptidases, the most potent by now.

NvCI is a novel exogenous proteinaceous inhibitor of metallo carboxypeptidases from the marine snail *Nerita versicolor*. The complex between human carboxypeptidase A4 and NvCI has been crystallized and determined at 1.7 Å resolution. The NvCI structure defines a distinctive protein fold basically composed of a two-stranded antiparallel β-sheet connected by three loops and the inhibitory C-terminal tail and stabilized by three disulfide bridges. NvCI is a tight-binding inhibitor that interacts with the active site of the enzyme in a substrate-like manner. NvCI displays an extended and novel interface with human carboxypeptidase A4, responsible for inhibitory constants in the picomolar range for some members of the M14A subfamily of carboxypeptidases. This makes NvCI the strongest inhibitor reported so far for this family. The structural homology displayed by the C-terminal tails of different carboxypeptidase inhibitors represents a relevant example of convergent evolution.

Metallo carboxypeptidases (MCPs) are exopeptidase enzymes involved in a great variety of processes, from digestion to blood coagulation/fibrinolysis, inflammation, and prohormone and neuropeptide processing among others, and have also been reported to be involved in the progress of certain cancers (1–3). Their activity normally takes place outside of the cell, by removing the C-terminal residues of polypeptidic chains. The activity of MCPs can be regulated in distinct organisms through the action of exogenous carboxypeptidase inhibitors (1, 3). Proteinaceous carboxypeptidase inhibitors can be found in a wide range of biological locations and in evolutionarily distant organisms such as potato, leech, ticks, and worms (4–7).

We have recently isolated a novel MCP inhibitor (NvCI) from the marine snail *Nerita versicolor*. It is a 53-residue protein that tightly binds to the M14A subfamily of carboxypeptidases (traditionally known as pancreatic-like carboxypeptidases). It displays an inhibition constant ($K_i$) in the picomolar range against several MCPs belonging to the abovementioned subfamily: bovine carboxypeptidase A1 (bCPA1), human CPA1 (hCPA1), and human carboxypeptidase A4 (hCPA4; which has been structurally characterized in this work). Such $K_i$ values are 3 orders of magnitude lower than for other known exogenous protein inhibitors of carboxypeptidases (4–7).

Only four exogenous proteinaceous inhibitors of carboxypeptidases with a similar mode of inhibition have been studied in detail: potato carboxypeptidase inhibitor (PCI) from *Solanum tuberosum* (4), leech carboxypeptidase inhibitor (LCI) from *Hirudo medicinalis* (5), tick carboxypeptidase inhibitor (TCI) from *Rhipicephalus bursa* (6), and *Ascaris* carboxypeptidase inhibitor (ACI) from *Ascaris suum* (7). A common feature of all of these protein inhibitors is their small size (39–75 residues) and their stabilization by several disulfide bridges. In all cases, the inhibition relies on the interaction of the C-terminal tail with the active site groove of the carboxypeptidase in a mechanism that mimics substrate binding (8–10). Although there are neither sequence nor three-dimensional structure similarities between the main body of these inhibitors, they all have effects on the key residues for the activity of the enzyme.
Remarkably, a protein carboxypeptidase inhibitor for humans, latexin, which is the only endogenous inhibitor isolated so far, displays a different mode of action, in a way reminiscent of the interaction of the prodomain with the carboxypeptidase, covering the active site of the enzyme (11).

NvCI is the first proteinaceous inhibitor of MCPs isolated and characterized from a marine organism. The marine Caribbean fauna is characterized by its richness and diversity, which make it a very attractive natural source for the identification of novel biomolecules with biological and biomedical interests. The potential of marine invertebrates as a source of these biomolecules has been reported in previous studies, particularly those focused on endoproteases such as serine and cysteine proteases and their inhibitors, some with exceptional structural and functional properties (2, 12–14).

Pro-CPA4 and its active form (CPA4), a counterpart used in this work, belong to the M14A subfamily of carboxypeptidases and have been implicated in different physiological processes (15, 16). Human pro-CPA4 was also identified as a gene product involved in prostate cancer (17).

In this work, we report the crystal structure of NvCI in complex with human CPA4 at 1.7 Å resolution. NvCI displays a different protein fold, and its interface with hCPA4 has been analyzed in detail and compared with the few reported structures of exogenous MCP inhibitors to rationally explain its exceptional ability (picomolar range) to inhibit certain MCPs.

**EXPERIMENTAL PROCEDURES**

**Heterologous Expression and Purification of Recombinant NvCI**—The NvCI amino acid sequence (UniProt ID P86912) was determined by a combination of Edman degradation and MALDI-TOF-MS. A synthetic gene encoding NvCI was designed and constructed to express this molecule in the *Pichia pastoris* system (GeneArt). The DNA sequence of NvCI was fused in-frame to the *Saccharomyces cerevisiae* prepro-α-factor signal in the XhoI site of the pPICZαA vector for secretion into the culture medium. Production of recombinant NvCI was carried out using a Zeocin hyper-resistant strain in an autoclavable bioreactor (Applikon Biotechnology). Production was monitored according to parameters such as wet cell weight, as well as by MALDI-TOF-MS, determination of the protein concentration in the supernatant by the BCA method (18), and bCPA1 inhibitory activity (19).

Purification of NvCI was performed using a combination of two ion exchange chromatographic methods: an initial weak cation exchange (Accell™ Plus CM, Waters) using 20 mM Tris-HCl (pH 7.0) and an ion strength gradient (up to 1 M NaCl), followed by a second step of anion exchange (TSKgel® DEAE-5PW, Tosoh Bioscience LLC) using a linear gradient of 0–100% 20 mM Tris-HCl (pH 8.5) containing 1 M NaCl. The purity of NvCI was determined by its molecular mass obtained by MALDI-TOF-MS, by Tris/Tricine/SDS-PAGE, and by its functional activity against bCPA1.

**Heterologous Expression and Purification of Recombinant hCPA4**—Human pro-CPA4 was overexpressed and secreted into the extracellular medium using the *P. pastoris* heterologous system as described (11). Production of hCPA4 was carried out and monitored in the same way as described above for NvCI.

Enzyme purification was performed using a combination of hydrophobic interaction chromatography with a TOYOPEARL® butyl-650M column (Sigma-Aldrich) and weak anion exchange chromatography using a TSKgel® DEAE-5PW column according to the purification process described previously (16). The purity of hCPA4 was determined by SDS-PAGE, and its functional activity was determined by hydrolysis of the synthetic substrate *N*-(4-methoxyphenylazoformyl)phenylalanine (19).

**Formation and Purification of NvCI-hCPA4 Complex**—The formation and capture of the NvCI-hCPA4 complex were performed by preincubation of both proteins for 30 min in 50 mM Tris-HCl (pH 8.5) containing 150 mM NaCl at 37 °C. For this purpose, 16 mg of hCPA4 were incubated with 5.5 mg of recombinant NvCI in a final reaction volume of 70 ml (equivalent to an enzyme/inhibitor molar ratio of 1:2). The complex was captured on a size exclusion chromatography column (HiLoad Superdex 75 26/60, GE Healthcare) equilibrated with the same buffer used for complex formation. Elution peaks corresponding to the complex, free enzyme, and inhibitor were analyzed by PAGE. The NvCI-hCPA4 complex was concentrated to 17.6 mg/ml using an Amicon Ultra-4 centrifugal filter (Millipore).

**Crystalization and Data Collection**—Crystals of the complex between NvCI and hCPA4 were obtained at 18 °C by sitting-drop vapor-diffusion methods. The reservoir solution contained 0.04 M ammonium nitrate and 25% (v/w) PEG 3350. Single crystals appeared after 4 days from equal volumes of protein solution (17.6 mg/ml in 5 mM Tris (pH 8.5) and 50 mM NaCl) and reservoir solution. Crystals were cryoprotected in reservoir buffer containing 12% glycerol and flash-frozen in liquid nitrogen prior to diffraction analysis. Diffraction data were recorded from cryo-cooled crystals (100 K) at Grenoble beamline ID23-2. Data were integrated and merged using XDS (20) and scaled, reduced, and further analyzed using CCP4 (21) (Table 1).

**Structure Determination and Refinement**—The structure of the NvCI-hCPA4 complex was determined from the x-ray data at 1.7 Å by molecular replacement using Protein Data Bank code 2PCU for hCPA4 as a model. The quality of the diffraction data allowed automatic building of the inhibitor using wARP (22). Manual building and improvement of the model were performed using Coot (23). Refinement utilized CNS (24) and PHENIX (25). Ramachandran analysis showed that 94.70% of the residues (661) are in allowed regions, 4.58% of the residues (32) are in allowed regions, and 0.72% of the residues (5) are in outlier regions for both complexes of NvCI-hCPA4 in the asymmetric unit. Refinement and data statistics are provided in Table 1.

**Determination of Inhibition Constants**—*Ki* values were determined according to the strategy described for tight-binding inhibitors (26). The experiments were performed at 37 °C and pH 7.5 by varying the inhibitor concentration in each assay with a fixed concentration of enzyme and substrate (0.1 mM *N*-(4-methoxyphenylazoformyl)phenylalanine) and preincubation time. *Ki* values were determined for bCPA1 (Sigma-
The structure of the hCPA4 enzyme in complex with NvCI is related to previously reported structures of hCPA4 alone and in complex with other inhibitors (11). The structure of the enzyme displays the classical carboxypeptidase fold, with eight α-helices and a mixed eight-stranded β-sheet forming a globular α/β-motif (Fig. 1). The coordination of the zinc atom is essentially conserved compared with other MCP structures. In the absence of inhibitor, the zinc atom of MCPs is coordinated to the “catalytic” water molecule and to carboxypeptidase residues His-69 and His-196 and, in a bidentate form, to Glu-72 (1). In the NvCI-hCPA4 complex, the catalytic water is not present, and it is substituted with a bidentate coordination to the zinc atom by the C-terminal carboxylate group of NvCI, which is buried in the active site groove of the enzyme (Fig. 1B). As observed in other carboxypeptidase complexes with inhibitors, the most dramatic change observed in the active site residues is the movement of the side chain of Tyr-248, almost 180° from the “up” to the “down” position in the complex. There are other minor movements in the active site residues of hCPA4 to accommodate the C-terminal tail of the inhibitor.

“Primary” Interaction Site in NvCI-hCPA4—Despite its small size, NvCI interacts extensively with hCPA4, with a total contact area between both proteins of 1875.1 Å² (Table 2). As observed with the other exogenous carboxypeptidase inhibitors (PCI, LCI, ACI, and TCI), inhibition of the enzyme can be attributed to a competitive interaction with the active site of the carboxypeptidase by occlusion of the active site subsites S1′, S1, and S2 (Fig. 2A). These sites are occupied by the C-terminal tail of NvCI (Tyr-52 and Ala-53) and constitute the primary contact region of the inhibitor. The “secondary” contact region, which is very extended and covers almost a complete face of the inhibitor, is discussed below.

The C-terminal tail of NvCI is shorter in comparison with other known carboxypeptidase inhibitors; it is composed of only two residues, Tyr-52 and Ala-53, but as discussed below, this short extension is sufficient to interact with the active site residues and zinc atom of the carboxypeptidase.

Aldrich) and for hCPA1, hCPA2, and hCPA4, which were produced by recombinant expression by our group (11, 27, 28).

**RESULTS**

Recombinant Protein Production and Complex Formation—The natural form of NvCI was detected, isolated from a crude extract of the marine snail _N. versicolor_, and _de novo_ sequenced by MALDI-TOF-MS and Edman degradation, which allowed the construction of a synthetic gene with an optimized _P. pastoris_ codon usage. A recombinant form of NvCI with the same mass and size as the natural one (5945.6 Da by MALDI-TOF-MS and 53 residues) was produced in _P. pastoris_, followed by chromatographic purification as summarized under “Experimental Procedures.” NvCI was overexpressed, yielding 329.7 mg of recombinant NvCI/liter of culture broth. In the case of the enzyme counterpart, hCPA4 was also overexpressed in _P. pastoris_ with an overall yield of 17.6 mg of hCPA4/liter of culture medium. The complex was formed and purified by gel filtration at a 1:2 molar ratio of hCPA4 to NvCI (see “Experimental Procedures” for details).

Crystal Structure of NvCI in Complex with hCPA4 at 1.7 Å—The polypeptide chain of hCPA4 and NvCI can be clearly and nearly completely traced in the electron density maps (Phe-6 to Asn-307 for hCPA4 and Val-3 to Ala-53 for NvCI) (Fig. 1). The crystal structure of NvCI-hCPA4 displays two complexes in the asymmetric unit. Both complexes are almost identical, showing a root mean square deviation of 0.8 Å and displaying similar overlapping for the hCPA4 and NvCI molecules. Based on results obtained by gel filtration chromatography, the biological unit can be considered to be a monomer, formed by only one binary complex between hCPA4 and NvCI.

The structure of NvCI displays a new extended globular protein fold, which is basically formed by a central two-stranded antiparallel β-sheet connected by three major loops and two short tails that extend at the N and C termini (Fig. 1). The β-strands and the three loops are cross-connected and stabilized by three disulfide bridges formed between Cys-9 and Cys-23, Cys-15 and Cys-51, and Cys-27 and Cys-38. Despite its small size, NvCI has a small hydrophobic core located next to the C terminus of the protein formed by non-polar interactions of the side chain of Trp-42, which is sandwiched between two disulfide bridges of the inhibitor (Fig. 1D). The β-structure of the inhibitor also contains a few bulky exposed hydrophobic residues to the solvent, Phe-25, Phe-34, and Phe-44, which probably contribute to the reduced solubility of the recombinant protein. The C-terminal tail of NvCI is formed by only two residues, Tyr-52 and Ala-53, but as discussed below, this short extension is sufficient to interact with the active site residues and zinc atom of the carboxypeptidase.

Crystallographic data

| Data collection | Space group |
|-----------------|-------------|
| Cell dimensions | P2₁ |
| α, β, γ (Å)     | 69.22, 71.98, 79.84 |
| Resolution ( Å )| 50–1.70 (1.70) * |

| Refinement      | Resolution (Å) | No. of reflections | Rmerge/Rwork | No. of atoms | Protein | Water | Nitrate | Zinc | Average B-factors (Å²) | r.m.s.d. | Bond lengths (Å) | Bond angles |
|-----------------|----------------|-------------------|--------------|--------------|---------|-------|---------|------|-----------------------|----------|-----------------|------------|
|                | 50–1.70       | 79,607            | 20.54/23.46  | 5903         | 702     | 306   | 6       | 2    | 17.25                 | 0.007    | 1.033           |

* Statistics for the highest resolution shell are shown in parentheses.

**Crystal Structure of NvCI-hCPA4**

**TABLE 1**

| Crystallographic data | |
|-----------------------|--|
| Data collection       | P2₁ |
| Space group           | |
| α, β, γ (Å)           | 69.22, 71.98, 79.84 |
| Resolution (Å)        | 50–1.70 (1.70)* |

| Refinement            | Resolution (Å) | No. of reflections | Rmerge/Rwork | No. of atoms | Protein | Water | Nitrate | Zinc | Average B-factors (Å²) | r.m.s.d. | Bond lengths (Å) | Bond angles |
|-----------------------|----------------|-------------------|--------------|--------------|---------|-------|---------|------|-----------------------|----------|-----------------|------------|
|                      | 50–1.70        | 79,607            | 20.54/23.46  | 5903         | 702     | 306   | 6       | 2    | 17.25                 | 0.007    | 1.033           |
The subsite of the carboxypeptidase is occupied by a nitrate molecule from the crystallographic buffer (Fig. 2A). The nitrate molecule is in contact with the guanidinium group of Arg-145 (at distances of 2.88 and 2.97 Å to the NH1 and NH2 atoms, respectively) and Asn-144 (at a distance of 2.79 Å to the ND2 atom). These residues belong to the S1'/H1 subsite and interact with the carboxylate group of the cleaved residue of a carboxypeptidase substrate. In the PCI and LCI complex structures, the cleaved and trapped C-terminal residue occupies a similar position as the nitrate molecule in NvCI.

Sequence and three-dimensional structure comparisons of the C-terminal tails of NvCI with those of the different exogenous carboxypeptidase inhibitors indicated strong similarities and identical conformations of the backbone and side chains for the P1 and P2 residues (Ala-53 and Tyr-52 for NvCI, respectively) (Fig. 2, B and C, and supplemental Fig. S1). P1 and P2 residues are oriented in a substrate-like manner in all reported structures of these competitive tight-binding inhibitors. Notably, in all of them, the chemical character of the residue forming the P1 subsite is aliphatic, whereas the preference is for aromatic residues for the P2 subsite (except Leu for ACI), which establishes stacking interactions with the aromatic ring of Tyr-248 (Fig. 2C).

As mentioned above, the C-terminal carboxylate group of Ala-53 coordinates the zinc atom in a bidentate form (distances of 2.28 and 2.44 Å, respectively), whereas the amino group of...
Ala-53 forms a hydrogen bond with the hydroxyl side chain oxygen of Tyr-248 (2.78 Å), which is disposed in the down or “closed” conformation (when bound to substrates). The S1 subsite in the enzyme is also composed of Glu-270 and Arg-127, both of which presumably participate in the polarization of the carbonyl group of the scissile peptide bond and in proton exchange (1). In addition to coordination of the zinc atom, each of the carboxylate C-terminal oxygens of P1 Ala-53 is at hydrogen bonding distance with Glu-270 and Arg-127 (2.54 and 2.79 Å, respectively).

TABLE 2

| Interaction site | NvCI | hCPA4 | Distance (Å) |
|------------------|------|-------|-------------|
| Primary interaction region | Cys-51 N | Glu-163 Oe1 | 3.06 |
| | Tyr-52 N | Glu-163 Oe2 | 3.08 |
| | Tyr-52 Cβ2 | Val-164 Cy1 | 3.59 |
| | Tyr-52 Cβ2 | Tyr-248 Cβ | 3.59 |
| | Tyr-52 O | Arg-71 Nη2 | 3.02 |
| | Tyr-52 O | Arg-127 Nη2 | 3.23 |
| | Tyr-52 O | Phe-279 Cβ | 3.20 |
| | Ala-53 N | Tyr-248 Oη | 3.78 |
| | Ala-53 Cβ | Glu-270 Oe2 | 3.26 |
| | Ala-53 O | Glu-270 Oe2 | 2.54 |
| | Ala-53 Ωr | Arg-127 Nη2 | 2.79 |

NvCI displays the strongest inhibitory constants (in the picomolar range) against most CPA-type forms: bCPA1, hCPA1, and hCPA4 with $K_i$ values of 5.8, 1.2, and 4.9 pm, respectively. An exception is hCPA2, for which the $K_i$ value is in the nanomolar range (Table 3). In such an enzyme variant, a major structural difference in the contact residues of the primary interaction site is the substitution of Glu-163 with Asp (9), which presumably is too distant to form any hydrogen bond (Fig. 2D). This fact suggests an explanation for the nanomolar value of $K_i$ displayed by NvCI against hCPA2 (similar to the other protein carboxypeptidase inhibitors (PCI, LCI, ACI, and TCI)) instead of the picomolar range of $K_i$ displayed against all other A-type carboxypeptidases with a glutamic acid in that position.

In summary, most of the active site residues in hCPA4 involved in substrate binding and catalysis are in contact with NvCI. The interaction with the enzyme and the conformation of the C-terminal tail in NvCI (composed only of Tyr-52 and Ala-53 (P2 and P1 positions, respectively)) are similar to those of other known exogenous protein carboxypeptidase inhibitors (PCI, LCI, TCI, and ACI), in which the inhibitor tail mimics substrate binding (see Fig. 2, B and C, for comparison). All of the three-dimensional protein structures of these inhibitors from evolutionarily distant organisms are unrelated and completely different (see Fig. 4 and supplemental Fig. S1). However, the only conserved motif in all of them is the structural conformation of the P1 and P2 residues of the C-terminal tail, which can be considered as a general feature, derived from a common functional mechanism for carboxypeptidase inhibition, and as a clear example of convergent evolution. As an exception, a relevant structural difference in the NvCI-hCPA4 complex is the main chain conformation of the P3 residue, which favors the formation of two extra hydrogen bonds with Glu-163 and presumably induces a reduction of the inhibition constant by stabilization of product formation (Fig. 2D).

Secondary Interaction Site in NvCI-hCPA4—The secondary interaction site of NvCI is composed mainly of contacts between residues from the two-stranded $\beta$-sheet and regions distant from the active site groove of hCPA4 (Fig. 3). As also suggested for other carboxypeptidase inhibitors, the secondary interaction site contributes substantially to the decrease in the inhibition constant (9, 10). The contacts are in large number and varied and fit with the unusual strong inhibitory power of NvCI against most CPA-like enzymes. Besides several van der Waals interactions, there are major specific polar contacts between the backbone and side chains of NvCI with hCPA4 (Table 2). Of special interest are the specific hydrogen bonds between the side chains of Glu-39 and Asn-123 (2.99 Å), Glu-37 and Arg-130 (2.86 and 2.86 Å between O1 and O2 with the NH1 and NH2 atoms, respectively), and Arg-7 and Asn-159, with a second nitrate molecule bridging both through two hydrogen bonds (2.77 and 2.79 Å, respectively) (Fig. 3). Four hydrogen bonds involving main chain atoms are also formed: between the carbonyl oxygens of Ile-10 and Asp-11 and the side chain of Asn-159 (3.30 and 3.71 Å, respectively), between the amide hydrogen of Cys-38 and the carbonyl group of Ser-137 (3.19 Å), and between the amide hydrogen of His-40 and the carbonyl group of Cys-161 (3.43 Å). A small hydrophobic core can also be distinguished in the secondary interface of NvCI with hCPA4,
basically formed by Phe-25 of the inhibitor, which is buried in a pocket created by two disulfide bridges, Cys-27 and Cys-38 of NvCI and Cys-138 and Cys-161 of hCPA4.

Structural comparisons indicated that the conformation of the residues of the enzyme (hCPA4 in this case) forming the secondary interaction surface is highly conserved in all A/B-type carboxypeptidases (M14A subfamily). This is also the case for the complex with NvCI, in particular considering the conservation and similar orientation of the specific side chain contacts described above, such as for Glu-37 and Gln-39. Thus, the lower $K_i$ values observed for NvCI regarding other MCP inhibitors can be attributed to both the primary and secondary interaction regions, which create an extended interface with the carboxypeptidase enzyme that minimizes the product release of the catalytic reaction.

**DISCUSSION**

The few reports that appeared in the last decade on the structure-function relationships of MCP inhibitors of exogenous origin (4–7), after the initial one from potatoes (8, 29), indicated that they share a similar substrate-like mechanism of inhibition. Thus, they suffer a trimming of their C termini, behaving as competitive tight-binding inhibitors, mimicking...
the interaction of a peptide substrate with the active site of the enzyme, and requiring only the S1 and S2 subsites to be covered to fully inhibit the enzyme. Thus, even though these exogenous MCP inhibitors are isolated from evolutionarily distant organisms, this is a good example of convergent evolution dictated by the architecture of the active site of the enzyme. However, the structure of NvCI with hCPA4 indicates that the trimming action is absent (i.e. a shorter two-residue tail is sufficient) and that the S3 subsite is also implicated, in addition to S1 and S2, in promoting stronger inhibition in the picomolar range.

In addition to this primary and essential C-terminal interaction, MCP inhibitors possess a second interface (distant from active site) that covers different regions of the carboxypeptidase and confers stability to the complex formation (Fig. 4). The NvCI interface with hCPA4 is quite extended in comparison with the other known inhibitors, covering a total of 1875 Å². In other known cases, for the protein inhibitors from leech (LCI), potato (PCI), and the Ascaris worm (ACI), the interface of CPA-like enzymes is clearly smaller: 1509, 1241, and 1426 Å², respectively. An exception would be the two-domain (exosite-behaving) tick carboxypeptidase inhibitor (TCI), which is the largest and covers a total interface of 2108 Å². Although the secondary interaction surface provided by all inhibitors is distinct, there is only a single way of positioning the C-terminal tail in the active site of the enzyme.

As mentioned above, the main body of each of the exogenous carboxypeptidase inhibitors has a completely different three-dimensional structure, with the C-terminal tail as the only similar structural motif (Fig. 2, B and C, and Fig. 4). The short C-terminal tail of NvCI, considered from the third disulfide bridge and formed by only two residues, Tyr-52 and Ala-53, is sufficient for a tight-binding inhibition of several types of carboxypeptidases. Interestingly, as mentioned above, in NvCI, the P3 position (Cys-51) also participates in the binding with a double main chain hydrogen bond with Glu-163 of hCPA4, presumably increasing the affinity and lowering the $K_i$ value for NvCI, which is in the picomolar range, 3 orders of magnitude lower than the other inhibitors. This fact suggests a reason for the strongest inhibitor being NvCI in comparison with the other described proteinaceous inhibitors of MCPs.

The different ways that nature acts along evolution to inhibit carboxypeptidases can be used as a valuable tool to elucidate the determinants for a general mechanism of inhibition of MCPs. In general, these proteases are secreted and their enzymatic action takes place normally in the extracellular space, except in the case of a novel subfamily of cytosolic carboxypeptidases, which have been recently described and are presumably involved in tubulin processing (30, 31). Knowledge of the control of interference with those mechanisms, by rational structure-based or other drug design approaches, can be potentially of great interest, especially for biotechnical and biomedical industrial purposes. In addition, the expansion of this strategy to the variety of forms found in the very diverse phyla of invertebrates, including marine organisms such as N. versicolor, is
revealing a rich source of discovery for novel scaffolds and lead compounds.

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