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ARTÍCULO DE REVISIÓN

Marine organisms: a source of biomedically relevant metallo M1, M2 and M17 exopeptidase inhibitors

Los organismos marinos: Fuente de inhibidores de exopeptidasas de tipo metalo M1, M2 y M17 de relevancia biomédica

Isel Pascual Alonso[1], Laura Rivera Méndez[1], Fabiola Almeida[1], Mario Ernesto Valdés Tresanco[1,2], Yarini Arrebola Sánchez[1], Aida Hernández-Zanuy[3], Luis Álvarez-Lajonchere[4], Dagmara Díaz[1], Belinda Sánchez[5], Isabelle Florent[6], Marjorie Schmitt[7], Miguel Cisneros[8], Jean Louis Charli[8]

[1] Center for Protein Studies, Faculty of Biology, University of Havana, Cuba.

[2] Department of Biological Sciences, University of Calgary, Calgary, Canada.

[3] Instituto de Ciencias del Mar, Agencia de Medio Ambiente, Cuba.

[4] Museo de Historia Natural "Felipe Poey", Facultad de Biología, Universidad de La Habana, Cuba.

[5] Centro de Inmunología Molecular, Habana, Cuba.

[6] Molécules de Communication et Adaptation des Microorganismes, Muséum National d'Histoire Naturelle, CNRS, France.

[7] Laboratoire d'Innovation Moléculaire et Applications-Université de Haute-Alsace, Université de Strasbourg, CNRS, France.

[8] Instituto de Biotecnología, Universidad Nacional Autónoma de México, México.

Autor para correspondencia:
isel@fbio.uh.cu

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ABSTRACT

Proteolytic enzymes, known as peptidases or proteases, are critical in all living organisms. They can act as exo- and/or endo-peptidases. Peptidases are segregated in classes that strongly depend on the chemical nature of the groups involved in catalysis. Peptidases control the activation, synthesis, and turnover of proteins and regulate most biochemical and physiological processes. They are consequently major regulators of homeostasis, ageing, diseases, and death. Proteases are also essential for propagation of infectious agents, being major contributors of pathogenesis in several infectious diseases, including the current coronavirus emergent pandemic COVID-19. Exopeptidases catalyze the cleavage of the N-terminal or C-terminal amino acids of proteins or peptide substrates. They are distributed in many phylla and play critical roles in physiology and pathophysiology. Most of them are metallo peptidases belonging to the M1, M2, and M17 families, among others. Some, such as M1 aminopeptidases N, A and thyrotropin-releasing hormone degrading ectoenzyme, M2 angiotensin converting enzyme and M17 leucyl aminopeptidase are targets for the development of therapeutic agents for human diseases including cancer, hypertension, central nervous system disorders, inflammation, immune system disorders, skin pathologies and infectious diseases, like malaria and coronavirus-induced syndromes. The relevance of exopeptidases has driven the search and identification of potent and selective inhibitors, as major tools to control proteolysis with impact in biochemistry, biotechnology, and biomedicine. The present contribution focuses on marine biodiversity as an important and promising source of inhibitors of metallo exopeptidases from different families, with biomedical applications in human diseases.

Keywords: exopeptidases, aminopeptidase N, aminopeptidase A, TRH -degrading ectoenzyme, angiotensin converting enzyme, leucyl aminopeptidase, inhibitors, marine organisms

RESUMEN

Las enzimas proteolíticas, conocidas como peptidasas o proteasas, son críticas en todos los organismos vivos. Pueden actuar como exo– o endopeptidasas. Las peptidasas se segregan en clases que dependen en gran medida de la naturaleza química de los grupos involucrados en la catálisis. Controlan la activación, síntesis y recambio de proteínas, y regulan la mayoría de los procesos bioquímicos y fisiológicos. En consecuencia, son los principales reguladores de la homeostasis, el envejecimiento, las enfermedades y la muerte. Las proteasas también son esenciales para la propagación de agentes infecciosos, ya que son los principales contribuyentes de la patogénesis en varias enfermedades infecciosas, incluida la actual pandemia de COVID-19. Las exopeptidasas son enzimas que catalizan la escisión de los aminoácidos N-terminal o C-terminal de las proteínas o sustratos peptídicos. Están ampliamente distribuidos en muchos filos y desempeñan papeles críticos en fisiología y fisiopatología. Son principalmente metalopeptidasas pertenecientes a las familias M1, M2 y M17, entre otras. Algunas de ellas, las aminopeptidasas M1 N, A y la ectoenzima que degrada la hormona liberadora de tirotropina, la enzima convertidora de angiotensina M2 y la leucil aminopeptidasa M17 son blancos actuales para el desarrollo de nuevos agentes terapéuticos para diferentes enfermedades humanas, que incluyen el cáncer, la hipertensión, trastornos del sistema nervioso central, inflamación, desordenes del sistema inmune y enfermedades infecciosas como la malaria. La relevancia de las exopeptidasas ha impulsado la búsqueda e identificación de inhibidores potentes y selectivos, como una herramienta importante para controlar la proteólisis con impacto en la bioquímica, la biotecnología y la biomedicina. Por estas razones, los inhibidores específicos de estas peptidasas son foco de estudio para el desarrollo de herramientas terapéuticas prometedoras en el tratamiento de diferentes trastornos. En particular, el presente trabajo centra su atención en la biodiversidad marina como una fuente importante y promisoria de inhibidores de serino y metalo exopeptidasas de diferentes familias, con aplicaciones biomédicas en enfermedades humanas.

Palabras clave*: exopeptidasas, aminopeptidasa N, aminopeptidasa A, enzima que degrada a la hormona liberadora de tirotropina, enzima convertidora de angiotensina, leucil aminopeptidasa, inhibidores, organismos marinos*

INTRODUCTION

Proteolytic enzymes, known as peptidases or proteases, are critical in all living organisms (Rawlings *et al*., 2018). Proteases can act as exo- and/or endo-peptidases. They are segregated in classes that strongly depend on the chemical nature of the groups involved in catalysis. The recognized mechanistic classes are: aspartic, cysteine, glutamic, metallo, asparagine, mixed, serine, threonine, and a group dedicated to unknown catalytic type (Rawlings *et al*., 2018).

Peptidases are one of the most abundant groups of enzymes in living organisms. They control the activation, synthesis and turnover of proteins, and regulate most biochemical and physiological processes, such as digestion, fertilization, growth, differentiation, cell signaling/ migration, immunological defense, wound healing, and apoptosis (Drag and Salvense, 2010; Deu *et al*., 2012; Rawlings *et al*., 2018). They are consequently major regulators of homeostasis, ageing, diseases, and death. Proteases are also essential for propagation of infectious agents, being major contributors of pathogenesis in several infectious diseases, including the current coronavirus emergent pandemic COVID-19 (Newman and Cragg, 2016; Rawlings *et al.,* 2018; Drinkwater *et al*., 2017; Drinkwater *et al*., 2019; Hoffmann *et al*., 2020; Zhang *et al*., 2020).

Exopeptidases are peptidases that act at the extremities of polypeptide chains producing free amino acids or small peptides. They can be subdivided into aminopeptidases or carboxypeptidases.

They are proteolytic enzymes that begin to hydrolyze the peptide bonds of the polypeptide chain from the N-terminus or C-terminus (Rawlings *et al*., 2018). They are widely distributed in many phylla and play critical roles in physiology and pathophysiology (Drinkwater *et al*., 2017; Salomon *et al.,* 2018; Rawlings *et al*., 2018; Drinkwater *et al*., 2019; Pujiastuti *et al.,* 2019). In mammals, they have a widespread organ distribution and are found in many subcellular organelles, in the cytoplasm, as integral membrane proteins or extracellularly (Albiston *et al*., 2004; Carl -McGrath *et al*., 2006; Mucha *et al*., 2010; Drinkwater *et al*., 2017). They are mainly metallo peptidases belonging to different families like the M1, M2, M17 ones (Rawlings *et al*., 2018), among others. Among the best representatives of aminopeptidases currently the focus of biomedical investigation, we can find the M1 family neutral aminopeptidase (APN, EC 3.4.11.2), glutamyl aminopeptidase (APA EC 3.4.11.7) and thyrotropin-releasing hormone degrading ectoenzyme (TRH-DE; EC 3.4.19.6), the M2 family angiotensin converting enzyme (ACE, 3.4.15.1), and the M17 neutral aminopeptidase also known as leucyl aminopeptidase (LAP, EC 3.4.11.21) (Fig. 1).

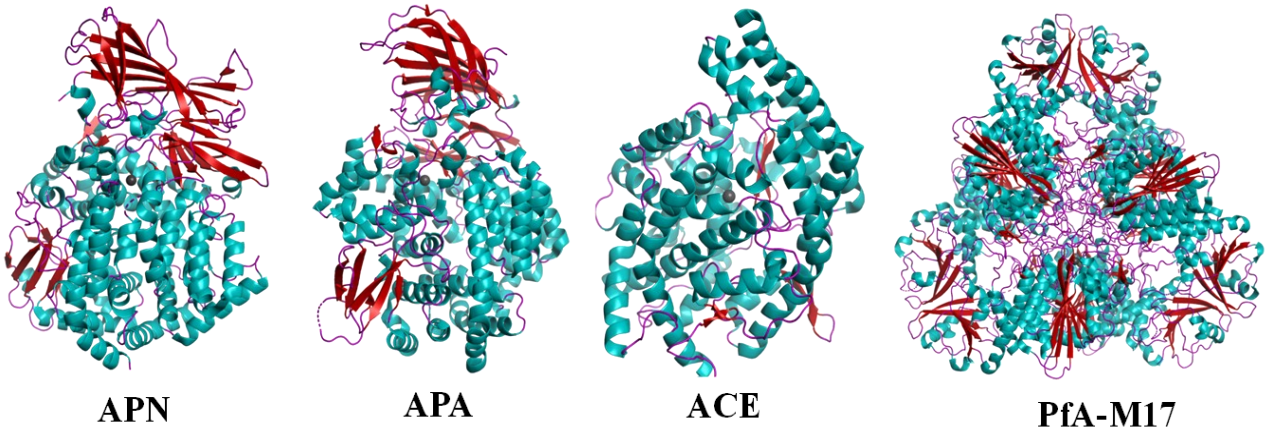

Figure 1. Ribbon representation of different aminopeptidases: APN (PDB ID: 4fyq), APA (PDB ID: 4kx7), ACE (PDB ID: 1o86) and PfA-M17 (PDB ID: 4r76). Colors: alpha-helices (light blue), beta sheets (red) and ribbons (violet).

Figura 1. *Representación en cintas de diferentes aminopeptidasas: APN (PDB ID: 4fyq), APA (PDB ID: 4kx7), ACE (PDB ID: 1o86) and PfA-M17 (PDB ID: 4r76). Colores: alfa-hélices (azul celeste), hojas beta (rojo) y lazos (violeta).*

These enzymes are implicated in relevant physiological and pathophysiological processes like cancer, hypertension, central nervous system disorders, inflammation, immune system disorders, skin pathologies and infectious diseases, like malaria, and are current targets for the development of new therapeutic drugs.

The biological relevance of peptidases has driven the search and identification of potent and selective inhibitors, as a major tool to control proteolysis. Natural and synthetic peptidase inhibitors are useful in biochemistry, biotechnology, and biomedicine (Turk *et al*., 2006). Thus, specific inhibitors of target peptidases are emerging as promising therapeutics for the treatment of hypertension, cancers, inflammation, immunological and respiratory disorders, and parasitic and viral infectious diseases (Drag and Salvesen, 2010; Deu *et al*., 2012; Deu *et al*., 2017; Amin *et al*., 2018; Roy *et al*., 2017; Li *et al*., 2018; Fung and Liu, 2019).

Many protease inhibitors have been isolated from natural sources and are characterized by high chemical diversity, biochemical specificity, molecular flexibility and other molecular properties that make them more favorable than synthetic inhibitors as lead structures for drug discovery (Newman and Cragg, 2014; Newman and Cragg, 2016).

Marine invertebrates are an abundant source of bioactive molecules such as toxins (Alvarez *et al*., 2009; 2017; 2020; Valle *et al*., 2015), antimicrobial peptides (Otero *et al*., 2010), enzymes and enzymes inhibitors (Nakao and Fusetani, 2007), and particularly peptidases (Alonso del Rivero *et al*., 2009; Pascual *et al*., 2020) and peptidases inhibitors of almost all mechanistic classes (Chávez *et al*., 1988; Delfín *et al*., 1994; 1996; Lenarčič *et al*., 1997; González *et al*., 2004; Reytor *et al*., 2011; Alonso del Rivero *et al*., 2012; Salas-Sarduy *et al*., 2013; González *et al*., 2016; Salas Sarduy *et al*., 2017; Covaleda *et al*., 2017; Hong *et al*., 2018; Cabrera *et al*., 2019).

These bioactive molecules have a great diversity of chemical structures, high potency and diverse specificity, including for inhibitors of metallo enzymes (Fig. 2- 4) (Ikegami *et al*., 1994; Kato *et al*., 1998; Fusetani *et al*., 1999; Fujita *et al*., 2002; 2003; Shim *et al*., 2004; Pascual *et al*., 2004; Alonso del Rivero *et al*., 2012; 2013; Covaleda *et al*. 2012; Cabrera *et al.,* 2019).

These biomolecules are involved in nutrition, reproduction, and communication. Additionally, since most invertebrates (e.g., sponges, bryozoans, tunicates, among others) lack morphological defense structures, peptidases inhibitors are also part of mechanisms related with protection against predators, infection, and competition (Hussain *et al*., 2012). In the present contribution we review and summarize the status of serine and metallo exopeptidases inhibitors isolated from marine organisms with a focus on M1, M2, M17 families enzymes targeted in biomedical studies.

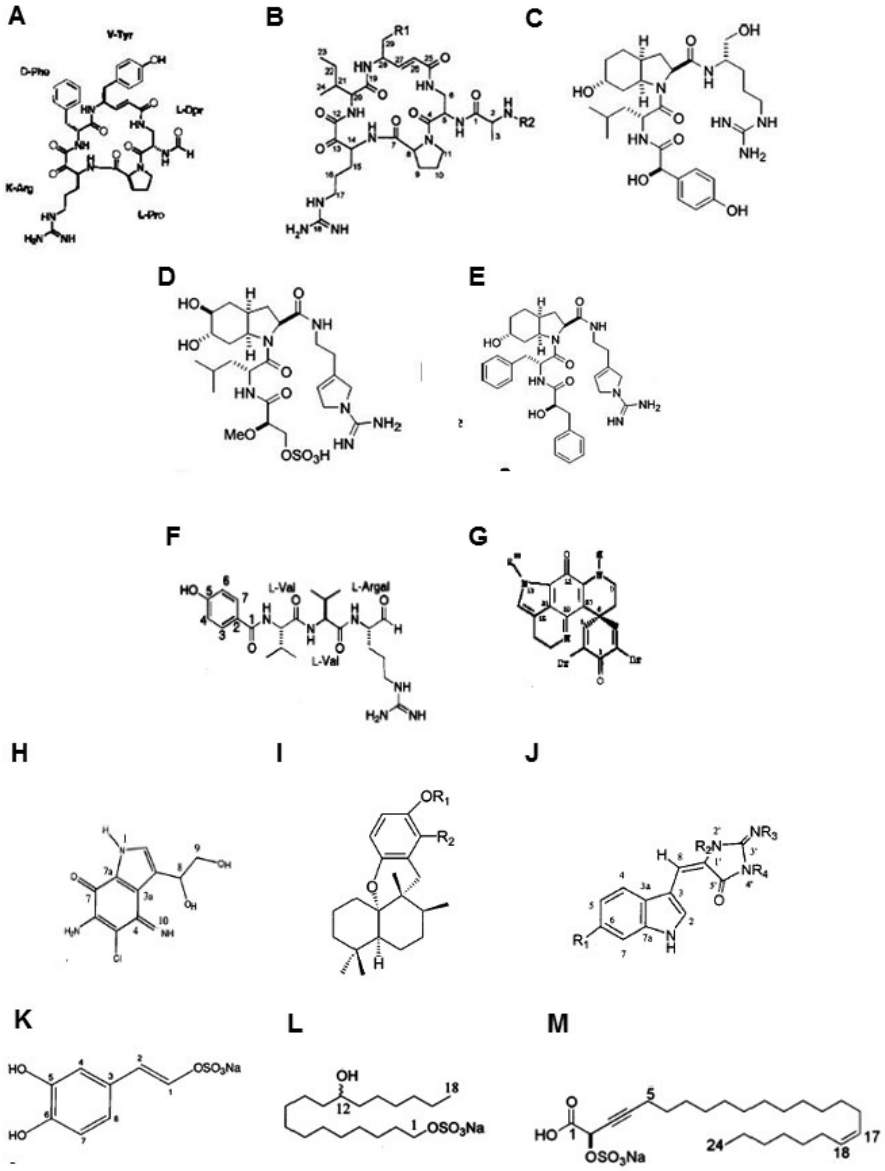

Figure 2. Structures of low molecular weight non-peptidic protease inhibitors of different mechanistic classes isolated from marine organisms. Serine peptidase inhibitors: A) cycloteonamide A (Nakao *et al.,* 1998), B) general structure of cycloteonamide E (Nakao *et al.,* 1998), C) aeruginosine 298-A, D) dinosine, E) oscilarina (Hanessian *et al.,* 2004); Cystein peptidases inhibitors: F) tokaramida A (Fusetani *et al.* 1999), G) discorhabdina P (Gunasekera *et al.,* 1999a), H) secobatzellina A (Gunasekera *et al.,* 1999b); Aspartic peptidases inhibitors: I) N,N-dimetiltiocarbamate, J) 6 Br-aplisinopsine (Hu *et al.,* 2002); Metallo endopeptidase inhibitors: K) jaspisin (Ikegami *et al.,* 1994); L) 1-12-hydroxyoctadecanil sodium sulphate (Fujita *et al.,* 2002); M) calisponginol sulphate (Fujita *et al.,* 2003).

Figura 2. *Estructuras de inhibidores de bajo peso molecular de proteasas de distintas clases mecanisticas aislados de organismos marinos. Inhibidores de proteasas tipo serina: A) cicloteonamida A (Nakao et al. 1998), B) Estructura general de la cicloteonamida E (Nakao et al. 1998), C) aeruginosina 298-A, D) dinosina, E) oscilarina (Hanessian et al., 2004); Inhibidores de proteasas tipo cisteina: F) tokaramida A (Fusetani et al. 1999), G) discorhabdina P (Gunasekera et al., 1999a), H) secobatzellina A (Gunasekera et al. 1999b); Inhibidores de proteasas tipo aspartico: I) N,N-dimetiltiocarbamato, J) 6 Br-aplisinopsine (Hu et al., 2002); Inhibidores de endoprotesasas tipo metallo: K) jaspisina (Ikegami et al., 1994); L) 1-12-hidroxioctadecanil sulfato de sodio (Fujita et al., 2002); M) sulfato de calisponginol (Fujita et al., 2003).*

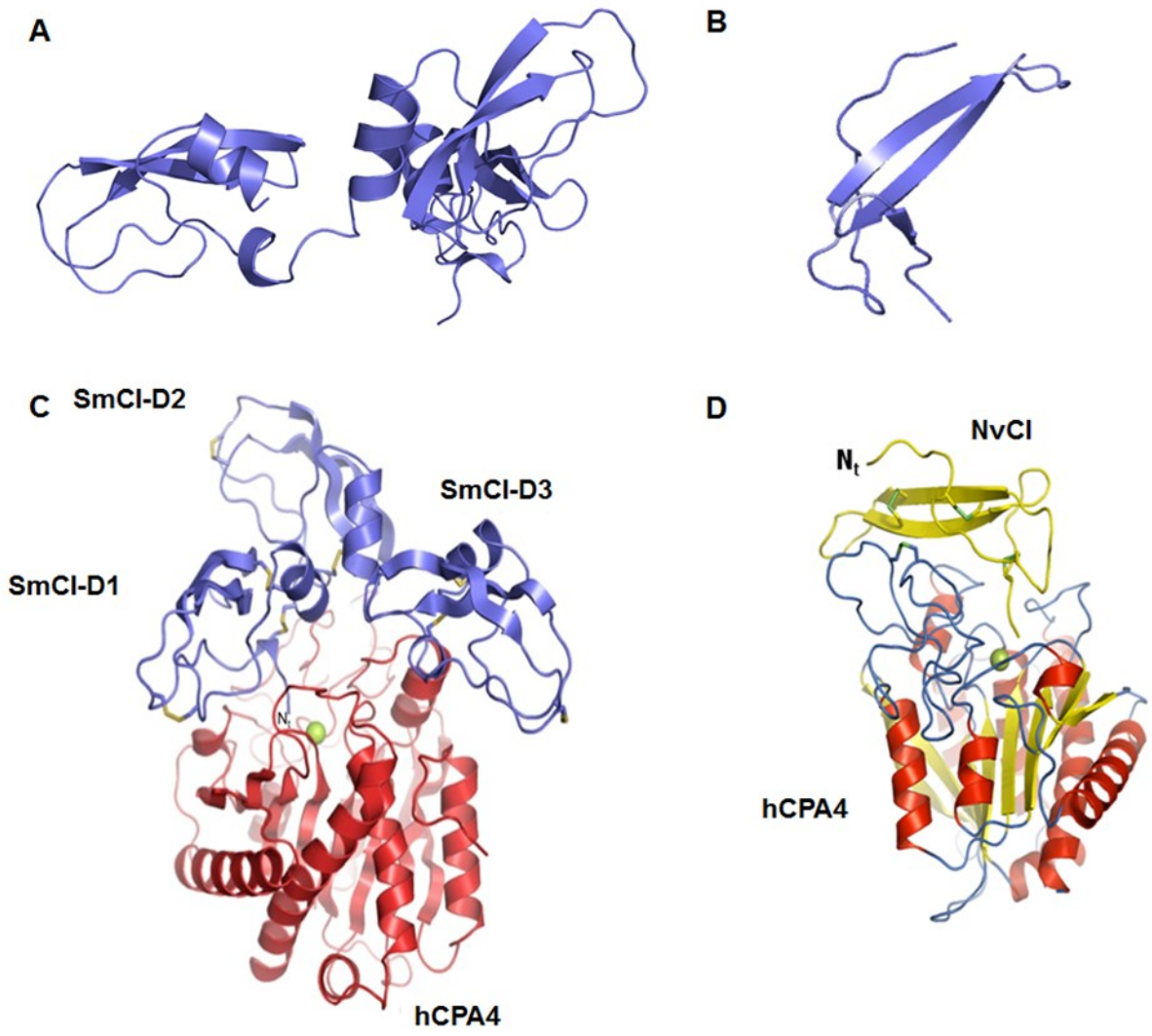

Figure 3. Structure of metallo carboxypeptidase inhibitors isolated from marine invertebrates. A) SmCI: carboxypeptidase A inhibitor isolated from marine annelide *Sabellastarte magnifica*, B) NvCI: carboxypeptidase A inhibitor isolated from marine snail *Nerita versicolor*, C) Structure of SmCI in complex with human carboxypeptidase A4 (hCPA4) (Alonso del Rivera *et al.* 2013), D) Structure of NvCI in complex with hCPA4 (Covaleda *et al.* 2012). Courtesy of Dr. Maday Alonso del Rivero Antigua.

Figura 3. *Estructura de inhibidores de metalo carboxipeptidasas aislados de invertebrados marinos. A) SmCI: inhibidor de carboxipeptidasa A aislado del anélido marino Sabellastarte magnifica, B) NvCI: inhibidor de carboxipeptidasa A aislado del molusco marino Nerita versicolor, C) Estructura de SmCI en complejo con la carboxipeptidasa A4 humana (hCPA4) (Alonso del Rivera et al. 2013), D) Estructura de NvCI en complejo con hCPA4 (Covaleda et al. 2012). Cortesía de la Dra. Maday Alonso del Rivero Antigua.*

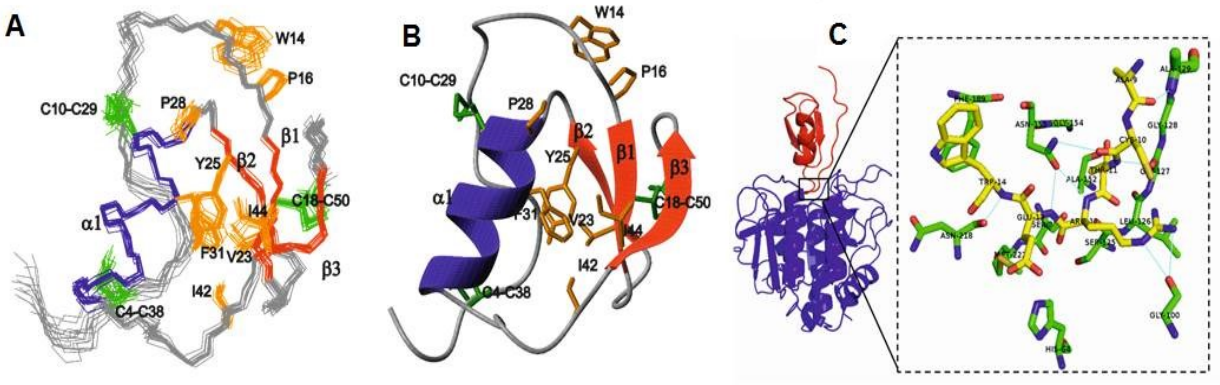

Figure 4. Structure of CmPI-II (a serine protease inhibitor isolated from the marine snail *Cenchritis muricatus*). A) Family of the 15 lowest energy structures of CmPI-II obtained by NMR. B) Tape representation of the lowest energy structure. The β strands (β1 – β3) and the α helix are represented in red and blue, respectively. Orange rods represent the side chains of the hydrophobic residues that form the nucleus of the protein and those that show chemical shift values that deviate from the expected values (Ile44 and Pro16) and green shows disulfide bridges. C) Prediction of the 3D structure of the CmPI-II / subtilisin A complex. CmPI-II is shown in red and subtilisin A in blue. The interaction interface is shown on the insert. Hydrogen bridges are shown in blue. Residues of the reactivating loop of CmPI-II are shown as bars where the carbon atoms are colored yellow while contact residues in the subtilisin are represented as bars where the carbon atoms are represented green. In both cases, the oxygen atoms are colored red, the nitrogen atoms blue and the sulfur atoms orange (Cabrera-Muñoz *et al.,* 2019). Courtesy of Dr. Aymara Cabrera-Muñoz.

Figura 4*. Estructura de CmPI-II (inhibidor de proteasas de tipo serino aislado del molusco marino Cenchritis muricatus). A) Familia de las 15 estructuras de menor energía de CmPI-II obtenidas por RMN. B) Representación en cinta de la estructura de menor energía. Las hebras β (β1–β3) y la hélice α se representan en rojo y azul, respectivamente. En naranja y en varillas se representan las cadenas laterales de los residuos hidrofóbicos que forman el núcleo de la proteína y aquellos que presentan valores de desplazamientos químicos que se desvían de los valores esperados (Ile44 y Pro16) y en verde los puentes disulfuro. C) Predicción de la estructura 3D del complejo CmPI-II/subtilisina A. CmPI-II se muestra en rojo y la subtilisina A en azul. La interfase de interacción se muestra en el inserto. Los puentes de hidrógenos se muestran en azul. Los residuos del lazo reactivo de CmPI-II están representados por barillas donde los átomos de carbono están coloreados de amarillo mientras que los residuos de contacto en la subtilisina están representados como barillas donde los átomos de carbono están representados en verde. En ambos casos los átomos de oxígeno están coloreados en rojo, los de nitrógeno en azul y los de azufre en anaranjado (Cabrera-Muñoz et al., 2019). Cortesía de la Dra. Aymara Cabrera-Muñoz.*

METALLO M1, M2 AND M17 EXOPEPTIDASES INHIBITORS ISOLATED FROM MARINE ORGANISMS

Metallo aminopeptidases. General characteristics and classification

Up to now, 15 clans of metallo peptidases have been described, with six of them comprising exo-peptidases. They are MA (with 94 families), MC (2 families), MD (2 families), ME (2 families), MF (1 family), MG (1 family), MH (3 families), MJ (1 family), MM (1 family), MN (1 family), MO (2 families), MP (1 family) and MQ (1 family), MS (1 family), and MU (4 families), clans MA and MF being two of the most characterized with enzymes from all living organisms (Rawlings *et al*., 2018).

Clan MA: subclan MA (E)

The clan MA is the largest of the metallopeptidases, with a total of 94 families (Rawlings *et al*., 2018), all consisting of enzymes that contain Zn^{2+} in their active sites. This clan is made up of both endopeptidases and exopeptidases comprising aminopeptidases (families M1, M2, M4, M5, M9, M13, M30, M36, M48 and M61), carboxypeptidases (M2, M32), peptidyl-dipeptidases (M2), oligopeptidases (M3, M13) and endopeptidases (families M4, M10, M12). In the enzymes of MA clan, the Zn^{2+} atom is linked to the protein through two His residues, which are part of the HEXXH consensus sequence. In addition to the His residues, the catalytic Zn^{2+} is coordinated by a water molecule and a third residue, the nature of which determines the clan's subdivision into the MA (E) and MA (M) sub-clans. In the subclan MA (M) the third ligand can be a residue of His or Asp within the HEXXHXXGXXH/D signature sequence, while in subclan MA (E) the third ligand is a residue of Glu, located at

least 14 residues after the carboxyl terminus of the HEXXH motif (Rawlings *et al*., 2018). The oxygen atom of the water molecule that acts as a metal ligand is the nucleophilic agent that attacks the carbonyl of the peptide bond to be hydrolyzed.

Thermolysin (EC 3.4.24.27) is the model enzyme of the MA clan and its structure, widely characterized, is a point of reference for the study of the enzymes of this clan due to the high structural similarity between them in terms of the organization of the active center (Rawlings *et al*., 2018). Among the most studied families of the subclan MA (E) is M1, which members show a wide distribution in the living world (Table 1); furthermore, they are involved in many functions that include cell maintenance, growth, development and defense (Drinkwater *et al*., 2017). This family includes enzymes of Gram (+) and Gram (-) bacteria, cyanobacteria, archaea, protozoa, fungi, animals, and plants (Drinkwater *et al*., 2017; Rawlings *et al*., 2018) (Table 1). The family M2 also belongs to subclan MA (E), and contains angiotensin-converting enzyme peptidase unit 1 (ACE), which until recently was the only known member of this family (Table 1) (Rawlings *et al*., 2018).

Table 1. Some members of the M1 and M2 families of the MA(E) subclan of metallopeptidases.

Tabla 1. *Algunos miembros de las familias M1 y M2 del subclan MA(E) de las metalopeptidasas.*

Enzyme	IUBMB* Nomenclature	Sources
Family M1		
Aminopeptidase N (APN)	EC 3.4.11.2	*Homo sapiens, Sus scrofa*
Aminopeptidase A (APA)	EC 3.4.11.7	*Homo sapiens*
Thyrotropin-releasing hormone-degrading ectoenzyme (PPII, TRH-DE)	EC 3.4.19.6	*Homo sapiens, Mus musculus, Rattus novergicus*
Insulin-regulated membrane aminopeptidase (IRAP)	EC 3.4.11.3	*Homo sapiens*
Leucin aminopeptidase derived from adipocytes (A-LAP)	EC 3.4.11.-	*Homo sapiens*
Puromycin-sensitive aminopeptidase (PSA)	EC 3.4.11.14	*Homo sapiens*
Ape2 aminopeptidase	-	*Saccharomyces cerevisiae*
Lysyl aminopeptidase	-	*Escherichia coli*
Aminopeptidase O (AP-O)	EC 3.4.11.-	*Homo sapiens*
Aminopeptidase B (APB)	EC 3.4.11.6	*Homo sapiens*
Leukotriene A4 hydrolase (LTA4H)	EC 3.3.2.6	*Homo sapiens*
Aminopeptidase Ey	EC 3.4.11.20	-
Aminopeptidase II (APII)	-	-
Endoplasmic reticulum aminopeptidase 1 ERAP-1	-	*Homo sapiens*
Endoplasmic reticulum aminopeptidase 2 ERAP-2	-	*Homo sapiens*
Tricorn interacting factor F2	-	*Thermoplasma acidophilum*
Tricorn interacting factor F3	-	*Thermoplasma acidophilum*
Cold-active aminopeptidase	-	*Colwellia psychrerythraea*
Family M2		
Angiotensin-converting enzyme peptidase unit 1	3.4.15.1	*Homo sapiens*
Peptidyl-dipeptidase Acer	3.4.15.1	*Drosophila melanogaster*
Peptidyl-dipeptidase Ance	3.4.15.1	*Drosophila melanogaster, Bombyx mori, Haematobia irritans exigua*
Angiotensin-converting enzyme peptidase unit 2	3.4.15.1	*Homo sapiens*
Angiotensin-converting enzyme-2	3.4.17.23	*Homo sapiens*

*IUBMB: International Union of Biochemistry and Molecular Biology

Inhibitors of M1 family isolated from marine organisms

M1 family aminopeptidases exist in monomeric or dimeric forms. In eukaryotes, they are generally membrane-associated enzymes such as mammalian APN, acidic aminopeptidase (APA), adipocyte-derived leucine aminopeptidase, and thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE) also known as pyroglutamyl peptidase II (Albiston *et al*., 2004). Some are cytosolic enzymes, such as leukotriene A4 hydrolase (bifunctional enzyme with aminopeptidase activity) (Haeggström *et al*., 2002) and aminopeptidase B (APB) (Fukasawa *et al*., 1999), or associated with the cell wall (Drinkwater *et al*., 2017) as the neutral aminopeptidase (APN, EC 3.4.11.2) of the yeast *Candida albicans* (Klinke *et al*., 2008).

The structure of the membrane-bound aminopeptidases of the M1 family, in general, comprises a short intracellular tail attached to the transmembrane domain and a large ectodomain formed, in turn, by 2 or 3 folded and conserved domains. Domain I, N-terminal, has a β-sheet nucleus that, although it is widely exposed to the solvent, contains a hydrophobic region that continues in an anchorage region in the membrane. Catalytic domain II, like that of thermolysin, contains the active site flanked by a mixed structure of β-sheet and α-helix that is highly conserved throughout the family. Domain III, which is composed of an immunoglobulin-like fold, does not appear in some family members (such as leukotriene A4 hydrolase).

Domain IV, C-terminal, is the most variable region within the family. It is completely helical, with such an arrangement that it covers the active site; it is also involved in the dimerization of the mammalian isoforms (Drinkwater *et al*., 2017). Disulfide bridges and abundant glycosylations are generally seen in this extracellular region, and some of these enzymes are surface antigens (Albiston *et al*., 2004; Rawlings *et al*., 2018).

In the M1 family, a well-conserved motif is the Gly-Ala/X-Met-Glu-Asn (GAMEN/GXMEN) sequence. This sequence, also known as the exopeptidase motif, infrequently shows variations in the first two residues (Dalal *et al*., 2013), and is very useful for the identification of family members (Albiston *et al*., 2004; Wong *et al*., 2012; Drinkwater *et al*., 2017).

Inhibitors of thyrotropin-releasing hormone degrading ectoenzyme/ pyroglutamyl aminopeptidase II isolated from marine organisms

Thyrotropin-releasing hormone (TRH, pGlu-His-ProNH_2) is a peptide mainly produced by brain neurons. If expressed by the paraventricular nucleus of the hypothalamus, TRH is an hypophysiotropic factor that increases the synthesis and release of thyroid stimulating hormone (TSH) and prolactin (PRL) from the adenohypophysis. In other central nervous system (CNS) circuits, it functions as a neurotransmitter and / or neuromodulator (Joseph-Bravo *et al*., 2015). This peptide has therapeutic properties in the treatment of brain and spinal damages and various neurodegenerative disorders (Lechan and Toni, 1990; Cone *et al*., 2003). However, TRH effects are of short duration, in part because the peptide is hydrolyzed in blood and extracellular space by TRH-DE, a M1 family metallopeptidase. TRH-DE is enriched in various brain regions but is also expressed in peripheral tissues including the anterior pituitary and the liver, which secretes a soluble form into blood.

Among the M1 metallopeptidases, TRH-DE is the only member with a very narrow specificity, hydrolyzing preferentially the pGlu-His bond of TRH, its best characterized biological substrate, making it a target for the specific manipulation of TRH activity. TRH-DE presents an anatomical location that correlates partially with TRH receptors in various regions and is very strictly regulated by different hormones and hypothalamic factors, as well as by various pharmacological and pathophysiological conditions that alter the transmission of TRH-mediated signals.

The regulation of TRH-DE activity may be very important for the adjustment of communication mediated by this peptide (Charli *et al*., 2020). Therefore, TRH -DE inhibitors are important tools for studying the physiological functions of this enzyme and TRH in the CNS, as well as for enhancing the different actions of TRH by protecting the degradation of endogenous TRH or exogenously administered analogues (Kelly *et al*., 1995).

TRH-DE inhibition may be used to enhance TRH activity in different pathologies (Fig. 5). Only a few synthetic PPII inhibitors have been described (Charli *et al*., 1989; Kelly *et al*., 2000; Kelly *et al*., 2005).

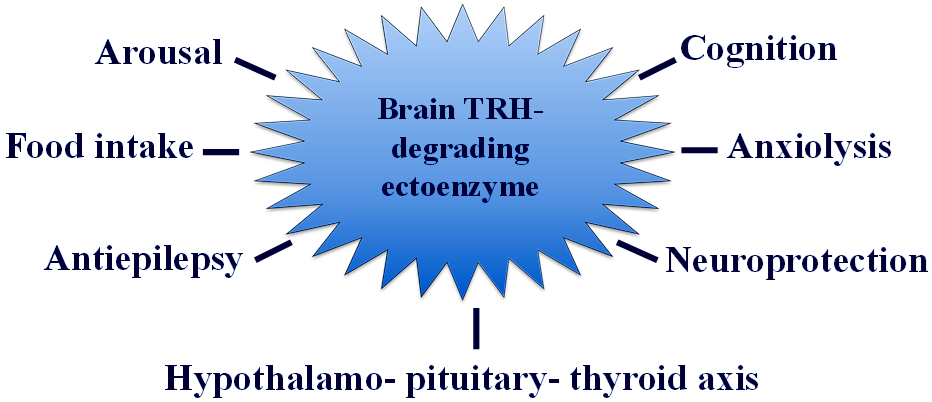

Figure 5. Potential therapeutic applications of targeting central TRH-degrading ectoenzyme activity.
Figura 5. *Aplicaciones terapéuticas potenciales de la inhibición de la ectoenzima de inactivación de la TRH en el cerebro.*

A joint project of the Faculty of Biology, University of Havana, Cuba with the Institute of Biotechnology of UNAM, Mexico, involving a screening in aqueous extracts from 26 Cuban coastline marine invertebrates (Table 2), resulted in the first natural inhibitor of TRH-DE identified and isolated from the marine annelid *Hermodice carunculata* (Fig. 6); it was named HcPI. As a result of this screening, we also detected inhibitory activities of porcine dipeptidyl peptidase IV in the species *Phallusia nigra, Mycale microsigmatosa, Condylactis gigantean, Stichodactyla helianthus* and *Palythoa caribaeorum*. HcPI is a 580 Da compound (molecular mass determined by ESI-TOF mass spectrometry), with a possible polymeric structure and the presence of bromine in its structure, as well as amide-type bonds. HcPI potently inhibits TRH-DE with a Ki value of 70.3 nM in a slow and reversible way, making it one of the most powerful inhibitors described against this enzyme (Pascual *et al.*, 2004).

Figure 6. Marine annelid *Hermodice carunculata*. Picture courtesy of Professor José Espinosa, PhD, ICIMAR (*Instituto de Ciencias del Mar*), CITMA, Cuba.
Figura 6. *Anélido marino Hermodice carunculata. Foto cortesía del Profesor José Espinosa, PhD, del ICIMAR* (*Instituto de Ciencias del Mar*)*, CITMA, Cuba.*

Table 2. Screening of inhibitory activity of TRH-DE and DPP-IV in aqueous extracts from marine invertebrates collected at the Havana coastline, Cuba. Adapted from Pascual *et al*. (2004).

Tabla 2. *Tamizaje de actividad inhibidora de PPII y DPP-IV en extractos acuosos de invertebrados marinos recolectados en las costas de la Habana, Cuba. Adaptado de Pascual et al. (2004).*

Species	Phyllum	Proteins (mg/mL)	Inhibitory activity of TRH-DE (U/mg)	Inhibitory activity of DPP-IV (U/mg)
Caulerpa racemosa	Chlorophyta	5.86	-	-
Dictyosphaeria cavernosa	Chlorophycota	8.73	-	-
Halimeda opuntia	Chlorophycota	14.72	-	-
Halimeda incrassata	Chlorophycota	10.23	-	-
Bidens pilosa	Magnoliophyta	22.78	-	-
Ascidia sidneyense	Chordata	57.27	-	-
Molgula occidentalis	Chordata	31.41	-	-
Pyura vittata	Chordata	58.53	-	-
Phallusia nigra	Chordata	25.90	-	93.00
Microcosmus gamus	Chordata	24.45	-	-
Tectitethya cripta	Porifera	0.90	-	-
Mycale microsigmatosa	Porifera	43.50	-	56.59
Lima scabra	Mollusca	51.55	-	-
Aplisia dactilomela	Mollusca	24.00	-	-
Zoanthus pullchelus	Cnidaria	15.57	-	-
Plexaura homomalla	Cnidaria	15.25	-	-
Condylactis gigantea	Cnidaria	36.60	-	79.46
Stichodactyla helianthus	Cnidaria	79.50	-	17.48
Cassiopea xamachana	Cnidaria	3.14	-	-
Physalia physalis	Cnidaria	13.84	-	-
Palythoa caribaeorum	Cnidaria	16.80	-	133.00
Bartholomea annulata	Cnidaria	56.50	-	
Hermodice carunculata	Annelida	62.41	24.00	-
Sabellastarte magnifica	Annelida	67.24	-	-
Holothuria floridiana	Echinodermata	18.84	-	-
Holothuria mexicana	Echinodermata	29.63	-	-

Inhibitory specificity studies carried out against proteases of all mechanistic classes indicate that HcPI is highly specific for TRH-DE. At a dose of 2 mg/mL, a concentration 300 times higher than the K_i value against rat TRH-DE, it does not inhibit other M1 aminopeptidases, like porcine kidney aminopeptidase A and B and only inhibits aminopeptidase N by 20%. It was further confirmed that HcPI inhibits, *in vitro*, thyroliberinase, a soluble version of TRH-DE that inactivates TRH in the bloodstream.

The inhibition is dose-dependent, with a K_i value of 51 nM, similar to that previously described for TRH-DE. The specificity results support the idea that HcPI should be useful to study the role of TRH -DE in different experimental models. HcPI is not toxic *in vivo* and its intraperitoneal injection in BalbC mice decreases TRH-DE activity in the pituitary and in different brain regions such as the hypothalamus, cerebellum, and olfactory bulb (Pascual *et al*., 2004).

The inhibition of TRH-DE *in vivo* in this experimental model causes a transient increase in the serum concentrations of prolactin (PRL) and thyrotropin (TSH), which indicates an *in vivo* enhancement of the actions of endogenous TRH, which degradation by TRH-DE is decreased. Additionally, studies on cells were performed. First, in primary cultures of adenohypophyseal cells, 45 minutes of incubation with HcPI produces a decrease in the activity of membrane-associated TRH-DE, highly dependent on the dose of inhibitor tested with an IC_{50} of 8.3 µg / mL. Incubation with 8 µg HcPI / mL decreases enzyme activity by 42% in 5 minutes, an effect stable for at least one hour. Once enzyme inhibition was demonstrated in cultures, the effect of the enzyme on TRH-mediated communication was evaluated, and it was detected that in the absence of TRH in the system, the presence of HcPI (50 µg / mL) does not change the basal levels of secretion of TSH, or PRL. On the other hand, in the presence of TRH (10 nM), the inhibition of TRH-DE by HcPI caused an increase in the levels of PRL released after 30 minutes by lactotrophs, specialized cells of the adenohypophysis.

These results were confirmed in parallel by inhibition of TRH-DE synthesis with the use of antisense RNA, which demonstrated for the first time in a direct way, the effects of the regulation of PPII activity on one of the functions of TRH (Cruz *et al*., 2008). Other experiments related to the role of PPII in TRH communication within the hypothalamic-adenohypophysis-thyroid axis were continued with the use of animal models. In these studies, HcPI was injected at the beginning of the experiment at a dose of 50 µg / g of animal weight to Wistar rats, dissolved in physiological saline (doses of 5-10 µg / g of animal weight strongly reduce PPII activity in the adenohypophysis and decrease it in CNS regions). Controls received only saline. Four groups of animals were subsequently treated with 1 ng / g animal weight TRH in saline or saline only and slaughtered by decapitation 15 minutes after the second treatment. Two additional groups were transferred to a cold room kept at 4 °C for 30 minutes and similarly sacrificed after the end of the experiment. Since cold stress rapidly activates the hypothalamic-adenohypophysis-thyroid axis by increasing concentrations of TRH in the portal hypothalamus-pituitary vessels, this paradigm was used in addition to exogenous administration of TRH, with the objective of evaluating the effects of inhibition of TRH-DE by HcPI on a naturally occurring surge of circulating TSH concentration.

Compared to animals that received a single injection of saline, TRH-DE activity is significantly decreased in the hypothalamus and in the pituitary of animals that receive a single dose of HcPI. Similar changes are observed in the activity of serum thyroliberinase. Inhibition of TRH-DE activity by HcPI has no effect on baseline TSH levels, as observed in primary adenohypophyseal cell cultures. However, in animals injected with exogenous TRH or exposed to ambient cold, inhibition of TRH-DE activity by HcPI is associated with a significant increase in serum TSH concentration when compared to control groups that only received saline (Sánchez -Jaramillo *et al*., 2009).

These results demonstrated for the first time the role that TRH-DE exerts on TRH activity and TSH secretion by the adenohypophysis, and made HcPI a very useful tool for further studies and potential biomedical applications.

Inhibitors of aminopeptidase N isolated from marine organisms

Neutral aminopeptidases are enzymes that catalyze the cleavage of neutral amino acids from the N-terminus of protein or peptide substrates. They have been classified in several metallopeptidase families such M1 and M17 (Drinkwater *et al*., 2017; Rawlings *et al*., 2018; Drinkwater *et al*., 2019). These enzymes are present in all living organisms, but the diversity of the functions in which they are involved is far from being entirely deciphered. Mammalian neutral aminopeptidase (APN, EC 3.4.11.2, M1 family) is the most extensively studied member of the M1-family of zinc-dependent aminopeptidases and catalyzes the cleavage of neutral and basic amino acids from the N-terminus of protein or peptide substrates. This enzyme is widely expressed on cell surfaces of tissues, such as intestinal epithelia and the nervous system. APN is a type II membrane protein generally found as a homodimer in several mammalian species. Full-length human APN consists of 967 amino acids with a short N-terminal cytoplasmic domain, a single transmembrane segment, and a large ectodomain containing two catalytic motifs highly conserved across the M1 family: the zinc-binding motif HEXXHX18E and the exopeptidase signature GXMEN (Wong *et al*., 2012; Pascual *et al*., 2015). APN plays pivotal roles in many physiological processes such as pain sensation, sperm motility, cell-cell adhesion, and blood pressure regulation (Fig. 7) (Mucha *et al*., 2010; Drinkwater *et al*., 2017).

This enzyme is also up-regulated in human pathologies such as coronavirus entry, inflammation, immune cell chemotaxis, tumor angiogenesis and metastasis in several types of cancer with a strong correlation between the level of APN expression of a cell and its resultant invasive capacity (Fig. 8). Dysregulation of APN expression evolves in almost all types of human malignancies, including breast cancer, cervical cancer, ovarian cancer, prostate cancer, non-small-cell lung cancer (NSCLC), liver cancer, colon cancer, scirrhous gastric cancer, pancreatic cancer, renal cell carcinoma (RCC), hepatocellular carcinoma (HCC), head and neck squamous cell carcinoma (SCC), melanoma, osteosarcoma, and thyroid cancer (Reviewed in Amin *et al*., 2018). This makes APN an attractive target for the treatment of diseases including cancer (Fig. 8) (Mucha *et al*., 2010; Drinkwater *et al*., 2017; Amin *et al*., 2018; Li *et al*. 2018).

Accordingly, strategies for its inhibition have been developed primarily for the treatment of pain (Schreiter *et al*., 2012; Bonnard *et al*., 2015), and less so for cancer and skin pathologies (Thielitz *et al*., 2008; Amin *et al*., 2018; Arrebola *et al*., 2019). Only Ubenimex (bestatin) is currently approved by FDA for it uses in human pathologies, mainly in cancer.

Natural inhibitors of APN are scarce and have been mainly described from microorganisms (Mucha *et al*., 2010), plants (Melzig and Bowman, 1999), marine invertebrates (Shim *et al*., 2004; Reytor *et al*., 2011, Pascual *et al*., 2017 a,b), and more recently from Cuban toads secretions (Pascual *et al*., 2019). In the next section we review the information available regarding marine organisms, promissory and still unexplored source of inhibitors of APN with biomedical relevance mainly in cancer.

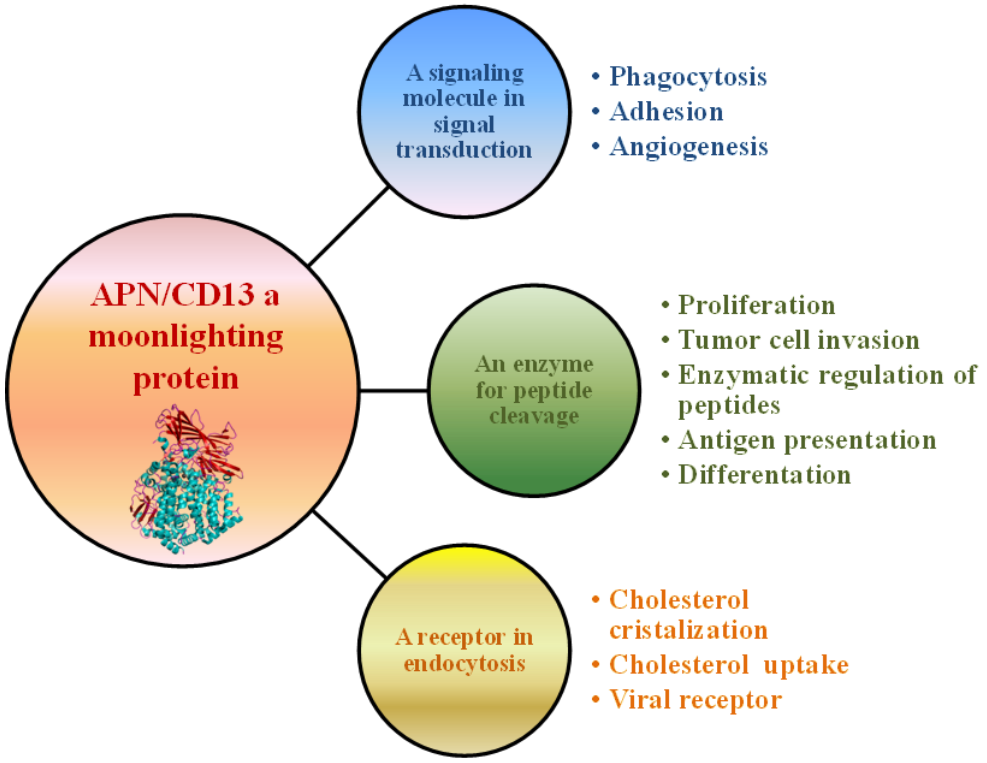

Figure 7. Functions of human APN/CD13. Adapted from Amin *et al.* (2018).
Figura 7. *Funciones de la APN/CD3 humana. Adaptado de Amin et al. (2018).*

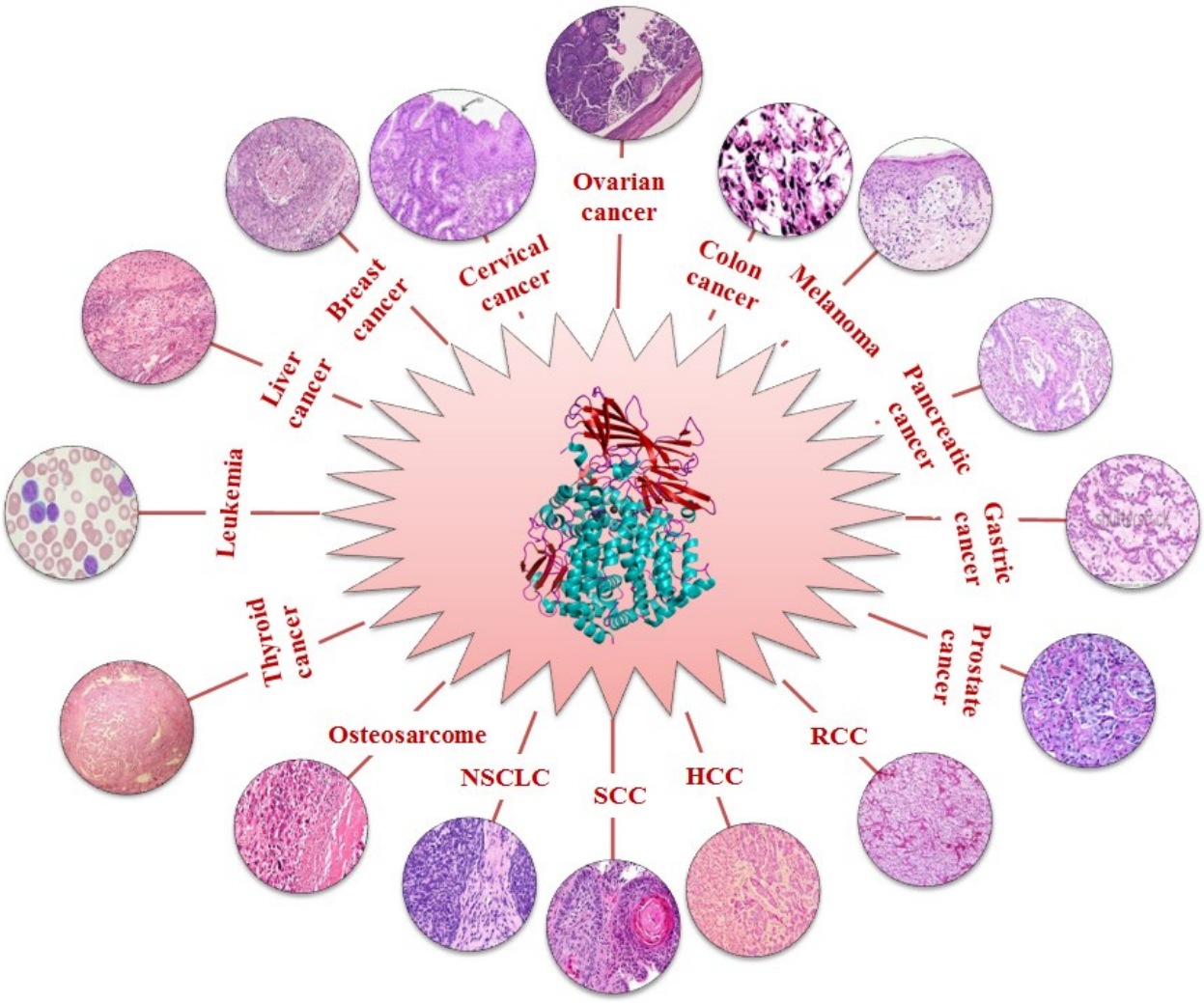

Figure 8. Up-regulation of APN in different cancers. Abbreviations: renal cell cancer (RCC), hepatocellular carcinoma (HCC), scamous cell carcinoma (SCC), non small cell lung carcinoma (NSCLC). Adapted from Amin *et al.* (2018).

Figura 8. *Regulación positiva de la APN en diferentes tipos de cáncer. Abreviaciones: Cáncer renal (RCC), carcinoma hepatocelular (HCC), carcinoma escamoso (SCC), carcinoma de pulmón de células no pequeñas (NSCLC). Adaptado de Amin et al. (2018).*

Psammaplin A

Psammaplin A (PsA) is a natural bromothyrosine derivative (Fig. 9) isolated from the association between two sponges, *Poecillastra* sp. *and Jaspis* sp. (Jung *et al*., 1995). Several biological activities have been described for this compound; it is an antibacterial mainly against *Staphylococcus aureus* (SA) and methicillin-resistant *Staphylococcus aureus* (MRSA), due to DNA gyrase inhibition and bacterial DNA synthesis arrest (Kim *et al*., 1999). PsA also inhibits chitinases that are common to fungi and are crucial to the control of ecdysis in insects (Tabudravu *et al*., 2002). In mammalian cells, PsA inhibits topoisomerase II, an enzyme catalyzing DNA relaxation, with a high IC_{50} of 18.8 mM (Kim *et al*., 1999). Additionally, the growth of several cancer cells, including P-glycoprotein-overexpressing multidrug resistant cell lines, is effectively inhibited by PsA implying that this compound can be developed as a novel anti-tumor agent. However, until 2004, detailed mechanism about the broad range of biological activities of PsA was largely unknown. In 2004, almost at the same time our group described HcPI as an inhibitor of TRH-

DE, Shim *et al*. (2004) found that PsA inhibits mammalian APN with a Ki value of 15 µM, in a non-competitive way. Structural analogues of PsA, in which phenolic hydroxyl groups were replaced, did not inhibit APN, indicating that these groups are crucial in the recognition and inhibition of this enzyme. This finding perfectly agreed with the effectiveness of bulky and hydrophobic groups in molecules targeting APN (Revelant *et al*., 2015). Furthermore, it was found that PsA efficiently inhibits the proliferation of various types of cancer and endothelial cells. Finally, in this study it was shown that PsA suppresses the invasion and tube formation of endothelial cells stimulated by the basic fibroblast growth factor (Shim *et al*., 2004). These results demonstrate that PsA is a new APN inhibitor that can be developed as a new antiangiogenic agent. In relation with cancer studies, PsA also acts as an histone deacetylase (HDAC) inhibitor. Ahn *et al*., (2008) studied in-depth the effect of PsA as an HDAC inhibitor in Ishikawa endometrial cancer cells.

PsA inhibits the proliferation of Ishikawa cells in a dose-dependent manner and markedly induces the expression of acetylated H3 and H4 histone proteins. In addition, PsA markedly up-regulates the expression of cyclin-dependent kinase inhibitor, p21WAF1, and down-regulates the expression of retinoblastoma protein, cyclins, and cyclin-dependent kinases, which leads to cell cycle arrest. Cell cycle analysis indicated that PsA treatment increases the proportion of cells in the G0/G1 and G2/M phases and decreases the ratio of cells in the S phase. These findings indicated that PsA treatment induces apoptosis, which was associated with p53 independent p21WAF1 expression, thus clarifying that PsA exhibits antiproliferative effects on endometrial cancer cells through selective induction of genes related to cell cycle arrest and apoptosis (Ahn *et al*., 2008).

Psammaplin A also possesses antiproliferative activities against various cancer cell lines, including triple-negative breast (TNBC, MDA-MB-231), doxorubicin-resistant human breast (MCF-7/adr), colon (HCT15), ovarian (SK-OV-3), lung (A549, LM4175), bone (BoM1833), endometria, skin (SK-MEL-2), and central nervous system (BrM-2a, XF498) cancer cell lines (Kim *et al*., 2015; Ratovitski, 2016; Zhou *et al.,* 2018). Moreover, PsA also exhibits potent enzyme inhibitory and antiproliferative activities under reduced conditions in cells, which indicates that PsA could be used as a natural prodrug (Kim *et al*., 2007). Although PsA possesses a broad spectrum of bioactivities, its in-depth study has been hindered due to the limited amount of the compound that can be isolated from marine microorganism sources, as well its poor physiological stability (Jing *et al*., 2019). Inspired by this, many research groups have carried out its total synthesis and synthesis of derivatives (Reviewed in Jing *et al*., 2019).

Identification of inhibitory activity of mammalian

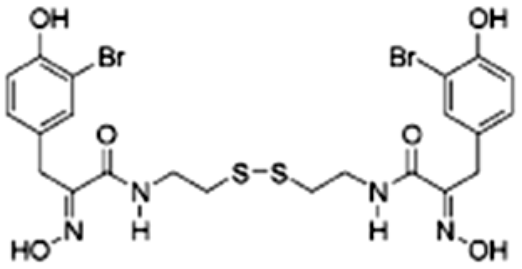

Figure 9. Structure of psammaplin A.
Figura 9. *Estructura de la psammaplina A.*

APN in marine invertebrates from Cuban coastline

Considering the identification of a highly specific inhibitor of TRH-DE in the marine annelide *Hermodice carunculata* in aqueous extracts from Cuban marine invertebrates, our group extended the screening to porcine and human APN as targets. As part of a first study carried out in 2011-2015 in aqueous extracts from marine invertebrates belonging to the Phylla Echinodermata, Chordata, Annelida and Bryozoa (Table 3), an APN inhibitory activity is detected in all the extracts evaluated except for *Lebrunia danae* and *Hermodice carunculata*, whose extracts present values of enzymatic activity higher than those of the control test of pAPN (Table 3, Fig. 10). The L-Leu-pNA substrate is also hydrolyzed when the assay is performed only in the presence of the extract and activity buffer, indicating the possible presence of a neutral aminopeptidase type activity in these two species. The extracts of the species: *Bryozoo* sp. 2, *Diplosoma listerianum, Lisoclinum verrilli, Eucidaris tribuloides* and *Ophiocoma echinata* were selected as the most promising in terms of the specific inhibitory activity of porcine APN as well as a dose-dependent inhibition behavior (Pascual *et al*., 2017a).

The extracts of the *Phallusia nigra, Ascidia sidneyense, Microcosmus guanus, Steinacidia turbinata* and *Poticlenum constellatum* species do not show inhibition at increasing concentrations, indicating that the initial result is probably due to a component of the extract that interferes with the correct determination of the enzyme activity. All the selected extracts, except that of *Lisoclinum verrilli*, show slow inhibition, in the order of minutes. On the other hand, for the latter, equilibrium is reached within 1 min of preincubation time, suggesting a fast interaction of the inhibitory components of the extract with the porcine APN.

The IC_{50} values are in the range of 0.11-2.39 mg/mL (Table 3), the highest efficiency being for the extracts of the two sea squirts (*Diplosoma listerianum* and *Lisoclinum verrilli*) and that of *Briozoo sp2*. The active extracts were submitted to clarification treatments (like 2.5% trichloroacetic acid and heat treatments) to eliminate contaminants (mainly proteins), and to promote dissociation from endogenous inhibitor-target complexes. Clarification increases the specific inhibitory activity of the extracts, suggesting that the procedure should be useful in future works dealing with the isolation of the inhibitory molecules (Pascual *et al*., 2017a).

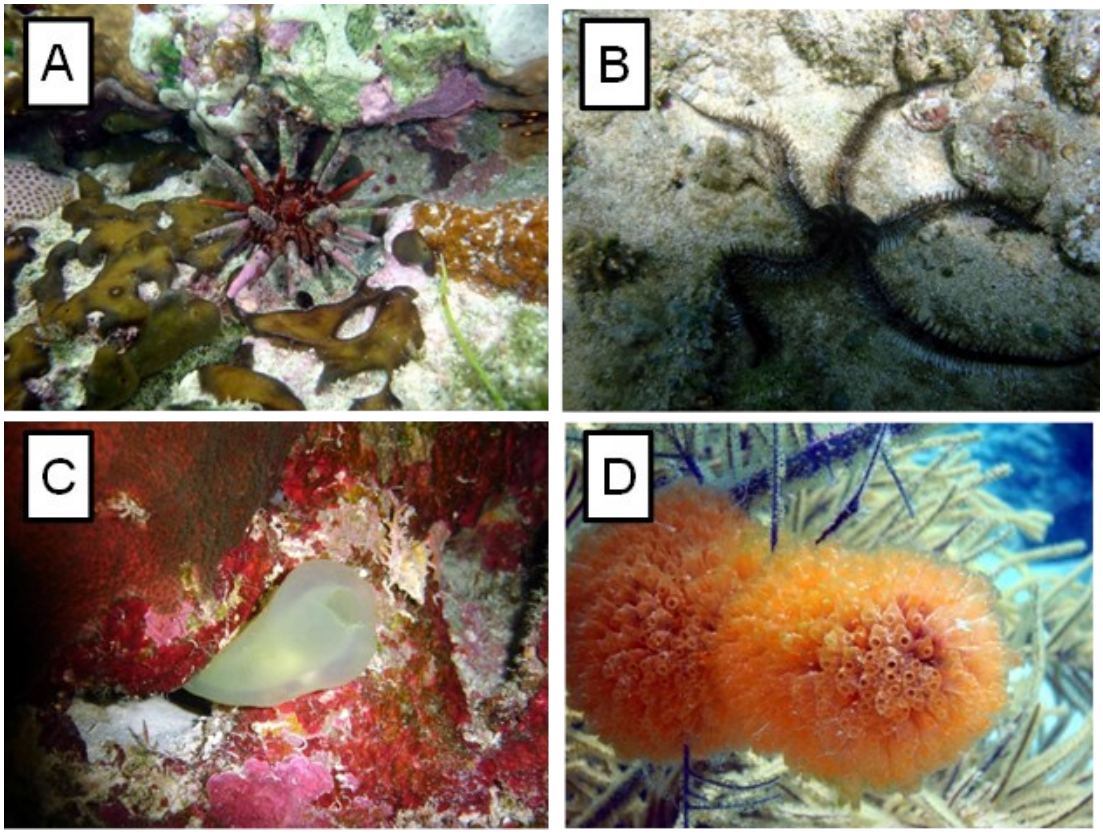

Figure 10. Some of the marine invertebrates screened for inhibitory activity of pAPN. A: *Eucidiaris tribuloides,* B: *Ophiocoma echinata*, C: *Ascidia sydneiensis*, D: *Esteinacidia turbinata*. Pictures courtesy of Professor José Espinosa, PhD from ICIMAR (*Instituto de Ciencias del Mar*), CITMA, Cuba.

Figura 10. *Algunos de los invertebrados marinos evaluados para actividad inhibidora de pAPN. A:* Eucidiaris tribuloides*, B:* Ophiocoma echinata*, C:* Ascidia sydneiensis*, D:* Esteinacidia turbinata*. Fotos cortesía del Profesor José Espinosa, PhD, del ICIMAR (Instituto de Ciencias del Mar), CITMA, Cuba.*

Table 3. Summary of the preliminary characterization of the porcine APN inhibitory activity detected in crude extracts of positive species (screening in the period 2011-2015). Adapted from Pascual *et al*. (2017a).

Tabla 3. *Resumen de la caracterización inicial de la actividad inhibidora de APN porcina detectada en los extractos crudos positivos (tamizaje en el periodo 2011-2015). Adaptado de Pascual et al. (2017a).*

Species	Phylum	pAPN inhibitory activity (U/mL)	pAPN specific inhibitory activity (U/mg)	Pre-incubation time (min)	IC_{50} (mg/mL)
Ascidia nigra	Chordata	2.3060	0.8363	-	-
Lisoclinum verrilli	Chordata	7.8770	0.7361	1	0.11 ± 0.06
Ascidia sidneyensis	Chordata	3.1760	0.6481	-	-
Microcosmus guanus	Chordata	7.1368	0.5366	-	-
Esteinacidia turbinata	Chordata	3.6340	0.5344	-	-
Diplosoma listerianum	Chordata	8.1300	0.4394	30	0.11 ± 0.26
Poticlenum constellatum	Chordata	3.2230	0.2984	-	-
Eucidaris tribuloides	Echinodermata	6.2526	0.3206	5	1.35 ± 0.19
Ophiocoma echinata	Echinodermata	8.2337	0.0874	60	2.39 ± 1.09
Lebrunia danae	Echinodermata	-	-	-	-
Bryozoo sp. 1	Bryozoa	5.7240	0.0560	-	-
Bryozoo sp. 2	Bryozoa	6.7600	1.1266	30	0.29 ± 0.05
Hermodice carunculata	Annelida	-	-	-	-

In a second study performed in 2015-2019, aqueous extracts from species belonging to the phylla Mollusca, Poriphera, Echinodermata and Cnidaria (Fig. 11) were screened using human placental APN as target (Table 4). The initial evaluations allowed detection of an inhibitory activity of hAPN only in the species *Cenchritis muricatus* and *Isostichopus badionotus.* Increased L-Leu-AMC hydrolysis rates over the control value are found instead of inhibitory activities for the rest of the species.

These results suggest either the presence of an activator of the target enzymes used in the assays, or the presence of neutral aminopeptidase-like enzymes hydrolyzing L-Leu-AMC, in the corresponding aqueous extract. The clarification of all aqueous crude extracts with a 2.5% TCA treatment increases, in all cases, the recovery of specific inhibitory activities as compared to their detection in positive crude extracts. The treatment also allows the identification of inhibitory activities from species that were negative after screening using aqueous crude extracts. This result indicates that clarification eliminates contaminants and/or induces dissociation from endogenous inhibitor-target complexes that do not allow the detection of inhibitory components in crude extracts.

The 2.5% TCA treated extracts from the species *Cenchritis muricatus, Nerita peloronta, Nerita versicolor, Lissodendoryx* (*Lissodendoryx*) *isodictyalis, Tripneustes ventricosus, Echinaster* (*Othilia*) *echinophorus, Isostichopus badionotus, Stichodactyla helianthus, Bunodosoma granuliferum* and *Physalia physalis* were used to continue the inhibition studies vs hAPN (Pascual *et al*., 2017b). Additionally, the enhanced activities over the control detected in some extracts are lost, in all cases, after the 2.5% TCA treatments, suggesting susceptibility to the chaotropic agent and/or to the acidic pH, of the molecule(s) responsible for these effects.

To test for the presence of neutral aminopeptidase-like enzymes in aqueous crude extracts, preliminary enzymatic assays were performed using different amounts of the samples (in absence of the initial target hAPN) in presence of L-Leu-AMC. A linear dependence of the initial rate versus the amount of crude extract in the assays is detected for the species *Nerita peloronta, Nerita versicolor, Lissodendoryx* (*Lissodendoryx*) *isodictyalis, Tripneustes ventricosus, Echinaster* (*Othilia*) *echinophorus, Stichodactyla helianthus, Bunodosoma granuliferum* and *Physalia physalis* (Pascual *et al*., 2017b). These neutral aminopeptidases like activities were recently characterized by a kinetic approach combining substrates, inhibitors and cations, showing for the first time a biochemical behavior indicative of the presence of M1 and M17 enzymes in these species (Pascual *et al*., 2020).

Table 4. Summary of the screening of phylla *Mollusca, Poriphera, Echinodermata* and *Cnidaria* for inhibitory activity against hAPN.

Tabla 4. *Resumen de la búsqueda de actividad inhibitoria contra la APN humana en los filos Mollusca, Poriphera, Echinodermata y Cnidaria.*

Species	NA-like activity ($x\ 10^4$ U/mg)	Crude extracts hAPN sIA (U/mg)	2.5% TCA treated extracts hAPN sIA (U/mg)
Cenchritis muricatus	ND	0.46	5.20
Nerita peloronta	6.95 ± 2.84	ND	5.06
Nerita versicolor	12.56 ± 1.70	ND	2.21
Lissodendoryx (Lissodendoryx) isodictyalis	1.51 ± 0.49	ND	171.92
Tripneustes ventricosus	6.39 ± 0.07	ND	56.86
Echinaster (Othilia) echinophorus	58.87 ± 12.62	ND	10.82
Isostichopus badionotus	ND	1.56	33.81
Stichodactyla helianthus	9.63 ± 2.68	ND	32.81
Bunodosoma granuliferum	39.44 ± 5.45	ND	4.31
Physalia physalis	2.04 ± 0.52	ND	13.13

hAPN inhibitory activities in aqueous crude and 2.5% TCA extracts are expressed as specific Inhibitory Activity (sIA) in U/mg. One unit of enzyme activity was defined as the amount of enzyme needed to produce one arbitrary unit of fluorescence per minute and inhibitory activities are expressed per mg of extract. The first column indicates tested species, the second column neutral aminopeptidase-like activity (NA), detected in aqueous crude extracts, using L-Leu-AMC as substrate, and the remaining columns refer to sIA. ND: not detected.

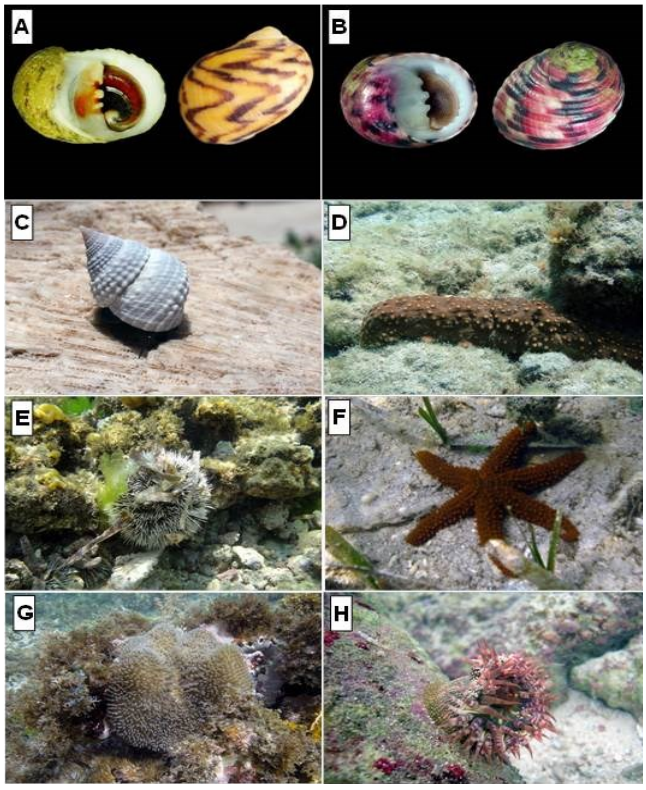

Figure 11. Some of the marine invertebrates which show inhibitory activity of hAPN and rPfA-M17. A: *Nerita peloronta,* B: *Nerita versicolor*, C: *Cenchritis muricatus*, D: *Isostichopus badionotus*, E: *Tripneustes ventricosus*, F: *Echinaster (Othilia) echinophorus*, G: *Stichodactyla helianthus*, H: *Bunodosoma granuliferum*. Pictures courtesy of Professor José Espinosa, PhD from ICIMAR(*Instituto de Ciencias del Mar*), CITMA, Cuba.

Figura 11. *Algunos de los invertebrados marinos con actividad inhibidora de hAPN y rPfA-M17. A: Nerita peloronta, B: Nerita versicolor, C: Cenchritis muricatus, D: Isostichopus badionotus, E: Tripneustes ventricosus, F: Echinaster (Othilia) echinophorus, G: Stichodactyla helianthus, H: Bunodosoma granuliferum. Fotos cortesía del Profesor José Espinosa, PhD, del ICIMAR (Instituto de Ciencias del Mar), CITMA, Cuba.*

Table 5 summarizes the results of the evaluation of the inhibitory potential displayed by the 2.5% TCA clarified extracts on native hAPN. The clarified extracts inhibit hAPN activity in a dose dependent manner and the inhibition is characterized by a concave behavior, indicating the reversibility of the inhibition and corroborating the presence of inhibitory molecules in the samples (and not artifacts interfering with the enzyme activity). IC_{50} values are in the range of 11.7-567.6 µg/mL. As a promissory result, hAPN is inhibited with IC_{50} values around or less than 100 µg/mL in four of the ten species tested (*Lissodendoryx* (*Lissodendoryx*) *isodictyalis, Tripneustes ventricosus, Isostichopus badionotus* and *Stichodactyla helianthus*); this inhibition is stronger than that produced by bestatin or amastatin (pure compounds) assayed in parallel as control. Once the inhibition of hAPN was corroborated, the effect of each treated extract on the viability of two cancer cell lines PC3 and 3LL was evaluated.

Neutral aminopeptidase activity in presence of L-Leu -AMC was reported for the first time (Pascual *et al*., 2017b). All treated extracts, and bestatin used as a positive control, have a dose-dependent effect on PC3 and 3LL cells viability. The higher effects on both cell lines, with IC_{50} values below 100 µg/mL are observed for the species showing the strongest hAPN inhibition. An IC_{50} value under 5 µg/mL for *L. isodictyalis* extract vs both cancer cells lines, similar to the effect displayed by bestatin, indicates that this species is promissory for the isolation of hAPN inhibitors, as defined by Mambu and Grellier (2008). In this work, the IC_{50} values for cell viability are in good agreement with the IC_{50} values for hAPN inhibition, including bestatin results. To the best of our knowledge, this work was the first to show concomitantly natural inhibitor potency on hAPN and indications of activity on hAPN expressing cells (Pascual *et al*., 2017b).

Table 5. Summary of the preliminary characterization of inhibitory activities against porcine APA and human APN in TCA 2.5% treated crude extracts marine invertebrates (screening in the period 2015-2019). Effect on 3LL and PC3 tumor cells viability. Adapted from Pascual *et al.* (2017b).

Tabla 5. *Resumen de la caracterización inicial de actividades inhibidoras de la actividad de APA porcina o APN humana extractos crudos de invertebrados marinos tratados con TCA 2.5% (tamizaje en el periodo 2015-2019). Efecto sobre la viabilidad de las líneas tumorales 3LL y PC3. Adaptado de Pascual et al. (2017b).*

Species	Phylum	IC_{50} vs pAPA (µg/mL)	IC_{50} vs hAPN (µg/mL)	IC_{50} vs 3LL viability (µg/mL)	IC_{50} vs PC3 viability (µg/mL)
Cenchritis muricatus	Mollusca	ND	450.20 ± 77.40	214.00 ± 46.50	352.90 ± 65.00
Nerita peloronta	Mollusca	5.07 ± 2.98	237.80 ± 20.90	273.30 ± 78.80	299.10 ± 31.70
Nerita versicolor	Mollusca	14.09 ± 20.45	370.00 ± 50.00	358.80 ± 70.20	289.70 ± 39.70
Lissodendoryx (*Lissodendoryx*) *isodictyalis*	Porifera	0.91± 0.32	11.70 ± 2.70	< 5.00	< 5.00
Tripneustes ventricosus	Echinodermata	6.57 ± 4.14	25.00 ± 3.10	39.90 ± 2.00	77.00 ± 3.90
Echinaster (*Othilia*) *echinophorus*	Echinodermata	1.82 ± 0.67	198.20 ± 27.20	265.70 ± 29.60	405.60 ± 50.40
Isostichopus badionotus	Echinodermata	10.05 ± 2.93	69.70 ± 10.00	57.10 ± 2.70	83.10 ± 3.00
Stichodactyla helianthus	Cnidaria	1.42± 0.31	103.60 ± 20.60	110.80 ± 13.20	58.10 ± 7.50
Bunodosoma granuliferum	Cnidaria	ND	567.60 ± 88.00	786.80 ± 37.10	711.30 ± 29.30
Physalia physalis	Cnidaria	ND	123.10 ± 21.30	257.30 ± 6.70	234.50 ± 5.00
Bestatin (positive control)	-	25.50 ± 2.35	6.70 ± 1.90	0.54 ± 0.01	3.15 ± 0.72
Amastatin (positive control)	-	75.45 ± 4.55	63.45 ± 7.61	ND	ND

Inhibitors of aminopeptidase A isolated from marine organisms

Recently, we extended the screening of marine organisms extracts to porcine aminopeptidase A, a M1 family enzyme that is a current target for the development of antihypertensive agents (Fig. 12). This enzyme is widely distributed in mammalian tissues. APA has been reported to have molecular weights ranging around 100 kDa, 109 kDa for the human and 108 kDa for the porcine enzyme (Rawlings *et al.*, 2018). APA's S1 pocket accommodates acid residue side chains, whereby this enzyme hydrolyzes aspartic and glutamic residues from peptide N-terminus (Yang *et al.*, 2013). APA performs fundamental functions in a wide range of physiological processes, since it participates in the metabolism of angiotensin II, involved in the renin-angiotensin system in the central nervous system and other anatomical locations, making it an important regulator of blood pressure (Pascual *et al.*, 2018). In addition, it is involved in the development of Alzheimer's disease, glomerulosclerosis and in the progression of cancer.

It is associated with the development of renal neoplasms, malignant trophoblasts, renal choriocarcinoma and colorectal cancer (Göhring *et al.*, 1998; Tonna *et al.*, 2008; Puertas *et al.*, 2013; Blanco *et al.*, 2014; Pascual *et al.*, 2018). We observed that extracts from the species *Nerita peloronta, Nerita versicolor, Lissodendoryx* (*Lissodendoryx*) *isodictyalis, Tripneustes ventricosus, Echinaster* (*Othilia*) *echinophorus, Isostichopus badionotus* and *Stichodactyla helianthus* displayed dose-dependent inhibition of porcine APA activity, with IC_{50} values in the range 0.91-14.09 µg/mL (Table 5), showing a higher selectivity for porcine APA than for human APN (unpublished data). Additionally, for all the extracts the inhibitory effects were stronger than that of bestatin used as control (Table 5, unpublished data). These results strongly support the exploration of marine fauna, as promissory sources of inhibitors of M1 family enzymes with potential biomedical applications.

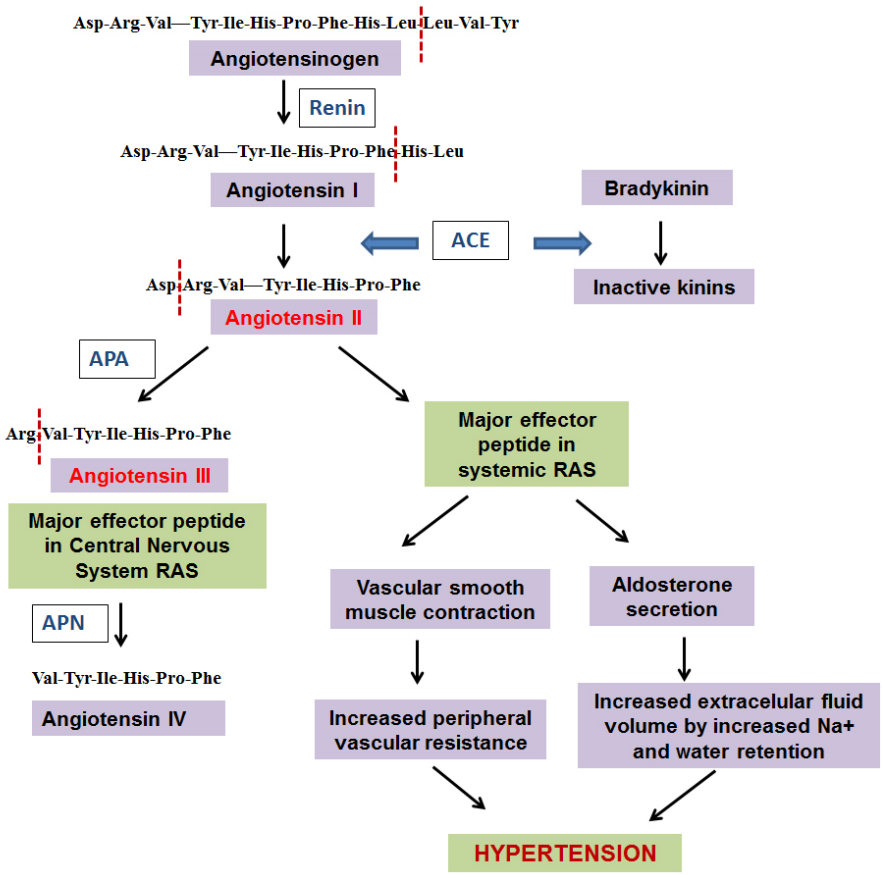

Figure 12. Role of APA and ACE in the renin angiotensin systems.
Figura 12. *Papel de APA y ACE en los sistemas renina angiotensina.*

Inhibitors of M2 family enzymes isolated from marine organisms

Family M2 includes the animal peptidyl-dipeptidase A, which removes C-terminal dipeptides. Its best-known biological function is the processing of angiotensin I and the peptidase is commonly known as angiotensin I-converting enzyme (Rawlings *et al*., 2018). The mammalian enzyme exists in two forms, both derived from the same gene by alternative modes of transcription. Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase and was originally isolated from horse blood (EC. 3.4.15.1) (He *et al*., 2013). It´s also known as Zinc ion (Zn^{2+})-dependent dipeptidyl carboxypeptidase (Pujiastuti *et al*., 2019). ACE shows a strict requirement for a chloride ion for activity on at least some substrates (Araujo *et al*., 2000). ACE is an ectoenzyme, with active site residues: H390, E391, H394 (matching with the HEXXH motif of clan MA) and E418 (according with clan MA(E) characteristics).

ACE is present in biological fluids, such as plasma and semen, and in many tissues, such as testis, intestinal epithelial cells, proximal renal tubular cells, brain, lungs, stimulated macrophages, vascular endothelium, and the medial and adventitial layers of blood vessel walls. In humans, ACE exists in two isoforms: somatic ACE (sACE) and germinal ACE (gACE). sACE is distributed in many types of endothelial and epithelial cells, whereas gACE occurs in germinal cells in the testis, and is therefore also known as testicular ACE (Pujiastuti *et al*., 2019).

Figure 12 shows the renin-angiotensin system (RAS). ACE catalyzes the conversion of angiotensin I (decapeptide) to angiotensin II (octapeptide) by removing the C-terminal dipeptide His–Leu. Angiotensin II is a potent vasoconstrictor that increases peripheral vascular resistance and the renal sodium reabsorption by the secretion of aldosterone; consequently, angiotensin II elevates arterial pressure (He *et al*., 2013). Moreover, angiotensin II acts as a neurotransmitter, and in the liver, it stimulates the generation of new glucose molecules from glycogen (glycogenolysis) and non-carbohydrates substrates (gluconeogenesis), which may potentially lead to the development of insulin resistance or diabetes mellitus (Manikkam *et al*., 2016). ACE also catalyzes the degradation of bradykinin, which is a known vasodilator (He *et al*., 2013). Therefore, inhibition of ACE activity leads to a decrease in the concentration of angiotensin II and increases the level of bradykinin, resulting in reduced blood pressure (He *et al*., 2013). The somatic form is a drug target for the control of hypertension (MEROPS, 2020).

The RAS is present in tissues such as the adrenal cortex, kidney, heart, liver, retina, pancreas, vascular smooth muscle, brain and the adipose tissue (Manikkam *et al*., 2016). It has long been established that the hyperactivity of the RAS plays an important role not only to promote hypertension but also in the onset of obesity and metabolic diseases. This is because angiotensin II also regulates the growth of adipose tissue, which affects the endocrine and metabolic systems. An increase in adipocyte angiotensinogen was observed in obese subjects in the study of Van Harmelen and colleagues (Manikkam *et al*., 2016). Moreover, angiotensin II may promote adipocyte hypertrophy by increasing the expression of fatty acid synthase (FAS) within fat cells (Jones *et al*., 1997). Angiotensin II enhances the accumulation of fatty acids and triglycerides within the body and promotes fat cell proliferation, which is a common causative factor for obesity and metabolic syndrome (Manikkam *et al*., 2016).

Therefore, inhibiting ACE activity may be therapeutically useful in lowering blood pressure, reducing body fat mass, and improving overall body composition (Manikkam *et al*., 2016). ACE inhibitors derived from natural sources (bioactive peptides) are stable and have minimal side effects compared with synthetic ACE inhibitor drugs (Lee and Hur, 2017). Naturally occurring peptides with ACE inhibitory activity were first obtained from snake venom (Ondetti *et al*., 1971) and the discovery of marine-derived ACE inhibitory peptides started in 1986, when they were isolated from sardine by a Japanese scientist (Suetsuna, 1986). In the early 1990s, bonito (Katsuobushi) was examined for its potential ACE-inhibitory peptides (Yokoyama *et al*., 1992). Since then, many other sources have been explored. In the next sections, we review the current knowledge regarding marine organisms as an abundant and promissory source of ACE inhibitors of different chemical nature with antihypertensive potentialities.

Angiotensin converting enzyme inhibitors isolated from marine organisms

Bioactive peptides

Extensive research and development in bioactive peptides began in the 1950s, when Mellander proposed that casein-derived phosphorylated peptides enhanced vitamin D-independent bone calcification in rachitic infants (Manikkam *et al*., 2016).

Since then, many investigative efforts have been devoted to the identification of functional peptides from diverse sources. Bioactive peptides can act as therapeutic agents and are characterized by high biological specificity, low toxicity, high structural diversity, high and wide spectrum of activity, and small size (2-20 amino acids) (Li *et al*., 2004), which implies that they have a low likelihood of triggering undesirable immune responses (Pujiastuti *et al*., 2019). Bioactive peptides are inert inside the original protein but become active once cleaved (Li *et al*., 2004). They are generally produced via enzymatic hydrolysis using digestive enzymes, fermentation using proteolytic starter cultures, or proteolysis using microorganism-or plant-derived enzymes. To generate short-chain functional peptides, enzymatic hydrolysis is used in combination with fermentation (Pujiastuti *et al*., 2019).

In order to exert a beneficial health effect, they must be released, resist the digestive conditions of the gastrointestinal tract and subsequently be absorbed through the intestine where they enter the blood circulatory system and reach their site of action (Manikkam *et al*., 2016). Dipeptides and tripeptides are easily absorbed in the intestine. Peptides isolated from food sources are structurally similar to endogenous peptides and therefore interact with the same receptors and play a prominent role as immune regulators, growth factors, and modifiers of food intake. Depending on the sequence of amino acids, these peptides can exhibit diverse activities, including antimicrobial, antioxidant, antithrombotic, opioid, immunomodulatory, and antihypertensive activity (Pujiastuti *et al*., 2019; Lee and Hur, 2017).

Depending on their inhibitory activity following preincubation with ACE, food peptides can be divided into three major groups. The first category is known as the "true inhibitor type" peptides since their IC_{50} value is not affected when pre-incubated with ACE. The "substrate type" inhibitors are the second group of peptides that are hydrolyzed by ACE resulting in weak inhibition. The third group includes the "pro-drug type" peptides, which are converted to "true inhibitor type" peptides by ACE or enzymes (proteases) of the digestive tract. *In vivo* studies have demonstrated that only peptides belonging to the true inhibitor or prodrug type groups reduce systolic blood pressure of spontaneously hypertensive rats (SHR) (Manikkam *et al*., 2016).

The favored structures of ACE inhibitory peptides reveal that amino acid residues with bulky side chain as well as hydrophobic side chains are more active for dipeptides. Meanwhile, for tripeptides, the most favorable residue for the C-terminus is an aromatic amino acid, while a positively charged amino acid in the middle, and an hydrophobic amino acid in the N-terminus (Pangestuti and Kim, 2017). Figure 13 shows the general favored structure for ACE inhibitory tripeptides descripted by Lee and Hur (2017). In agreement, many studies have shown that peptides with high ACE inhibitory activity contain Trp, Phe, Tyr or Pro at the C-terminus and branched aliphatic amino acids at the N-terminus (Pujiastuti *et al*., 2019).

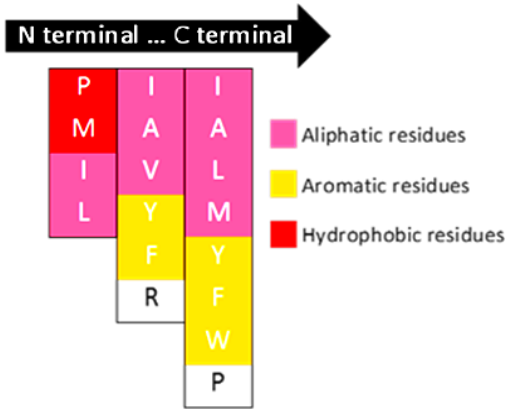

Figure 13. Preferred amino acids in ACE inhibitory tripeptides.
Figura 13. *Aminoácidos preferidos en la estructura de tripéptidos inhibitorios de la ACE.*

For some peptides longer than tripeptides, hydroxyproline (Hyp) is important for the binding of peptides to the active site of ACE. In general, one of the most conserved characteristics in ACE-inhibitory peptides is that they have hydrophobic amino acids at their ends. Moskowitz *et al*. (2002), explained the clinical superiority of hydrophobic- compared to hydrophilic- based ACE inhibition. All ACE inhibitors bind to the C-terminal catalytic site, but only hydrophobic ones bind and block the N-terminal catalytic site (Lee and Hur, 2017).

Several studies have reported that ACE-inhibitory peptides can come from animal products, marine organisms, and plants, derived with hydrolyzing enzymes such as: pepsin, chymotrypsin, and trypsin and microbial enzymes such as: alcalase, thermolysin, flavourzyme, and proteinase K (Lee and Hur, 2017). Table 6 shows an abstract of the main methods by which hydrolysate-derived peptides are obtained.

Table 6. Optimal hydrolysis treatments for bioactive peptide generation according to source.
Tabla 6. *Tratamientos hidrolíticos óptimos para la generación de péptidos bioactivos de acuerdo a la fuente.*

Source	Optimal hydrolysis treatments	IC_{50} values
Animal products	- Pepsin, frequently used for peptides from eggs - Microbial fermentations are frequently used for dairy products	1 and 10 micromolar range
Marine organisms	- Pepsin, alcalase and microbial fermentation using *Bacillus* spp.	1 and 10 micromolar range
Plants	- Microbial substrates using *Bacillus* spp. and *Lactobacillus* spp.	1, 10 and 100 micromolar range

Most of the reported peptides act as competitive inhibitors of ACE (Pangestuti and Kim, 2017, Pujiastuti *et al*., 2019). However, there is no correlation between competitive inhibitor potency and high ACE inhibitory activity. Several non-competitive inhibitors show high ACE inhibitory activity (Pujiastuti *et al*., 2019).

The structure of the peptides may be closely related with their ACE-inhibitory effects. Generally, inhibition of ACE is performed by hydrophobic peptides that exert high affinity toward the active site of ACE (Li *et al.*, 2004). These hydrophobic amino acids in the peptide sequence are thought to improve the solubility of the peptide in lipid-based conditions such as in the cell membrane. This could help them exert a greater antihypertensive effect (Lee and Hur, 2017). Furthermore, to exert antihypertensive effects, bioactive peptides must reach the target cells after absorption through the intestine (Pujiastuti *et al*., 2019).

Marine species comprising approximately one-half of the global biodiversity, the ocean offers a wonderful resource for novel compounds, which may serve in improving the health of the population (Manikkam *et al*., 2016).

Natural ACE inhibitory peptides have been isolated from different food proteins such as cod frame, pollack skin, sea bream scales, yellowtail bone and scales, yellow sole frame, tuna frame and clam, krill, mussel, oyster, shrimp and others. Numerous *in vivo* studies of anti-hypertensive peptides derived from marine organisms in spontaneously hypertensive rats (SHR) have shown potent ACE inhibitory activity. In general, the reduction in systolic blood pressure (SBP) following oral administration (10 mg/kg of body weight) of peptides was on average 25 mm Hg compared to controls (Ngo *et al*., 2012).

Marine-derived bioactive peptides have thus potential for use as functional ingredients in nutraceuticals and pharmaceuticals due to their effectiveness in both prevention and treatment of hypertension.

Moreover, cost effective and safe natural health products can be produced from marine bioactive peptides (Kim and Wijesekara, 2010). Table 7 summarizes examples of peptides from marine organisms, their biological effect, as well as their IC_{50} value for ACE inhibition.

Li and colleagues (Li *et al*., 2014) identified and synthesized two peptides derived from *Rhopilema esculentum* (jellyfish), VKP and VKCFR. Both peptides show ACE inhibitory activity with IC_{50} values of 1.3 µM and 34.5 µM, respectively and results from docking simulation studies suggest that the peptides bind ACE through coordination with the active site Zn^{2+} atom. On the other hand, they found that these peptides protect endothelial cells against oxidative damage, possibly via quenching the hydroxyl radical and enhancing antioxidant enzyme activity (Li *et al*. 2014). But the relation between ACE inhibition and antioxidant activity of the peptides is not well established. However, limited information is available on the effects of fish-derived bioactive peptides, mainly with ACE-inhibitory potential on the markers of metabolic syndrome, including obesity, glucose intolerance and body composition improvement (Manikkam *et al*., 2016).

Marine organisms have adapted excellently to diverse extreme environmental conditions, such as high salt concentration, low or high temperature, high pressure, and low-nutrient availability. Therefore, the composition and primary sequences of amino acids of these marine proteins are different from those of land proteins (He *et al*., 2013). The ACE inhibitory activities of marine-derived bioactive peptides were higher than those of ACE inhibitory peptides derived from terrestrial food sources (i.e., milk, chicken muscle and bovine) (Pangestuti and Kim, 2017).

Table 7. Bioactive peptides derived from marine organisms, their biological effect, as well as their IC_{50} value for ACE inhibition.
Tabla 7. *Péptidos bioactivos derivados de organismos marinos, su efecto biológico y valor de IC_{50} para la ACE.*

Source	Peptide sequence	IC_{50} (μM)	Biological effect	Reference
Skate skin (*Okamejei kenojei*)	M-V-G-S-A-P-G-V-L	3.1	Antihypertensive effect in SHR	Ngo *et al.* (2015)
	L-G-P-L-G-H-N	4.2		
	P-G-P-L-G-L-T-G-P	95		Lee *et al.* (2011)
	N-L-G-F-L-G-P-R	148		
Tuna dark muscle	W-P-E-A-A-E-L-M-M-E-V-D-P	21.6	Antihypertensive effect after oral administration in SHR	Qian *et al*. (2007)
Tuna frame	G-D-L-G-K-T-T-T-V-S-N-W-S-P-P-K-Y-K-D-T-P	11.28	Potent anti-hypertensive activity after oral administration in SHR	Pangestuti and Kim (2017) Lee *et al.* (2010)
Yellowfin tuna (*Neothunnus macropterus*)	P-T-H-I-K-W-G-D	2		Kohama *et al.* (1988)
Salmon muscle (*Oncorhynchus gorbuscha*)	I-W	1.2	Strong inhibitory activity against hypertension	Enari *et al.* (2008)
Atlantic salmon	A-P	356.9		Gu *et al.* (2011)
	V-R	1301.1		
Chum salmon (*Oncorhynchus keta*) muscle	V-W	2.5		Ono *et al.* (2003)
Izumi shrimp	V-W-Y-H-T	28.30	Decreased blood pressure in stroke-prone SHR (Single oral administration, 10 mg/kg of body weight)	Nii *et al.* (2008)
	V-W	6.6		
Mantle (*Argopecten irradians*)	V-L-I-V-P	19.7	Antihypertensive effects on SHR following oral administration	Wu *et al.* (2017)
Oyster	T-A-Y	16.7	Decreased systolic blood pressure of SHR	Xie *et al.* (2014)
	V-K	29.0		
	K-Y	51.5		
	F-Y-N	68.2		
	Y-A	93.9		
	V-V-Y-P-W-T-Q-R-F	66	Anti-hypertensive effect when orally administered in SHR at a dose of 20 mg/kg	Wang *et al.* (2008)
	L-F	126000		Matsumoto *et al.* (1994)
Pearl oyster (*Pinctada fucata martensii*)	L-V-E	14200		Suetsuna (2002)
	A-L-A-P-E	167.5		
Shrimp (*Acetes chinensis*)	F-C-V-L-R-P	12.3	ACE inhibitory activity	Hai-Lun *et al.* (2006)
	I-F-V-P-A-F	3.4		
	K-P-P-E-T-V	24.1		
	L-H-P	3.4	Oral administration (6 mg/kg of body weight) reduces blood pressure in SHR	Cao *et al.* (2010)
	D-F	2.15		Wang *et al.* (2008)
	G-T-G	5.54		
	S-T	4.03		
Jellyfish (*Rhopilema esculentum*)	V-R	0.332 mg/mL	-ACE inhibition (*in vivo*) -Hypotensive at 200 mg/kg, 400 mg/kg and 800 mg/kg. -Antihyperlipidemic at 10–15wks, male SHR	Liu *et al.* (2012)
	Q-P-G-P-T	80.67		Liu *et al.* (2013)
Sardine muscle	K-W	1.63	-Hypotensive - Lower blood glucose level *(in vivo*) - Decrease ACE activity in kidney, aorta, mesentery (1g/kg/d)	Otani *et al.* (2009) Matsufuji *et al.* (1994)
Bonito bowels	I-R-P-V-E	1.4		Hideaki *et al.* (1993)
Bonito muscle	L-K-P	0.32	Maximum decline in blood pressure after 2 h of administration	Fujita and Yoshikawa (1999)
Bonito	I-K-W	0.4		Hasan *et al.* (2007)
	I-K-P	1.70		Yokoyama *et al.* (1992)
	I-W	2.00		
	L-Y-P	6.60		

Table 7. Bioactive peptides derived from marine organisms, their biological effect, as well as their IC_{50} value for ACE inhibition. (Continued).

Tabla 7. *Péptidos bioactivos derivados de organismos marinos, su efecto biológico y valor de IC_{50} para la ACE. (Continuación)*

Source	Peptide sequence	IC_{50} (μM)	Biological effect	Reference
Wakame seaweed (*Undaria pinnatifida*)	Y-N-K-L	21.00		Suetsuna and Nakano (2000)
	A-I-Y-K	213		
	Y-K-Y-Y	64.2		
	K-F-Y-G	90.5		
	I-W	1.50		Sato *et al.* (2002)
	V-Y	35.2		
	A-W	18.8		
	F-Y	42.3		
	V-W	3.3		
	L-W	23.6		
	I-Y	2.7	Single (50mg/kg body weight) and repeated (10mg/day/kg body weight) oral administration decreases blood pressure in SHR.	Suetsuna *et al.* (2004)
Seaweed (*P. yezoensis*)	A-K-Y-S-Y	1.52		Suetsuna (1998)
Seaweed pipefish muscle protein	T-F-P-H-G-P	0.62 mg/mL		Wijesekara *et al.* (2010)
Shark meat	M-F	0.92		Wu *et al*. (2008)
	E-Y	2.68		
Hard clam (*Meretrix lusoria*)	Y-N	51.00		Tsai *et al.* (2008)
Microalgae *(Chlorella vulgaris)*	I-V-V-E	315.3		Suetsuna and Chen (2001)
	A-F-L	63.8		
	F-A-L	26.3		
	A-E-L	57.1		
	V-V-P-P-A	79.5		
	V-E-C-Y-G-P-N-R-P-Q-F	29.60		Sheih *et al.* (2009)
Cyanobacteria *(Spirulina platensis)*	I-A-E	34.7		Suetsuna and Chen (2001)
	I-A-P-G	11.4		
	V-A-F	35.8		
Microalgae (*Chlorella ellipsoidea*)	V-E-G-Y	128.4		Ko *et al.* (2012)
Mollusc, Sea cucumber (*Acaudina molpadioidea*)	M-E-G-A-Q-E-A-Q-E-D	15.90		Zhao *et al.* (2009)
Mollusc, Squid gelatin	G-P-L-G-L-L-G-F-L-G-P-L-G-K-S	90.03		Alemán *et al.* (2011)
Mollusc, Squid (*Dosidicus gigas*) skin collagen	G-R-G-S-V-P-A-P-G-P	47.78		Alemán *et al.* (2013)
Mollusc, Cuttlefish (*Sepia officinalis*)	V-E-L-Y-P	5.22		Balti *et al.* (2015)
	G-I-H-E-T-T-Y	25.66		
	E-K-S-Y-E-K-P	14.41		
	V-Y-A-P	6.1		Balti *et al.* (2010)
	V-I-I-F	8.7		
	M-A-W	16.32		
Mollusc, *Corbicula fluminea*	V-K-P	3.7		Tsai *et al.* (2006)
	V-K-K	1045		
Rotifer (*Brachionus rotundiformis*)	D-D-T-G-H-D-F-E-D-T-G-E-A-M	9.64		Lee *et al.* (2009)
Yellow fin sole (*Limanda aspera*)	M-I-F-P-G-A-G-G-P-E-L	268.3		Jung *et al.* (2006)
Blue mussel *(Mytilus edulis)*	E-V-M-A-G-N-L-Y-P-G	18.4		Je *et al.* (2005)
Trevally (*Pseudocaranx* sp.)	A-R	570.78		Salampessy *et al.* (2015)
	A-V	956.28		
	A-P-E-R	530.21		

Table 7. Bioactive peptides derived from marine organisms, their biological effect, as well as their IC_{50} value for ACE inhibition. (Continued).

Tabla 7. *Péptidos bioactivos derivados de organismos marinos, su efecto biológico y valor de IC_{50} para la ACE. (Continuación)*

Source	Peptide sequence	IC_{50} (μM)	Biological effect	Reference
Lizard fish	M-K-C-A-F	45.70		Lan *et al.* (2015)
	R-V-C-L-P	175		Wu *et al.* (2015)
Skipjack tuna (*Katsuwonus pelamis*)	L-R-P	1		Matsumura *et al.* (1993)
	D-L-D-L-R-K-D-L-Y-A-N	67.4		Intarasirisawat *et al.* (2013)
	M-C-Y-P-A-S-T	58.7		
	M-L-V-F-A-V	3.07		
Small-spotted catshark (*Scyliorhinus canicula*)	V-A-M-P-F	0.44		García-Moreno *et al.* (2015)
Pelagic thresher (*Alopias pelagicus*) muscle	I-K-W	0.54		Nomura *et al.* (2002)
Sipuncula (*Phascolosoma esculenta*)	A-W-L-H-P-G-A-P-K-V-F	135000		Du *et al.* (2013)
Grass carp	V-A-P	19.9		Chen *et al.* (2012)
Pacific cod	G-A-S-S-G-M-P-G	6.9		Ngo *et al.* (2016)
	L-A-Y-A	14.5		
Paralichthys alivaceus	M-E-V-F-V-P	79		Ko *et al.* (2016)
	V-S-Q-L-T-R	105		
Channa striatus	V-P-A-A-P-P-K	0.45		Ghassem *et al*. (2011)
	N-G-T-W-F-E-P-P	0.63		

Unfortunately, not all the sea resources are adequately used. In recent years, over-exploitation of fishery resources has become a major concern worldwide. Many commercially important fish species are being overfished and existing valued species are becoming exhausted. The annual amount of unintentional catch discarded is 7.3 million tons (Food and Agriculture Organization, 2005), a serious marine conservation problem. Notably, much of these wasted and discarded stocks are highly nutritious, comprising high-quality proteins, vitamins, omega-3 fatty acids, and important novel compounds, the bioactive peptides (Manikkam *et al*., 2016).

For instance, sea bream scales were used to obtain ACE-inhibitory peptides. Similarly, Je *et al.* (2004) hydrolyzed Alaska Pollack (saltwater fish of the Cod family), which is usually discarded as an industrial by-product in the Korean fish processing plant. Tilapia and catfish, commercially important aquatic species, have been used as a potential source of bioactive peptides, including ACE-inhibitors, because of the large quantities of waste generated, on a yearly basis (Manikkam *et al*., 2016). Table 8 shows peptide sequences, IC_{50} values and biological effect of bioactive peptides derived from marine wasted and discarded stocks.

Table 8. Bioactive peptides derived from marine organisms (wasted and discarded stocks), their biological effect, as well as their IC_{50} value respect ACE inhibition.

Tabla 8. *Péptidos bioactivos derivados de desechos de organismos marinos, su efecto biológico y valor de IC_{50} para la ACE.*

Source	Peptide sequence	IC_{50} (μM)	Biological effect	Reference
Sea bream scales	G-Y	265	Ability to exert hypotensive effects in SHR.	Fahmi *et al*. (2004)
	V-Y	16		
	G-F	708		
	V-I-Y	7.50		
Alaska Pollack skin (*Theragra chalcogramma*)	G-P-L	2.6		Byun and Kim (2001)
	G-P-M	17.3		
	F-G-A-S-T-R-G-A	14.7		Je *et al.* (2004)

Although the ACE-inhibitory peptides are not as efficient as the drugs commonly used in the treatment of hypertension, they are naturally derived from marine protein sources that are consumed daily, and are considered to be milder and safer without side effects. Moreover, these peptides usually have multifunctional properties and are easily absorbed. Therefore, these marine-derived ACE-inhibitory peptides show great promise in the development of novel physiologically functional foods for preventing hypertension as well as for therapeutic purposes. In addition, it is convenient and economic to synthesize these ACE-inhibitory peptides through a chemical method. With the development of bioprocess engineering technologies and screening methods, marine sources may serve as inexpensive raw materials for generating high-value antihypertensive peptides in the future (Liu *et al*., 2013). In Japan, some of the marine-derived peptides and hydrolysates have been approved as "foods for specified health uses" (FOSHU) by the Japanese Ministry of Health, Labor, and Welfare. Bonito oligopeptides are incorporated in blood pressure lowering capsules and sold as nutraceuticals worldwide (Pangestuti and Kim, 2017).

Research on discovering novel marine-derived ACE-inhibitors and their possible functions would benefit health care. However, bioactive peptides are not the only ACE-inhibitors isolated from marine sources; other marine-derived ACE inhibitors are chitooligosaccharide derivatives and phlorotannins (Wijesekara and Kim, 2010).

Chitooligosaccharide derivatives

Chitin is the second most abundant biopolymer on earth after cellulose, and one of the most abundant polysaccharides. It is a glycan of β (1→4)-linked N-acetylglucosamine units and it is widely distributed in crustaceans and insects as the protective exo-skeleton, and as cell walls of most fungi. Chitin is usually prepared from the shells of crabs and shrimps. Chitosan, a partially deacetylated polymer of N-acetylglucosamine, is prepared by alkaline deacetylation of chitin. Chitooligosaccharides (COS) are chitosan derivatives (polycationic polymers comprised principally of glucosamine units) and can be generated via either chemical or enzymatic hydrolysis of chitosan. COS have been the subject of increased attention in terms of their pharmaceutical and medicinal applications, due to absence of toxicity and high solubility as well as positive physiological effects such as ACE activity inhibition, antioxidant, antimicrobial, anticancer, immuno-stimulant, antidiabetic, hypocholesterolemic, hypoglycemic, anti-Alzheimer's, anticoagulant and anti-adipogenic (Wijesekara and Kim, 2010).

Marine-derived chitooligosaccharide (COS) derivatives such as hetero-chitooligosaccharides (different molecular weight COS), aminoethyl chitooligosaccharides, chitin derivatives, chitosan trimer oligomers and carboxylated chitooligosaccharides are potent ACE inhibitors (Wijesekara and Kim, 2010). Chitosan trimer is more effective in lowering blood pressure compared to other oligomers (Hong *et al*., 1998). ACE inhibitory activity of hetero-COS was dependent on the degree of deacetylation of chitosan (Wijesekara and Kim, 2010). Table 9 shows some examples of chitooligosaccharide derivatives that are ACE inhibitors.

Table 9. ACE inhibitory COS derived from marine resources: derivatives and IC_{50} values. Adapted from Wijesekara and Kim (2010).
Tabla 9. *Inhibidores de la ACE de tipo COS derivados de fuentes marinas: derivados y valores de IC_{50} para la ACE. Adaptado de Wijesekara and Kim (2010).*

COS derivative	IC_{50}	Reference
Chitosan trimer	0.9 μM	Hong *et al.* (1998)
Chitosan oligosaccharides	1.22 mg/mL	Park *et al.* (2003)
Chitin derivatives		
AEC	0.064 μM	Je *et al.* (2006)
AEC 50	0.038 μM	Je *et al.* (2006)
AEC 90	0.103 μM	Je *et al.* (2006)
Aminoethyl-COS (AE-COS)	0.80 μg/mL	Ngo *et al.* (2008)

In vivo studies have shown that three chitin derivatives: aminoethyl-chitin with 10% deacetylation (IC_{50}, 0.064 μM), aminoethyl-chitin with 50% deacetylation (IC_{50}, 0.038 μM), and aminoethyl chitin with 90% deacetylation (IC_{50}, 0.103 μM), effectively decrease the systolic blood pressure of spontaneously hypertensive rats in a dose-dependent manner. When comparing with the IC_{50} values of previous studies, these three water-soluble chitin derivatives have superior ACE inhibitory activity than that of chitosan oligosaccharide derivatives and captopril (IC_{50}, 0.1 μM (Je *et al*., 2006) and IC_{50}, 0.05 μg/mL (Liu *et al*., 2003; Wijesekara and Kim, 2010).

Phlorotannins

Phlorotannins are phenolic compounds formed by the polymerization of phloroglucinol or defined as 1,3,5-trihydroxybenzene monomer units, and biosynthesized through the acetate-malonate pathway. They are highly hydrophilic components with a wide range of molecular sizes ranging between 126–650.000 Dalton. Marine brown algae and red algae accumulate a variety of phloroglucinol-based polyphenols, as phlorotannins of low, intermediate and high molecular weight containing both phenyl and phenoxy units. Among marine algae, *Ecklonia cava*, an edible brown alga is the richest source of phlorotannins.

In addition to *E. cava*, other marine algae such as *E. stolonifera*, *Hizikia fusiforme, Eisenia bicyclis*, and *Eisenia arborea* contain various phlorotannins. Phlorotannins have several health beneficial biological activities including antioxidant, anti-HIV, antiproliferative, anti-inflammatory, radioprotective, antidiabetic, anti-Alzheimer's disease (acetyl cholinesterase and butyryl cholinesterase inhibitory), antimicrobial and antihypertensive, or ACE inhibitory activities (Wijesekara and Kim, 2010). Table 10 shows some examples of ACE inhibitory phlorotannins derived from marine resources, and their IC_{50} values.

Table 10. ACE inhibitory phlorotannins derived from marineresources: phlorotannin/fraction and IC_{50} value. Adapted from Wijesekara and Kim (2010).

Table 10. *Phlorotaninos derivados de organismos marinos que inhibin a la ACE: fraccion con phlorotannin y valor de IC_{50}. Adaptado de Wijesekara and Kim (2010).*

Phlorotannin/fraction	IC_{50}	Reference
Phlorofucofuroeckol A	12.74 µM	Nagayama *et al.* (2002)
Flavourzyme digest fraction of *E. cava*	0.3 µg/mL	Liu *et al.* (2003)
Methanolic extract of *A. flabelliformis*	13.8 µg/mL	Athukorala and Jeon (2005)

Although the current knowledge about the relationship between the structure and activity of the active phlorotannins is limited, a closed ring dibenzo-1,4-dioxin moiety may be crucial to the above ACE inhibitory effect. In addition, the ACE inhibitory activity may depend on the degree of polymerization of phlorotannin derivatives. Polyphenolic compounds inhibit ACE activity through sequestration of the enzyme metal factor, Zn^{2+} ion. Therefore, it has been assumed that phlorotannins might form a complex associated with proteins or glycoproteins to inhibit the ACE activity (Wijesekara and Kim, 2010).

Marine-derived phlorotannins are valuable bioactive compounds and could be introduced for the preparation of novel pharmaceuticals as well as functional foods, and their selection is also a good approach for the treatment or prevention of hypertension (Wijesekara and Kim, 2010).

Clan MF: Family M17

Clan MF contains aminopeptidases that require co-catalytic metal ions for activity. The clan contains only the single family M17, a family of leucyl aminopeptidases (Rawlings *et al*., 2018). The diverse M17 aminopeptidases utilize two divalent metal ion cofactors to catalyze the removal of selected N-terminal amino acids from short peptide chains. M17 aminopeptidases are found in all kingdoms of life, wherein they possess a characteristic homo-hexameric arrangement, and play roles in a wide range of cellular processes. The proteolytic reaction contributes to intracellular protein turnover, a fundamental housekeeping process across all living organisms (Matsui *et al*., 2006). However, a wide range of additional functions beyond aminopeptidase activity has also been attributed to M17 family members.

M17 aminopeptidases from plants possess chaperone activity (Scranton *et al*., 2012), which might contribute to their function in the stress response pathway (Chao *et al*., 1999), while in bacteria they play roles in site-specific DNA recombination (Stirling *et al*., 1989, Alén *et al*., 1997), and further, can moderate transcription of key virulence factors (Behari *et al*., 2001). Therefore, the family of M17 aminopeptidases is multifunctional, capable of performing diverse organism-specific functions far beyond peptide hydrolysis (Fig. 14).

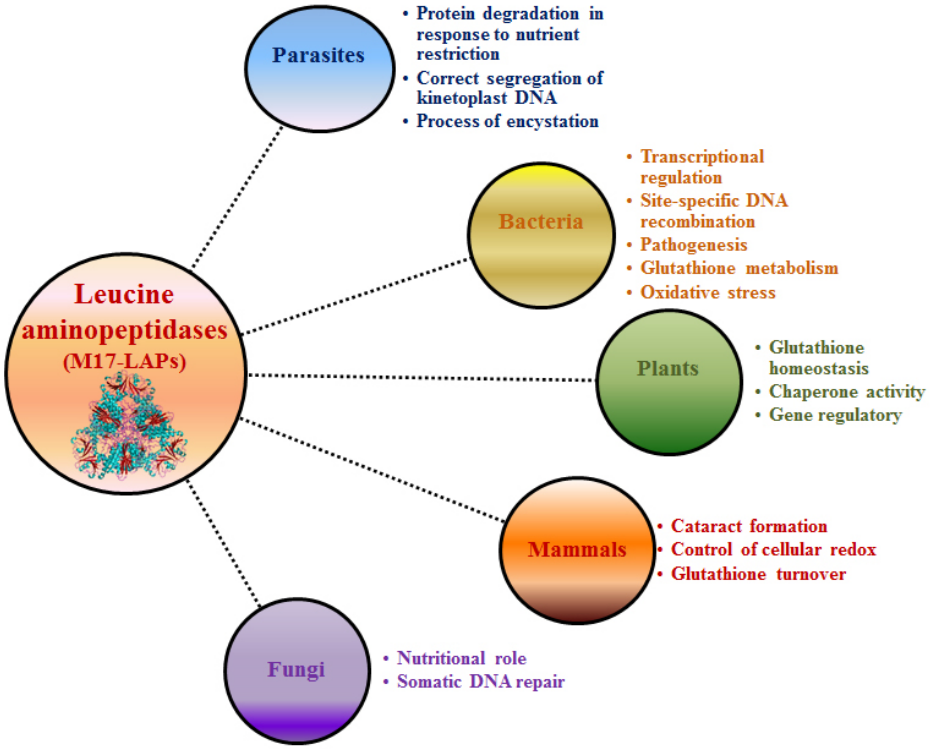

Figure 14. Functions of M17 aminopeptidases from different groups of organisms.
Figura 14. *Funciones de las aminopeptidasas M17 en diferentes grupos de organismos.*

Leucyl aminopeptidases are also distributed in Apicomplexan protist parasites like *Plasmodium falciparum*, the main agent of malaria in humans. Malaria remains the deadliest human parasitic disease in many parts of the world, especially in Sub Sahara Africa, and is responsible for over 405 000 deaths per year. In 2018, more than 228 million people had malaria (WHO report). Malaria is caused by five *Plasmodium* species transmitted by the bite of female Anopheles mosquitoes, *Plasmodium falciparum* being by far the most lethal species of *Plasmodium*. The widespread appearance of drug-resistant parasites, even to newly-developed second and third generation therapeutics such as artemisinin and its derivatives, illustrates the need to design the next generation of anti-malarial drugs to inhibit biochemical pathways critical for parasite survival and/or transmission (Hoffman *et al*., 2015). The most important clinical stage of the complex *P. falciparum* life cycle (Aly *et al*., 2009), which has attracted the highest attention for the development of antimalarials, takes place in the human erythrocyte where significant hemoglobin degradation occurs under the concerted action of endo and exo peptidases (Goldberg, 2013; 2015). PfA-M17 is involved in the final steps of hemoglobin digestion (Skinner-Adams *et al*. 2010) and is currently a promising chemotherapeutic target due to its inhibitors can kill parasites *in vitro* and *in vivo* (Fig. 14) (Dalal and Klemba, 2007; Harbut *et al*., 2011; Drinkwater *et al*., 2016; 2019).

Inhibitors of M17 leucyl aminopeptidases from marine organisms

In the work of Pascual *et al*. (2017b), the screening of inhibitory activities, in aqueous extracts from species belonging to the phylla Mollusca, Poriphera, Echinodermata and Cnidaria from the Cuban coastline also involved human LAP (hLAP), and a recombinant form of PfA-M17 (rPfA-M17). As a result of preliminary assays, inhibitory activity vs hLAP was detected in the species *Cenchritis muricatus, Lissodendoryx* (*Lissodendoryx*) *isodyctialis, Isostichopus badionotus* and *Stichodactyla helianthus.* Inhibitory activity against rPfA-M17 was detected in the species *Cenchritis muricatus, Echinaster* (*Othilia*) *echinophorus, Isostichopus badionotus, Physalia physalis, Stichodactyla*

helianthus and *Bunodosoma granuliferum* (Specific Inhibitory activity values are summarized in Table 11, Fig. 11). The clarification of all aqueous crude extracts with a 2.5% TCA treatment increases the recovery of specific inhibitory activities as compared to their detection in positive crude extracts. The treatment also allows the identification of inhibitory activities from species that were negative after screening using aqueous crude extracts. This result indicates that this clarification step is useful in the elimination of contaminants and/or induces dissociation from endogenous inhibitor-target complexes that do not allow the detection of inhibitory components in crude extracts.

Table 11. Summary of the screening of inhibitory activity against malarial rPfA-M17 and hLAP in aqueous extracts from marine invertebrates from the Cuban coastline.
Tabla 11. *Resumen de la búsqueda de actividad inhibitoria contra la rPFA-M17 y la hLAP en extractos acuosos de invertebrados marinos de la costa cubana.*

Species	rPfA-M17 sIA (U/mg)	hLAP sIA (U/mg)	rPfA-M17 sIA (U/mg)	hLAP sIA (U/mg)
Cenchritis muricatus	0.40	0.16	7.56	4.30
Nerita peloronta	ND	ND	10.61	6.48
Nerita versicolor	ND	ND	7.17	5.58
Lissodendoryx (*Lissodendoryx*) *isodictyalis*	ND	0.27	256.13	312.01
Tripneustes ventricosus	ND	ND	41.85	43.31
Echinaster (*Othilia*) *echinophorus*	0.11	ND	9.10	7.85
Isostichopus badionotus	0.18	1.24	55.83	27.86
Stichodactyla helianthus	0.55	0.68	38.15	18.67
Bunodosoma granuliferum	0.19	ND	5.45	4.08
Physalia physalis	0.40	ND	19.21	11.00

Inhibitory activities found in aqueous crude and 2.5% TCA extracts are expressed as specific Inhibitory Activity (sIA) in U/mg. One unit of enzyme activity was defined as the amount of enzyme needed to produce one arbitrary unit of fluorescence (AUF) per minute and inhibitory activities are expressed per mg of extracts. The first column indicates the species, and the remaining columns refer to specific sIA. ND: not detected.

These 2.5% TCA treated extracts were used to evaluate their inhibitory potential on malarial rPfA-M17 and native human LAP (Table 12, Fig. 11). We were able to detect inhibitory activities against both M17 aminopeptidases, in all TCA clarified extracts. These activities have a concave dose-response behavior, corroborating the presence of reversible inhibitory molecules with IC_{50} values in the range of 15.3-509.2 µg/mL and 66.3-12 429.6 µg/mL, for rPfA-M17 and hLAP, respectively (Table 12). Inhibition of rPfA-M17 with IC_{50} values up to ~100 µg/mL is detected for 6 of the 10 extracts (those of *Cenchritis muricatus, Nerita perolonta, Lissodendoryx* (*Lissodendoryx*) *isodictyalis, Tripneustes ventricosus, Isostichopus badionotus* and *Stichodactyla helianthus*). Comparing the inhibitions on rPfA-M17 and hLAP, in all cases the plasmodial enzyme is more susceptible than its human counterpart, with ratios of selectivity between 1.87 and 60 times. The most selective extract is from *Nerita versicolor*, an attractive result even if its IC_{50} value for the malarial enzyme is moderate. With the exception of *Cenchritis muricatus, Stichodactyla helianthus* and *Bunodosoma granuliferum*, which display an IC_{50} value greater than 400 µg/mL, all of the treated extracts display a dose-dependent effect on a choroquine resistant *Plasmodium falciparum* strain FcB1 cells viability (IC_{50} values in the range of 0.24 -325.5 µg/mL). The best effects are obtained for *Tripneustes ventricosus* and *Lissodendoryx* (*Lissodendoryx*) *isodictyalis*. These results are the first and still only report of inhibition of M17 enzymes (human and plasmodial) by marine invertebrates' species extracts.

Table 12. Summary of the IC_{50} determination for the 2.5% TCA clarified extracts against rPfA-M17 and hLAP. Preliminary inhibitory effects on the growth of *Plasmodium falciparum* FcB1 strain. Adapted from Pascual *et al*. (2017b).
Tabla 12. *Resumen de los valores de IC_{50} de los extractos de TCA 2.5% contra rPfA-M17 y hLAP. Efectos preliminares sobre el crecimiento de* Plasmodium falciparum *(cepa FcB1). Adaptado de Pascual et al. (2017b).*

Species	IC_{50} value vs rPfA-M17 (µg/mL)	IC_{50} vs hLAP (µg/mL)	IC_{50}hLAP/ IC_{50}rPfA-M17	IC_{50} Pf FcB1 (µg/mL)
Cenchritis muricatus	113.40 ± 3.00	341.00 ± 110.00	3.00	> 400
Nerita peloronta	22.20 ± 2.70	329.50 ± 100.00	14.80	291.80 ± 38.50
Nerita versicolor	207.00 ± 30.60	12 429.60 ± 633.00	60.00	325.50 ± 0.80
Lissodendoryx (*Lissodendoryx*) *isodictyalis*	27.30 ± 9.40	66.30 ± 27.60	2.42	2.60 ± 0.60
Tripneustes ventricosus	84.80 ± 7.30	607.70 ± 300.50	7.16	0.24 ± 0.01
Echinaster (*Othilia*) *echinophorus*	127.50 ± 82.10	308.70 ± 100.00	2.42	201.60 ± 162.10
Isostichopus badionotus	86.70 ± 32.60	272.70 ± 63.50	3.13	183.70 ± 155.70
Stichodactyla helianthus	15.30 ± 6.20	234.90 ± 34.60	15.35	> 400
Bunodosoma granuliferum	509.20 ± 100.90	1171.00 ± 92.10	2.29	> 400
Physalia physalis	293.70 ± 100.00	550.00 ± 85.00	1.87	206.00 ± 84.00
Bestatin (positive control)	0.15 ± 0.02	11.83 ±2.61	78.86	1.14 ± 0.27
Amastatin (positive control)	60.70 ± 19.84	158.05 ± 28.44	2.60	ND

ND: not detected.

CONCLUSIONS

Marine biodiversity is an important and promising source of inhibitors of metallo exopeptidases from different families, in particular M1, M2 and M17 enzymes with biomedical applications in human diseases. The results reviewed in present contribution support and encourage further studies with inhibitors isolated from marine species in different biomedical models associated to the activity of these families of exopeptidases.

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