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Aqueous extracts of marine invertebrates from Cuba coastline display neutral aminopeptidase inhibitory activities and effects on cancer cells and *Plasmodium falciparum* parasites

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Neutral aminopeptidases are enzymes distributed in all living organisms. By hydrolyzing biologically active peptides in tissues and biological fluids, they are involved in the control of many physiological processes. They became established targets for new therapeutic agents in cancer, but also in parasitic diseases like malaria. Marine organisms are promising sources for biomolecules but few examples of neutral aminopeptidase inhibitors are described. The goal of this work was to search in Cuban marine invertebrates, for inhibitory activities of neutral aminopeptidases of biomedical relevance, belonging to the M1 and M17 metallopeptidase families. The screening of inhibitory activities was performed using aqueous crude extracts and their 2.5 % TCA treatments. The treatments with 2.5 % TCA increased the recovery of inhibitory activities versus all enzymes tested and from all of marine species. These inhibitory activities were dose-dependent in all cases, with certain selectivity for PfA-M17 regarding hLAP, and good inhibition of hAPN. Interestingly, some TCA treated extracts displayed promising effect on either *Plasmodium* parasite growth as well as on PC3 and 3LL cells. This contribution is the first report identifying inhibitory activities from marine invertebrates, directed against human and malarial neutral aminopeptidases, suggesting a potential for biomedical applications for the corresponding marine species.

Keywords: Cancer, Inhibitors, Malaria, Marine invertebrates, Neutral aminopeptidases, Screening.

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Introduction

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 (like M1 and M17 families)[1,2]. 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.

In humans, neutral aminopeptidases are distributed in several tissues and found in various subcellular organelles, in the cytoplasm, and as integral membrane proteins. In particular, neutral metallo-aminopeptidase (APN, EC 3.4.11.2, M1 family) catalyzes the cleavage of neutral and basic amino acids from the N-terminus of protein or peptide substrates and is involved in the degradation of enkephalin, now a target for the development of new pain management strategies[3,4]. This enzyme is also up-regulated in human pathologies such as various types of cancers, inflammation, and skin diseases[5-7]. Strategies for inhibition of APN[8] have been developed primarily for the treatment of pain[4,9] and less so for cancer and skin pathologies. Mammalian leucyl aminopeptidase (LAP, EC 3.4.11.21, M17 family) is cytosolic and involved in the breakdown of

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peptide products of intracellular proteinases[1,2]. It is one of the enzymes that trim proteasome-produced peptides for class I antigen presentation and is also associated with tumor cell proliferation, invasion and/or angiogenesis[10,11].

Neutral aminopeptidases are also distributed in Apicomplexan protist parasites like *Plasmodium falciparum*, the main agent of malaria in humans. Malaria remains the most deadly human parasitic disease in many parts of the world, especially in the Sub Sahara Africa, and is responsible for over 438,000 deaths. In 2015, more than 214 million people had malaria. 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[12]. The most important clinical stage of the complex *P. falciparum* life cycle[13], 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[14,15]. These enzymes include PfA-M1 (M1 family) and PfA-M17 (M17 family), two neutral metallo-aminopeptidases involved in the final steps of hemoglobin digestion[16]. Both enzymes are promising chemotherapeutic targets because their inhibitors can kill parasites *in vitro* and *in vivo*[17-27].

While natural products are a major source of drug lead compounds, the tropical marine sources of aminopeptidases inhibitors, in particular from the Caribbean invertebrate fauna, remain largely unexplored and have the advantages of their tremendous richness and diversity. Two inhibitors of the M1 family enzymes have been isolated from marine organisms, PsammaplinA[28] and HcPI[29]. However, there are no available reports in the literature of inhibitors isolated from marine sources that are targeting the M17 family of neutral aminopeptidases.

The aim of the present study was to investigate whether Cuban marine invertebrates represent valuable sources of promising inhibitors of M1 and M17 neutral aminopeptidases of medical relevance for either cancer or malaria.

Materials and Methods

Materials

Human placenta from the Institute of Research on Placental Histotherapy was kindly donated by Institute Oncology and Radiobiology, Cuba. DEAE Sephacel was purchased from Amersham Biosciences. L-Leu-AMC, bestatin and amastatin were purchased from Sigma-Aldricht. Leu-pNA was purchased from Bachem. The rest of the reagents were of analytical grade. PC3 (CRL 1435) and 3LL (CRL-1642) tumor cell lines were from ATCC (American Type Culture Collection, EEUU) and were kindly donated by the Center for Molecular Immunology, Cuba. The media and supplements for tumor cell cultures were purchased from Gibco. Marine invertebrates used in the present study were identified by Aida Hernandez-Zanuy, PhD, from the National Institute of Oceanology (CITMA, Cuba) (Table 1, Plate1).

Aminopeptidase N and Leucyl aminopeptidase preparations from human placenta

Human Aminopeptidase N (hAPN) was prepared according to Byzia *et al*.[30] in the form of microsomes by differential centrifugation, but without Triton X-100. The microsomal fraction was resuspended in 50 mM Tris-HCl pH 7.5 (buffer A) for use in enzymes assays. Human Leucyl

Table 1 — Marine invertebrate's species from the Havana coastline studied in the present contribution.

Species	Phyla: Class: Family
Cenchritis muricatus (Linnaeus, 1758)	Mollusca: Gastropoda: Littorinidae
Nerita peloronta (Linnaeus, 1758)	Mollusca: Gastropoda: Neritidae
Nerita versicolor (Gmelin, 1791)	Mollusca: Gastropoda: Neritidae
Lissodendoryx (Lissodendoryx) isodictyalis (Carter, 1882)	Porifera: Demospongiae: Coelosphaeridae
Tripneustes ventricosus (Lamarck, 1816)	Echinodermata: Echinoidea: Toxopneustidae
Echinaster (Othilia) echinophorus (Lamarck, 1816)	Echinodermata: Asteroidea: Echinasteridae
Isostichopus badionotus (Selenka, 1867)	Echinodermata: Holothuroidea: Stichopodidae
Stichodactyla helianthus (Ellis, 1768)	Cnidaria: Anthozoa: Stichodactylidae
Bunodosoma granuliferum(Le Sueur, 1817)	Cnidaria: Anthozoa: Actiniidae
Physalia physalis (Linnaeus, 1758)	Cnidaria: Hydrozoa: Physaliidae

Species names were validated on the reference WORMS database (http://www.marinespecies.org/index.php).

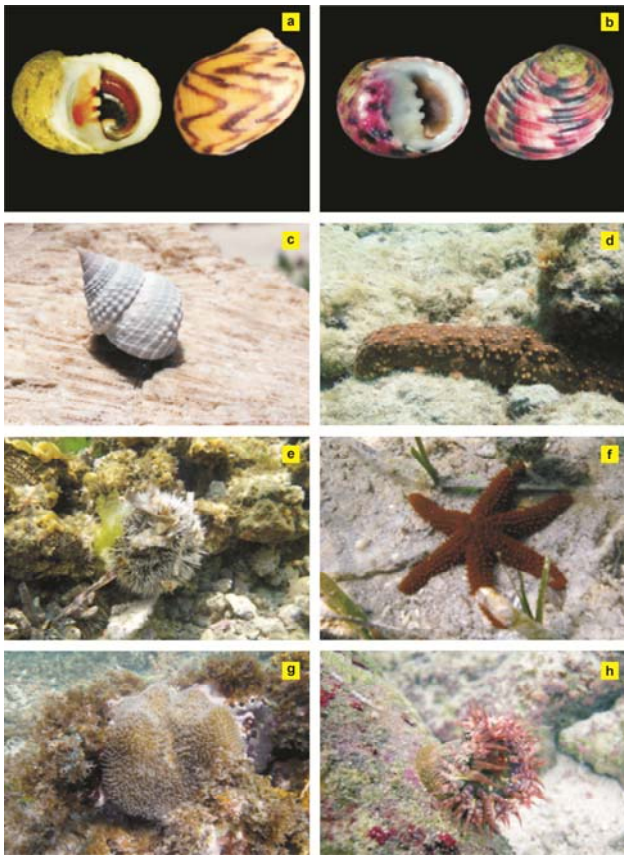

Plate 1 — Some of the marine invertebrates assayed in the present contribution: a) *Nerita peloronta,* b) *Nerita versicolor*, c) *Cenchritis muricatus*, d) *Isostichopus badionotus*, e) *Tripneustes ventricosus*, f) *Echinaster (Othilia) echinophorus*, g) *Stichodactyla helianthus*, and h) *Bunodosoma granuliferum*. Pictures were supplied by Professor Jose Espinosa, PhD from the National Institute of Oceanology, CITMA, Cuba.

aminopeptidase (hLAP) was partially purified from the soluble fraction of human placenta homogenates obtained from the first centrifugation at 10 000 × g. This supernatant was extensively dialyzed in presence of buffer A, using dialysis membranes with a 10 000 Da cut off (Spectrapor). The dialyzed sample was applied onto a DEAE Sephacel column (12 × 1.6 cm) previously equilibrated with the same buffer A. The column was washed with 3 column volumes of buffer A to wash out the non-retained material. Then, elution was carried out by a stepwise salt increase using 0.15, 0.3, 0.5, 0.75 and 1.0 mol/L NaCl concentrations in buffer A with 3 column volumes of buffer each step. Active fractions (0.15 mol/L NaCl) were collected, concentrated via ultrafiltration using a membrane of 10 000 Da of cutoff, and used for enzyme activity.

Preparation of recombinant forms of malarial PfA-M1 and PfA-M17 aminopeptidases

Both malarial enzymes were produced as recombinant, enzymatically active proteins, in *Escherichia coli.* For PfA-M1, a genomic DNA fragment encoding residues 192-1085 of native PfA-M1[31,32]was amplified from genomic DNA of the FcB1 strain of *P. falciparum,* with primers appending a N-terminal hexahistidine tag followed by cleavage site for the tobacco etch virus (TEV) protease (ENLYFQS), and was cloned into the *BamH*1 and *Not*I sites of the pET45b vector (Novagen). For PfA-M17, a synthetic gene (Genecust, Luxembourg) encoding a cleavage site for the TEV protease fused to residues 84-605 of native *P. falciparum* leucyl aminopeptidase PfA-M17 (PlasmoDB PF3D7_1446200), was cloned into *Bam*HI and *Sal*I sites of the pET45b vector (Novagen), which appended an N-terminal hexahistidine tag. Both plasmids were transformed into *Escherichia coli* BL21(DE3) Rosetta 2 (Novagen) after appropriate validations by Sanger sequencing (Beckman Coulter Genomics). Bacterial cultures were grown in auto-induced LB medium (Merck) supplemented with appropriate antibiotics (carbenicillin 50 µg/mL, chloramphenicol 34 µg/mL) for 24 h at 25 °C under vigorous agitation. The clarified lysates were loaded onto Ni^{2-} charged HisTrap column (GE Healthcare) equilibrated in phosphate buffer supplemented with 20 mM imidazole, extensively washed in phosphate buffer supplemented with 20 mM imidazole, and bound recombinant proteins were eluted in respectively
80 mM imidazole in phosphate buffer (rPfA-M1) and 200 mM imidazole in phosphate buffer (rPfA-M17). Eluted fractions were extensively dialyzed at 4 °C into respectively 50 mM Tris-HCl, 200 mM NaCl, 10 µM $ZnCl_2$, pH 7.4 (rPfA-M1)[21] and 50 mM Tris-HCl, 200 mM NaCl, 10 µM $ZnCl_2$ pH 8 (rPfA-M17)[33].

Preparation of crude extracts from marine invertebrates

Organisms were collected along the coast of Havana (Cuba), classified, transported alive on ice, and stored at –20 °C until used for aqueous extract preparation. Specimens for each species were thawed, washed with distilled water, dried on paper filter, weighted, cut into small pieces with sterile scissors and homogenized in distilled water 2:1 (mL/g) at 4 °C in a warring blender (10 sec for 3 repeated cycles) as described earlier[29,34]. Distilled water was preferred as extracting solvent for extract preparation with the aim

to have a crude extract enriched in molecules soluble and stable in aqueous conditions. This selection was based on their potential future application in different biomedical models, all of them requiring physiological conditions. Homogenates were centrifuged at 10 000 g for 30 min at 4 °C. The supernatants were filtered on fiberglass to yield aqueous crude extracts. These extracts were lyophilized and kept at -20 °C until being suspended in the correspondent buffer to assay for neutral aminopeptidase inhibitory activities.

Trichloroacetic clarification treatment of marine crude extracts

About 200 mg of each lyophilized crude extract was suspended in 5 mL buffer A and treated with 2.5 % final concentration of the chaotropic agent trichloroacetic acid (TCA) for 15 min at 4 °C following procedure described earlier[29,34]. The aim of this clarification step was to eliminate contaminants (mainly proteins susceptible to this condition), and to promote dissociation from endogenous inhibitor-target complexes that do not allow the detection of inhibitory components in crude extracts[29,34]. The TCA clarified and dialyzed extracts were then kept at -20 °C until assayed for neutral aminopeptidase inhibitory activities.

Protein concentration determination for marine extracts

The protein concentration was determined at 280 nm as described by Scopes[35] using one unit as an arbitrary extinction coefficient (0.1 %, 1 cm) and bovine serum albumin as standard in a GENESYS 10S UV-Vis spectrophotometer (Thermo Scientific) similar to previously described[29,34].

Monitoring of human and malarial enzymatic activities and inhibitions

The activities of the neutral aminopeptidases used in the present work were measured using the substrate L-Leu-AMC in specific conditions established for each enzyme. The substrate concentration in each assay was equal or lower than one K_M value for each enzyme to avoid competition. In all cases kinetic assays were run at 37 °C during 40 min in 96 well plates and the AMC fluorescence was measured at 460 nm (bandwidth: 40) upon excitation at 360 nm (bandwidth: 40) in a BioTek FL600 fluoromark spectrofluorometer[31,32].

Human placental aminopeptidase N activity (0.25 mg/mL) was measured using buffer A, in presence of 0.030 mmol/L of L-Leu-AMC in a final assay volume of 200 µL. Human placental Leucyl aminopeptidase activity (0.84 mg/mL) was measured using buffer A, in presence of 0.200 mmol/L of L-Leu-AMC in a final assay volume of 200 µL. rPfA-M1 activity (666 nmol/L) was measured using buffer A, in presence of 0.160 mmol/L of L-Leu-AMC in a final assay volume of 100 µL. rPfA-M17 activity (280 nmol/L) was measured using buffer Tris HCL 50 mmol/L pH 8, 1 mmol/L Cobalt (Buffer B) in presence of 0.050 mmol/L of L-Leu-AMC in a final assay volume of 100 µL. One unit of enzyme activity was defined as the amount of enzyme needed to produce one arbitrary unit of fluorescence (AUF) per minute in the specified conditions.

Inhibition by aqueous crude and 2.5 % TCA cleared extracts from marine invertebrates was determined by quantifying the decrease in activity of each enzyme (hAPN, hLAP and rPfA-M1, rPfA-M17) pre-incubated with the extract sample for 30 min at 37 °C prior to addition of L-Leu-AMC substrate, regarding the control assay. One unit of inhibitory activity was defined as the amount of the extract (in terms of protein amount) needed to inhibit one unit of enzyme activity and was calculated using the formula

$$sIA\ (U/mg) = (v_o - v_i) * V_{assay} / {}_{mg\ prot\ sample}$$

where sIA is specific inhibitory activity, v_o is initial rate of the control, v_i is initial rate in presence of the inhibitory sample, and V_{assay} is assay volume.

Based on the increment of the specific inhibitory activity detected for 2.5 % TCA cleared extracts, dose response studies were carried out in these cases in the range of 0.1-1000 µg/mL of each extract (n=3). IC_{50} (half maximal inhibitory concentration) values were determined by non-linear regression adjustment of the dose-response curves (residual activity vs protein concentration of each treated extract) to the IC_{50} equation included in the GRAFIT 6.0 software and was defined as the amount of the extract (in terms of protein concentration) needed to inhibit the control enzyme activity by 50 %. Dose-response studies in presence of bestatin (1-100 µM, equivalent to 0.34-34.40 µg/mL) and amastatin (4-400 µM, equivalent to 1.89-189.00 µg/mL) were done in parallel as positive control of inhibition for each target aminopeptidase.

Evaluation of the effects on tumor cell lines cultures growth

Detection of the APN activity in the PC3 and 3LL tumor cell lines cultures

The APN activity present in the membrane of the tumor cells in study was measured spectrophotometrically

according to the work done by Ashmun and Look[36] using Leu-pNA as substrate. A total of $5x10^4$ cells per well were seeded, in flat end 96 well plates (Costar, High Binding, EEUU) and incubated in DMEM-F12 with 10 % of SFT, during 24 h at 37 °C at 5 % of CO_2. After this, the medium was removed and 90 µL of DMEM-F12 was added to the intact cells. To begin the enzymatic reaction, 10 µL of Leu-pNA (3 mmol/L in the assay) previously incubated at 37 °C, were added to each well. The reaction was carried out at 37 °C for 1 h. The formation of pNA was followed by absorbance at 405 nm in a 96 well plate kinetic spectrometer (iMark Biorad), using the "M6M" software supplied by the manufacturer. The enzyme activity was determined using the equation given below and expressed as nmol/h/10^6 cells. Assays were run in triplicates using in parallel a standard free of cell and a control free of substrate.

$$EA\ (nmol/h/10^6\ cells) = (\Delta DO/\Delta t) \times (1/\xi) \times (Va/(No.Cel./10^6\ cells)) \quad (1)$$

where, EA is enzymatic activity; $\Delta DO/\Delta t$ (λ= 405 nm) is variation in the absorbance at 405 nm after 1 h; Va is total volume assay (mL); No.Cel. is number of cell per well; ξ is extinction coefficient of pNA at 405 nm ([8800 (mol/L)

Effects on mammalian cancer cell viability

The viability of each cell line in presence of each 2.5 % TCA cleared and dialyzed extracts were determined by a modification of the colorimetric assay based on MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazodium bromide) method previously described by Mosmann[37]. Flat-bottomed 96-well microculture plates (Costar) were seeded with PC3 (human prostatic adenocarcinoma) or 3LL (mouse lung Lewis carcinoma) cell line in 90 µL (10^4 cell)/well and grown in DMEM-F12 with 10 % SFT and kept at 37 °C with 5 % CO_2, for 24 h before the addition of the inhibitor sample diluted in 10 µL of media. After 72 h (for PC3) and 48 h (for 3LL) of incubation at 5 % of CO_2, cells viability was measured by the addition of MTT at 1 mg/mL. All the tests were performed in triplicates. Cells without treatment were included as a maximum cell growth point. Bestatin was included as positive control of inhibition. Percentage of viable cells in test wells was calculated using the following formula:

Viable cells (%) = (O.D.540 nm-620 nm of treated cells/O.D.540 nm-620 nm of non-treated cells) × 100

Effect on *P. falciparum* growth in cultures of the 2.5 % TCA cleared extracts displaying inhibitory activities on malarial rPfA-M17

The general procedure was similar to that previously described (Flipo *et al.*)[18]. Antimalarial assays were performed using the *P. falciparum* FcB1 strain, grown *in vitro* according to Trager and Jensen[38]. Parasite culture medium contains RPMI 1640 medium (Life Technologies, Inc.), 25 mM Hepes, 27.5 mM $NaHCO_3$, and 11 mM glucose (pH 7.4) and is supplemented with 8 % (v/v) compatible heat-inactivated human serum. Human red blood cells (RBC) were added at hematocrit of 2 %, and the parasite culture was maintained at 37 °C under an atmosphere of 3 % CO_2, 6 % O_2, and 91 % N_2, with daily medium changes. The effect of selected TCA clarified extracts on the intraerythrocytic development of *P. falciparum* was tested on asynchronous cultures containing mixtures of all stages at a final parasitemia of ~1 % that were distributed in 96-well microplates. Serial dilutions of selected extracts were added to the wells, in parasite culture medium, in triplicates. Parasites were allowed to grow in a candle jar system and after 24 h in culture, ^{3}H-hypoxanthine was added (0.5 µCi/well). After an additional 24 h incubation period at 37 °C in the candle jar system, parasites were harvested on filters after a freeze-thawing cycle. Dried filters were submerged in a liquid scintillation mixture (OptiScintHisafe, Perkin Elmer) and counted in a 1450 Microbeta counter (Wallac, Perkin Elmer). Parasite growth inhibition (IC_{50}) was calculated from the parasite-associated radioactivity (incorporated into nucleic acids) in treated cultures compared with control cultures performed in the presence of equivalent amounts of buffer A as described earlier[18,39]. Bestatin was included as positive control of parasite growth inhibition.

Results

Inhibition of hAPN, hLAP, rPfA-M1 and rPfA-M17 by aqueous extracts from marine invertebrates

In the present study, initially, the inhibitory activity of crude extracts from 10 species of marine invertebrates from the Cuban coastline (Table 1), against four neutral aminopeptidases: hAPN, hLAP and the malarial rPfA-M1 and rPfA-M17 was evaluated. These species belong to the phyla Mollusca, Poriphera, Echinodermata, and Cnidaria. The studies were performed using the substrate L-Leu-AMC at a final concentration equivalent to their respective Michaelis constant (K_M) values,

determined in the present work, which were: 25.53 µmol/L (hAPN), 238.00 µmol/L (hLAP), 69.40 µmol/L (rPfA-M1), and 43.33 µmol/L (rPfA-M17), to avoid substrate competition during inhibition assays.

The initial evaluations performed allowed detection of inhibitory activity of the target enzymes in the aqueous crude extracts as follow: hAPN: *Cenchritis muricatus* and *Isostichopus badionotus;* hLAP: *C. muricatus, Lissodendoryx (Lissodendoryx) isodyctialis, I. badionotus* and *Stichodactyla helianthus*; rPfA-M17: *C. muricatus, Echinaster (Othilia) echinophorus, I. badionotus*, *Physalia physalis, S. helianthus*, and *Bunodosoma granuliferum* (Specific Inhibitory activity values are given in Table 2). Screening performed against recombinant malarial PfA-M1 (rPfA-M1) failed to detect any inhibitory activity from either extract (data not shown). Interestingly, in some assays with aqueous crude extracts, increased L-Leu-AMC hydrolysis rates over the control value were found instead of inhibitory activities of the tested enzymes. 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.

Clarification of all the aqueous crude extracts with a 2.5 % TCA treatment increased, in all cases, the recovery of specific inhibitory activities as compared to their detection in positive crude extracts. The treatment also allowed the identification of inhibitory activities from species that were negative after screening using aqueous crude extracts. This result indicates that this clarification step was useful in the elimination of contaminants and/or induced dissociation from endogenous inhibitor-target complexes that did not allow the detection of inhibitory components in crude extracts. These 2.5 % TCA treated extracts were used to continue the inhibition studies.

Additionally, the enhanced activities over the control detected in some extracts were 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 in the concerned extract. To test for the possible presence of potential neutral aminopeptidase-like enzymes in aqueous crude extracts (which could be responsible for these observations), enzymatic assays were performed using different amounts of the samples (in absence of the initial target aminopeptidases) in presence of L-Leu-AMC, in buffer A. A linear dependence of the initial rate versus the amount of crude extract in the assays was detected for the species *Nerita peloronta, N. versicolor, L. (L.) isodictyalis, Tripneustes ventricosus, E. (O.) echinophorus, S. helianthus, B. granuliferum*, and *P. physalis.* This allowed the determination of the specific enzyme activity in each case (Table 2). These results suggest the presence of neutral aminopeptidase activities in the aqueous crude

Table 2 — Summary of the screening of inhibitory activity against hAPN, malarial rPfA-M17 and hLAP.

Species	Crude extracts				2.5% TCA cleared extracts		
	NA-like activity (x 10^4 U/mg)	rPfA-M17 sIA (U/mg)	hLAPsIA(U /mg)	hAPNsIA (U/mg)	rPfA-M17 sIA (U/mg)	hLAPsIA (U/mg)	hAPNsIA (U/mg)
Cenchritis muricatus	ND	0.40	0.16	0.46	7.56	4.30	5.20
Nerita peloronta	6.95±2.84	ND	ND	ND	10.61	6.48	5.06
Nerita versicolor	12.56±1.70	ND	ND	ND	7.17	5.58	2.21
Lissodendoryx (Lissodendoryx) isodictyalis	1.51±0.49	ND	0.27	ND	256.13	312.01	171.92
Tripneustes ventricosus	6.39±0.07	ND	ND	ND	41.85	43.31	56.86
Echinaster (Othilia) echinophorus	58.87±12.62	0.11	ND	ND	9.10	7.85	10.82
Isostichopus badionotus	ND	0.18	1.24	1.56	55.83	27.86	33.81
Stichodactyla helianthus	9.63±2.68	0.55	0.68	ND	38.15	18.67	32.81
Bunodosoma granuliferum	39.44±5.45	0.19	ND	ND	5.45	4.08	4.31
Physalia physalis	2.04±0.52	0.40	ND	ND	19.21	11.00	13.13

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 in the specified conditions and inhibitory activities are expressed per mg of corresponding extracts. The first column indicates tested species, the second column neutral aminopeptidase-like activity (NA), detected in aqueous crude extracts only, using L-Leu-AMC as substrate in Buffer A and the remaining columns refer to sIA on corresponding enzymes. ND: not detected.

extracts of all of these species. Characterizing of these in future is planned.

Dose response studies of inhibition of hAPN by 2.5 % TCA clarified extracts: Preliminary effect on PC3 and 3LL cancer cell lines

Table 3 summarizes the results of the evaluation of the inhibitory potential displayed by the 2.5 % TCA clarified extracts onnative hAPN. Once the crude extracts were treated with TCA at 2.5 % and extensively dialyzed, inhibitory activity of hAPN were detected in all treated extracts. All the clarified extracts inhibited hAPN in a dose dependent manner. The inhibition was characterized by a concave behavior observed in all the dose-response curves (Supplementary Fig. 1), 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 were in the range of 11.7-567.6 µg/mL. Inhibition of hAPN with IC_{50} values around or less than 100 µg/mL was detected in four of the ten species tested (*L. (L.) isodictyalis, T. ventricosus, I. badionotus*, and *S. helianthus*). IC_{50} values for bestatin and amastatin were obtained in parallel as positive controls of inhibition (6.70±1.90 and 63.45±7.61 µg/mL, respectively). The IC_{50} values for the TCA extract from *L. (L.) isodictyalis* and *T.ventricosus* were similar to that of bestatin. 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. As first step, we determined the neutral aminopeptidase activity in these cells in presence of L-Leu-AMC. PC3 and 3LL were characterized by an activity of 87.78±3.14 and 27.53±2.27 nmol/h/10^6 cells, respectively. All of the treated extracts and bestatin used as positive control displayed a dose-response effect on PC3 and 3LL cell viability, with the best effects i.e. lowest IC_{50} values (below 100µg/mL) obtained was for *L. (L.) isodictyalis* (strongest effect, IC_{50} value below of 5µg/mL, similar to bestatin effect), *T. ventricosus, I. badionotus* and *S. helianthus*, on both cell lines. Globally, the IC_{50} values of cell viability were in good agreement with the IC_{50} values on hAPN inhibition, including bestatin results (IC_{50} on 3LL and PC3: 0.54±0.01 and 3.15±0.72 µg/mL, respectively). The rest of the TCA clarified extracts displayed moderately interesting IC_{50} values in the range of 234.5-711.3 µg/mL.

Dose response studies of inhibition of malarial rPfA-M17 and hLAP: Preliminary effects on the growth of *P. falciparum* FcB1 strain

Table 4 summarizes the results of the evaluation of the inhibitory potential of the 2.5 % TCA clarified extractson malarial rPfA-M17 and native human LAP. We were able to detect inhibitory activities of both tested M17 aminopeptidases, in all TCA clarified extracts. These activities were characterized by a concave dose-response behavior corroborating the presence of reversible inhibitory molecules in the samples (Supplementary Fig. 2) 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 4). Similar behavior was detected in the presence of bestatin and amastatin as positive controls of inhibition vs both enzymes with IC_{50} values

Table 3 — Summary of the IC_{50} determination for the 2.5 % TCA clarified extracts against hAPN. Preliminary effects on growth of cancer cell lines 3LL and PC3.

Species	IC_{50} value vs hAPN (µg/mL)	IC_{50} value vs 3LL (µg/mL)	IC_{50} value vs PC3 (µg/mL)
Cenchritis muricatus	450.20±77.40	214.00±46.50	352.90±65.00
Nerita peloronta	237.80±20.90	273.30±78.80	299.10±31.70
Nerita versicolor	370.00±50.00	358.80±70.20	289.70±39.70
Lissodendoryx (Lissodendoryx) isodictyalis	11.70±2.70	< 5.00	< 5.00
Tripneustes ventricosus	25.00±3.10	39.90±2.00	77.00±3.90
Echinaster (Othilia) echinophorus	198.20±27.20	265.70±29.60	405.60±50.40
Isostichopus badionotus	69.70±10.00	57.10±2.70	83.10±3.00
Stichodactyla helianthus	103.60±20.60	110.80±13.20	58.10±7.50
Bunodosoma granuliferum	567.60±88.00	786.80±37.10	711.30±29.30
Physalia physalis	123.10±21.30	257.30±6.70	234.50±5.00
Bestatin (positive control)	6.70±1.90	0.54±0.01	3.15±0.72
Amastatin (positive control)	63.45±7.61	ND	ND

Bestatin and amastatin were tested in parallel as positive control. ND: not determined.

Table 4 — 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

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

Bestatin and amastatin were tested in parallel as positive control. ND: not determined.

of: rPfA-M17: 0.15±0.02 and 60.70±19.84 µg/mL, respectively and hLAP: 11.83±2.61 and 158.05± 28.44 µg/mL, respectively. We detected inhibition of rPfA-M17 with IC_{50} values up to ~100 µg/mL for 6 of the 10 extracts (those of *C. muricatus, N. peloronta, L. (L.) isodictyalis, T. ventricosus, I. badionotus* and *S. helianthus)*. Comparing the inhibitions on rPfA-M17 and hLAP, in all cases the plasmodial enzyme was more susceptible than its human counterpart, with ratios of selectivity between 1.87 and 60 times. The most selective extract was from *N. versicolor*, an attractive situation even if its IC_{50} value on the malarial enzyme is moderate. Once the inhibition of rPfA-M17 was corroborated, we evaluated the effect of each extract on *P. falciparum* growth in cultures. With the exception of *C. muricatus*, *S. helianthus* and *B. granuliferum*, which displayed an IC_{50} value greater than 400 µg/mL, all of the treated extracts displayed a dose-response effect on FcB1 cells (IC_{50} values in the range of 0.24-325.5 µg/mL). Bestatin tested in parallel as positive control displayed an IC_{50} value of 1.14±0.27 µg/mL. The best effects were obtained for *T. ventricosus* (IC_{50} value of 0.24 µg/mL, one order lower than bestatin effect indicating strongest effect for this extract) and *L. (L.) isodictyalis* (IC_{50} value of ~2.8 µg/mL, similar to bestatin effect).

Discussion

Peptidases are one of the most abundant groups of enzymes in living organisms, and they are involved in a wide variety of biochemical and physiological processes such as digestion, fertilization, growth, differentiation, cell signaling/migration, immunological defense, wound healing, and apoptosis. Peptidases are also involved in pathological outcomes in many human diseases including propagation of infectious agents and resulting pathogenesis and infectious diseases. For these reasons specific inhibitors of target peptidases are emerging as promising therapeutics uses both in the treatment of metabolic disorders (like type II diabetes mellitus, hypertension, cancers, inflammation, immunological and respiratory disorders and parasitic infectious diseases)[40-42]. Metallo-aminopeptidases catalyze the cleavage of amino acids from the N-terminus of protein or peptide substrates. They belong to different families, the M1 and M17 families being the best characterized[1,2,6]. Among M1 family enzymes, APN has been extensively investigated in mammals, with recent important findings about its structure-function relationships[43,44] and its implication in tumorigenesis, immune system and pain management[4,8,9]. PfA-M17 from *P. falciparum* is one of the best characterized M17 family members and current target for the development of new antimalarials because of the importance of this enzyme for parasite survival[25,27,45-49].

Marine invertebrates are an abundant source of bioactive molecules such as toxins[50,51], peptidases[52] and peptidase inhibitors of different mechanistic classes. They are characterized by a great diversity of chemical structures, high potency and diverse specificity including metallo inhibitors[28,29,53-56]. These biomolecules are involved in nutrition, reproduction, and communication processes. Additionally, most invertebrates (like sponges, bryozoans, tunicates,

among others) lack morphological defense structures. These biomolecules are also part of mechanisms related with protection against predator, infection, and competition[57]. Currently, identification of APN inhibitors from marine sources remains scarce with only two reports of molecules behaving as inhibitors of the porcine enzyme[28,29] and inhibitory activity reported from one ascidian species[58]. However, marine peptidase inhibitors that may interact with human and plasmodial M1 and M17 enzymes have not yet been described.

As result of the screening of inhibitory activity of neutral aminopeptidases of biomedical relevance in the present contribution, the present findings are the first to show the presence of inhibitory activity of human APN in the 2.5 % TCA clarified and dialyzed extracts from *C. muricatus, N. peloronta, N. versicolor, L. (L.) isodictyalis, T. ventricosus, E. (O.) echinophorus, I. badionotus, S. helianthus, B. granuliferum*, and *P. physalis*. In all the cases, inhibition was highly dependent on the dose.The higher inhibitions characterized by promising IC_{50} values (around 100 µg/mL or lesser) were obtained for *L. isodictyalis, T. ventricosus, S. badionatus*, and *S. helianthus.* The IC_{50} values for almost all the ten species were lower than the data previously described for both TCA and heat clarified extracts from the ascidian *Diplosoma listerianum* vs pAPN in similar assay conditions but in the presence of susbtrate Leu-pnitroanilide[58]. Additionally, for *L. isodictyalis* and *S. helianthus* inhibition of porcine pancreatic elastase (EPP) and human neutrophil elastase (HNE), by heat treated extracts have been recently reported[59]. HNE is a serine peptidase that contributes to the onset and progression of many inflammatory diseases with severe impact on organ tissue integrity (like pulmonary emphysema, rheumatoid arthritis, and cystic fibrosis) that is now viewed as a promising target for the development of new therapeutics[60]. The EPP inhibition was characterized by IC_{50} values in the same order of hAPN inhibition identified in the present contribution for these two species. Findings show that that EPP/HNE and hAPN belong to different peptidases clans with completely different catalytic mechanisms[1,2]. Further, the clarification treatments employed in each work were different. It is very likely that the molecules responsible for the hAPN and EPP inhibition in *L. isodictyalis* and *S. helianthus* are not the same molecular entities. The present results support these two species as potential new sources of inhibitors for biomedical relevant human peptidases like hAPN and HNE. All the clarified extracts had effect on 3LL and PC3 cancer cell characterized by the presence of APN activity reported in the present study. The higher effects with IC_{50} values below 100 µg/mL were observed for the same species that were also displaying the strongest hAPN inhibition. In particular, the identification of an IC_{50} value under 5 µg/mL for *L. isodictyalis* extract vs both cancer cells lines, similar to the effect displayed by bestatin (a pure compound), indicates that we are in presence of a potentially very good extract for the isolation of hAPN inhibitors, as defined by Mambu and Grellier[61].

A wide variety of anticancer activities and isolated compounds with a large diversity of chemical structures (alkaloids, steroids, macrolides, polyketides, depsipeptides among others) have been identified from a large diversity of marine organisms[57,62,63]. Some are currently in clinical and late preclinical development[64]. To the best of our knowledge, this is the first time concomitant indications of activity on hAPN target are also provided.

As a second part of the screening of inhibitory activity for neutral aminopeptidases performed in the present study, inhibition of rPfA-M17 in all the 2.5 % TCA cleared extracts assayed was identified. The inhibition was also dose-dependent, with six species with IC_{50} values lower than 100 µg/mL. All the species also inhibited the human LAP, but the inhibition was higher for the malarial enzyme indicating certain degree of selectivity of the entities responsible for rPfA-M17 inhibition. These results constitute the first report of inhibition of M17 enzymes (human and plasmodial) by marine invertebrates' species extracts. With the exception of *C. muricatus*, *S. helianthus* and *B. granuliferum*, all the treated extracts showing inhibition of rPfA-M17 also have an inhibitory effect on the growth of FcB1 strain of *P. falciparum*. The extracts from *L. isodictyalis* and *T.ventricosus* were characterized by the most promising activity in terms of IC_{50} value under 3 µg/mL. These results indicate that we are in presence of two very good extracts as defined Mambu and Grellier[61]. Particularly attractive are the *T. ventricosus* extracts that displayed an effect 300 times more potent on FcB1 strain of *P. falciparum* regarding the human cancer cells, indicating parasite effect specificity. Additionally, this effect of *T. ventricosus* extract on FcB1 strain was stronger than bestatin effect (a pure compound). Due to the fact that IC_{50} for *L. isodyctialis* and

T. ventricosus is lower on FcB1 strain of *P. falciparum* than on PfA-M17 enzymes, it is very likely that these extracts may contain not only PfA-M17 inhibitors but also other compounds active on other malarial targets. For example, *L. isodyctialis*, inhibition of subtilisin from *Bacillus licheniformes* with IC_{50} value of 3 µg/mL has been recently described[59]. Another interesting result is the selectivity for rPfA-M17 regarding hLAP of *N. versicolor* extract showing effects on parasite growth with IC_{50} in the same order of enzyme inhibition, indicating that this species is also attractive.

More than 50 natural compounds isolated from marine organisms have been described with antimalarial activity, most of them from sponges. The molecular mechanism of the effect has been identified for only two of these natural compounds[65,66]. Sponges are an abundant source of compounds with activity against protozoa responsible for the so called "neglected diseases" (such as leishmaniasis, amoebiasis, trichomoniasis, African sleeping sickness and Chagas disease)[66]. The present results are similar in that good antimalarial activity vs FcB1 strain of *P. falciparum* (IC_{50} of 2.8 µg/mL) in the 2.5 % TCA extract from the Caribbean sponge *L. (L.) isodictyalis*, which also displayed a good inhibition of rPfA-M17 (IC_{50} 27.3 µg/mL) was identified. For this species, Mendiola *et al.*[67] previously identified inhibition of F32/Tanzania *P. falciparum* strain in an aqueous crude extract prepared without any treatment, with a MIC of 50 µg/mL. In the same work the researchers described inhibition of the same F32/Tanzania *P. falciparum* strain by aqueous extracts from three species of Ascidians (*Phallusia nigra, Ascidia sidneiensis and Microcosmus goanus*) and two echinoderms (*Holothuria* sp. and *E. (O.) echinophorus*). The last species was also tested in the present contribution but the aqueous extract was characterized by a high aminopeptidase activity in presence of L-Leu-AMC at pH 7.5 that did not allow the detection of inhibition of enzymes tested. The TCA treated extract showed that inhibition of rPfA-M17 and the effect of FcB1 *P. falciparum* cells (IC_{50} 232.22 µg/mL) was higher than the one reported for the aqueous crude extract of this species (500 µg/mL) by Mendiola *et al*.[67], probably due to a more clarified extract in the present study. The results of the present study supports that sponges as well as marine invertebrates from other Phyla such as mollusks, echinoderms, and cnidarians are a good and unexplored source of potential anticancer and antimalarial compunds associated with the inhibition of neutral aminopeptidases from M1 and M17 families, involved in these human pathologies.

Conclusion

The present study constitutes the first report of inhibitory activity of human and plasmodial neutral aminopeptidases in aqueous extracts from marine species. The results strongly suggest that all of the species in this study have potential as sources of inhibitors of hAPN that could be applied in future studies on cancer models involving this enzyme. *L. isodictyalis, T. ventricosus, S. badionatus* and *S. helianthus* are the most promising in terms of IC_{50} vs both enzyme and cell models. Although all the species extracts inhibited rPfA-M17, the most promising of them for future applications in malaria models are *L. isodictyalis* and *T. ventricosus* characterized by the higher effects on growth of FcB1 *P. falciparum* strain cells and with effects on rPfA-M17. The potentialities of the remaining tested species in the malaria field will depend whether the molecules responsible for the inhibition detected vs hAPN and rPfA-M17 are different, and in all cases, whether the purified compounds are not toxic vs normal human cells i.e. vs host. The present results strongly support that marine fauna, in particular the invertebrates, are an important and unexplored source of inhibitors of metallo-aminopeptidase with promising applications in cancer and malaria treatment.

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References

1 Rawlings N D, Waller M, Barrett A J and Bateman A, MEROPS: The database of proteolytic enzymes, their substrates and inhibitors, *Nucleic Acids Res*, 2014, **42**, D503-D509.

2 Rawlings N D, Barrett A J and Finn R D, Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors, *Nucleic Acids Res,* 2016, **44**, D343-D350.

3 Poras H, Bonnard E, Dangé E, Fournié-Zaluski M C and Roques B P, New orally active dual enkephalinase inhibitors (denkis) for central and peripheral pain treatment, *J Med Chem,* 2014, **57**, 5748−5763.

4 Bonnard E, Poras H, Nadal X, Maldonado R, Fournié-Zaluski M C and Roques B P, Long-lasting oral analgesic effects of N-protected aminophosphinic dual ENKephalinase inhibitors (DENKIs) in peripherally controlled pain, *Pharmacol Res Perspect,* 2015, **3,** e00116.

5 Luan Y and Xu W, The structure and main functions of aminopeptidase N, *Curr Med Chem,* 2007, **14**, 639-647.

6 Mucha A, Drag M, Dalton J P and Kafarski P, Metallo-aminopeptidase inhibitors, *Biochimie*, 2010, **92**, 1509-1529.

7 Wickstrom M, Larsson R, Nygren P and Gullbo J, Aminopeptidase N (CD13) as a target for cancer chemotherapy, *Cancer Sci,* 2011, **3**, 501–508.

8 Zhang X, Fang H, Zhang J, Yuan Y and Xu W, Recent advance in aminopeptidase N (APN/CD13) inhibitor research, *Curr Med Chem,* 2011, **18**, 5011-5021.

9 Schreiter A, Gore C, Labuz D, Fournie-Zaluski M C, Roques B P, Stein C, *et al.*, Pain inhibition by blocking leukocytic and neuronal opioid peptidases in peripheral inflamed tissue, *FASEB J,* 2012, **26**, 5161-5171.

10 Matsui M, Fowler J H and Walling L L, Leucine aminopeptidases: Diversity in structure and function, *Biol Chem,* 2006, **387**, 1535-1544.

11 He X, Huang Q, Qiu X, Liu X, Sun G, Guo J, *et al*., AP3 promotes glioma progression by regulating proliferation, migration and invasion of glioma cells, *Int J Biol Macromol*, 2015, **72**, 1081-1089.

12 Hoffman S L, Vekemans J, Richie T L and Duffy P E, The march toward malaria vaccines, *Vaccine*, 2015, **49**, S319-S333.

13 Aly A S I, Vaughan A M and Kappe S H I, Malaria parasite development in the mosquito and infection of the mammalian host, *Ann Rev Microbiol*, 2009, **63**, 195-221.

14 Goldberg D E, Hemoglobin degradation, *In*: CTMI 295, Springer-Verlag Berlin Heidelberg, 2005, 275–291.

15 Goldberg D E, Complex nature of malaria parasite hemoglobin degradation, *Proc Natl Aca Sci,* 2013, **110**, 5283-5284.

16 Skinner-Adams T S, Stack C M, Trenholme K R, Brown C L, Grembecka J, Lowther J, *et al*., *Plasmodium falciparum* neutral aminopeptidases: New targets for anti-malarials, *Trends Biochem Sci*, 2010, **35**, 53-61.

17 Flipo M, Florent I, Grellier P, Sergheraert C and Deprez-Poulain R F, Design, synthesis and antimalarial activity of novel, quinoline-based, zinc metallo-aminopeptidase inhibitors, *Bioorg Med Chem Lett,* 2003, **13**, 2659-2662.

18 Flipo M, Beghyn T, Leroux V, Florent I, Deprez B P and Deprez-Poulain R F, Novel selective inhibitors of the zinc plasmodial aminopeptidase PfA-M1 as potential antimalarial agents, *J Med Chem*, 2007, **50**, 1322-1334.

19 Dalal S and Klemba M, Roles for two aminopeptidases in vacuolar hemoglobin catabolism in *Plasmodium falciparum*, *J Biol Chem*, 2007, **282**, 35978-35987.

20 McGowan S, Porter C J, Lowther J, Stack C M, Golding S J, Skinner-Adams T S, *et al.*, Structural basis for the inhibition of the essential *Plasmodium falciparum* M1 neutral aminopeptidase, *Proc Natl Acad Sci,* 2009, **106**, 2537-2542.

21 Harbut M B, Velmourougane G, Dalal S, Reiss G, Whisstock J C, Onder O, *et al.*, Bestatin-based chemical biology strategy reveals distinct roles for malaria M1- and M17-family aminopeptidases, *Proc Natl Acad Sci,* 2011, **108**, E526-E534.

22 Velmourougane G, Harbut M B, Dalal S, McGowan S, Oellig C A, Meinhardt N, *et al.*, Synthesis of new (-)-bestatin-based inhibitor libraries reveals a novel binding mode in the S1 pocket of the essential malaria M1 metalloaminopeptidase, *J Med Chem*, 2011, **54**, 1655-1666.

23 Deprez-Poulain R, Flipo M, Piveteau C, Leroux F, Dassonneville S, Florent I, *et al*., Structure−activity relationships and blood distribution of antiplasmodial aminopeptidase-1 inhibitors, *J Med Chem,* 2012, **55**, 10909-10917.

24 McGowan S, Working in concert: The metalloaminopeptidases from *Plasmodium falciparum, Curr Opinion Struct Biol*, 2013, **23**, 828–835.

25 Mistry S N, Drinkwater N, Ruggeri C, Sivaraman K K, Loganathan S, Fletcher S, *et al.,* Two-pronged attack: Dual inhibition of *Plasmodium falciparum* M1 and M17 metalloaminopeptidases by a novel series of hydroxamic acid-based inhibitors, *J Med Chem,* 2014, **57**, 9168-9183.

26 Paiardini A, Bamert R S, Kannan-Sivaraman K, Drinkwater N, Mistry S N, Scammells P J *et al*., Screening the medicines for malaria venture "malaria box" against the *Plasmodium falciparum* aminopeptidases, M1, M17 and M18, *PLoS One,* 2015, **10**(2), e0115859

27 Drinkwater N, Vinh N B, Mistry S N, Bamert R S, Ruggeri C, Holleran J P, *et al*., Potent dual inhibitors of *Plasmodium falciparum* M1 and M17 aminopeptidases through optimization of S1 pocket interactions, *Eur J Med Chem*, 2016, **110**, 43-64.

28 Shim J S, Lee H S, Shin J and Kwon H J, Psammaplin A, a marine natural product, inhibits aminopeptidase N and suppresses angiogenesis *in vitro*, *Cancer Lett,* 2004, **3**, 163-169.

29 Pascual I, Sh G, Cisneros M, Joseph-Bravo P, Diaz J, Possani L, *et al*., Purification of a specific inhibitor of pyroglutamyl aminopeptidase II from the marine annelid *Hermodice carunculata*. *In vivo* effects in rodent brain, *Int J Biochem Cell B,* 2004, **36**, 138-152.

30 Byzia A, Szeffler A, Kalinowski L and Drag M, Activity profiling of aminopeptidases in cell lysates using afluorogenic substrate library, *Biochimie*, 2016, **122**, 31-37.

31 Florent I, Derhy Z, Allary M, Monsigny M, Mayer R and Schrével J, A *Plasmodium falciparum* aminopeptidase belonging to the M1 familiy of zinc-metallopeptidase is expressed in erythrocytic stages. *Mol Biochem Parasitol,* 1998, **97**, 149-160.

32 Allary M, Schrével J and Florent I, Properties, stage-dependent expression and localization of *Plasmodium falciparum* M1 family zinc-aminopeptidase, *Parasitol,* 2002,**125**, 1-10.

33 Stack C M, Lowther J, Cunningham E, Donnelly S H, Gardiner D L, Trenholme K R, *et al*., Characterization of the *Plasmodium falciparum* M17 Leucyl Aminopeptidase: A

protease involved in amino acid regulation with potential for antimalarial drug development, *J Biol Chem*, 2007, **282**, 2069-2080.

34 Pascual I, Gómez H, LopézChappe M A, Saroyán A, González Y, Cisneros M, *et al*., Screening of inhibitors of dipeptidyl peptidase IV activity in aqueous extracts from marine organisms, *Enzyme Microb Tech,* 2007, **40**, 414-419.

35 Scopes R, Protein purification: Principles and practice, Springler- Verlag Series, 1982, 329.

36 Ashmun R and Look A, Metalloprotease activity of CD13/aminopeptidase N on the surface of human myeloid cells, *Blood,* 1990, **75**, 462–469.

37 Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *J Immunol Methods,* 1989, **65**, 55–63.

38 Trager W and Jensen J B, Human malaria parasite in continuous culture, *Science,* 1976, **193**, 673-675.

39 Desjardin R E, Canfield C J, Haynes J D and Chulay J D, Quantitative assessment of anti-malarial activity in vitro by semiautomated microdilution technique, *Antimicrob Agents Chem,* 1979, **16**, 710- 718.

40 Drag M and Salvesen G S, Emerging principles in protease-based drug discovery, *Nat Rev Drug Discov*, 2010, **9**, 690–701.

41 Deu E, Verdoes M and Bogyo M, New approaches for dissecting protease functions to improve probe development and drug discovery, *Nat Struct Mol Biol*, 2012, **19**, 9-16.

42 Rascon A A and McKerrow J H, Synthetic and natural protease inhibitors provide insights into parasite development, virulence and pathogenesis, *Curr Med Chem*, 2013, **20**, 3078-3102.

43 Wong A, Zhou D and Rini J M, The X-ray crystal structure of human aminopeptidase N reveals a novel dimer and the basis for peptide processing, *J Biol Chem,* 2012, **44**, 36804–36813.

44 Chen L, Ling Y L, Peng G and Li F, Structural basis for multifunctional roles of mammalian aminopeptidase N, *Proc Natl Acad Sci,* 2012, **44**, 17966–17971.

45 McGowan S, Oellig C A, Birru W A, Caradoc-Davies T T, Stack C M, Lowther J, *et al*., Structure of the *Plasmodium falciparum* M17 aminopeptidase and significance for the design of drugs targeting the neutral exopeptidases, *Proc Natl Acad Sci,* 2010, **107**, 2449-2454.

46 Poreba M, McGowan S, Skinner-Adams T S, Trenholme K R, Gardiner D L, Whisstock J C, *et al*., Fingerprinting the substrate specificity of M1 and M17 aminopeptidases of human malaria *Plasmodium falciparum*, *PLoS One*, 2012, **7**, e31938.

47 Sivaraman K, Paiardini A, Sieńczyk M, Ruggeri C, Oellig C A, Dalton J P, *et al*., Synthesis and structure–activity relationships of phosphonic arginine mimetics as inhibitors of the M1 and M17 aminopeptidases from *Plasmodium falciparum*, *J Med Chem,* 2013, **56**, 5213-5217.

48 Drinkwater N, Bamert R S, Sivaraman K K, Paiardini A and McGowan S, X-ray crystal structures of an orally available aminopeptidase inhibitor, Tosedostat, bound to anti-malarial drug targets PfA-M1 and PfA-M17, *Proteins*, 2015, **83**, 789-795.

49 Ruggeri C, Drinkwater N, Sivaraman K K, Bamert R S, McGowan S and Paiardini A, identification and validation of a potent dual inhibitor of the *P. falciparum* M1 and M17 aminopeptidases using virtual screening, *PloS One*, 2015, **10**, e0138957.

50 Alvarez C, Mancheno J M, Martínez D, Tejuca M, Pazos F and Lanio M E, Sticholysins, two pore-forming toxins produced by the Caribbean Sea anemone *Stichodactyla helianthus*: Their interaction with membranes, *Toxicon*, 2009, **54**, 1135–1147.

51 Valle A, Alvarado-Mesén J, Lanio M E, Álvarez C, Barbosa J A and Pazos I F, The multigene families of actinoporins (part I): Isoforms and genetic structure, *Toxicon*, 2015, **103**, 176-187.

52 Alonso del Rivero M, Trejo S, Rodríguez de la Vega M, González Y, Bronsoms S, Canals F, *et al*., A novel metallocarboxypeptidase like enzyme isolated from the marine annelid *Sabellastarte magnifica* a step into the invertebrate world of proteases, *FEBS J*, 2009, **276**, 4875-4890.

53 Fusetani N and Tokaramide A, A new cathepsin B inhibitor from the marine sponge *Theonella* aff. *mirabilis*, *Bioorg Med Chem Lett,*1999, **9**, 3397-3402.

54 Chao Y, Fan C, Liang Y, Gao B and Zhang S, A Novel serpin with antithrombin-like activity in *Branchiostoma japonicum*: Implications for the presence of a primitive coagulation system, *PLos One,* 2012, **7**, e32392.

55 Alonso del Rivero M, Trejo S A, Reytor M L, Rodriguez de la Vega M, Delfin J, Diaz J, *et al*., Tri-domain bifunctional inhibitor of metallocarboxypeptidases A and serine proteases isolated from marine annelid *Sabellastarte magnifica*, *J Biol Chem*, 2012, **287**, 15427-15438.

56 Covaleda G, Alonso delRivero M, Chávez M A, Avilés F X and Reverter D, Crystal structure of novel metallocarboxypeptidase inhibitor from marine mollusc *Nerita versicolor* in complex with human carboxypeptidase A4, *J Biol Chem*, **2012**, 287, 9250-9258.

57 Hussain F, Fareed S, Ansari S and Sahid Khan M, Marine natural products: A lead of anticancer, *Indian J Geo-Mar Sci*, 2012, **41**, 27-39.

58 Reytor M L, González Y, Pascual I, Hernández A, Chávez M A and Alonso delRivero M, Screening of protease inhibitory activity in extracts of five Ascidian species from Cuban coasts, *Biotecnología Aplicada*, 2011, **28**, 77-82.

59 González L, Sánchez R E, Rojas L, Pascual I, García-Fernández R, Chávez M A, *et al*., Screening of protease inhibitory activity in aqueous extracts of marine invertebrates from Cuban Coast, *Am J Anal Chem*, 2016, **7**, 319-331.

60 von Nussbauma F and Li M J, Neutrophil elastase inhibitors for the treatment of (cardio) pulmonary diseases: Into clinical testing with pre-adaptive pharmacophores, *Bioorg Med Chem Lett*, 2015, **25**, 4370–4381.

61 Mambu L and Grellier P, Antimalarial compounds from traditionally used medicinal plants, *In*: Bioactive natural products: Detection, isolation, and structural determination, CRC Eds, 2007, 491-530.

62 Cragg G M and Pezzuto J M, Natural products as a vital source for the discovery of cancer chemotherapeutic and chemopreventive agents, *Med Princ Pract*, 2015, **25** (Suppl. 2), 41-59.

63 Newman D J and Cragg G M, Drugs and drug candidates from marine sources: An assessment of the current "State of play", *Planta Med*, 2016, **82**, 775-789.

64 Newman D J and Cragg G M, Marine-sourced anti-cancer and cancer pain control agents in clinical and late preclinical development, *Mar Drugs*, 2014, **12**, 255-278. .

65 Mayer A M S, Rodríguez A D, Berlinck R G S and Fusetani N, Marine pharmacology in 2007–8: Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous system, and other miscellaneous mechanisms of action, *Comp Biochem Phys*, 2011, **153,** 191–222.

66 Mayer A M S, Rodríguez A D, Taglialatela-Scafati O and Fusetani N, Marine pharmacology in 2009–2011: Marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action, *Drugs*, 2013, **11**, 2510-2573.

67 Mendiola J, Hernandez H, Sariego I, Rojas L, Otero A, Ramırez A, *et al.*, Antimalarial activity from three ascidians: An exploration of different marine invertebrate phyla, *Trans R Soc Trop Med Hyg*, 2006, **100**, 909-916.