Fish and Shellfish Immunology 86 (2019) 559–570

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

# Evidence for antimicrobial and anticancer activity of pituitary adenylate cyclase-activating polypeptide (PACAP) from North African catfish (*Clarias gariepinus*): Its potential use as novel therapeutic agent in fish and humans

Juana Maria Lugo[a,b], Carolina Tafalla[b], Ayme Oliva[c], Tirso Pons[d], Brizaida Oliva[e], Carolina Aquilino[b], Reynold Morales[a], Mario Pablo Estrada[a,*]

[a] Animal Biotechnology Department, Aquatic Biotechnology Group, Center for Genetic Engineering and Biotechnology, Havana, 10600, Cuba
[b] Fish Immunology and Pathology Group, Animal Health Research Center (CISA-INIA), Valdeolmos, 28130, Madrid, Spain
[c] Animal Biotechnology Department, Veterinary Clinical Research Group, Center for Genetic Engineering and Biotechnology, Havana, 10600, Cuba
[d] Structural Biology and BioComputing Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain
[e] Pharmaceutical Department. Laboratory of Cancer Biology. Center for Genetic Engineering and Biotechnology, Havana, 10600, Cuba

ARTICLE INFO

*Keywords:*
pacap
Fish
Antimicrobial peptides
Anticancer peptides
Cell-penetrating peptides
Bacterial and fungal pathogens
Cytotoxic activity
*In silico* studies

ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a regulatory neuropeptide that belongs to the secretin/glucagon superfamily, of which some members have shown antimicrobial activities. Contrasting to mammals, published studies on the action of PACAP in non-mammalian vertebrate immune system remain scarce. Some of our recent studies added this peptide to the growing list of mediators that allow cross-talk between the nervous, endocrine and immune systems in teleost fish. Regulation of PACAP and expression of its receptor genes has been demonstrated during an immune response mounted against acute bacterial infection in fish, though the direct effect of PACAP against fish pathogenic bacteria has never been addressed. Current work provides evidence of antimicrobial activity of *Clarias gariepinus* PACAP against a wide spectrum of Gram-negative and Gram-positive bacteria and fungi of interest for human medicine and aquaculture, in which computational prediction studies supported the putative PACAP therapeutic activity. Results also indicated that catfish PACAP not only exhibits inhibitory effects on pathogen growth, but also affects the proliferation of human non-small cell lung cancer cell line H460 in a dose-dependent manner. The observed cytotoxic activity of catfish PACAP against human tumor cells and pathogenic microorganisms, but not healthy fish and mammalian erythrocytes support a potential physiological role of this neuropeptide in selective microbial and cancer cell killing. All together, our findings extend the mechanisms by which PACAP could contribute to immune responses, and open up new avenues for future therapeutic application of this bioactive neuropeptide.

## 1. Introduction

The increased emergences of multi-resistant human pathogenic bacteria have become a worldwide problem [1]. Another global concern is the rise in the incidence of cancer [2–4]. Lung cancer is one of the most diagnosed forms of this disease [5]. Moreover, the intersection between infection and cancer is highlighted by the number of cancer deaths and new occurrences that are related to treatment or chronic infections [6]. This phenomenon is not only related to human healthcare, but can also be observed in agriculture and veterinary applications [7]. Fish are constantly challenged by a variety of pathogens, which not only shows detrimental effects on their health but antibiotic treatment of these disease outbreaks also increases the risk of pathogens becoming resistant to conventional antibiotics, which severely affects the aquaculture industry [8–10]. In recent years, a promising new class of molecules has been discovered that has some advantages against both of the above major world health concerns, such as better biocompatibility and target selectivity over conventional drugs (reviewed in Refs. [6,11]). Antimicrobial peptides (AMPs) are one such molecule (reviewed in Ref. [6]). These peptides are ancient defense molecules considered to be one of the key protective components of all evolutionary branches, providing the first line of defense against a wide range of microbial pathogens. AMPs possess a broad spectrum of antibiotic activity, as well as anti-inflammatory and immunomodulatory properties (reviewed in Refs. [7,12,13]). More recently, anticancer activity was also described for some of these peptides (reviewed in Ref.

* Corresponding author.
*E-mail address:* mario.pablo@cigb.edu.cu (M.P. Estrada).

https://doi.org/10.1016/j.fsi.2018.11.056
Received 22 August 2018; Received in revised form 19 November 2018; Accepted 23 November 2018
Available online 24 November 2018
1050-4648/ 

**Table 1**
Database and web tool information.

| Database/web tool | Uniform Resource Locator (URL) |
|---|---|
| PSI-BLAST | https://www.ebi.ac.uk/Tools/sss/psiblast |
| APD3 | http://aps.unmc.edu/AP/prediction/prediction_main.php |
| LAMP | http://biotechlab.fudan.edu.cn/database/lamp/tools.php |
| CAMPr3 | http://www.camp.bicnirrh.res.in/seqDb.php |
| AVPpred | http://crdd.osdd.net/servers/avppred/avpblast.php |
| AntiBP2 | http://www.imtech.res.in/raghava/antibp2/ |
| iAMPpred | http://cabgrid.res.in:8080/amppred/ |
| CancerPPD and AntiCP | http://crdd.osdd.net/raghava/anticp/ |
| ExPASy | http://web.expasy.org/compute_pi/ |
| I-TASSER | https://zhanglab.ccmb.med.umich.edu/I-TASSER/ |
| WHEEL | http://rzlab.ucr.edu/scripts/wheel/wheel.cgi |
| CellPPD | http://crdd.osdd.net/raghava/cellppd/index.html |
| CCPpred | http://bioware.ucd.ie/~testing/biowareweb/Server_page/cpppred.php |

[14]).

Neuropeptides are ancient signaling molecules, which are conventionally defined as peptide neurotransmitters but have recently been shown to be pleiotropic molecules that are integral components of the nervous and immune system [15]. Similarities in size, cationic charge or amphipathic design between some neuropeptides and AMPs suggest that they might serve an additional function in antimicrobial immunity [15]. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a multifunctional neuropeptide that belongs to the secretin/glucagon superfamily [16], of which some members has shown direct antimicrobial activity [17–20]. The vasoactive intestinal peptide (VIP)/PACAP system consists of two peptides and three receptors: VPAC1, VPAC2 and PAC1, belonging to family 2 of the G-protein coupled receptors [21]. PACAP plays many roles as a hypophysiotropic peptide, neurotransmitter, neuromodulator, vasodilator and neurotropic factor in mammals [22]. Its sequence has been remarkably conserved throughout evolution, from fish to mammals, suggesting that this peptide fulfils important biological functions in a broad spectrum of organisms [23].

Despite diverse studies concerning the immune functions of PACAP in mammals, where PACAP appears as an important endogenous immunomodulatory molecule exerting pro- [24–30] and anti-inflammatory properties (reviewed in Refs. [31,32]), information regarding the immune function of PACAP in aquatic organisms is still scarce [33–40]. In past studies, our research group demonstrated that administration of recombinant catfish PACAP, by either immersion bath or intraperitoneal injection, influences immune functions in larval and juvenile teleost fish [33,34]. Our published studies described, for the first time, PACAP immunoreactivity and mRNA expression in peripheral blood leucocytes from North African catfish (*Clarias gariepinus*) and Nile tilapia (*Oreochromis niloticus*) [34]. A different anatomical distribution of the two PACAP transcriptional splicing variants and its receptors in diverse lymphoid organs of both rainbow (*Oncorhynchus mykiss*) and brown (*Salmo trutta*) trout were also demonstrated [35,37]. Our research provided evidence of PACAP expression not only in the brain and intestine as have been previously reported in fish but also in other tissues related with the immune system, including in the central (thymus) and peripheral (spleen) lymphoid organs. PACAP receptor expression was demonstrated in primary immune tissues and in tissues that constitute the first barriers against pathogens in fish (skin and gills). All together, these results confirmed the hypothesis of PACAP action by an autocrine/paracrine mechanism to mediate immune function in fish, and added this peptide to the growing list of mediators that allow cross-talk between the neuroendocrine and immune systems in aquatic organisms [34,35,37,40]. We have also reported a regulation of the VIP/PACAP system during live bacterial challenge experiments in brown trout (*S. trutta*) [37], though the direct antimicrobial effect of PACAP or PACAP-family members against aquatic pathogens has never been published. As well, their activity as anticancer peptides (ACPs) remains to be properly investigated.

The present study aims to investigate the prospective antimicrobial and anticancer activity of the neuropeptide PACAP from North African catfish (*C. gariepinus*). PACAP minimal inhibitory concentration (MIC) and its half maximal inhibitory concentration ($IC_{50}$) were determined for a broad spectrum of pathogenic microorganisms of interest for human medicine and aquaculture. The antiproliferative effect of PACAP was evaluated and compared with the action of well-characterized therapeutic drugs in a model cell line derived from lung cancer. Based on the calculation of drug potency, the direct effect of PACAP on tumor cell growth was assessed. Computational prediction studies were conducted to the antimicrobial and anticancer activity of PACAP. Finally, their cytotoxic activity in healthy mammalian and fish red blood cells was also assessed. Overall, the current study contributes to a better understanding of PACAP function on the immune system, and stimulates renewed interest in PACAP as a new therapeutic agent for the treatment of microbial and cancer diseases.

## 2. Materials and methods

### 2.1. Computational analyses of catfish PACAP-38

The amino acid sequence of North African catfish (*C. gariepinus*) PACAP-38 was submitted to different webservers for an *in silico* characterization. Database and web tools used in this study are given in Table 1. Sequence analysis was done using PSI-BLAST [41] to search for sequence similarity against the UniProt database. We also searched for sequences similar to PACAP in anti-bacterial, antiviral, antifungal, and anticancer peptide databases (i.e., APD3 [42], LAMP [43], CAMPr3 [44], AVPpred [45], AntiBP2 [46], iAMPpred [47] and CancerPPD [48]).

The total net charge, theoretical isoelectric point (pI) and theoretical molecular mass of catfish PACAP-38 were calculated using the APD3 and Compute pI/Mw from ExPASy, given in Table 1. The three-dimensional (3D) structure of *C. gariepinus* PACAP was predicted using the I-TASSER method [49]. The Shiffer-Edmundson helical wheel diagram was predicted using a web tool (WHEEL), specified in Table 1.

Prediction of the catfish PACAP-38 regions with similarity to cell-penetrating peptides (CPP) was done by using the CellPPD method [50]. To complement the CellPPD results the predicted PACAP38 regions were also submitted to an independent neural-network method, CPPpred [51]. CPPpred limits the input to peptide sequences with 5–30 residues in length. A CPPpred score of 1 means very likely to be cell penetrating peptide. A CPPpred score of 0 means very unlikely to be cell penetrating peptide.

### 2.2. Peptides and chemotherapeutic agents

The sequence of the North African catfish (*C. gariepinus*) PACAP

used in this study was HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYR-QRFRNK-$NH_2$, an amidated form of the peptide with 38 amino acids [52]. Catfish PACAP38 was purchased from Cuseko International CO., LTD (Seongnam, South Korea). In brief, PACAP was synthesized on a solid phase support and purified by reverse phase high performance liquid chromatography (RP-HPLC) to > 75% purity and confirmed by ion-spray mass spectrometry. The peptide was re-purified by RP-HPLC with a purity ≥90%, complying with the batch release criteria approved for this peptide by the Quality Control Department of the Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba. In short, analytical separation was achieved in a RP C18 column (Vydac, 4.6 × 150 $mm^2$, 5 μm). A linear gradient from 5 to 60% of solvent B for 35 min at 0.8 ml/min flow rate, with UV detection at 226 nm, was used. Solvent A was 0.1% (v/v) TFA in water. Solvent B was 0.05% (v/v) TFA in acetonitrile. The software UNICORN 4.11 (GE Healthcare, USA) was used for processing the data from RP-HPLC chromatograms. Additionally, an *in vivo* growth experiment assay using fish larvae was also conducted successfully, as one of the standard biological release criteria approved for this substance (as described in Ref. [52]).

CIGB-552, an anticancer peptide in phase I clinical trials by the CIGB and Heber Biotec (Havana, Cuba), with sequence Ac-HARIKPTFRRLKWKYKGKFW (where proline and leucine are D-amino acids, and the N-terminal was blocked by acetylation) was synthesized at CIGB (Havana, Cuba) at analytical scale. The peptide synthesis was carried out using manual parallel Fmoc/tBu solid phase chemistry, with the peptide then purified by RP-HPLC to > 95% purity using an acetonitrile/$H_2O$-trifluoroacetic acid gradient and confirmed by ion-spray mass spectrometry [53].

The clinical grade chemotherapeutic drugs cisplatin and paclitaxel were kindly provided by the Oncology Service of the National Institute of Oncology and Radiology, Havana, Cuba.

### 2.3. Antimicrobial assays

#### 2.3.1. Maintenance of microorganisms

All microorganisms used in this study were prepared and grown following supplier instructions for biological risk 1 (non-pathogenic) and 2 (pathogenic) groups.

*2.3.1.1. Human host bacteria and fungi.* All human host bacteria and fungi were provided by the Central Collection of Microorganisms, CIGB, Cuba. *Bacillus subtilis* (ATCC 6333), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739) and *Salmonella* sp. strains were maintained on Müller-Hinton agar plates (BioCen, Cuba). *Candida albicans* (ATCC 10231) was maintained on Sabouraud dextrose agar plates (Oxoid, UK). For liquid culture, the bacteria were grown in Tryptic Soy broth and *C. albicans* was grown in Sabouraud dextrose broth.

*2.3.1.2. Fish host bacteria.* All fish host bacteria were provided by the Central Veterinary Laboratory, Algete, Madrid, Spain. *Yersinia ruckeri* (4319/03), *Aeromonas hydrophila* (4387/12), *Aeromonas sobria* (02/12), *Aeromonas salmonicida* (4387/12) strains were maintained on Luria-Bertani (LB) agar plates (Sigma-Aldrich, Germany). For liquid culture, the bacteria were grown in LB broth.

#### 2.3.2. Broth microdilution method

The minimal inhibitory concentration (MIC) of PACAP was measured for each strain of microorganism listed above by a broth microdilution method [54]. The MIC is defined as the lowest concentration of an antimicrobial agent at which bacterial growth is not detected. Briefly, logarithmic phase microorganism cultures were diluted in the broth according to the microorganism to an optical density at 600 nm (OD600) of 0.001, which is approximately equivalent to $10^5$ cfu $ml^{-1}$. Diluted microorganisms (90 μl) were mixed with 10 μl of water (negative control), antibiotic (positive control) or PACAP in wells of polypropylene microtiter plates (Greiner Bio-One, Germany). PACAP was two-fold serially diluted. The growth was monitored, after an overnight incubation at 37 °C or 28 °C according to the microorganism, by measuring the change in the absorbance of the culture at 600 nm using a microplate reader. Visual verification of microbial sedimentation as well as absorbance readings (600 nm) confirmed the MICs. In addition, we determined the half maximal inhibitory concentration ($IC_{50}$). $IC_{50}$ is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process by half. The MIC and IC50 were expressed as the absolute value of the mean of at least two determinations in triplicate.

### 2.4. In vitro cell cytotoxicity assay

#### 2.4.1. Fish and human peripheral blood extraction

Juveniles of Nile tilapia (*O. niloticus*) were provided by the Mamposton Aquaculture Research Station, Havana, Cuba, and were kept alive in aerated freshwater, between 28 °C and 30 °C, under natural photoperiod. Animals were anesthetized with methanosulfonate salt of 3-aminobenzoic ethyl ester (Sigma, USA) dissolved in water, and blood was collected from the caudal vessels using a syringe. Blood was also collected from anonymous human donors. Venipuncture and blood collection were performed by a trained phlebotomist in order to minimize the risk to the donors. All procedures were previously approved by the Ethics Committee of the CIGB, Havana, Cuba.

#### 2.4.2. Hemolytic assay

The ability of PACAP to lyse erythrocytes was determined using human and fish red blood cells as follows. Freshly packed human or fish erythrocytes (5 ml) were washed with phosphate-buffered saline (pH 7.4) until the supernatant was colorless and re-suspended in phosphate-buffered saline (50 ml) supplemented with glucose (0.2%, w/v). PACAP (serially diluted in phosphate buffered saline) were added to 90 μl of a 1% erythrocyte suspension (1:10 dilution of washed erythrocytes) in microcentrifuge tubes. The samples were incubated for 30 min at 37 °C with gentle shaking and centrifuged for 10 min at 3500 rpm at room temperature. The supernatants (70 μl) were transferred to a microtiter plate, and the OD was determined at 405 nm. We used samples with erythrocytes without peptide treatment (control of basal hemolysis) and samples with erythrocytes incubated with 0.1% of sodium dodecyl sulfate (SDS) (control of 100% hemolysis). Two individual experiments using triplicate samples were conducted. We calculated the percentage of hemolysis using the formula: % hemolysis = (absorbance units of the erythrocytes exposed to peptide × 100)/absorbance units of the erythrocytes exposed to SDS.

### 2.5. In vitro cell proliferation assay

#### 2.5.1. Cell culture and peptide/drug treatment

The non-small cell lung cancer cell line H460 (ATCC, HTB-117) was maintained at 37 °C and 5% $CO_2$ in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone, USA) plus 30 μg $ml^{-1}$ of gentamycin (Gibco, USA). H460 cells were seeded at 4 × $10^4$ cells per well on 24-well culture plates (Corning Costar) and incubated for 24 h. On the next day, peptide concentrations ranging from 150 to 9.37 μM were added to the plates and incubated for 48 h at 37 °C in 5% $CO_2$. Cisplatin and paclitaxel chemotherapeutic drugs were used as cell proliferation controls. The effect of each peptide on cell proliferation was monitored by Crystal Violet Assay.

#### 2.5.2. Crystal violet assay

Crystal violet is a triarylmethane dye that can bind to ribose type molecules such as DNA in nuclei. Normally, dead adherent cells will detach from cell culture plates and will be removed from the viable cell

population during washing steps. Crystal violet staining can be used to quantify the total DNA of the remaining population and thus determine cell viability. In brief, a crystal violet staining solution was added to each well and cells were stained for 20 min at room temperature. After washing, the plates were dried for at least 2 h at room temperature. Subsequently, 500 µl of methanol were added to each well, and incubated for 20 min at room temperature on a bench rocker with a frequency of 20 oscillations per minute. Finally, the OD of each well was measured at 570 nm (OD570) with a plate reader. Background corresponding to the absorbance of wells without cells was subtracted. Each sample point was done in duplicate, and experiments were carried out twice. The percentage of cytotoxicity (cell growth inhibition) was calculated using the formula below: % Cytotoxicity = [OD570 (negative control) - OD570 (sample)] × 100%/OD (negative control). Where: OD570 (negative control) is the OD at 570 nm of the negative control after background correction and OD570 (sample) is the OD at 570 nm of the tested agents after background correction.

### 2.6. Data analysis

All data analysis and chart creation was performed using either GraphPad Prism Statistical Software version 4.00 (GraphPad Software Inc., San Diego CA, USA), or CalcuSyn software (Biosoft, Cambridge, UK). The Mann Whitney *U* test was used to evaluate differences between PACAP and negative control (untreated microorganism). A Kruskal-Wallis analysis followed by a Dunn's test was used to evaluate differences among PACAP, CIGB-552 and negative control (untreated H460 cells). Differences were considered to be significantly different if $p < 0.05$.

## 3. Results

### 3.1. In silico approaches to predict antimicrobial, anticancer and cell-penetrating features of the C. gariepinus PACAP molecule

#### 3.1.1. Searching for PACAP sequence similarity against different antimicrobial and anticancer databases

PSI-BLAST sequence similarity searches for African catfish (*C. gariepinus*) PACAP, against UniProt, revealed 43 proteins with scores better than threshold (E-value lower than 1.0e-03), coverage of the query sequence > 60% and sequence identity > 20%, after five iterations (Fig. 1A). Computational results confirm that *C. gariepinus* PACAP exhibits a high sequence identity with other members of the secretin/glucagon superfamily. Catfish PACAP shows a high sequence identity with VIP (more than 50% identity), thus identifying PACAP as a member of the same family of regulatory peptides (Fig. 1A). As is known, the sequence of this bioactive peptide has been remarkably conserved during evolution. In past studies, we disclosed that the amino acid sequence of the *C. gariepinus* PACAP is highly homologous (more than 80% identity) to sequences previously identified from tunicates to mammals [52]. The positively charged C-terminal extension, PACAP (28–38), is the more variable. However, the strong preservation of the N-terminal primary sequence (residues 1–27) of PACAP throughout evolution clearly suggests an important role of this region for its biological activity. In accord with this observation, we identified that PACAP-38 residues $Val^{19}$, $Leu^{23}$, $Val^{26}$ and $Leu^{27}$ are positioned in a highly conserved region on the same hydrophobic surface of an α-helix, according to the antimicrobial database, APD3, predictions. A second conserved region coincides with the N-terminal (residues $His^{1}$, $Gly^{4}$, $Phe^{6}$, $Asp^{8}$) of PACAP-38 (Figs. 1 and 2A). We also identified similar sequences in antibacterial, antiviral, antifungal, and anticancer peptide databases (i.e., APD3, LAMP, CAMPr3, AVPpred, and CancerPPD). Multiple sequence alignment of the similar peptides is shown in Fig. 1B. PACAP from *C. gariepinus* aligned well (67.9% identity) to human VIP (ID: AP01477, L02A001477 and CAMPSQ3268 from antimicrobial databases APD3, LAMP and CAMPr3, respectively) (Fig. 1B). PACAP also aligned (14.3% identity) to human cathelicidin LL-37 (ID: AVP_0305 from anti-viral database AVPpred) (Fig. 1B). As well, a 36.8% identity was observed with respect to the Tat-a5 anticancer peptide (ID: Tat-a5 from anticancer peptide database CancerPPD) (Fig. 1B). Thus, different web tools suggested that PACAP-38 possesses antimicrobial (CAMPr3 score: 0.67; AntiBP2 score: 1.08; iAMPpred score: 0.79) and anticancer (AntiCP score: 0.73) activities (score > 0.5 means peptide sequence predicted in positive class and otherwise in the negative class). In addition, the 'Design-Peptide' module of AntiCP suggests single mutations in the amino terminal of PACAP-38 that could increase the anticancer activity of this peptide (residues in bold in Fig. 1C). The AntiCP SVM-score of 0.75 is the highest score among all possible single mutations generated by the 'Design-Peptide' module. Indeed, the AntiCP SVM-score for the wild-type sequence of PACAP-38 is 0.73.

#### 3.1.2. Structural and physicochemical analysis

Analysis of *C. gariepinus* PACAP-38 primary structure reveals the high cationic nature of the peptide, which displays a net charge of +9 at physiological pH. The theoretical molecular mass of the peptide was 4667.38 Da and theoretical pI was 11.03, reinforcing the observation that it is a strongly basic peptide. Surface electrostatic potential representation of catfish PACAP-38 is shown in Fig. 2C. The three-dimensional (3D) structure of catfish PACAP-38 was predicted by the I-TASSER method (Fig. 2A). Eight of the top ten alternative alignments used by I-TASSER corresponded to the NMR structure of human PACAP-38 (PDB ID: 2d2p; UniProt ID: PACA_HUMAN), which shares 89% sequence identity in the modeled region. The estimated RMSD value for the 3D model was 1.8 ± 1.5 Å. Catfish PACAP was predicted to adopt an α-helical conformation (Fig. 2A and C). In addition, Shiffer-Edmundson helical wheel modeling was used to predict hydrophobic and hydrophilic regions in the secondary structure of catfish PACAP-38. This mature peptide showed hydrophobic and hydrophilic residues on opposite sides of the α-helix (Fig. 2C).

#### 3.1.3. Prediction of the catfish PACAP regions with cell-penetrating properties

Prediction of the catfish PACAP-38 regions with similarity to CPP, using the CellPPD method, disclosed four putative regions (Fig. 2A). The predicted regions were also submitted to an independent neural-network method, CPPpred. The CPPpred scores for the predicted CellPPD PACAP-38 regions are shown in parenthesis: LPAVL (0.515), RRYRQRFRNK (0.849), GRRYRQRFRN (0.773), and LGRRYRQRFR (0.818). A CPPpred score near to 1 means peptide sequence very likely to be cell penetrating, while 0 means very unlikely to be cell penetrating. Overall CPP prediction results, together to the high polycationic nature of PACAP, as well as its tendency to adopt an α-helical conformation, constitute key CPP elements supporting the notion that PACAP-38 has cell-penetrating properties.

### 3.2. Antimicrobial activity of C. gariepinus PACAP

The antimicrobial activity of *C. gariepinus* PACAP against each strain of microorganism listed in Table 2 was determined. PACAP was tested against Gram-negative and Gram-positive bacteria for bactericidal activity. Bacteria were chosen to represent both fish and human pathogens. PACAP was also tested against an opportunistic pathogenic yeast *C. albicans*. Results show that this peptide is active against both Gram-negative and Gram-positive bacteria and fungi (Table 2). PACAP over a range of concentrations up to 300 µM displayed variable degrees of antimicrobial activity against all the microorganisms tested, with the exception of *S. aureus* (Table 2). Most notably, we observed that fish pathogenic bacteria *Y. ruckeri*, *A. salmonicida* and *A. sobria*, non-pathogenic bacterium *B. subtilis* and human opportunistic yeast *C. albicans* were the most sensitive to PACAP, while fish pathogenic bacterium *A. hydrophila* and human pathogens *S. aureus*, *E. coli*, *P. aeruginosa* and *Salmonella* sp were the most resistant (Table 2). In summary, *C.*

**(A) Sequence similarities from UniProt**

| ID | ACC | | SEQUENCES | | IDENTITY |
|---|---|---|---|---|---|
| PACAP_38 | | 1 | HSDGIFTDSYSRYRKQMA**V**KKY**L**AA**VL**GRRYRQRFRNK | 38 | |
| PACA_MOUSE | O70176 | 131 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNKG-- | 169 | 89,5% |
| PACA_PIG | P41535 | 132 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNKG-- | 170 | 89,5% |
| PACA_HUMAN | P18509 | 132 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNKG-- | 170 | 89,5% |
| PACA_BOVIN | Q29W19 | 132 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNKG-- | 170 | 89,5% |
| PACA_RAT | P13589 | 131 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNKG-- | 169 | 89,5% |
| PACA_PELRI | Q09169 | 127 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRIKNKG-- | 165 | 89,5% |
| PACA_CHICK | P41534 | 131 | HIDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNKG-- | 169 | 86,8% |
| PACA_CLAMA | P48144 | 130 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNKGR | 169 | 100% |
| PACA_SHEEP | P16613 | 132 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNKG-- | 170 | 89,5% |
| PACA_ONCNE | P41585 | 129 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNKG-- | 167 | 94,7% |
| PACA_HELSU | P0DJ95 | 24 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQ-------- | 56 | 93,9% |
| PACA_URAJA | P81039 | 1 | HSDGIFTDSYSRYRKQMAVQKYLAAVLGRRYRQRVRNK--- | 38 | 94,7% |
| SECR_PIG | P63298 | 30 | HSDGTFTSELSRLRDSARLQRLLQGLVGKR-SQQDPE---- | 65 | 37,8% |
| SECR_HUMAN | P09683 | 28 | HSDGTFTSELSRLREGARLQRLLQGLVGKR-SEQDAE---- | 63 | 37,8% |
| SECR_RAT | P11384 | 33 | HSDGTFTSELSRLQDSARLQRLLQGLVGKR-SEEDTENIPE | 72 | 32,4% |
| SECR_MOUSE | Q08535 | 32 | HSDGMFTSELSRLQDSARLQRLLQGLVGKR-SEQDTENIPE | 71 | 35,1% |
| SECR_CHICK | P01280 | 1 | HSDGLFTSEYSKMRGNAQVQKFIQNLM-------------- | 27 | 40,7% |
| SLIB_RAT | P09916 | 31 | HADAIFTSSYRRILGQLYARKLLHEIMNRQQGERNQEQ--R | 69 | 34,2% |
| SLIB_MOUSE | P16043 | 31 | HVDAIFTTNYRKLLSQLYARKVIQDIMNKQ-GERIQEQ--R | 68 | 21,1% |
| SLIB_BOVIN | P63292 | 31 | YADAIFTNSYRKVLGQLSARKLLQDIMNRQQGERNQEQGAK | 71 | 29,0% |
| SLIB_HUMAN | P01286 | 32 | YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGAR | 72 | 26,3% |
| SLIB_MESAU | Q60549 | 31 | YADAIFTSSYRKVLGQLSARKLLQDIMSRQQGERNQEQGPR | 71 | 29,0% |
| SLIB_CYPCA | P42692 | 1 | HADGMFNKAYRKALGQLSARKYLHTLMAKRVGGGSMIEDDN | 41 | 31,3% |
| SLIB_SHEEP | P07217 | 1 | YADAIFTNSYRKILGQLSARKLLQDIMNRQQGERNQEQGAK | 41 | 29,0% |
| SLIB_CAPHI | P63293 | 1 | YADAIFTNSYRKVLGQLSARKLLQDIMNRQQGERNQEQGAK | 41 | 29,0% |
| SLIB_PIG | P01287 | 1 | YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGAR | 41 | 29,0% |
| EXE1_HELSU | P04203 | 1 | HSDATFTAEYSKLLAKLALQKYLESILGSSTSPRPPSS--- | 38 | 46,4% |
| EXE2_HELSU | P04204 | 47 | HSDAIFTEEYSKLLAKLALQKYLASILGSRTSPPPPSR--- | 84 | 42,1% |
| VIP_BOVIN | P81401 | 125 | HSDAVFTDNYTRLRKQMAVKKYLNSILNGKRSSE------- | 158 | 51,4% |
| VIP_MOUSE | P32648 | 125 | HSDAVFTDNYTRLRKQMAVKKYLNSILNGKRSSE------- | 158 | 51,4% |
| VIP_RAT | P01283 | 125 | HSDAVFTDNYTRLRKQMAVKKYLNSILNGKRSSE------- | 158 | 51,4% |
| VIP_HUMAN | P01282 | 125 | HSDAVFTDNYTRLRKQMAVKKYLNSILNGKRSSE------- | 158 | 51,4% |
| VIP_MELGA | P45644 | 129 | HSDAVFTDNYSRFRKQMAVKKYLNSVLTGKRSQEELNPA-- | 167 | 62,2% |
| VIP_CHICK | P48143 | 129 | HSDAVFTDNYSRFRKQMAVKKYLNSVLTGKRSQEELNPA-- | 167 | 62,2% |
| VIP_PIG | P01284 | 45 | HSDAVFTDNYTRLRKQMAVKKYLNSILNGKR---------- | 75 | 61,3% |
| VIP_RABIT | P32649 | 45 | HSDAVFTDNYTRLRKQMAVKKYLNSILN------------- | 72 | 67,9% |
| VIP_CANFA | P63289 | 1 | HSDAVFTDNYTRLRKQMAVKKYLNSILN------------- | 28 | 67,9% |
| VIP_DIDVI | P39089 | 1 | HSDAVFTDSYTRLLKQMAMRKYLDSILN------------- | 28 | 60,7% |
| VIP_AMICA | P84771 | 1 | HSDAIFTDNYSRFRKQMAVKKYLNSVLT------------- | 28 | 81,5% |
| VIP_SCYCA | P09685 | 1 | HSDAVFTDNYSRIRKQMAVKKYINSLLA------------ | 28 | 67,9% |
| VIP_CAVPO | P04566 | 45 | HSDALFTDTYTRLRKQMAMKKYLNSVLN------------- | 72 | 67,9% |
| VIP_ALLMI | P48142 | 1 | HSDAVFTDNYSRFRKQMAVKKYLNSVLT------------- | 28 | 77,8% |
| VIP_GADMO | P09684 | 1 | HSDAVFTDNYSRFRKQMAAKKYLNS----------------- | 25 | 72,0% |

**(B) Anti-Gram$^{+}$/Gram$^{-}$, antiviral, antifungal, and anticancer peptides**

| ID | DATABASE | SEQUENCES | IDENTITY | PubMed ID |
|---|---|---|---|---|
| AP01477 | APD3 | HSDAVFTDNYTRLRKQMAVKKYLNSILN | 67.9% | PMID: 18603306 |
| L02A001477 | LAMP | HSDAVFTDNYTRLRKQMAVKKYLNSILN | 67.9% | PMID: 18603306 |
| CAMPSQ3268 | CAMPr3 | HSDAVFTDNYTRLRKQMAVKKYLNSIL | 67.9% | PMID: 18603306 |
| AVP_0305 | AVPpred | ----LLGDFFRKSKEK**ir**IKDFLRNLVPRTES------ | 14.3% | PMID: 16020269 |
| Tat-a5 | CancerPPD | ---------------KAQIRAMECNILGRKKRRQRRR- | 36.8% | PMID: 18656352 |

**(C) Anticancer peptides predictions by antiCP tool**

| ID | SEQUENCES | POS | Score |
|---|---|---|---|
| PACAP-38 | HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | - | 0.73 |
| Mutant-1 | **F**SDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 1 | 0.75 |
| Mutant-2 | **I**SDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 1 | 0.75 |
| Mutant-3 | **K**SDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 1 | 0.75 |
| Mutant-4 | **R**SDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 1 | 0.75 |
| Mutant-5 | HSD**A**IFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 4 | 0.75 |
| Mutant-6 | HSD**C**IFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 4 | 0.75 |
| Mutant-7 | HSD**L**IFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 4 | 0.75 |
| Mutant-8 | HSD**M**IFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 4 | 0.75 |
| Mutant-9 | HSD**V**IFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 4 | 0.75 |
| Mutant-10 | HSD**W**IFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 4 | 0.75 |
| Mutant-11 | HSDGI**E**TDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 6 | 0.75 |
| Mutant-12 | HSDGI**R**TDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 6 | 0.75 |
| Mutant-13 | HSDGIFT**E**SYSRYRKQMAVKKYLAAVLGRRYRQRFRNK | 8 | 0.75 |

*(caption on next page)*

**Fig. 1.** Multiple sequence alignment of *C. gariepinus* PACAP-38 and similar sequences annotated in databases. PACAP-38: *C. gariepinus* PACAP having 38 amino acids. PACA: PACAP. SECR: secretin. SLIB: Somatoliberin or Growth hormone-releasing hormone. EXE: exendin or VIP-like peptide. Numbers in the SEQUENCES column indicate the start and end positions of the peptides. Highlighted amino acids in *C. gariepinus* PACAP-38 ($Val^{19}$, $Leu^{23}$, $Val^{26}$, $Leu^{27}$) correspond to residues on the same hydrophobic surface of the α-helix. The bold and lower-case letters '**ir**' in AVP_0305 indicates an insertion of 9 residues (**i**GKEFKRIVQ**r**) in the peptide sequence. ID: peptide identifier. ACC: accession number in UniProt. SEQUENCES: amino acid sequence of the peptide. IDENTITY: percent of sequence identity. Mutant (1–13): single-mutations in the amino terminal of PACAP-38 that could increase the anticancer activity of this peptide. The mutant amino acids are representing in bold. POS: position of the mutation in the amino acid sequence of PACAP-38. Score: AntiCP SVM-score.

*gariepinus* PACAP was more active against fish Gram-negative pathogenic bacteria (Fig. 3) than it was for human pathogenic microorganisms, with the exception of *C. albicans*.

### 3.3. Hemolytic activity of C. gariepinus PACAP against fish and human red blood cells

To estimate the potential cytotoxic effect of *C. gariepinus* PACAP against fish and mammalian red blood cells, a hemolytic assay was performed, at the same concentrations tested in the antimicrobial assays. Interestingly, catfish PACAP was less cytotoxic for human cells than for fish cells. In any case, the hemolytic activity reached 50% hemolysis (Fig. 4A and B). PACAP was not hemolytic for fish red blood cells at concentrations below 18.75 μM (0% hemolysis). At concentration between 18.75 μM and 150 μM, the hemolytic activity was less than 20%. PACAP reached a top value of 39% hemolysis at the highest concentration analyzed (300 μM) (Fig. 4A). On the other hand, PACAP was not hemolytic for human erythrocytes at concentrations below 75 μM (0% hemolysis). At concentration between 75 μM and 150 μM the hemolytic activity was less than 3%. PACAP reached a top value of 6% hemolysis at the highest concentration analyzed (300 μM) (Fig. 4B).

### 3.4. Effect of C. gariepinus PACAP on cell proliferation in human lung cancer cells H460

To investigate the putative selectivity of neuropeptide PACAP for

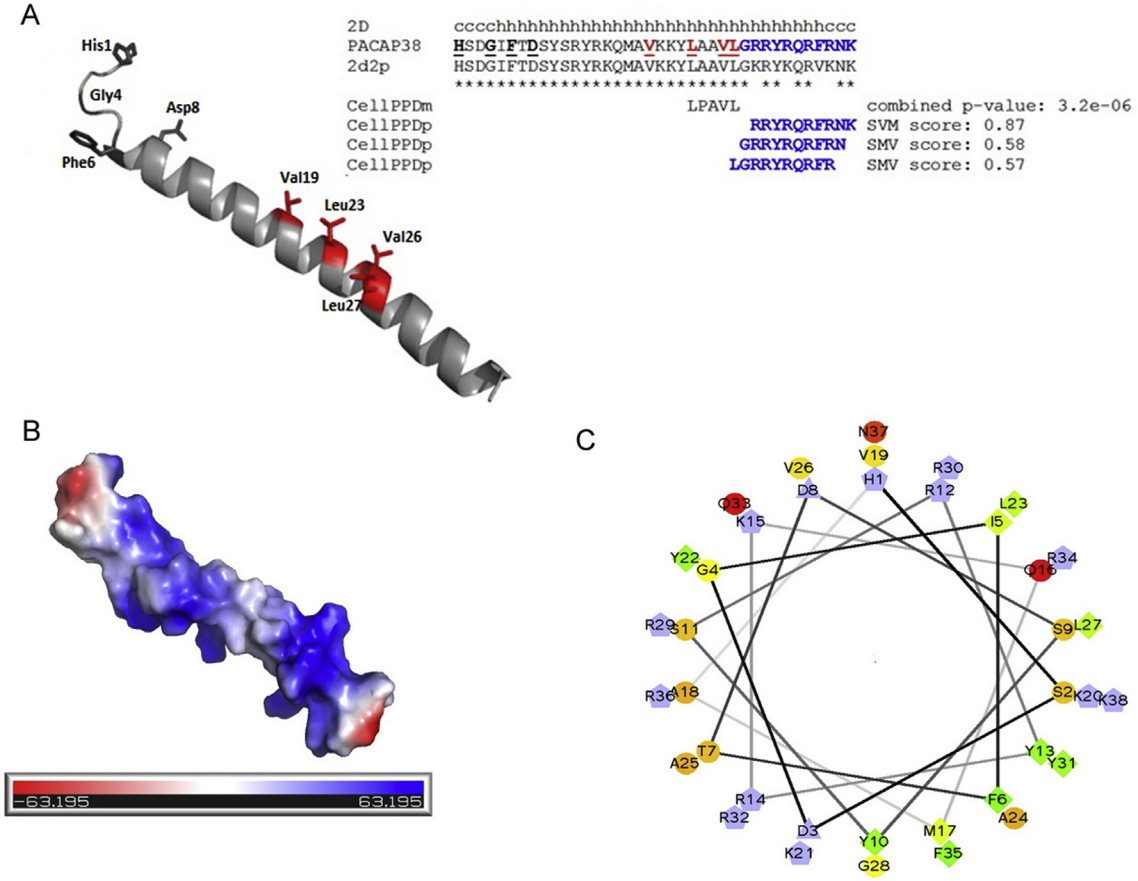


**Fig. 2.** Structural and physicochemical analyses of *C. gariepinus* PACAP-38. (A) Ribbon representation of the three-dimensional structural (3D) model and structure-based sequence alignment by the I-TASSER method. (Inset in A) Amino acids in red and underlined ($Val^{19}$, $Leu^{23}$, $Val^{26}$, $Leu^{27}$) correspond to residues on the same hydrophobic surface of the α-helix. Amino acids in black and underlined ($His^{1}$, $Gly^{4}$, $Phe^{6}$, $Asp^{8}$) correspond to another conserved region with putative AMP significance at the N-terminal of PACAP-38. Amino acids in blue correspond to catfish PACAP-38 region with similarity to cell-penetrating peptides, predicted by using the CellPPD method. The Motif Scanning option (CellPPDm) and Protein Scanning option (CellPPDp) scores are shown. (B) Surface electrostatic potential representation of catfish PACAP-38. The surface is colored according to the electrostatic potential: negative regions (in red), positive regions (in blue) and neutral regions (in gray). The orientation of the surface electrostatic potentials is the same as that in the ribbon representation. We also provide a color intensity scale to better represent the electrostatic potential. These figures were prepared with the PyMOL Molecular Graphics System, Schrödinger, LLC. (C) A Schiffer-Edmundson helical wheel diagram for catfish PACAP-38. Residues are numbered consecutively from the N-terminus to the C-terminus. Circles: hydrophilic residues. Diamonds: hydrophobic residues. Triangles: potentially negatively charged residues. Pentagons: potentially positively charged residues. Hydrophobicity is color coded as well: the most hydrophobic residue is green, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red. The potentially charged residues are light blue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Table 2**
Antimicrobial spectrum of the African catfish (*Clarias gariepinus*) PACAP against Gram-negative and Gram-positive bacteria and fungi from both fish and humans. The half maximal inhibitory concentration ($IC_{50}$) was calculated from each cell survival curve using GraphPad Prism software version 4.00, (GraphPad Software Inc., San Diego CA, USA). Data represents the absolute value of the mean of at least two independent experiments in triplicate.

| Organism hosts | Microorganisms | Most common diseases | PACAP as antimicrobial agent (μM) | |
|---|---|---|---|---|
| | | | MIC[a] | $IC_{50}$ |
| Fish | *Yersinia ruckeri* (Gram −) | Enteric red mouth disease | 50 | 0.023 |
| | *Aeromonas hydrophila* (Gram −) | Hemorrhagic septicemia | 300 | 7.2 |
| | *Aeromonas sobria* (Gram −) | Septicemia | 100 | 28.6 |
| | *Aeromonas salmonicida* (Gram −) | Furunculosis | 50 | < 3.125 |
| Human | *Bacillus subtilis* (Gram +) | Is not a disease causing agent | 5 | 0.76 |
| | *Staphylococcus aureus* (Gram +) | Pneumonia, meningitis, osteomyelitis, endocarditis, bacteremia, sepsis | > 300 | NE[b] |
| | *Pseudomonas aeruginosa* (Gram −) | Airway, urinary tract, burns, wounds and blood infections | 300 | 54.4 |
| | *Escherichia coli* (Gram −) | Gastroenteritis, urinary tract infections, neonatal meningitis | > 300 | 46.6 |
| | *Salmonella* sp. (Gram −) | Gastrointestinal disease | 300 | 116.8 |
| | *Candida albicans* | Candidiasis, nosocomial infections | 75 | 36.9 |

[a] The minimal inhibitory concentration.
[b] Not effect in bacteria growth was observed.

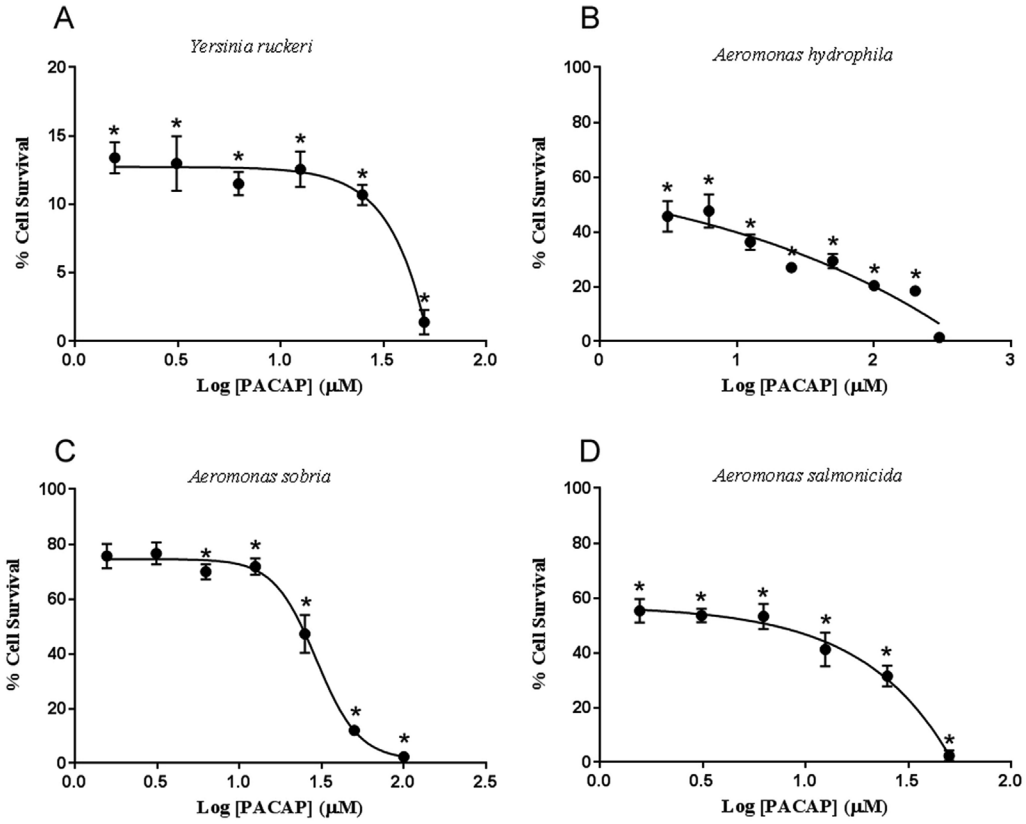


**Fig. 3.** PACAP dose-response curve against fish pathogenic bacteria. (A) *Y. ruckeri*, (B) *A. hydrophila*, (C) *A. sobria* and (D) *A. salmonicida*. Percentage of cell survival was plotted versus the logarithm of treatment concentrations. Data were evaluated using a Mann Whitney *U* test. * Statistically significant differences between PACAP-treated bacteria and untreated bacteria ($p < 0.05$). Data points represent the mean ± SD of at least two determinations in triplicate.

tumor cells, we evaluated the effect of *C. gariepinus* PACAP on the growth of human lung cancer cell line H460 and compared it with the action of the anticancer peptide CIGB-552 and two different chemotherapeutic drugs used in the treatment of lung cancer (cisplatin and paclitaxel). Our results indicated that PACAP not only exhibits inhibitory effects on pathogen growth, but also affects the proliferation of human lung cancer cell line H460 (Fig. 5 and Table 3). As shown in Fig. 5A, the fraction of tumor cells affected by PACAP (Fa) increased in a dose-dependent manner. The fitting of the dose-response antiproliferative curve to the median-effect equation, using the CalcuSyn software, allowed us to estimate the $IC_{50}$, also referred to as drug potency, and the steepness (m) of the curve (PACAP $IC_{50}$ = 13.17 μM and m: 0.70). Thus, whereas CIGB-552 showed more than 50% of inhibition of cell growth at ≥ 37.5 μM (66.8% inhibition) ($p < 0.05$), PACAP exhibited an even greater effect on cell growth at ≥ 18.75 μM (72.9% inhibition) ($p < 0.05$) (Fig. 5B). Both peptides showed an equivalent activity at 75 μM (79.5% and 79.9% cell growth inhibition by PACAP and CIGB-552, respectively). Conventional drugs, cisplatin and paclitaxel inhibited cell proliferation at ≥ 20 μM (81.6% and 87.2%, respectively) (data not shown). The $IC_{50}$ value was also calculated from dose-response antiproliferative curves generated by fitting the data derived from the crystal violet assay (growth inhibition % versus concentration data) using GraphPad Prism software. As expected, due to the diverse nature of the tested compounds, the results showed that these drugs differ in their potency against human lung cancer H460 cell growth (Table 3). Interestingly, catfish PACAP showed comparable potency as CIGB-552 anticancer peptide (Table 3). We obtained similar $IC_{50}$ estimated values for PACAP when using either CalcuSyn or

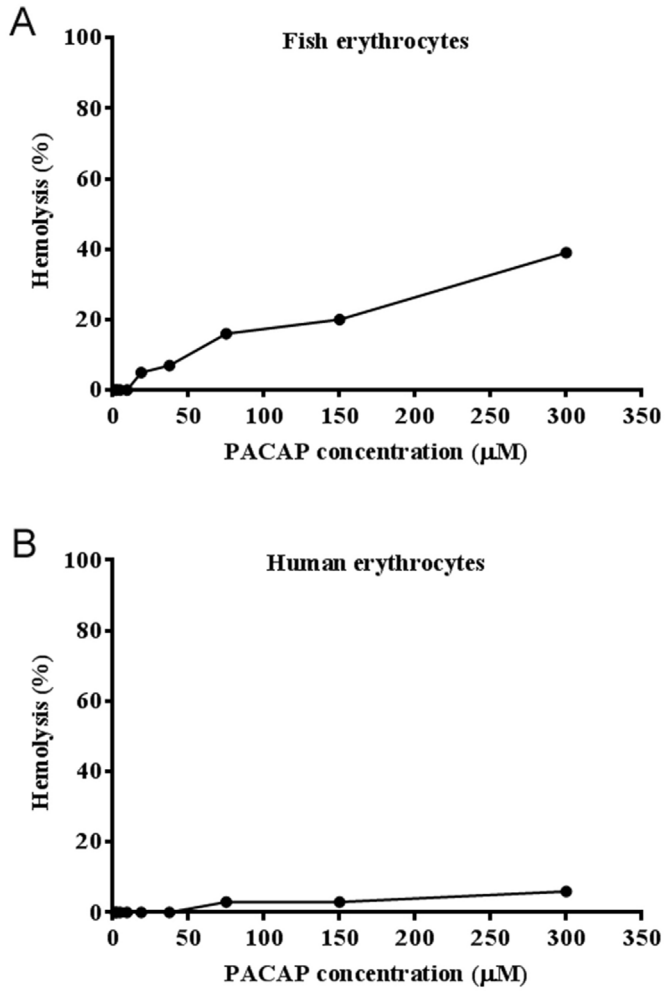


**Fig. 4.** Hemolytic activity of *C. gariepinus* PACAP against fish (A) and human (B) red blood cells. Two individual experiments using triplicate samples were conducted. The percentage of hemolysis was defined relative to the hemolysis obtained with the erythrocyte suspension treated with 0.1% SDS (100% hemolysis).

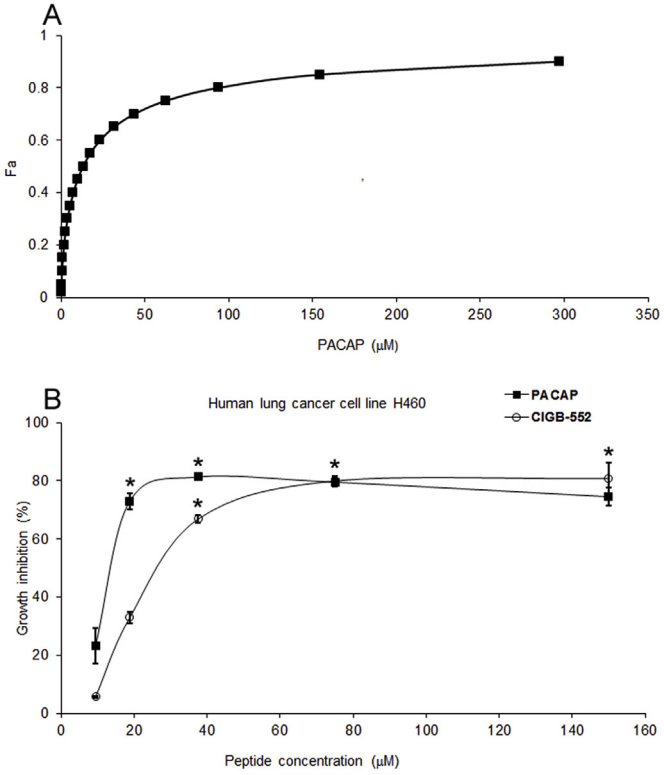


**Fig. 5.** Antiproliferative dose-response curves for *C. gariepinus* PACAP, CIGB-552 anticancer peptide and conventional chemotherapeutic drugs assessed as single agents in the human non-small cell lung cancer cell line H460. Catfish PACAP at concentrations ranging from 150 to 9.37 μM was incubated for 48 h, and its effect in tumor cells evaluated by the crystal violet-based assay. (A) Dose-response curve estimated for PACAP using the fraction of cells affected (Fa) versus concentration data. The curve was generated by fitting the data derived from the crystal violet assays to the median-effect equation using the CalcuSyn software (Biosoft, Cambridge, UK). (B) Dose-response curves estimated for PACAP and also for CIGB-552, as a positive control of peptide with demonstrated anticancer activity. The curves were generated by fitting the data derived from the crystal violet assay (% growth inhibition versus concentration data) using GraphPad Prism software version 4.00, (GraphPad Software Inc., San Diego CA). Data were evaluated using a Kruskal-Wallis analysis followed by a Dunn's test. * Statistically significant differences between PACAP/CIGB552-treated H460 cells and untreated cells ($p < 0.05$). Data points represent the mean ± SD of two determinations in duplicate.

GraphPad Prism software ($IC_{50}$ = 13.17 μM and $IC_{50}$ = 14.97 μM, respectively). The quality of the fitting was assessed by the $R^2$ coefficient, where an $R^2 > 0.9$ was obtained for all curves (Table 3).

## 4. Discussion

Microbial resistance to conventional antibiotics and the unique mode of action of host defense peptides have brought the latter into the forefront of research, where they are promising candidates for the development of a new class of antibiotics [1]. In the past decade, some neuropeptides have been associated with this group of molecules. Prospective roles for neuropeptides in the local tissue microenvironment, in addition to their traditional modulation roles in central and peripheral neural systems, include those generally associated with conventional host defense peptides, such as direct antimicrobial action against microorganisms or immunomodulatory effects on host cells [15].

Our research group recently reported, for the first time, the existence of diverse mechanisms of modulation of the immune functions in fish mediated by the VIP/PACAP system [33–35,37,40]. These findings, in connection with the preceding scientific information in mammals (reviewed in Ref. [32]), suggested a potential involvement of the neuropeptide PACAP in host defense through its immunoregulatory properties. In the current work, we demonstrated a direct involvement of PACAP in antimicrobial immunity, providing evidence of antibacterial/antifungal activity for PACAP from the North African catfish (*C. gariepinus*) against a broad spectrum of pathogenic microorganisms of interest for human medicine and aquaculture. Accordingly, our *in silico* studies searching for PACAP sequence similarity with entries in various antimicrobial databases strengthened the idea that PACAP may have therapeutic activity. We observed that PACAP from *C. gariepinus* shares some structural and physicochemical characteristics with AMPs, such as: rich in basic amino acids (lysine and arginine), a net positive charge, small size and the ability to adopt α-helical and amphipathic structures containing clusters of hydrophobic and cationic residues. It is known, that a net positive charge enables AMPs electrostatic attraction to the negatively charged microbial membranes. Hydrophobicity is another important characteristic of AMPs, which enables them to induce membrane lysis [55]. Furthermore, an increase in the hydrophobicity of AMPs correlates with their low selectivity and toxicity toward mammalian cells (reviewed in Ref. [12]). Our results demonstrated that even at high micromolar concentration, PACAP showed limited cytotoxicity against both human and fish red blood cells, supporting a potential physiological role for this peptide in selective killing of microbes.

**Table 3**
Antitumor activity of African catfish (*Clarias gariepinus*) neuropeptide PACAP on non-small cell lung cancer cell line H460. Drug potency ($IC_{50}$) and the quality of the fitting data ($R^2$ coefficient) were calculated from each dose-response curve using GraphPad Prism software version 4.00, (GraphPad Software Inc., San Diego CA, USA). Data represents the mean ± SD of two determinations in duplicate.

| Tested compound | Classification | Compound range in μM (serial two-fold dilution) | Lung cancer cell line H460 | |
|---|---|---|---|---|
| | | | $IC_{50}$ (μM)[c] | $R^{2}$[d] |
| PACAP | Multifunctional neuropeptide, neurotransmitter, immunomodulator | From 150 to 9.37 | 14.97 ± 1.16 | 0.9957 ± 0.0008 |
| CIGB 552[a] | NFkB inhibitor peptide, cell-penetrating capacity | From 150 to 9.37 | 19.45 ± 1.09 | 0.9995 ± 0.0003 |
| Cisplatin[b] | Alkylating | From 100 to 3.125 | 8.57 ± 0.59 | 0.9731 ± 0.0161 |
| Paclitaxel[b] | Antimitotic | From 20 to 0.625 | 6.15 ± 1.06 | 0.9749 ± 0.0102 |

[a] CIGB 552: an anticancer peptide in phase I clinical trials by CIGB and Heber Biotec, Cuba.
[b] Cisplatin and paclitaxel: conventional chemotherapy drugs.
[c] $IC_{50}$: concentration of the tested compound that was required for 50% inhibition of the cell growth during 48 h.
[d] $R^2$: coefficient of determination.

During recent years, the role of PACAP in immunoregulation has been partially elucidated in mammals (reviewed in Refs. [31,32]). Their involvement in viral infections has been demonstrated [56–59]. For example, it is known that PACAP has potent regulatory activity for herpes simplex virus activation [56]. In addition, increased plasma PACAP-38 levels were observed in patients with chronic hepatitis B following lamivudine-induced elimination of viremia [58]. Treating macrophages infected with the human immunodeficiency virus (HIV-1) with VIP and PACAP diminished viral production. The underlying mechanisms seem to be related to the regulation of the chemokine axis but also implicates the induction of interleukin-10 (IL-10) secretion by macrophages [59]. *In vitro* and *in vivo* studies conducted by us in fish, provided evidence of the involvement of PACAP in the antiviral response in salmonids. We have demonstrated a differential regulation of PACAP transcriptional variants together with their receptors in spleen and head kidney leukocytes of the rainbow trout (*O. mykiss*) and in the monocyte/macrophage cell line RTS11, at different time points after infection with important pathogens for salmonid aquaculture, such as viral hemorrhagic septicemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV) [60,61]. In this connection, we also established that PACAP induces transcriptional activation of cytokines involved in antiviral host defense in peripheral blood leukocytes of naïve rainbow trout [60,61]. More recently, we also demonstrated a specific transcription modulation of genes encoding PACAP, as well as VIP/PACAP receptors, after septicemic infection with VHSV or the Gram-negative bacterium *Y. ruckeri*, in another fish species the brown trout (*S. trutta*). Kidney and spleen, the main lymphopoietic organs, were screened for PACAP-system gene expression over the course of viral (VHSV-Ia) and bacterial (*Y. ruckeri*, serotype O1 biotype 2) septicemic infections. PACAP splicing variants were strongly induced during the pathogenesis of both diseases. Our results confirmed an involvement of the PACAP system during active immune responses elicited by both viral and bacterial etiological agents [37]. In this work, although a direct antiviral action of PACAP was not addressed, our computational studies searching for PACAP sequence similarity against entries in antiviral peptide databases showed that PACAP has a 14.3% identity to human cathelicidin (LL-37), from antiviral database AVPpred. Cathelicidin (LL-37) is a 37-residue human cationic peptide that has been shown to have antiviral activity against herpes simplex virus (HSV), vaccinia virus and adenovirus Ad19. It has been reported to play a major role in protecting humans against naturally occurring respiratory diseases [62,63]. In this direction, further research is needed to fully characterize PACAP function in antiviral immunity.

Disease issues are of great concern in the aquaculture industry, since the appearance of pathogenic bacteria having multi-drug resistance to conventional treatments severely affects this sector [8]. *Y. ruckeri*, the etiological agent of Enteric Red Mouth disease, still causes significant economic losses to salmonid aquaculture [64]. The disease is still widespread in many countries as a consequence of the recent emergence of new bacterial strains [65]. *Aeromonas* species are increasingly being reported as important pathogens for lower vertebrates and for humans [66]. In this study, we demonstrated that fish pathogenic bacteria *Y. ruckeri*, *A. salmonicida* and *A. sobria* are very susceptible to catfish PACAP. In contrast, we observed that fish pathogenic bacterium *A. hydrophila* was more resistant, leading to a growth inhibition action at low concentration of PACAP more than a bactericidal effect. Consistently, PACAP did not affect *A. hydrophila*-stimulated cytokine gene expression *in vivo* in grass carp head kidney [38]. Transcriptional regulation studies of PACAP and its receptors after bacterial infection in teleost fish has been sparsely reported and no previous studies existed that demonstrated a direct antimicrobial effectiveness of PACAP or PACAP-family members against fish pathogenic microorganisms. From our published studies [37,40,60], only one report describes a transcriptional regulation of the PACAP precursors in Japanese flounder *Paralichthys olivaceus* challenged with a pathogen *Edwardsiella tarda* [39].

The role of PACAP in acute inflammation induced by LPS, a major component of the outer membrane of Gram-negative bacteria, has been investigated in mammals [67]. Both *in vivo* and *in vitro* studies demonstrated that PACAP inhibits LPS-stimulated production of pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), while it further enhances LPS-stimulated production of anti-inflammatory cytokine IL-10 in murine macrophages [31,67]. In agreement with this, grass carp PACAP attenuated LPS-stimulated head kidney leukocytes. This finding suggested that PACAP, in fish as in mammals, can work as an anti-LPS factor and protect against over-response to LPS [38]. All together, the literature reports suggest that the pro- and anti-inflammatory activities described for PACAP might be the result of an immune balance between a systemic anti-inflammatory effect induced by PACAP to protect the organism from the detrimental consequences of pro-inflammatory cytokines, and a local neuropeptide-associated pro-inflammatory effect necessary for controlling the infections [24–32]. Consistently with the results obtained here, we have also previously demonstrated an up-regulation of the pro-inflammatory cytokines IL-1β and TNF-α transcription by PACAP, in peripheral blood leukocytes of healthy/unexposed rainbow trout [60]. Similar results were obtained by Wang et al. in head kidney leukocytes and head kidney in grass carp [38]. This class of cytokines induces a number of different responses including a decreasing of bacterial load [68,69]. Our studies identify PACAP as a prominent target molecule with the potential to stimulate fish antimicrobial immunity.

In contrast, catfish PACAP did not kill *S. aureus* at any concentration. Also, it was observed that human pathogens *E. coli*, *P. aeruginosa* and *Salmonella* sp were most resistant to PACAP. It is known, that *S. aureus* and *P. aeruginosa* belong to the so-called *ESKAPE* pathogens, which show a high propensity to develop treatment resistance, even resistance to some cationic host defense peptides [6]. El Karim et al., in 2008 obtained results similar to our own when they tested different

neuropeptides against a range of microorganisms from the skin and the oral, respiratory and gastrointestinal tract. They observed a resistance of the S. *aureus* strain to all neuropeptides tested [18]. Likewise, Ohta et al., in 2011 obtained similar data when they examined the bactericidal effects of orexin B and VIP alone or combined with cationic AMPs, such as LL-37, on *E. coli*, *P. aeruginosa*, *Streptococcus mutans* and *S. aureus*. VIP had bactericidal activity against *E. coli*, *P. aeruginosa* and *S. mutans* in a dose-dependent manner. However, *S. aureus* showed resistance to VIP [20]. In contrast, non-pathogenic Gram-positive bacterium *B. subtilis* and human opportunistic yeast *C. albicans* were very sensitive to catfish PACAP. To date, to the best of our knowledge, the direct antimicrobial effect of PACAP against *B. subtilis* and *C. albicans* has never been examined. From a therapeutic point of view, the killing effect against Gram-positive bacteria is especially relevant because these bacteria have a thick cell wall and develop sophisticated resistance mechanisms [55,70]. In this sense, the observed bactericidal effect of *C. gariepinus* PACAP against *B. subtilis* could be due to the high cationic nature of the peptide and hydrophobicity, since both characteristics seem to be critical in the susceptibility of some Gram-positive bacteria to many antimicrobial peptides [55,70]. Certainly, the mechanisms involved in this effect of PACAP are still unknown.

In addition, our AMP prediction results showed that *C. gariepinus* PACAP exhibits a high sequence identity with human VIP (67.9% identity), a member of the same family of regulatory peptides that has recently been identified as a potential AMP [17–20]. In agreement with our results, it was demonstrated that VIP51, a metabolically stable analogue of VIP generated by substitutions/additions of critical residues, and an N-terminal truncated derivative of VIP51, named as VIP51(6–30), were more potent than native VIP as antimicrobial peptides against various non-pathogenic and pathogenic Gram-positive and Gram-negative bacteria, including *E. coli* and *P. aeruginosa*. Interestingly, the VIP derivatives, but not VIP, were able to efficiently kill Gram-positive bacteria. Moreover, both VIP derivatives, but not native VIP, were able to kill tropical protozoal parasites from the genus *Leishmania* [19]. Besides the increase in their stability, the chemical changes made in VIP51 and VIP51(6–30) improved their function as antimicrobial peptides [19]. In this direction, additional studies are needed to investigate a probable enhanced antimicrobial activity and stability of PACAP derivatives molecules.

In recent studies, it was also demonstrated that VIP almost completely loses its antibacterial effect against *E. coli* and *P. aeruginosa* in a physiological saline environment [20]. In addition, it was demonstrated that the antimicrobial activities of VIP and its derivatives in Gram-negative but not in Gram-positive bacteria depend on the pH of the medium. Interestingly, the three peptides gained and lost effectiveness in killing *E. coli* at basic and acidic pH conditions, respectively [19]. Taken together, these results indicated that the bactericidal activities of the AMP are strongly influenced by the ionic strength and pH of the medium. The effects of such specific factors on antibacterial activity of catfish PACAP deserve a new line of investigation. In this sense, the different levels of antimicrobial PACAP effectiveness observed in this study between fish and human host bacteria could be explained as due to the different growth conditions of microorganisms such as optimal growth temperature and the composition, ionic strength and pH of the culture medium affecting PACAP antimicrobial activity, or by the fact that the bacterial killing mechanism developed by PACAP depends on species.

As mentioned above, the fact that evolutionary pressure has acted to strongly preserve the primary sequence of the N-terminal domain (residues 1–27) of PACAP supports an important role of this region for its biological activity [23]. Accordingly, we documented here two conserved regions at the N-terminal of PACAP-38 sequences ($His^{1}$, $Gly^{4}$, $Phe^{6}$, $Asp^{8}$) and ($Val^{19}$, $Leu^{23}$, $Val^{26}$, $Leu^{27}$) with predictive antimicrobial significance. The positively charged C-terminal extension, PACAP (28–38), most likely has evolved and developed PACAP-species specific functions. In agreement with this proposition, we demonstrated a putative involvement of the C-terminal region of catfish PACAP-38 in the cell-penetrating feature of this neuropeptide. This finding could be relevant for explaining the observed attenuation in the killing activity of catfish PACAP against human host microbes. In line with our finding, in recent studies, it was demonstrated for the first time that both PACAP isoforms (PACAP-27 and PACAP-38) are able to cross the cellular membrane by a complementary receptor-independent mechanism in order to activate its cytosolic and nuclear binding sites. Noteworthy, the significant uptake difference observed between PACAP-38 and PACAP-27 suggested a role of the C-terminal (28–38) segment in the translocation process of PACAP through the plasma membrane [71]. Since the plasma membrane restricts transport of bioactive substances into cells, the potential cell-penetrating regions of the catfish PACAP-38 sequence predicted by us, together with the preceding information, has brought them into focus for use as transport vectors for drug delivery. This issue will be an interesting future research direction.

One of the main reasons for the increasing interest in peptides and peptide receptors in cancer is the possibility of receptor targeting, because the peptide receptors are expressed in many primary human cancers [72]. In addition, because of the increased level of phosphatidylserine (negatively charged) on the surface of cancer cells, as compared to normal cells, cationic amphipathic peptides could be an effective source of anticancer agents that are both selective and refractory to current resistance mechanisms [73]. PACAP receptors have been identified in numerous human tumors of the brain, breast, prostate, lung, colon, urinary bladder, cardiac muscle, and large intestine as well as in cancer cell lines and in chemically induced tumors in rodents, suggesting that PACAP may play important roles in the modulation of growth of many human tumors (reviewed in Ref. [74]). In the current study, the amino acid sequence of catfish PACAP-38 was submitted to different anticancer databases for an *in silico* characterization. The results reinforced PACAP's putative anticancer activity. As well, we found that the peptide strongly reduced survival of human lung cancer cell line H460 in a dose-dependent manner (> 70% of cell growth inhibition at micromolar concentrations). Surprisingly, PACAP reduced H460 cells viability with a similar potency as a known anticancer peptide, CIGB-552 [53,75–78]. By contrast, Zia et al., in 1995 reported that PACAP significantly stimulates growth of non-small cell lung cancer line NCI-H838 [79]. In addition, it was demonstrated that PACAP-38 stimulated the proliferation of C6 glioma cells after 24 and 72 h of treatment [80]. Contrary to this investigation, both PACAP and VIP were also shown to markedly reduce proliferation of the T98G human glioma cell line [81]. More recently, it was also demonstrated that serum starvation triggers the antiproliferative effects of both PACAP and VIP in glioma cells, suggesting that calorie restriction can be effective to improve peptide-based glioma cancer therapy [82]. In line with our results, Wojcieszak and Zawilska in 2014 found that both PACAP-38 and PACAP(6–38), a selective PAC1 receptor antagonist, did not affect human retinoblastoma Y79 cell viability at nanomolar concentration, but when used at high micromolar concentrations reduced cell survival in a dose-dependent manner [83]. It has also previously been reported that PACAP suppresses the proliferation of human κ and λ light chain-secreting multiple myeloma-derived cells, suggesting that PACAP could be used as a cytoprotective agent in the treatment of renal tubule injury in multiple myeloma [84]. Also, it was observed that 60% of the PACAP-deficient KO mice developed experimental colorectal tumors with an aggressive-appearing pathology. Consistent with these data, dextran sulfate sodium (DSS)-treated WT mice did not develop such tumors [85]. Likewise, deletion of a single copy of PACAP increased medulloblastoma incidence approximately 2.5-fold, to 66%, thereby demonstrating that PACAP exerts a powerful inhibitory action on the induction, growth and survival of these tumors [86]. In summary, studies of the effects of PACAP and VIP on the proliferation activity of neoplastic cells have shown that they exert different influences in cell growth, depending on the type of cancer cell line and also the species from which those cell lines were derived, on the culture medium

composition, time of incubation, and peptide concentration used [82,83]. The question why these peptides exert pro- or antimitogenic effects presently remains unanswered [82]. Definitely, additional studies are needed in order to define the molecular and cellular mechanisms of catfish PACAP-38-induced cytotoxicity in human non-small cell lung cancer cell line H460, and to elucidate whether our result, in the same or different conditions, are reproducible in other tumor cell lines. The potential therapeutic benefit of combining PACAP with different cytotoxic compounds, with are firmly established in clinical oncology could also be an interesting line of investigation.

## 5. Conclusions

Collectively, the findings described here demonstrate a dual antimicrobial and anticancer activity of PACAP from North African catfish (*C. gariepinus*). It is noteworthy not only from a therapeutic point of view but also from an evolutionary perspective. It appears that the ancient role of this neuropeptide was closely related to the innate immune system as a host defense peptide, which coevolved with the pathogens and acquired additional functions during its evolution. In summary, the new functions given in this study to catfish PACAP as a molecule of the innate host defences and as an anticancer/cell-penetrating peptide, in combination with its already demonstrated immunomodulatory effects, could be extremely useful in disorders in which inflammation and infection coexist. Thus, our results contribute to a better understanding of PACAP effects on the immune system in vertebrates and opens up new avenues for future application in the treatment of microbial and cancer diseases. Certainly, despite the promising results found here, further biological research is needed, including *in vivo* investigations, looking toward a medicinal use of this bioactive peptide.

## Funding

This work was supported by Center for Genetic Engineering and Biotechnology, Havana, Cuba. This study was also supported by the project AGL2011-29676 from the Spanish Ministry of Economy and Competitiveness (MINECO), Madrid, Spain to Tafalla C. Mobility between laboratories was supported by the grant from the Pan-American Marine Biotechnology Association (PAMBA) to Lugo JM.

## Acknowledgements

The authors thank Dr. John van der Meer for his valuable help in polishing the English in the manuscript. We would also like to thank members of the Central Veterinary Laboratory, Madrid, Spain for generously providing fish pathogenic bacteria and the Oncology Service of the National Institute of Oncology and Radiology, Havana, Cuba for kindly providing chemotherapeutic drugs. Lugo JM would like to thank members of Dr Tafalla's group for their support during her work at the Animal Health Research Center (CISA-INIA), Madrid, Spain. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

## References

[1] C.A. Arias, B.E. Murray, Antibiotic-resistant bugs in the 21st century–a clinical super-challenge, N. Engl. J. Med. 360 (5) (2009) 439–443.
[2] J. Ferlay, H.R. Shin, F. Bray, D. Forman, C. Mathers, D.M. Parkin, Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008, Int. J. Canc. 127 (12) (2010) 2893–2917.
[3] F. Pesola, J. Ferlay, P. Sasieni, Cancer incidence in English children, adolescents and young people: past trends and projections to 2030, Br. J. Canc. 117 (12) (2017) 1865–1873.
[4] L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal, Global cancer statistics, Ca - Cancer J. Clin. 65 (2) (2012) 87–108 2015.
[5] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, Int. J. Canc. 136 (5) (2015) E359–E386.
[6] M.R. Felicio, O.N. Silva, S. Goncalves, N.C. Santos, O.L. Franco, Peptides with dual antimicrobial and anticancer activities, Front Chem Feb 21 (5) (2017) 5, https://doi.org/10.3389/fchem.2017.00005. eCollection 2017 Review.
[7] Y.J. Gordon, E.G. Romanowski, A.M. McDermott, A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs, Curr. Eye Res. 30 (7) (2005) 505–515.
[8] G.M. Lalumera, D. Calamari, P. Galli, S. Castiglioni, G. Crosa, R. Fanelli, Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy, Chemosphere 54 (5) (2004) 661–668.
[9] R. Hirsch, T. Ternes, K. Haberer, K.L. Kratz, Occurrence of antibiotics in the aquatic environment, Sci. Total Environ. 225 (1–2) (1999) 109–118.
[10] B. Halling-Sorensen, S. Nors Nielsen, P.F. Lanzky, F. Ingerslev, H.C. Holten Lutzhoft, S.E. Jorgensen, Occurrence, fate and effects of pharmaceutical substances in the environment–a review, Chemosphere 36 (2) (1998) 357–393.
[11] B. Gomes, M.T. Augusto, M.R. Felicio, A. Hollmann, O.L. Franco, S. Goncalves, N.C. Santos, Designing improved active peptides for therapeutic approaches against infectious diseases, Biotechnol. Adv. (2018 Jan 9), https://doi.org/10.1016/j.biotechadv.2018.01.004 pii: S0734-9750(18)30004-1.
[12] S.J. Kang, S.J. Park, T. Mishig-Ochir, B.J. Lee, Antimicrobial peptides: therapeutic potentials, Expert Rev. Anti Infect. Ther. 12 (12) (2014) 1477–1486.
[13] D. Andreu, L. Rivas, Animal antimicrobial peptides: an overview, Biopolymers 47 (6) (1998) 415–433.
[14] D. Gaspar, A.S. Veiga, M.A. Castanho, From antimicrobial to anticancer peptides. A review, Front. Microbiol. 4 (2013) 294.
[15] D. Augustyniak, J. Nowak, F.T. Lundy, Direct and indirect antimicrobial activities of neuropeptides and their therapeutic potential, Curr. Protein Pept. Sci. 13 (8) (2012) 723–738.
[16] A. Arimura, Pituitary adenylate cyclase activating polypeptide (PACAP): discovery and current status of research, Regul. Pept. 37 (3) (1992) 287–303.
[17] E. Gonzalez-Rey, A. Chorny, M. Delgado, VIP: an agent with license to kill infective parasites, Ann. N. Y. Acad. Sci. 1070 (2006) 303–308.
[18] I.A. El Karim, G.J. Linden, D.F. Orr, F.T. Lundy, Antimicrobial activity of neuropeptides against a range of micro-organisms from skin, oral, respiratory and gastrointestinal tract sites, J. Neuroimmunol. 200 (1–2) (2008) 11–16.
[19] J. Campos-Salinas, A. Cavazzuti, F. O'Valle, I. Forte-Lago, M. Caro, S.M. Beverley, M. Delgado, E. Gonzalez-Rey, Therapeutic efficacy of stable analogues of vasoactive intestinal peptide against pathogens, J. Biol. Chem. 289 (21) (2014) 14583–14599.
[20] K. Ohta, M. Kajiya, T. Zhu, H. Nishi, H. Mawardi, J. Shin, L. Elbadawi, N. Kamata, H. Komatsuzawa, T. Kawai, Additive effects of orexin B and vasoactive intestinal polypeptide on LL-37-mediated antimicrobial activities, J. Neuroimmunol. 233 (1–2) (2011) 37–45.
[21] A.J. Harmar, A. Arimura, I. Gozes, L. Journot, M. Laburthe, J.R. Pisegna, S.R. Rawlings, P. Robberecht, S.I. Said, S.P. Sreedharan, S.A. Wank, J.A. Waschek, International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide, Pharmacol. Rev. 50 (2) (1998) 265–270.
[22] A. Arimura, Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems, Jpn. J. Physiol. 48 (5) (1998) 301–331.
[23] B.J. Gonzalez, M. Basille, D. Vaudry, A. Fournier, H. Vaudry, [Pituitary adenylate cyclase-activating polypeptide], Ann. Endocrinol. 59 (5) (1998) 364–405.
[24] N. El Zein, F. Corazza, E. Sariban, The neuropeptide pituitary adenylate cyclase activating protein is a physiological activator of human monocytes, Cell. Signal. 18 (2) (2006) 162–173.
[25] N. El Zein, B. Badran, E. Sariban, The neuropeptide pituitary adenylate cyclase activating polypeptide modulates Ca2+ and pro-inflammatory functions in human monocytes through the G protein-coupled receptors VPAC-1 and formyl peptide receptor-like 1, Cell Calcium 43 (3) (2008) 270–284.
[26] I. Harfi, S. D'Hondt, F. Corazza, E. Sariban, Regulation of human polymorphonuclear leukocytes functions by the neuropeptide pituitary adenylate cyclase-activating polypeptide after activation of MAPKs, J. Immunol. 173 (6) (2004) 4154–4163.
[27] I. Harfi, F. Corazza, S. D'Hondt, E. Sariban, Differential calcium regulation of proinflammatory activities in human neutrophils exposed to the neuropeptide pituitary adenylate cyclase-activating protein, J. Immunol. 175 (6) (2005) 4091–4102.
[28] M. de la Fuente, M. Delgado, M. del Rio, E. Garrido, J. Leceta, A. Hernanz, R.P. Gomariz, Vasoactive intestinal peptide modulation of adherence and mobility in rat peritoneal lymphocytes and macrophages, Peptides 15 (7) (1994) 1157–1163.
[29] Y. Cai, X. Xin, G.J. Shim, Y. Mokuno, H. Uehara, T. Yamada, T. Agui, K. Matsumoto, Pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) stimulate interleukin-6 production through the third subtype of PACAP/VIP receptor in rat bone marrow-derived stromal cells, Endocrinology 138 (6) (1997) 2515–2520.
[30] C. Martinez, M. Delgado, D. Pozo, J. Leceta, J.R. Calvo, D. Ganea, R.P. Gomariz, VIP and PACAP enhance IL-6 release and mRNA levels in resting peritoneal macrophages: in vitro and in vivo studies, J. Neuroimmunol. 85 (2) (1998) 155–167.
[31] M. Delgado, C. Abad, C. Martinez, M.G. Juarranz, J. Leceta, D. Ganea, R.P. Gomariz, PACAP in immunity and inflammation, Ann. N. Y. Acad. Sci. 992 (2003) 141–157.
[32] D. Ganea, M. Delgado, Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) as modulators of both innate and adaptive immunity, Crit. Rev. Oral Biol. Med. 13 (3) (2002) 229–237.
[33] Y. Carpio, J.M. Lugo, K. Leon, R. Morales, M.P. Estrada, Novel function of recombinant pituitary adenylate cyclase-activating polypeptide as stimulator of innate immunity in African catfish (*Clarias gariepinus*) fry, Fish Shellfish Immunol. 25

(4) (2008) 439–445.
[34] J.M. Lugo, Y. Carpio, A. Oliva, A. Morales, M.P. Estrada, Pituitary adenylate cyclase-activating polypeptide (PACAP): a regulator of the innate and acquired immune functions in juvenile fish, Fish Shellfish Immunol. 29 (3) (2010) 513–520.
[35] J.M. Lugo, C. Tafalla, J. Leceta, R.P. Gomariz, M.P. Estrada, Differential expression pattern of pituitary adenylate cyclase-activating polypeptide (PACAP) alternative splicing variants and its receptors in the immune system of rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 30 (2) (2011) 734–738.
[36] J.M. Lugo, Y. Carpio, R. Morales, T. Rodriguez-Ramos, L. Ramos, M.P. Estrada, First report of the pituitary adenylate cyclase activating polypeptide (PACAP) in crustaceans: conservation of its functions as growth promoting factor and immunomodulator in the white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 35 (6) (2013) 1788–1796.
[37] B. Gorgoglione, Y. Carpio, C.J. Secombes, N.G. Taylor, J.M. Lugo, M.P. Estrada, Viral and bacterial septicaemic infections modulate the expression of PACAP splicing variants and VIP/PACAP receptors in brown trout immune organs, Fish Shellfish Immunol. 47 (2) (2015) 923–932.
[38] X. Wang, H. Wei, T. Zhao, X. Zhu, X. Yang, D. Chen, H. Zhou, Evidence for pituitary adenylate cyclase-activating peptide as a direct immunoregulator in teleost head kidney, Fish Shellfish Immunol. 34 (1) (2013) 265–272.
[39] B.H. Nam, J.Y. Moon, Y.O. Kim, H.J. Kong, W.J. Kim, D.G. Kim, Y.J. Jee, S.J. Lee, Structural and functional characterization of pituitary adenylyl cyclase-activating polypeptide (PACAP)/PACAP-related peptide (PRP) and its receptor in olive flounder (*Paralichthys olivaceus*), Comp. Biochem. Physiol. B Biochem. Mol. Biol. 164 (1) (2013) 18–28.
[40] J.M. Lugo, C. Tafalla, A. Gonzalez-Granja, B. Abós, J. Leceta, R.P. Gomariz, R. Castro, B. Gorgoglione, C.J. Secombes, J. Velázquez, Y. Carpio, M.P. Estrada, Recent discoveries of Pituitary adenylate cyclase-activating peptide (PACAP) function in the teleost fish adaptative immunity, Fish Shellfish Immunol. 53 (2016) 87–88.
[41] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (17) (1997) 3389–3402.
[42] G. Wang, X. Li, Z. Wang, APD3: the antimicrobial peptide database as a tool for research and education, Nucleic Acids Res. 44 (D1) (2016) D1087–D1093.
[43] X. Zhao, H. Wu, H. Lu, G. Li, Q. Huang, LAMP: a database linking antimicrobial peptides, PloS One 8 (6) (2013) e66557.
[44] F.H. Waghu, R.S. Barai, P. Gurung, S. Idicula-Thomas, CAMPR3: a database on sequences, structures and signatures of antimicrobial peptides, Nucleic Acids Res. 44 (D1) (2015) D1094–D1097.
[45] N. Thakur, A. Qureshi, M. Kumar, AVPpred: collection and prediction of highly effective antiviral peptides, Nucleic Acids Res. 40 (2012) W199–W204 Web Server issue.
[46] S. Lata, N.K. Mishra, G.P. Raghava, AntiBP2: improved version of antibacterial peptide prediction, BMC Bioinf. 11 (Suppl 1) (2009) S19.
[47] P.K. Meher, T.K. Sahu, V. Saini, A.R. Rao, Predicting antimicrobial peptides with improved accuracy by incorporating the compositional, physico-chemical and structural features into Chou's general PseAAC, Sci. Rep. 7 (2017) 42362.
[48] A. Tyagi, A. Tuknait, P. Anand, S. Gupta, M. Sharma, D. Mathur, A. Joshi, S. Singh, A. Gautam, G.P. Raghava, CancerPPD: a database of anticancer peptides and proteins, Nucleic Acids Res. 43 (2014) D837–D843 Database issue.
[49] A. Roy, A. Kucukural, Y. Zhang, I-TASSER: a unified platform for automated protein structure and function prediction, Nat. Protoc. 5 (4) (2010) 725–738.
[50] A. Gautam, K. Chaudhary, R. Kumar, A. Sharma, P. Kapoor, A. Tyagi, G.P. Raghava, In silico approaches for designing highly effective cell penetrating peptides, J. Transl. Med. 11 (2013) 74.
[51] T.A. Holton, G. Pollastri, D.C. Shields, C. Mooney, CPPpred: prediction of cell penetrating peptides, Bioinformatics 29 (23) (2013) 3094–3096.
[52] J.M. Lugo, A. Rodriguez, Y. Helguera, R. Morales, O. Gonzalez, J. Acosta, V. Besada, A. Sanchez, M.P. Estrada, Recombinant novel pituitary adenylate cyclase-activating polypeptide from African catfish (*Clarias gariepinus*) authenticates its biological function as a growth-promoting factor in low vertebrates, J. Endocrinol. 197 (3) (2008) 583–597.
[53] J.R. Fernandez Masso, B. Oliva Arguelles, Y. Tejeda, S. Astrada, H. Garay, O. Reyes, L. Delgado-Roche, M. Bollati-Fogolin, M.G. Vallespi, The antitumor peptide CIGB-552 increases COMMD1 and inhibits growth of human lung cancer cells, J. Amino Acids 2013 (2013) 251398.
[54] L. Otvos, M. Cudic, Broth microdilution antibacterial assay of peptides, Methods Mol. Biol. 386 (2007) 309–320.
[55] J. He, R. Eckert, T. Pharm, M.D. Simanian, C. Hu, D.K. Yarbrough, F. Qi, M.H. Anderson, W. Shi, Novel synthetic antimicrobial peptides against *Streptococcus mutans*, Antimicrob. Agents Chemother. 51 (4) (2007) 1351–1358.
[56] R.J. Danaher, A.D. Savells-Arb, S.A. Black Jr., R.J. Jacob, C.S. Miller, Herpesvirus quiescence in neuronal cells IV: virus activation induced by pituitary adenylate cyclase-activating polypeptide (PACAP) involves the protein kinase A pathway, J. Neurovirol. 7 (2) (2001) 163–168.
[57] P.B. Bokaei, X.Z. Ma, D. Sakac, D.R. Branch, HIV-1 integration is inhibited by stimulation of the VPAC2 neuroendocrine receptor, Virology 362 (1) (2007) 38–49.
[58] I.S. Elefsiniotis, I. Ketikoglou, G. Kafiri, K.D. Pantazis, A. Moulakakis, C. Mavrogiannis, Plasma pituitary adenylate cyclase activating polypeptide (PACAP) levels in chronic hepatitis B patients under lamivudine treatment, Eur. J. Gastroenterol. Hepatol. 15 (11) (2003) 1209–1216.
[59] J.R. Temerozo, R. Joaquim, E.G. Regis, W. Savino, D.C. Bou-Habib, Macrophage resistance to HIV-1 infection is enhanced by the neuropeptides VIP and PACAP, PloS One 8 (6) (2013) e67701.
[60] J.M. Lugo, C. Tafalla, Y. Carpio, R.P. Gomariz, B. Gorgoglione, C.J. Secombes, M.P. Estrada, Pituitary Adenylate Cyclase-Activating Peptide (PACAP) as potent modulator of innate and adaptive immune responses in fish, Fish Shellfish Immunol. 34 (2013) 1721–1722.
[61] M.P. Estrada, J.M. Lugo, Y. Carpio, C. Tafalla, Use of PACAP for Treating Viral Infections in Aquatic Organisms, (2012) WO2012072055A1.
[62] M.D. Howell, J.F. Jones, K.O. Kisich, J.E. Streib, R.L. Gallo, D.Y. Leung, Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum, J. Immunol. 172 (3) (2004) 1763–1767.
[63] Y.J. Gordon, L.C. Huang, E.G. Romanowski, K.A. Yates, R.J. Proske, A.M. McDermott, Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity, Curr. Eye Res. 30 (5) (2005) 385–394.
[64] E. Tobback, A. Decostere, K. Hermans, F. Haesebrouck, K. Chiers, *Yersinia ruckeri* infections in salmonid fish, J. Fish. Dis. 30 (5) (2007) 257–268.
[65] T.J. Welch, D.W. Verner-Jeffreys, I. Dalsgaard, T. Wiklund, J.P. Evenhuis, J.A. Cabrera, J.M. Hinshaw, J.D. Drennan, S.E. LaPatra, Independent emergence of *Yersinia ruckeri* biotype 2 in the United States and Europe, Appl. Environ. Microbiol. 77 (10) (2011) 3493–3499.
[66] G.H. Dar, S.A. Dar, A.N. Kamili, M.Z. Chishti, F. Ahmad, Detection and characterization of potentially pathogenic *Aeromonas sobria* isolated from fish Hypophthalmichthys molitrix (Cypriniformes: cyprinidae), Microb. Pathog. 91 (2016) 136–140.
[67] W. Bik, E. Wolinska-Witort, J. Pawlak, K. Skwarlo-Sonta, M. Chmielowska, L. Martynska, A. Baranowska-Bik, B. Baranowska, PACAP 38 as a modulator of immune and endocrine responses during LPS-induced acute inflammation in rats, J. Neuroimmunol. 177 (1–2) (2006) 76–84.
[68] T. Kielian, E.D. Bearden, A.C. Baldwin, N. Esen, IL-1 and TNF-alpha play a pivotal role in the host immune response in a mouse model of Staphylococcus aureus-induced experimental brain abscess, J. Neuropathol. Exp. Neurol. 63 (4) (2004) 381–396.
[69] C.A. Dinarello, Anti-cytokine therapeutics and infections, Vaccine 21 (Suppl 2) (2003) S24–S34.
[70] R.F. Epand, M.A. Schmitt, S.H. Gellman, A. Sen, M. Auger, D.W. Hughes, R.M. Epand, Bacterial species selective toxicity of two isomeric alpha/beta-peptides: role of membrane lipids, Mol. Membr. Biol. 22 (6) (2005) 457–469.
[71] N.D. Doan, D. Chatenet, M. Letourneau, H. Vaudry, D. Vaudry, A. Fournier, Receptor-independent cellular uptake of pituitary adenylate cyclase-activating polypeptide, Biochim. Biophys. Acta 1823 (4) (2012) 940–949.
[72] J.C. Reubi, Peptide receptors as molecular targets for cancer diagnosis and therapy, Endocr. Rev. 24 (4) (2003) 389–427.
[73] B. Deslouches, Y.P. Di, Antimicrobial peptides with selective antitumor mechanisms: prospect for anticancer applications, Oncotarget 8 (28) (2017) 46635–46651.
[74] T.W. Moody, B. Nuche-Berenguer, R.T. Jensen, Vasoactive intestinal peptide/pituitary adenylate cyclase activating polypeptide, and their receptors and cancer, Curr. Opin. Endocrinol. Diabetes Obes. 23 (1) (2016) 38–47.
[75] A. Rodriguez-Ulloa, J. Gil, Y. Ramos, L. Hernandez-Alvarez, L. Flores, B. Oliva, D. Garcia, A. Sanchez-Puente, A. Musacchio-Lasa, J. Fernandez-de-Cossio, G. Padron, L.J. Gonzalez Lopez, V. Besada, M. Guerra-Vallespi, Proteomic study to survey the CIGB-552 antitumor effect, BioMed Res. Int. 2015 (2015) 124082.
[76] M.G. Vallespi, G. Pimentel, A. Cabrales-Rico, J. Garza, B. Oliva, O. Mendoza, Y. Gomez, T. Basaco, I. Sanchez, C. Calderon, J.C. Rodriguez, M.R. Markelova, I. Fichtner, S. Astrada, M. Bollati-Fogolin, H.E. Garay, O. Reyes, Antitumor efficacy, pharmacokinetic and biodistribution studies of the anticancer peptide CIGB-552 in mouse models, J. Pept. Sci. 20 (11) (2014) 850–859.
[77] S. Astrada, Y. Gomez, E. Barrera, G. Obal, O. Pritsch, S. Pantano, M.G. Vallespi, M. Bollati-Fogolin, Comparative analysis reveals amino acids critical for anticancer activity of peptide CIGB-552, J. Pept. Sci. 22 (11–12) (2016) 711–722.
[78] S. Astrada, J.R. Fernandez Masso, M.G. Vallespi, M. Bollati-Fogolin, Cell penetrating capacity and internalization mechanisms used by the synthetic peptide CIGB-552 and its relationship with tumor cell line sensitivity, Molecules 23 (4) (2018).
[79] F. Zia, M. Fagarasan, K. Bitar, D.H. Coy, J.R. Pisegna, S.A. Wank, T.W. Moody, Pituitary adenylate cyclase activating peptide receptors regulate the growth of non-small cell lung cancer cells, Cancer Res. 55 (21) (1995) 4886–4891.
[80] P. Sokolowska, J.Z. Nowak, Effects of PACAP and VIP on cAMP-generating system and proliferation of C6 glioma cells, J. Mol. Neurosci. 36 (1–3) (2008) 286–291.
[81] P. Vertongen, I. Camby, F. Darro, R. Kiss, P. Robberecht, VIP and pituitary adenylate cyclase activating polypeptide (PACAP) have an antiproliferative effect on the T98G human glioblastoma cell line through interaction with VIP2 receptor, Neuropeptides 30 (5) (1996) 491–496.
[82] A.G. D'Amico, S. Scuderi, S. Saccone, A. Castorina, F. Drago, V. D'Agata, Antiproliferative effects of PACAP and VIP in serum-starved glioma cells, J. Mol. Neurosci. 51 (2) (2013) 503–513.
[83] J. Wojcieszak, J.B. Zawilska, PACAP38 and PACAP6-38 exert cytotoxic activity against human retinoblastoma Y79 cells, J. Mol. Neurosci. 54 (3) (2014) 463–468.
[84] M. Li, S. Cortez, T. Nakamachi, V. Batuman, A. Arimura, Pituitary adenylate cyclase-activating polypeptide is a potent inhibitor of the growth of light chain-secreting human multiple myeloma cells, Cancer Res. 66 (17) (2006) 8796–8803.
[85] N. Nemetz, C. Abad, G. Lawson, H. Nobuta, S. Chhith, L. Duong, G. Tse, J. Braun, J.A. Waschek, Induction of colitis and rapid development of colorectal tumors in mice deficient in the neuropeptide PACAP, Int. J. Canc. 122 (8) (2008) 1803–1809.
[86] J.R. Cohen, D.Z. Resnick, P. Niewiadomski, H. Dong, L.M. Liau, J.A. Waschek, Pituitary adenylyl cyclase activating polypeptide inhibits gli1 gene expression and proliferation in primary medulloblastoma derived tumorsphere cultures, BMC Canc. 10 (2010) 676.