

## Peptide Tyrosine Arginine, a potent immunomodulatory peptide isolated and structurally characterized from the skin secretions of the dusky gopher frog, *Rana sevosa*

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### Abstract

An octadecapeptide was isolated from the skin secretions of the dusky gopher frog (*Rana sevosa*) on the basis of histamine release from rat peritoneal mast cells. This peptide was purified to homogeneity by HPLC and found to have the following primary structure, YLKGWCWTKSYPPKPCFSR, using both Edman degradation chemistry and peptide sequencing using high-resolution mass spectrometry (Q-TOF MS). The peptide, named peptide Tyrosine Arginine (pYR) shares 77.8% homology with peptide Leucine Arginine (pLR). The effects of the natural amidated peptide, non-amidated peptide and C-loop region of pYR on granulopoiesis and neutrophil apoptosis were investigated. All three analogues inhibited the early development of granulocyte macrophage colonies from bone marrow stem cells but did not induce apoptosis of the end stage granulocytes, the mature neutrophil. Thus, pYR is a novel member of an important and emerging new class of amphibian peptides with hemopoietic actions.

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### 1. Introduction

Amphibian skin secretions represent a rich source of diverse peptides with various pharmacological properties. Several different classes of peptides have been isolated from the skins of ranid frog species, which have structural analogues in mammalian neuroendocrine systems. These include the bradykinin related peptides, bombesin-like peptides, ranatensin and thyrotropin-releasing hormone [2,5,8,14,15,20,23]. Hemolytic peptides have also been identified in ranid frogs and the release of histamine from rat

peritoneal mast cells may be utilized as a screen for putative bioactive peptides. Several peptides have been identified in this manner, including peptide Leucine Arginine (pLR) [21] and granuliberin-R from *Rana rugosa* [25]. The release of histamine from rat peritoneal mast cells has also been used as a screen to isolate bioactive peptides from the venom of insects from the order Hymenoptera [3,4,19].

Neuropeptides have been shown to exert an effect on the hemopoietic system; human CD34<sup>+</sup> hematopoietic progenitor cells express functional receptors for the neuropeptide calcitonin gene-related peptide (CGRP). Abolition of the neuropeptides, substance P (SP) and CGRP in mouse by capsaicin treatment inhibits normal hemopoiesis [6]. In this study we investigated immunomodulatory peptides in the skin secretion of the dusky gopher frog, *Rana sevosa*. A potent new immunomodulatory peptide was isolated, named peptide

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Tyrosine Arginine (pYR). This is the second member of a new class of amphibian peptides that affect hemopoiesis. Peptide YR shares high sequence homology (77.8%) with peptide Leucine Arginine (pLR) isolated from the Northern leopard frog *Rana pipiens* and it is likely that these peptides belong to a wider family of regulatory peptides with immune function.

In the present study we investigate the effects of the natural amidated peptide, non-amidated peptide and C-loop region of pYR on granulopoiesis and neutrophil apoptosis. Peptide YR exerted a dose-dependent inhibition of normal granulocyte macrophage colony forming unit (CFU-GM) formation. None of the three analogous peptides induced neutrophil apoptosis and in the inhibition of granulopoiesis, the C-loop and the amidated analogues were not as potent as the free C-terminal carboxyl form of the peptide. This is the second pLR-like peptide to be characterized in this manner with a possible mammalian counterpart involved in the regulation of the immune system as yet to be identified.

## 2. Materials and methods

### 2.1. Materials

All solvents were of HPLC grade. Methanol and acetonitrile were obtained from BDH (Poole Dorset, UK) and Milli-Q water (Millipore, Gloucestershire, UK) was used throughout. Nitrogen gas for the LCQ<sup>TM</sup> was delivered from a Whatman nitrogen generator (Whatman Inc., Haverhill, MA, USA). Chemicals employed in the operation of the 491-procise gas phase sequencer were supplied by Applied Biosystems, UK. Actinomycin D, Bisbenzimidazole H (Hoechst 33258 dye), phosphate-buffered saline (0.01 M, pH 7.4), Histopaque 1119 and Histopaque 1077 were obtained from Sigma (Dorset, UK). The ApoAlert<sup>TM</sup> Mitosensor<sup>TM</sup> kit was obtained from BD Biosciences Clontech (Oxford, UK). All other chemicals used were of analytical reagent quality.

### 2.2. Collection of skin secretions

Male adult *R. sevosia* frogs ( $n=5$ ) were massaged at multiple sites on the dorsal skin. The frogs readily secreted. The skin secretions were collected in a chilled beaker containing glacial acetic acid (5 ml) by washing the frog's skin with distilled water. Following this the collected skin secretions were pooled and subjected to lyophilization.

### 2.3. Purification of *R. sevosia* peptides

The HPLC system used was supplied by ThermoQuest and comprised a P4000 pump, AS 3000 autosampler, 200  $\mu$ l injector loop, on line UV 1000 UV detector and an SCM 1000 vacuum membrane degasser. The column used for chromatography separations was a Phenomenex (Phenomenex, Cheshire, UK) Luna C<sub>18</sub> column 250 mm  $\times$  4.6 mm. A security guard cartridge (Phenomenex, Cheshire, UK) was positioned just before the analytical column. For mass spec-

trometric studies the HPLC system was interfaced with a ThermoFinnigan electrospray ion trap mass spectrometer (LCQ<sup>TM</sup>) instrument. Skin secretions (200  $\mu$ l) were subjected to reverse phase HPLC. The column was equilibrated in 0.05% TFA/water (solution A) and elution of the peptides was achieved by increasing the concentration of solution B (0.05% TFA in 80% acetonitrile/water) from 0 to 100% B in 80 min at a flow rate of 0.5 ml/min. Absorbance was monitored at 214 nm.

### 2.4. Peptide sequencing

Automated Edman degradation was employed for the sequencing of the isolated purified peptides. Microsequencing was performed on a 491-procise sequencer (Applied Biosystems, Warrington, Cheshire, UK) with pulsed-liquid phase delivery. The limit for detection of phenylthiohydantoin (PTH) amino acids was 0.5 pmol. The primary structures of the peptides were compared with those deposited in SWISSPROT<sup>TM</sup> database. The protein database screening for similar sequences was performed using the FASTA program available in the bioinformatic program GCG<sub>10</sub> package at the human genome management program available at <http://www.hgmp.mrc.ac.uk>.

### 2.5. Peptide synthesis

The peptides pYR, pYR-amide and the C-loop of the peptide (C<sup>5</sup>WTKSYPPKPC<sup>15</sup>) were synthesized automatically using the solid phase method (Wang resin, ABI 433A peptide synthesizer, Applied Biosystems, Warrington, Cheshire, UK) and standard Fmoc chemistry. Couplings were performed by use of 2(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (Applied Biosystems, Warrington, Cheshire UK). The final cleavage/deblocking was performed using trifluoroacetic acid, phenol, water, thioanisole and ethanedithiol (82.5:5:5:5:2.5 v/v/v/v/v). The crude peptides were precipitated from 50 ml of *t*-butyl methyl ether, washed and repeated three times. The final purification was performed by Phenomenex Luna C<sub>18</sub> column, (250 mm  $\times$  4.6 mm). The column was equilibrated in 0.05% TFA/water (solution A) with elution of the peptide achieved by increasing the concentration of solution B (0.05% TFA in 80% acetonitrile/water) from 0 to 100% B in 80 min at a flow rate of 0.5 ml/min. Absorbance was monitored at 214 nm.

### 2.6. Mass spectrometry

The masses of the natural and synthetic peptides were determined by MALDI-TOF mass spectrometry and Q-TOF analysis. MALDI-TOF mass spectrometry was performed on a Voyager MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA). Mass was recorded as a mass/charge ratio against abundance. MS and MS/MS characterization of the natural and synthetic peptides was carried out using a Q-TOF<sup>TM</sup> mass spectrometer (Micromass, UK)

utilizing nanospray electrospray ionisation (ESI). Samples were introduced by means of a syringe pump located on the instrument at 5  $\mu\text{l}/\text{min}$ . The spray voltage was maintained at 3.5 kV and the cone voltage was set at 80 V. The source temperature was 80 °C and the desolvation temperature was 150 °C. The collision energy was set at 30 eV and the machine was operated in V mode with the TOF voltage set at 9.1 kV. API gas (nitrogen) was run at 300 L/h and argon at 50 L/h.

### 2.7. Histamine release assay

Male Hooded Lister rats (150–250 g body weight) were lightly anaesthetized with CO<sub>2</sub> and then killed by cervical dislocation and exsanguination. Mixed peritoneal cells were obtained as described by Cross et al. [9]. The cells were washed twice in Tyrode's buffer (NaCl (137 mM), glucose (5.6 mM), HEPES (10 mM), KCl (2.7 mM), MgCl<sub>2</sub>·6H<sub>2</sub>O (1 mM), CaCl<sub>2</sub>·2H<sub>2</sub>O (1 mM) and NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O (0.4 mM), pH 7.4) and recovered by centrifugation (100  $\times$  g, 4 °C, 2 min). Isolated peritoneal cells (100  $\mu\text{l}$ ) were aliquoted into conical polystyrene test tubes and prewarmed to 37 °C for 5 min. Lyophilized aliquots of chromatographic fractions were reconstituted in Tyrode's buffer (100  $\mu\text{l}$ ) and added to cells suspensions. Following incubation (10 min, 37 °C), the reaction was quenched by addition of ice-cold Tyrode's (2.8 ml). The cell suspensions were centrifuged as above and the supernatants removed for histamine assay. The remaining pellets were resuspended in buffer (3 ml) and then placed in a boiling water bath (10 min) to release the residual histamine. The histamine content was determined in both the supernatants and the cell pellets using the fluorimetric method based on Shore et al. [22]. Histamine release was expressed as the percentage of total content and values were corrected for spontaneous release. Spontaneous histamine-release in the absence of peptides did not exceed 5%.

### 2.8. Measurement of granulopoiesis by colony forming units-granulocyte macrophage formation (CFU-GM)

Semi-solid agar cultures were performed utilizing normal human bone marrow obtained with informed consent from thoracotomy rib specimens [11]. The assay was performed in 35 mm petri dishes (Nunc, Rochester, NY) with 1.5% agar base and 0.9% agar top. Bone marrow mononuclear cells ( $1 \times 10^5$ ) were cultured in the upper layer of triplicate cultures (1 ml), with human umbilical cord conditioned medium in the lower layer as the source of colony stimulating activity [12]. Synthetic pYR was incorporated into both layers of the cultures in the range of 0–5  $\mu\text{M}$ . CFU-GM colonies (>20 cells) were scored using an inverted microscope after incubation (37 °C, 7 days, 5% CO<sub>2</sub>/air). Colony inhibition in the presence of pYR, pYR·amide and pYR<sup>5–15</sup> was expressed as percentage inhibition of granulopoiesis compared with that observed in the absence of any peptide. These experiments were carried out in triplicate.

### 2.9. Measurement of neutrophil apoptosis

Neutrophils were prepared by layering human peripheral blood on an equal volume of Histopaque 1119 and Histopaque 1077 and centrifuging at 700  $\times$  g for 30 min. Cells were washed in phosphate-buffered saline (0.01 M, pH 7.4, Sigma) and resuspended in RPMI 1640 (Life Technologies, Inc. Paisley, UK) containing 10% (v/v) fetal calf serum (Life Technologies, Inc. Paisley, UK), and enumerated using a hemocytometer. A suspension of neutrophils ( $1 \times 10^6/\text{ml}$ ) was cultured in the presence of synthetic pYR (0–5  $\mu\text{M}$ ) in 5% CO<sub>2</sub>/95% air at 37 °C in six-well multi-dishes (Nunc, Rochester, NY). After 24 h the neutrophils were removed, and the cell viability and apoptotic status was evaluated. Cell viability was estimated by staining with trypan blue dye. Equal volumes of trypan blue stain (0.4%, Invitrogen Ltd., UK) and cell suspension were added together and 10  $\mu\text{l}$  of this was loaded on to a hemocytometer. The cells were examined under a light microscope for uptake of the dye. Apoptotic status was determined using Hoechst 33258 and propidium iodide to examine morphological changes. An aliquot of 500  $\mu\text{l}$  of the 24 h culture was incubated with 10  $\mu\text{g}/\text{ml}$  of Hoechst 33258 for 15 min at 37 °C in 5% CO<sub>2</sub>. Cells were centrifuged at 1500  $\times$  g for 5 min and resuspended in 10  $\mu\text{g}/\text{ml}$  propidium iodide solution in PBS. Cells were then examined for evidence of apoptosis under a fluorescence microscope with a band-pass DAPI filter and at  $\times 40$  magnification. A minimum of 200 cells were scored per sample.

Apoptosis was additionally assessed using the ApoAlert™ Mitochondrial Membrane Sensor Kit (Clontech UK, Hampshire, UK). This kit is based on the collapse in the mitochondrial inner transmembrane potential ( $\Delta\psi_m$ ) following the induction of apoptosis [18]. This change in  $\Delta\psi_m$  facilitates the release of caspase-activating proteins from the mitochondria into the cytosol. The cationic dye, Mitosensor™, is taken up by the mitochondria of non-apoptotic cells where it forms aggregates that fluoresce red; due to the altered transmembrane potential apoptotic cells can not take up the dye and fluoresce green. An aliquot of 450  $\mu\text{l}$  of the 24 h culture was centrifuged at 1500  $\times$  g for 15 min. Cells were then resuspended in 1 ml of Mitosensor™ incubation buffer, containing 1  $\mu\text{l}$  of Mitosensor™ reagent and 1 ml of incubation buffer to give a final concentration of Mitosensor™ reagent of 5  $\mu\text{g}/\text{ml}$ . Cells were incubated for 15 min at 37 °C in 5% CO<sub>2</sub>. Cells were then centrifuged for 5 min at 1500  $\times$  g and resuspended in 10  $\mu\text{l}$  of PBS. Cells (minimum 200 cells) were then examined under a fluorescence microscope using a band-pass filter to detect fluorescein and rhodamine at  $\times 40$  magnification.

### 2.10. Statistical analysis

Results are expressed as mean  $\pm$  S.E.M. Values were compared using Student's unpaired *t*-test. Groups of data were considered to be significantly different if  $P < 0.05$ .

### 3. Results

#### 3.1. HPLC purification of *R. sevos*a peptides

Peptides in the skin secretions of *R. sevos*a frogs were separated by HPLC (Fig. 1). Aliquots were subsequently screened for their ability to release histamine from rat peritoneal mast cells. The chromatogram shows a sharp peak at 26 min in both the TIC and UV profiles, this corresponds to the fraction later shown to contain pYR. The majority of large peaks appear on the chromatogram between 34 and 46 min, all peaks in this region displayed strong TIC and UV profiles and were identified as histamine-releasing antibacterial peptides (data not shown).

#### 3.2. Histamine-releasing activity of HPLC purified fractions

Three main areas of histamine release were evident in HPLC purified fractions (Fig. 2). Fractions 38–50 displayed strong histamine-releasing activity, these peptides also possessed antibacterial activity (data not shown). Fractions 26–27, contained a 2160 Da peptide and fraction 35 contained three peptides of masses 2220, 2255 and 2489 Da (as yet uncharacterized). The peptide in fraction 26, later found to be pYR, was pure as determined by electrospray and MALDI-TOF mass spectrometry. This peptide elicited 34% release of the total cellular histamine.

#### 3.3. Structural characterization of pYR

The primary structure of the histamine releasing peptide in fraction 26 was established using automated Edman degra-

dation and the amino acid sequence of this peptide was determined as: YLKGCVTKSYPPKPCFSR-amide. MALDI-TOF and Q-TOF mass spectroscopy of the isolated peptide indicated a molecular mass of 2160 Da  $[M + H]^+$ . The peptide sequence was confirmed using Q-TOF MS/MS. The ESI-MS profile of the natural and synthetic peptides indicated that the natural peptide was amidated. Automated Edman degradation established the identity of residues through 18 cycles with two blanks in positions 5 and 15. De novo sequencing of the peptide by Q-TOF MS/MS and deconvolution spectrum confirmed cysteines residues at position 5 and 15 (Fig. 3).

#### 3.4. Effect of pYR and related analogues on CFU-GM formation

pYR exerted a dose-dependent inhibition of normal CFU-GM formation (Fig. 4). At 1  $\mu\text{g/ml}$  pYR inhibited granulopoiesis by  $47 \pm 3\%$ . Near maximal inhibition,  $84 \pm 1\%$ , was achieved by pYR at a concentration of 10  $\mu\text{g/ml}$  (Fig. 4a). In contrast, the amidated form pYR-amide evoked a  $56 \pm 3\%$  inhibition of granulopoiesis at a concentration of 10  $\mu\text{g/ml}$  (Fig. 4b). At 5  $\mu\text{g/ml}$  the C-loop of the peptide pYR<sup>5–15</sup> evoked a  $52 \pm 1\%$  inhibition of granulopoiesis (Fig. 4c). Thus, the amidated and the C-loop portion of pYR were not as potent as the non-amidated peptide in terms of maximal effects and the concentration required for 50% inhibition of granulopoiesis

#### 3.5. Effect of pYR and related analogues on neutrophil apoptosis

pYR, pYR-amide and pYR<sup>5–15</sup> had no effect on either viability or apoptosis of neutrophils at concentrations up to

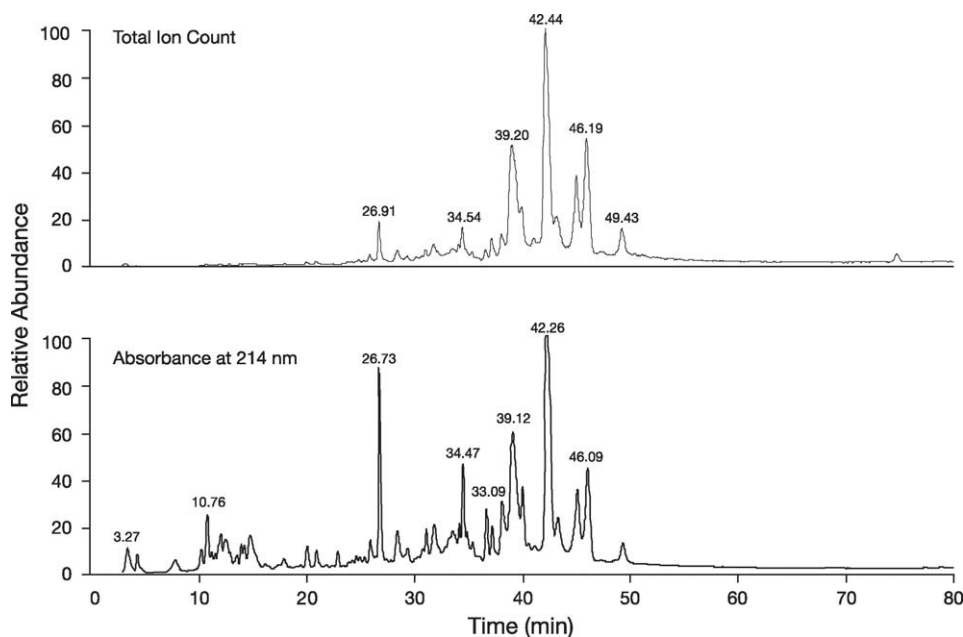


Fig. 1. Reverse phase HPLC purification of *R. sevos*a skin secretions. Pooled skin secretions (200  $\mu\text{l}$ ) from *R. sevos*a frogs ( $n = 5$ ) were applied to a Phenomenex Luna C18 column and eluted at a flow rate of 0.5 ml/min.

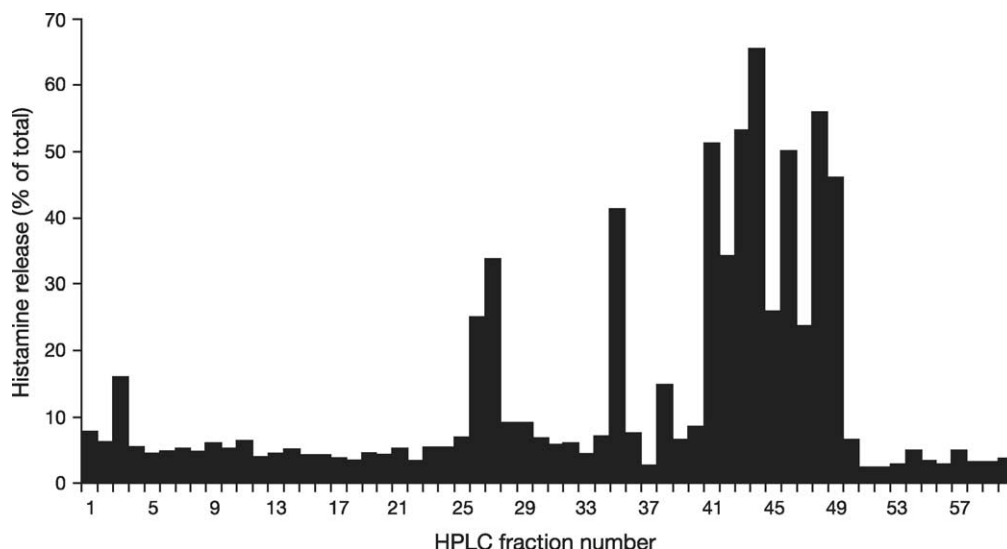


Fig. 2. Screen for histamine releasing activity of the HPLC purified fractions of *R. sevos*a. Lyophilized aliquots of each fraction were reconstituted in buffer and used in the histamine release assay.

10 µg/ml. Apoptotic status was determined using two distinct methods, staining with a combination of Hoechst 33258 and propidium iodide to morphological changes and staining with the cationic dye Mitosensor™ that detects changes in mitochondrial membrane permeability. In contrast, as a positive control, cells were treated with actinomycin D (10 µg/ml) resulting in 81 ± 4% of the cells in the apoptotic state as assessed by the Mitosensor™ staining.

**4. Discussion**

This study describes the isolation and characterization of pYR from the skin secretions of *R. sevos*a. The 18 amino acid

pYR shares 77.8% homology with peptide Leucine Arginine (pLR) and like pLR, it is a potent immunomodulatory peptide exerting effects on mast cells and granulopoietic progenitor cells [21]. Similar to pLR, the peptide displays the rigid polyproline motif Pro<sup>11</sup>-Pro<sup>12</sup>-Lys<sup>13</sup>-Pro<sup>14</sup> and two cysteines at residues 5 and 15. It is reasonable to assume that pYR, like its analogue pLR, will have a flexible N- and C-terminus with a rigid loop region governed by an intradisulphide bond between the two cysteine residues. Mass spectrometry is a highly sensitive and precise technique that has been used for structure and molecular weight determination. With advances in instrumentation, this technology has been successfully used to demonstrate the position of cysteine at residues 5 and 15 and that the natural pYR peptide is amidated. Ed-

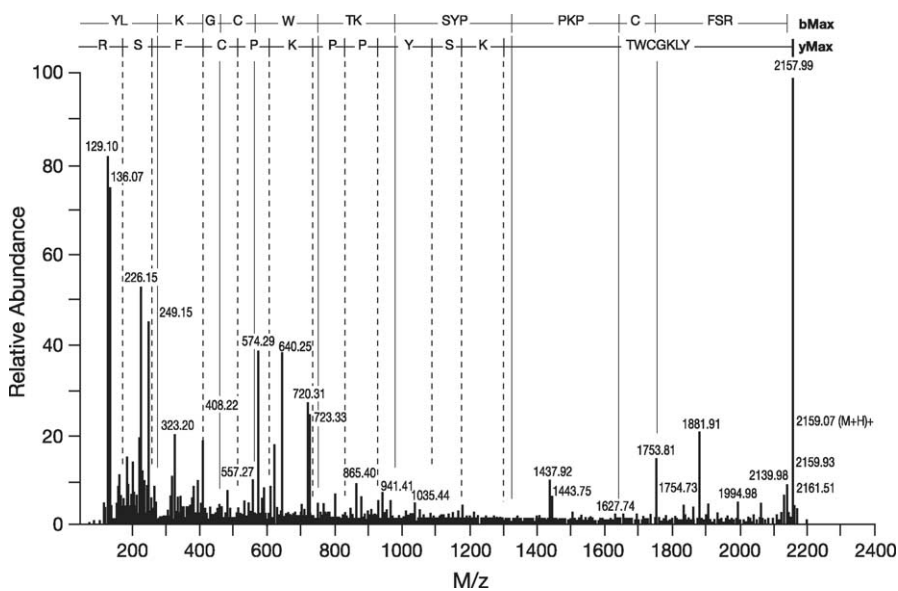


Fig. 3. Deconvolution spectrum generated from Q-TOF-MS/MS data for the natural pYR peptide, found in *R. sevos*a skin secretion. Spectrum shows amino acid sequence generated from the b- and y-ion profiles and indicated that the natural peptide is amidated.

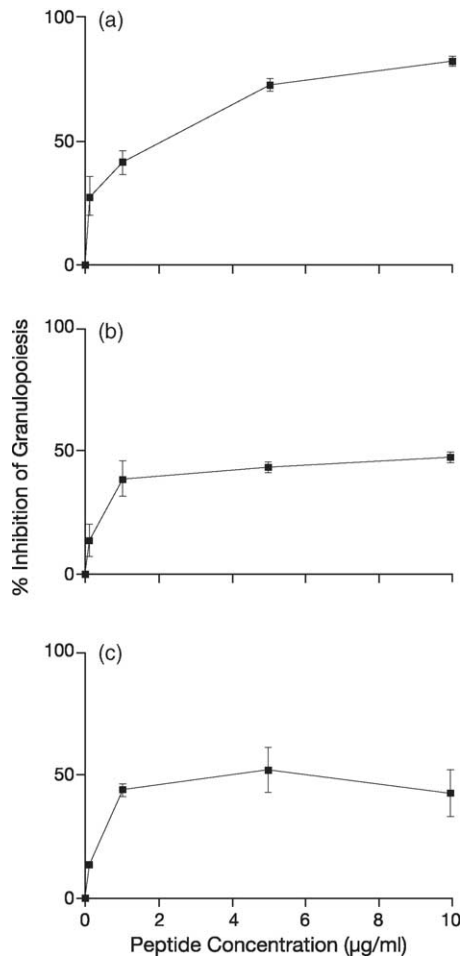


Fig. 4. Dose-dependent inhibitory effect of (a) pYR, (b) pYR-amide and (c) pYR<sup>5-15</sup> expressed as % inhibition of granulopoiesis compared to control. Increasing concentrations of the peptide were tested for the ability to inhibit granulopoiesis of human CFU-GM after 7 days incubation with bone marrow-derived stem cells. Data are presented as the means  $\pm$  S.E.M. for three independent experiments.

man degradation chemistry cannot determine amidation nor can it resolve cysteine residues unless the cysteine residues are reduced and modified. The high mass resolution afforded by Q-TOF mass spectrometry allows the difference of 1 Da to be readily detected enabling identification of C-terminal amidation.

PeptideYR interacts with mast cells resulting in degranulation. Mast cells are located throughout vascularised tissue, particularly at host–environmental interfaces such as the skin, lung and gastrointestinal tract and are important effectors cells of the immune system [24]. The release of cytokines and other mediators, such as histamine and serotonin, from mast cells has the potential to influence many aspects of the pathophysiology of allergic diseases at the site of mast cell degranulation e.g. airway hyper-reactivity, connective tissue changes and enhanced mucus production. The initial interaction between the mast cell and pYR is most likely electrostatically driven, with the polycationic peptide interaction with the anionic sialic acid residues on the mast cell membrane

[7]. The primary structure of pYR suggests that it is likely to be devoid of  $\alpha$ -helical regions as the peptide contains the helical breaking residues proline and glycine. This is consistent with previous studies that indicate that histamine-releasing ability is independent of  $\alpha$ -helicity [21,9].

PeptideYR exerted a dose-dependent inhibition of normal CFU-GM formation. This effect was not significantly different over the concentration range tested, to that observed and reported previously for pLR [21]. Synthesis of most peptide hormones from their precursors generally involves post-translational processing steps, which include signal peptide removal, endoproteolytic cleavage and the addition of post-translational modifications such as amidation and the establishment of disulfide bond [13]. Many bioactive peptide hormones require C-terminal amidation, with the amide group being absolutely required for activity of peptides such as gastrin, calcitonin and oxytocin [17]. However in the case of the pYR, the free C-terminal carboxy acid analogue was more potent in inhibiting normal CFU-GM formation. The free carboxy acid form of pLR was also shown to be a potent inhibitor of granulopoietic progenitor cells. This is extremely unusual, since in peptides where amidation is relatively unimportant, such as VIP and GHRF, amidation does not decrease their effectiveness [17]. The C-loop analogue was not as potent as the full-length peptide indicating that the flexible terminal regions may influence the conformation of the peptide and possibly have a putative role to play in any ligand recognition by receptors. Peptide YR did not induce any apoptotic change in mature neutrophil cultures over a 24 h period in comparison to cultures without the addition of the peptide indicating that immunomodulatory effects of pYR are stage specific. Since pYR directly inhibits CFU-GM formation in vitro, this suggests that the peptide may cause neutropenia in vivo, through its ability to suppress bone marrow progenitor cells.

Other peptides have been shown to exert an effect on granulocytes that is temporally regulated. A putative role for the human calcitonin gene-related peptide (CGRP), a potent vasoactive neuropeptide, has been suggested in the regulation of hematopoiesis in the bone marrow. The CGRP receptor is expressed on CD34<sup>+</sup> hematopoietic progenitor cells. CGRP directly acts on CD34<sup>+</sup> cells to promote formation of granulocyte-macrophage colonies. The effect of CGRP exerts on GFU-GM is not seen in mature granulocytes [10]. Similarly, pYR receptor expression may be restricted to early progenitor cells and lost as cells differentiate.

Other pLR-like peptides have been discovered in *Rana* frog's skin secretions such as ranacyclins isolated from *Rana esculenta* skin secretions and peptides from *Rana areolata* [16,1]. These peptides have not been assessed for their potential to inhibit granulopoiesis. Peptide YR is the second pLR like peptide to be isolated and characterized in the present manner. From these studies, it appears that pLR-like peptides are conserved within *Rana* frogs. Further research is needed to discover the true physiological function of this new family of peptides as both pYR and pLR have been shown to

be highly biologically potent and able to modulate immune function. Our recent studies indicate that this novel class of amphibian peptides may have potential as chemotherapeutic agents [21].

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## References

- [1] Ali MF, Lips KR, Knoop FC, Fritsch B, Miller C, Conlon JM. Antimicrobial peptides and protease inhibitors in the skin secretions of the crawfish frog, *Rana areolata*. *Biochim Biophys Acta* 2002;1601(1):55–63.
- [2] Anastasi A, Erspamer V, Bertaccini G. Occurrence of bradykinin in the skin of *Rana temporaria*. *Comp Biochem Physiol* 1965;14: 43–52.
- [3] Argiolas A, Pisano J. Isolation and characterization of two new peptides, mastoparan C and crabrolin, from the venom of the European hornet, *Vespa crabro*. *J Biol Chem* 1984;259(16):10106–11.
- [4] Argiolas A, Pisano J. Bombolitins, a new class of mast cell degranulating peptides from the venom of the bumblebee *Megabombus pennsylvanicus*. *J Biol Chem* 1985;260(3):1437–44.
- [5] Basir YJ, Knoop FC, Dulka J, Conlon JM. Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from skin secretions of the pickerel frog, *Rana palustris*. *Biochim Biophys Acta* 2000;1543(1):95–105.
- [6] Broome CS, Whetton AD, Miyan JA. Neuropeptide control of bone marrow neutrophil production is mediated by both direct and indirect effects on CFU-GM. *Br J Haematol* 2000;108(1):140–50.
- [7] Cocchiara R, Bongiovanni A, Albegiani G, Azzolina A, Lampiasi N, Di Blasi F, et al. Inhibitory effect of neuraminidase on SP-induced histamine release and TNF- $\alpha$  mRNA in rat mast cells: evidence of a receptor-independent mechanism. *J Neuroimmunol* 1997;75(1–2):9–18.
- [8] Conlon JM, Aronsson U. Multiple bradykinin-related peptides from the skin of the frog, *Rana temporaria*. *Peptides* 1997;18(3): 361–5.
- [9] Cross LJ, Ennis M, Krause E, Dathe M, Lorenz D, Krause G, et al. Influence of alpha-helicity, amphipathicity and D-amino acid incorporation on the peptide-induced mast cell activation. *Eur J Pharmacol* 1995;291(3):291–300.
- [10] Harzenetter MD, Keller U, Beer S, Riedl C, Peschel C, Holzmann B. Regulation and function of the CGRP receptor complex in human granulopoiesis. *Exper Hematol* 2002;30(4):306–12.
- [11] Irvine AE, French MA, Bridges JM, Crockard AD, Desai ZR, Morris TC. Normal unstimulated lymphocytes produce granulopoietic inhibitory activity. *Exper Hematol* 1991;19(2):106–9.
- [12] Irvine AE, Morris TCM, Kennedy H, Wisdom GB, Bridges JM. Human umbilical cord conditioned medium (HUCCM): a stimulus for human CFU-G. *Exper Haematol* 1984;12:19–24.
- [13] Iwai N, Martinez A, Miller MJ, Vos M, Mulshine JL, Treston AM. Autocrine growth loops dependent on peptidyl  $\alpha$ -amidating enzyme as targets for novel tumor cell growth inhibitors. *Lung Cancer* 1999;23(3):209–22.
- [14] Kim JB, Halverson T, Basir YJ, Dulka J, Knoop FC, Abel PW, et al. Purification and characterization of antimicrobial and vasorelaxant peptides from skin extracts and skin secretions of the North American pig frog *Rana grylio*. *Regul Pept* 2000;90(1–3):53–60.
- [15] Krane I, Naylor S, Helin-Davis D, Chin W, Spindel E. Molecular cloning of cDNAs encoding the human bombesin-like peptide neuromedin B. Chromosomal localization and comparison to cDNAs encoding its amphibian homolog ranatensin. *J Biol Chem* 1998;263(26):13317–23 [published erratum appears in *J Biol Chem* 1990 Apr 25;265(12):7091].
- [16] Mangoni ML, Papo N, Mignogna G, Andreu D, Shai Y, Barra D, et al. Ranacyclins, a new family of short cyclic antimicrobial peptides: biological function, mode of action, and parameters involved in target specificity. *Biochemistry* 2003;42(47):14023–35.
- [17] Merkler DJ. C-Terminal amidated peptides: production by the in vitro enzymatic amidation of glycine-extended peptides and the importance of the amide to bioactivity. *Enzyme Microbiol Technol* 1994;16(6):450–6.
- [18] Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM. An induced proximity model for caspase-8 activation. *J Biol Chem* 1998;273:2926–30.
- [19] Nakajima T, Yasuhara T, Uzu S, Wakamatsu K, Miyazawa T, Fukuda K, et al. Wasp venom peptides; wasp kinins, new cytotoxic peptide families and their physico-chemical properties. *Peptides* 1985;6(3):425–30.
- [20] Ravazzola M, Brown D, Leppaluoto J, Orzi L. Localisation by immunofluorescence of thyrotropin-releasing hormone in the cutaneous glands of the frog, *Rana ridibunda*. *Life Sci* 1979;25(15):1331–4.
- [21] Salmon AL, Cross LJM, Irvine AE, Lappin TRJ, Dathe M, Krause G, et al. Peptide Leucine Arginine, a potent immunomodulatory peptide isolated and structurally characterized from the skin of the Northern leopard Frog, *Rana pipiens*. *J Biol Chem* 2001;276(13):10145–52.
- [22] Shore A, Burkhalter A, Cohn VH. A method for the fluorometric assay of histamine in tissues. *J Pharmacol Exp Ther* 1959;127:182–6.
- [23] Simmaco M, De-Biase D, Severini C, Aita M, Erspamer GF, Barra D, et al. Purification and characterization of bioactive peptides from skin extracts of *Rana esculenta*. *Biochim Biophys Acta* 1990;1033(3):318–23.
- [24] Wedemeyer J, Tsai M, Galli SJ. Roles of mast cells and basophils in innate and acquired immunity. *Curr Opin Immunol* 2000;12(6):624–31.
- [25] Yasuhara T, Ishikawa O, Nakajima T. The studies on the active peptide in the skin of *Rana rugosa* II. The structure of ranatensin-R, the new ranatensin analogue, and granulin-R, the new mast cell degranulating peptide. *Chem Pharm Bull* 1979;27(2):492–8.