# Broad spectrum antibiotic activity of skin-PYY

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Received 29 November 1995

**Abstract Neuropeptide Y (NPY) and polypeptide YY (PYY) are two ubiquitous neuropeptides, found in brain and intestines, respectively, where they exert important regulatory functions. In this study, a new member of the YY family recently isolated from amphibian skin, skin-PYY (SPYY), is reported to inhibit irreversibly the proliferation of a broad spectrum of pathogenic microorganisms. NPY and PYY are shown to be endowed with the same activity. Their potency is similar to that of other antibacterial peptides which have been shown to exert their function by disintegrating the bacterial membrane. These findings and the fact that the C-terminal alpha-helical domain SPYY14–36, which is highly conserved among the family members, was responsible for killing microorganisms and for permeation of phospholipid vesicles, suggested that the antibiotic activity may emerge via a membrane permeation mechanism. These findings also raise the question whether NPY and PYY exert in vivo a similar function in mammals.**

*Key words:* Neuropeptide Y; Polypeptide YY; Amphibian skin; Antimicrobial peptide; Membrane lysis

## 1. Introduction

NPY [1] and PYY [2] are two 36 residues related peptides, found at high concentrations in the brain and in the lining of the gastrointestinal tract, respectively. They possess common features of tertiary structure, known as the PP-fold [3]. The PP-fold, as characterized by X-ray diffraction analysis of crystals, consists of two antiparallel helices: an N-terminal polyproline helix spanning residues 1–14, and a long amphipathic C-terminal alpha-helix. In mammals, these peptides are involved in a variety of important regulatory functions, e.g. sympathetic vascular control, central regulation of endocrine and autonomic function, food intake, circadian rhythm, etc., and are believed to induce their various biological effects by activating specific membrane bound receptors, $Y_1$ and/or $Y_2$ [4]. However, numerous experimental discrepancies raised the possibility that additional subtypes of Y-receptors exist [5,6]. Likewise, various atypical ($Y_1$- and $Y_2$-independent) activities remain ill-understood phenomena such as, histamine release from isolated mast cells, or increase of intracellular $Ca^{2+}$ in many cell types.

Recently, during the search for new antibiotic agents, chromatography of skin extract of the South American tree-frog *Phyllomedusa bicolor*, displayed three distinct antifugal fractions [7]. The activity of two of these fractions was shown to be due to two closely related peptides belonging to the dermaseptin family of antimicrobial peptides [7–10]. Surprisingly however, the antifugal activity of the third fraction was due to a peptide whose pharmacological and structural properties closely resembled those of NPY and PPY (72% and 94% amino acid positional identity, respectively) and thus termed Skin-PYY (SPYY) [11].

To confirm this unexpected antimicrobial activity, we investigated in this study, the ability of synthetic SPYY to affect the viability of various prokaryotic and eukaryotic cells in culture media. The data established that at micromolar concentrations SPYY behaves as a large spectrum antibiotic agent. Moreover, the concept was extended to other SPYY related peptide, NPY and PYY, which exhibited similar antimicrobial properties. A possible mode of action is discussed.

## 2. Materials and methods

### 2.1. Peptides

Peptides were prepared by stepwise solid-phase synthesis using fluoren-9-ylmethoxycarbonyl (Fmoc) polyamide active ester chemistry on a Milligen 9050 pepsynthesizer as described [11]. Cleavage of peptidyl-resin and side-chains deprotection were carried out with a mixture composed of trifluoroacetic acid, *para*-cresol, thioanisol, water and ethyl-methyl-sulfide (82.5,5,5,5 and 2.5 v/v). After filtering to remove the resin, and ether extraction, the crude peptides were purified by a combination of Sephadex gel filtration, ion exchange chromatography and preparative HPLC. Homogeneity of the synthetic peptides was assessed by analytical HPLC, amino acid analysis, solid phase sequence analysis and mass spectrometry. Human NPY and PYY were a generous gift of Dr. Hubert Vaudry from University of Rouen, France.

### 2.2. Biological assays

Antimicrobial assays were performed in sterilized 96-well plates (Nunc F96 microtiter plates, Denmark) as described [10]. Briefly, the synthetic peptides were weighted in a microbalance and solubilized in water at the desired primary dilution. Serial 2-fold peptide dilutions in water were added to suspensions containing $10^6$ spores or microconidia/ml in Sabouraud glucose broth, or $10^6$ bacteria/ml in Luria Bertani (LB) culture medium. Inhibition of growth was determined by measuring optical density at 492 nm with a Titertek Multiskan MCC after an incubation time of 24 h at 30°C. Bacteria were incubated at 37°C.

Reversibility of inhibition was assessed by incubating suspensions containing $1 \times 10^6$ cells/ml in culture media in presence of peptide concentration of 0.2 mg/ml. After various incubation periods, aliquots were centrifuged at $900 \times g$, the pellet washed and reincubated for 24 h in fresh culture medium.

The effect on leishmania parasites was assessed after peptide exposure by counting living cells following trypan blue inclusion. Promastigotes (strain MRHO/SU/59/Neal P.) at the stationary phase of growth ($1 \times 10^5$ cells/ml) were cultured at 26°C in RPMI 1640 complete medium. Amastigotes ($1 \times 10^4$ cells/ml) were purified from cutaneous lesions of infected Balb/c mice [12] and cultured at 37°C. Promastigote proliferation was assessed after 24 h incubation at 26°C in presence or absence of SPYY, followed by count of motile cells.

Hemolytic activity of the synthetic peptides was assayed with heparinized fresh human blood rinsed three times with PBS by centrifugation for 15 min at $900 \times g$. Red blood cells ($10^8$/ml) were then incubated under agitation at 37°C in distilled water for 100% hemolysis, in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7) for control or in PBS

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*SSDI* S0014-5793(96)00050-6

containing various concentrations of the peptide in a final volume of 0.2 ml. Release of hemoglobin was monitored after centrifugation at 900 × *g* by measuring the absorbance of 100 μl of supernatant at 541 nm after up to 24 h of incubation.

Toxicity against murine macrophages: macrophages were obtained by wash of peritonal cavity of Balb/c mice with 10 ml Dulbecco's modified Eagle's medium (DMEM, Gibco) as described [13]. Resident cells were allowed to adhere for 3 h at 37°C–5% $CO_2$ in 8-well plates (Lab-Tek) at $2 \times 10^5$ cells/well, then thoroughly washed to remove non-adherent cells and incubated with various SPYY concentrations. After 24 h incubation, the viability of treated macrophage was assessed after trypan blue inclusion by counting the live cells.

*2.3. Membrane permeation*

Small unilamellar vesicles (SUV) were prepared by sonication of PC/PS (1:1 w/w) and cholesterol (10% by weight) as described [14]. Membrane permeation was assessed using the diffusion potential assay [15] as described [14]. Increasing concentrations of the peptide were mixed with SUV that had been pretreated with the fluorescent potential-sensitive dye (diS-$C_2$-5) and valinomycin. Recovery of fluorescence was monitored as a function of time and usually occurred within 1 to 10 min. Maximal activity of the peptides was plotted versus peptide/lipid molar ratio.

## 3. Results

The peptide's ability to inhibit cell proliferation is reported in terms of minimal inhibitory concentration (MIC), defined as the lowest peptide dose at which 100% inhibition of growth was observed after 24 h of incubation. As shown in Table 1, at peptide concentrations ranging between 10 and 100 μg/ml SPYY inhibited the proliferation of a large spectrum of pathogenic microorganisms, including bacteria, yeasts and filamentous fungi. In fact, SPYY had a similar though non-identical spectrum of activity as dermaseptin S1, a well established antibiotic peptide from frog skin (Table 1). Whereas, the two peptides displayed similar MIC against *A. cavias*, *E. faecalis* and *A. fumigatus*, SPYY was less efficient than dermaseptin S1 against *E. coli* and *A. niger*, and SPYY was clearly more efficient against *N. brasiliensis*, *M. canis*, *T. rubrum* and *A. simii*.

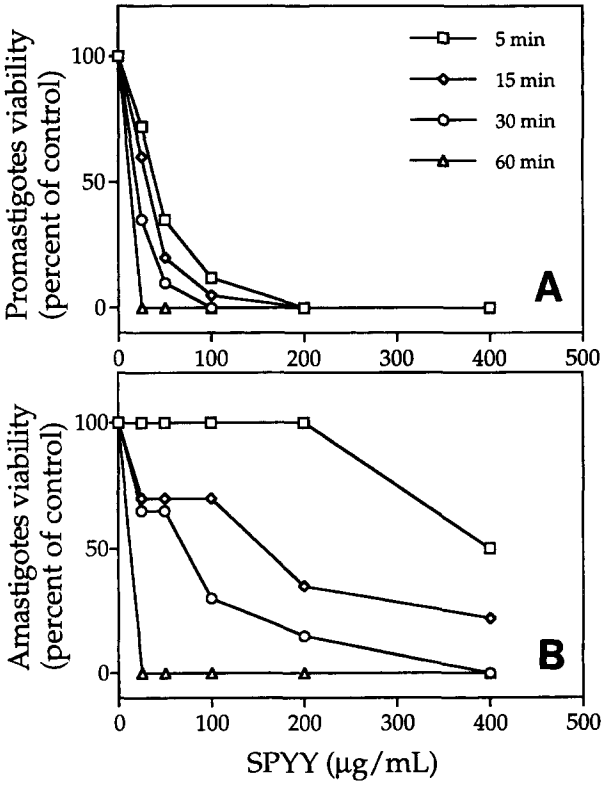


Fig. 1. Dose-dependent kinetics of the leishmanicidal effect. Each point represents the mean from at least 2 independent experiments performed in duplicates. Standard deviations were ≤ 10%.

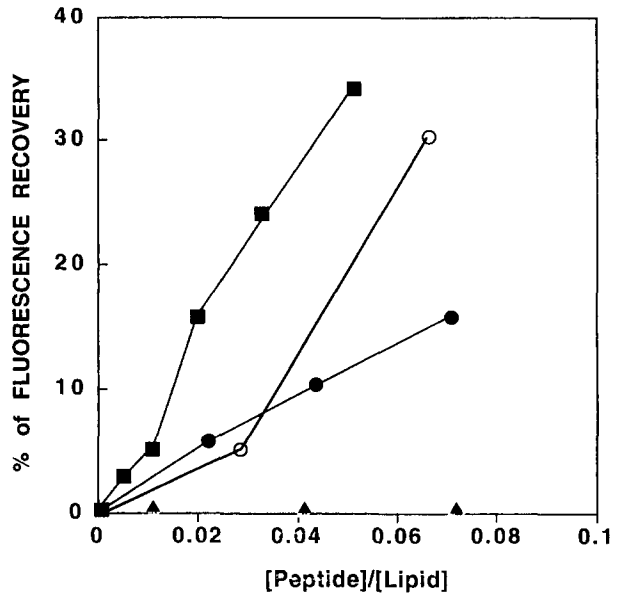


Fig. 2. Membrane permeation effect on acidic PC/PS vesicles. SPYY, filled circles; $SPYY_{14-36}$, squares; Cecropin B1, empty circles; control peptide (see legend of Table 2), triangles. Maximal activity of the peptides was plotted versus peptide/lipid molar ratio. Each point represents the mean of 3 to 6 separate experiments with standard deviation of ±5%.

To verify the reversibility of inhibition, treated suspensions were thoroughly washed at various periods and reincubated in fresh, SPYY-free culture medium. Washed microorganisms that were exposed to SPYY for 24 h, did not proliferate after additional 48 h of incubation. These results remained unchanged when suspensions were exposed to SPYY for 1 h or for 10 min. This indicated that the effect was rapid and irreversible.

The antibiotic effect of SPYY was further investigated using *Leishmania major*, the protozoan parasite that is responsible for worldwide diseases in man and animals. The parasite's life cycle consists of an extracellular promastigote stage, found in gut of sandflies, and an intracellular amastigote stage that occurs within the phagolysosomes of macrophages.

The effect of SPYY on the promastigote form was easily observable under light microscope. Upon SPYY addition, the motile parasites rapidly ceased to move and became round. With time, many cellular debris were observable although the shape of the promastigote was not dramatically altered (not shown). Yet, 100% promastigotes were found non viable within 1 h incubation at 25 μg/ml (Fig. 1A). For shorter incubation periods, the killing effect was dose dependent. At these concentrations, SPYY inhibited the proliferation of promastigotes as determined after 24 h incubation. Moreover, the effect was irreversible since treated parasites did not recover motility, nor did they proliferate after a thorough wash and reincubation over 48 h in SPYY-free fresh culture medium.

Table 1
Spectrum of antimicrobial activity and toxicity in culture media

| Organism | MIC[a] (μg/ml) | | | | |
|---|---|---|---|---|---|
| | SPYY | NPY | PYY | $SPYY_{14-36}$ | DS[c] |
| *Aeromonas caviae* | 60 | – | – | 40 | 50 |
| *Escherichia coli* | 15 | – | – | 10 | 5 |
| *Enterococcus faecalis* | 20 | – | – | 10 | 25 |
| *Nocardia brasiliensis* | 30 | – | – | 20 | 100 |
| *Cryptococcus neoformans* | 25 | 30 | 25 | 20 | 15 |
| *Candida albicans* | 25 | 25 | 25 | 15 | 60 |
| *Microsporum canis* | 10 | – | – | 10 | 50 |
| *Tricophyton rubrum* | 15 | – | – | 15 | 100 |
| *Arthroderma simii* | 15 | 20 | 15 | 10 | 100 |
| *Aspergillus fumigatus* | 100 | – | – | 80 | 100 |
| *Aspergillus niger* | >100 | – | – | >100 | 100 |
| | Toxicity[b] (μg/ml) | | | | |
| Human erythrocytes | >100 | >100 | >100 | >100 | >100 |
| Murine macrophages | >100 | >100 | >100 | >100 | >100 |

[a] Minimal inhibitory concentrations for cell proliferation were determined after 24 h incubation.
[b] Toxicity was determined after 1 h incubation. Each value was determined from at least 2 independent experiments performed in duplicate. Variations were <20%. –, not determined.
[c] The spectrum of dermaseptin (DS) is shown for comparison [8].

In addition, a similar profile was observed in the activity of SPYY against the amastigotes form of the parasite (Fig. 1B). Albeit, SPYY was less potent against amastigotes than against promastigotes, at incubation period shorter than 1 h.

Interestingly, toxicity was not observed for mammalian erythrocytes or macrophages up to peptide concentration of 100 μg/ml (Table 1), a concentration that killed a large variety of pathogenic microorganisms.

To further confirm these findings, antimicrobial activity was investigated for 2 SPYY-related peptides, NPY and PYY, under the same experimental conditions. Despite differences in primary structure that are located mostly within the N-terminal segment (Table 2), both peptides exhibited antibiotic activity against microorganisms at comparable concentrations, with no toxicity for mammalian cells (Table 1).

Moreover, a short peptide version of SPYY ($SPYY_{14-36}$) representing the C-terminal alpha-helical portion which is highly conserved among the PP family members showed that the truncature of the N-terminal 13 residues did not alter the peptide's antimicrobial properties (Table 1). In fact, $SPYY_{14-36}$ displayed a molar potency comparable to that observed for the parent molecule, against most microorganisms assayed. Conversely, the N-terminal fragment $SPYY_{1-14}$ had no antimicrobial activity against any of the microorganisms assayed.

Finally, to gain insight into the mechanism of antimicrobial action, we used the Dissipation of Diffusion Potential Assay to examine the efficacy of SPYY and its shorter version in perturbing the lipid packing and causing leakage of vesicular contents, a property that is characteristic of well defined antimicrobial peptides such as cecropin [16], magainin [17] or dermaseptin [18,19].

Albeit with different potencies, both SPYY and $SPYY_{14-36}$ permeated the phospholipid vesicles with a comparable or higher efficiency than that observed for cecropin (Fig. 2).

Table 2
Primary structures of YY peptides assayed[a]

| | |
|---|---|
| NPY | YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-$NH_2$ |
| PYY | YPIKPEAPGEAASPEELNRYYASLRHYLNLVTRQRY-$NH_2$ |
| SPYY | YPPKPESPGEDASPEEMNKYLTALRHYINLVTRQRY-$NH_2$ |
| $SPYY_{14-36}$ | PEEMNKYLTALRHYINLVTRQRY-$NH_2$ |
| $SPYY_{1-14}$ | YPPKPESPGEDASP |

[a] To control for any compounds carried over in the peptide preparation, a helical polyanionic peptide (EEEKRENEDEEKQDDEQSEM) was prepared and used in parallel in all experiments. The effect of the acidic peptide was generally equivalent to the untreated control experiments.

## 4. Discussion

Peptide synthesis and characterization of the final product were reported in detail [11] where the identity between the synthetic product and the natural SPYY was established. In this study, the synthetic replicate was used to confirm the antifungal activity observed for natural SPYY [7]. Such activity, was indeed confirmed and SPYY was furthermore found to have antibiotic action over a large spectrum of pathogens.

Previous studies have reported that peptide sequences corresponding to neuropeptide or hormonal fragments, such as Gastric Inhibitory $Polypeptide_{7-42}$ or Diazepam-Binding $Inhibitor_{32-86}$, were endowed with antimicrobial activity [20]. However, the synthetic replicates of the said peptides or their parent molecules were devoid of such activity. This study reports thus for the first time convincing evidence that antibiotic activity is associated with otherwise notoriously known gastro-intestinal and brain mammalian neuropeptides. It should be noted that antimicrobial activity was reported concerning another neuroactive skin peptide, adenoregulin, a 33-residue amphipathic-polycationic peptide that shares no homology with YY peptides, and that enhances binding of agonists to the A1 adenosine receptors [21]. Like the YY peptides, adenoregulin also acts at specific receptor sites with affinity in the nanomolar range, yet adenoregulin exhibited potent antibiotic properties at micromolar concentrations [7].

It seems unlikely that the mechanism of antimicrobial action exhibited by YY peptides, is mediated by activation of specific receptors. Rather, inherent structural properties may be involved in a concentration dependent manner that may explain

other atypical effects of YY peptides. Namely, Shen et al. proposed that histamine release from isolated mast cells as induced by NPY, is associated with non-receptor mediated direct activation of G-proteins [22]. However, this does not account for cell lysis. On the other hand, studies performed with antimicrobial peptides such as the dermaseptins, cecropin, or magainin indicated that the basicity and amphiphilicity of their alpha-helical structure are the responsible features for inducing cells lysis [23,24]. According to this hypothesis, upon association of the peptide at the membrane surface via electrostatic interactions, the amphipathic helix penetrates in the membrane and perturbs its structure, which results in rapid osmolysis. Moreover, since peptide-membrane interactions are governed by their respective physico-chemical properties (amphipathy, peptide length, charge distribution, lipid composition) it was postulated that lack of toxicity towards mammalian cells is due to the weak- or lack of interactions between these peptides and mammalian membranes [25,26]. Therefore, one expects that mild perturbation of the membrane structure may simply lead in some cases to non-fatal cross-membrane leakage of ions and other small molecules from high- to low concentration. We propose that SPYY, and related YY peptides, may act in a similar manner. Concordant with this view are the following observations: (i) the antibiotic action of SPYY did not require the N-terminal sequence, suffice the C-terminal domain which is an amphipathic alpha-helical and polycationic structure. In addition, whereas the C-terminal fragment usually induces the opposite effect of the parent molecule [5], in this study, SPYY and $SPYY_{14-36}$ has the same activity; (ii) both SPYY and $SPYY_{14-36}$ were able to perturb the structure of PS/PC vesicles and to permeated them, yet $SPYY_{14-36}$ was more efficient than the parent peptide; (iii) their potency is similar to that of other antibacterial peptides such as dermaseptin, cecropin and magainin which have been shown to exert their function by disintegrating microbial membrane; (iv) the time required to induce their antibiotic effect was short (<10 min) and irreversible.

In conclusion, SPYY and its related peptides may represent a promising alternative in the search for new non-toxic, large spectrum antibiotic agents. Nevertheless, skin secretions of amphibians have long been considered as a rich source for biologically active peptides including hormones and neuropeptides [27–29]. Although many of these peptides have identical or closely related counterparts in mammalian tissues, their physiological role in the skin is generally unclear. The results reported in this study suggest a physiological role for SPYY in the protection of amphibians against invading microbes and, by extension, raise the question whether SPYY like neuropeptides, i.e. NPY and PYY, do exert a similar function in mammalians and thereby contribute, to an extent, to control germ dissemination in their respective tissues of origin where they are largely expressed. Interestingly, NPY was found present at very high concentrations in brain of normal humans (about 10 $\mu$g/g wet tissue), exceeding those of CCK, VIP or somatostatin, hitherto considered to be the most abundant peptides in the nervous system [30]. Therefore, the fact demonstrated in this study, that at the low $\mu$g/ml level NPY killed 100% of the $10^6$ microorganisms in artificial culture medium, suggests that physiologically, the peptide could reach high concentrations that are effective for antimicrobial activity. However, such physiological role for YY peptides remains to be demonstrated.

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