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# Mechanism of action of puroindoline derived tryptophan-rich antimicrobial peptides

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ABSTRACT

A tryptophan (Trp)-rich region in the wheat endosperm protein, puroindoline A, was previously shown to possess potent antimicrobial activity against Gram-positive and Gram-negative bacteria and this was attributed to the peptide inducing membrane instability. In the present work, the antimicrobial activity of the corresponding Trp-rich region in the puroindoline B isoform was examined and its antimicrobial activity was characterized. Unexpectedly, the puroindoline B Trp-rich peptide (PuroB) was relatively inactive compared to the related puroindoline A peptide (PuroA), despite strong sequence similarity. Using the sequence of PuroA as a template, a series of PuroB variants were synthesized and the antimicrobial activity was restored. Interestingly, all of these PuroB peptides preferentially interacted with negatively charged phospholipids, but unlike PuroA, they did not disrupt the integrity of lipid bilayers. This suggests that the primary mode of action of the PuroB peptides involves an antimicrobial target other than the bacterial membrane. Further tests revealed that all of the puroindoline derived peptides bind deoxyribonucleic acid (DNA) and block macromolecular synthesis *in vivo*. Based on these results, it appears that the interaction between puroindoline derived peptides and membranes is only an initial step in the mode of action and that binding to intracellular targets, such as DNA and ribonucleic acid (RNA), contributes significantly to their antimicrobial mode of action.



## 1. Introduction

The problem of antibiotic resistance has spurred continued interest in antimicrobial peptide research. Naturally occurring antimicrobial peptides continue to be discovered in a variety of organisms and *de novo* synthetic peptides have also been generated through combinatorial chemistry, for example [1]. A different approach to identifying antimicrobial peptides involves excising peptide fragments from larger proteins to generate novel sequences. This strategy has been used to identify a number of antimicrobial sequences including lactoferricin [2,3] and lactoferrampin [4], both antimicrobial peptides derived from the iron binding protein, lactoferrin. Other examples of antimicrobial peptides isolated from larger proteins include peptides derived from lysozyme [5] and histones [6,7]. In this study, we focus on a series of antimicrobial peptides based on sequences from wheat puroindolines.

Puroindolines are highly basic, Trp- and Cys-rich proteins found in wheat (*Triticum aestivum*) endosperm [8,9]. In fact, the name puroindoline is derived from *puros*, the Greek word for wheat, and the indole ring found in the side chain of Trp residues [10]. The pinA and pinB genes are both located in the hardness locus of the wheat genome and they encode the puroindoline A and puroindoline B proteins respectively [11,12]. These two puroindoline proteins share 55% sequence homology with each other and are unique because of the presence of 5 disulfide bonds, a large number of basic residues and a tryptophan-rich region [9]. In addition to controlling the hardness of the wheat kernels, puroindolines also exhibit antimicrobial and antifungal properties [13–16]. Part of this antimicrobial activity is thought to be related to the ability of full length puroindoline to induce pores in lipid bilayers [10,17], a feature which is attributed to the Trp-rich loop in the native protein. This is of particular interest because this region resembles other Trp-rich cathelicidin antimicrobial peptides such as bovine indolicidin [18] and porcine tritrpticin [19,20]. In an effort to understand the antimicrobial and lipid binding properties of the puroindolines, we sought to examine peptide fragments based on the Trp-rich loops from puroindoline A and B.

Our group previously studied a 13-residue peptide corresponding to the Trp-rich region in puroindoline A (FPVTWKWWKWWKG-$NH_2$).

*Abbreviations:* PuroA, puroindoline A derived peptide; PuroB, puroindoline B derived peptide; DSC, differential scanning calorimetry; ePC, Egg derived L-α-phosphatidylcholine; ePG, egg derived L-α-phosphatidylglycerol; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DiPoPE, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine; $T_{hex}$, hexagonal phase transition temperature; PLE, *E. coli* polar lipid extract; DPC, dodecylphosphocholine; diSC3-5, 3,3′-Dipropylthiadicarbocyanine iodide; Kd, dissociation constant; Ksv, Stern–Volmer constant; LUV, large unilamellar vesicle; MIC, minimum inhibitory concentration

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This peptide exhibited potent antimicrobial activity against Gram-positive and Gram-negative bacteria and we concluded that this 13-mer (PuroA) constitutes the antimicrobial active center of puroindoline A [21]. The NMR structure of PuroA in the presence of SDS micelles adopts a partially helical amphipathic structure. In addition, PuroA preferentially interacts with negatively charged membranes and causes calcein leakage from negatively charged liposomes [21]. Based on these observations, it was concluded that PuroA employs a lytic mechanism to exert its antimicrobial effect.

The corresponding Trp-rich region in puroindoline B is a 12-mer (FPVTWPTKWWKG-$NH_2$) with remarkable sequence similarity to PuroA. However, when tested for antimicrobial activity, this 12-mer (PuroB1) was inactive compared to PuroA (see below). In this work, a series of peptides based on the PuroB1 sequence were synthesized in an attempt to increase the antimicrobial potency of this peptide. Three of these PuroB derivatives demonstrated antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* at similar concentrations to those seen for PuroA. Interestingly, biophysical characterization of the interactions between the PuroB peptides and lipids suggests that the principal mechanism of action does not involve the disruption of the bacterial membrane. Instead, our results suggest that the PuroB peptides cross the bacterial cytoplasmic membrane and bind to nucleic acids within the cell, blocking macromolecular synthesis of DNA, RNA and proteins. In addition, continued investigations of the PuroA peptide revealed that the membrane destabilizing effects of this peptide contribute to a more complex mode of action that also includes an intracellular mode of action.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All the phospholipids (ePC, ePG, DPPG, DPPC, DiPoPE and PLE) as well as DPC were obtained from Avanti Polar Lipids (Alabaster, AL) as stock solutions dissolved in chloroform. SDS was obtained from EMD Chemicals Inc. (Gibbstown, NJ). 3,3′-Dipropylthiadicarbocyanine iodide (diSC3-5) was purchased from AnaSpec Inc. (Fremont, CA). Deuterated $d_{25}$-SDS was purchased from Cambridge Isotopes Laboratories (Andover, MA). [methyl-$^3$H]-thymidine (6.7 Ci/mmol) and [5-$^3$H]-uridine (25.5 Ci/mmol) were obtained from Perkin Elmer (Waltham, MA) while [ring-2,5-$^3$H]L-histidine (47.7 Ci/mmol) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA). All other reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

### 2.2. Peptide sources

The puroindoline derived peptides were synthesized by AnaSpec (Fremont, CA) or at the University of Western Ontario peptide synthesis facility (London, Ont. Canada). All peptides were obtained at a purity of >95% as determined by HPLC and were used without further purification. The identity of each peptide was confirmed by mass spectrometry. All of the peptides were amidated at their C-terminus to remove the negative charge of the C-terminal carboxyl group. The sequences and net charges of the puroindoline peptides are shown in Table 1. Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-$NH_2$), purified from honey bee venom, was purchased from Sigma-Aldrich.

### 2.3. Peptide concentration determination

Peptide concentrations were determined based on the absorbance at 280 nm of a diluted peptide stock solution and the concentration was calculated according to Beer's law using theoretical extinction coefficients of 27500 $M^{-1}$ $cm^{-1}$, 16500 $M^{-1}$ $cm^{-1}$ and 5500 $M^{-1}$ $cm^{-1}$ for PuroA, all the PuroB peptides and melittin respectively. Theoretical extinction coefficients were generated with the ProtParam tool on the ExPASY proteomics server [22].

**Table 1**
Sequences and net charges of the puroindoline peptides.

| Peptide | Sequence | Net charge |
|---|---|---|
| PuroB1 | FPVTWPTKWWKG-$NH_2$ | +3 |
| PuroB2 | FPVTW**R**TKWWKG-$NH_2$ | +4 |
| PuroB3 | F**R**VTW**R**TKWWKG-$NH_2$ | +5 |
| PuroB4 | F**A**VTW**A**TKWWKG-$NH_2$ | +3 |
| PuroB5 | F**K**VTW**K**TKWWKG-$NH_2$ | +5 |
| PuroA | FPVTWKWWKWWKG-$NH_2$ | +4 |

### 2.4. Antimicrobial and hemolytic activity

The antimicrobial and hemolytic activities of the PuroB peptides were determined using the same procedure described for PuroA [21]. Briefly, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were grown to the exponential phase in 2% Bacto Peptone water (Difco 1807-17-4). The cell suspensions were diluted to $2 \times 10^6$ cfu/ml ($3.8 \times 10^8$ cfu/ml = 1U of $A_{600}$) in 2% Bacto Peptone Water and then added to the wells of 96-well polystyrene plates. Peptides were added to wells at concentrations ranging from 300 to 1.0 μg/ml and the plates were incubated overnight at 37 °C. The change in turbidity at 540 nm was measured and the MIC was defined as the lowest concentration of peptide that inhibited growth. All peptides were tested in triplicate.

To determine the hemolytic activity, human erythrocytes were isolated from heparinized human blood by centrifugation followed by three washes in phosphate-buffered saline (5 mM phosphate buffer, 150 mM NaCl, pH 7.4). Cell suspensions containing ~$10^7$ cells/ml were incubated with peptide for 30 min at 37 °C with gentle mixing. The cells were centrifuged and the absorbance at 540 nm was measured. Zero percent hemolysis was measured by adding phosphate buffer to the erythrocyte suspension while 100% hemolysis was achieved by adding 1% Triton X-100 to the erythrocytes.

### 2.5. Tryptophan emission fluorescence

Spectra were acquired on a Varian Cary Eclipse Fluorimeter (Varian Inc., Palo Alto, CA) equipped with a temperature control device set to 25 °C. Tryptophan emission spectra were collected between 300 and 450 nm using an excitation wavelength of 295 nm and excitation and emission slit widths of 10 nm. All samples contained 1 μM peptide in buffer (10 mM Tris, 150 mM NaCl. 1 mM EDTA, pH 7.4). Samples containing SDS and DPC were prepared to a final detergent concentration of 25 mM. Samples containing large unilamellar vesicles (LUVs, see calcein leakage section for details on LUV preparation) were made to a final lipid concentration of 30 μM.

Lipid binding was measured by titrating aliquots of *E. coli* polar lipid extract LUVs into 0.5 μM peptide solutions in buffer. After each addition of lipid, an emission spectrum was recorded between 300 and 400 nm using an excitation wavelength of 280 nm. Blank spectra were recorded for each addition of LUV into buffer and subtracted in the final analysis. The wavelength maxima (λmax) were determined by taking the slope of the emission spectra and using the wavelength where this value was closest to zero. The λmax values were plotted as a function of lipid concentration and the dissociation constants (Kd) were calculated in CaLigator [23] using a least-squares fit algorithm and a one-site binding model. The CaLigator software is typically used to determine calcium binding constants to proteins but it can also be used to evaluate the binding of any two interacting partners.

### 2.6. Acrylamide quenching of emission fluorescence

Stern–Volmer constants (Ksv) [24] were calculated as described previously [25,26]. Briefly, 5 μl aliquots of a 4 M acrylamide solution