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Bactericidal Activity Engineered on Human Pancreatic Ribonuclease and Onconase†

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Abstract: Ribonucleases belonging to the pancreatic-type family exhibit a variety of biological activities that make them potential candidates as chemotherapeutic agents. Among them are remarkable the selective cytotoxicity against tumor cells, exhibited by onconase, and the bactericidal activity presented by the eosinophil cationic protein (ECP). In the past years, based on what is known about the cytotoxic mechanism of ribonucleases, a lot of work has been performed to switch non-naturally cytotoxic ribonucleases to potent toxins. Most of the efforts have been devoted to the production of ribonucleases endowed with selective cytotoxicity against tumor cells. In the present paper, however, we have used two nonbactericidal ribonucleases, onconase and the human pancreatic ribonuclease, as scaffolds onto which to engineer bactericidal activity. To this end, the main bactericidal determinant described for ECP (YRWR) has been introduced to these proteins either in an internal position or as an extension of the C-terminal end. The ribonucleolytic activity, thermostability, cytotoxicity against eukaryotic cells and the antibacterial activity against Gram-positive and Gram-negative strains have been determined for all the variants produced. The results show that we have endowed both ribonucleases with antibacterial activity against Gram-negative and Gram-positive bacteria. In addition, we show that this activity is, at least in part, dependent on the ribonucleolytic activity of the enzymes. Remarkably, we have developed a human pancreatic ribonuclease variant with *de novo* acquired selective antibacterial which is not cytotoxic to mammalian cells.

Keywords: Bactericidal activity; onconase; human pancreatic ribonuclease

Introduction

Ribonucleases (RNases) can be cytotoxic and thus have noteworthy potential as chemotherapeutic agents. For example, onconase (ONC) a member of the ribonuclease A (RNase A) superfamily, which can be obtained from oocytes or early embryos of Northern Leopard frog (*Rana pipiens*), presently is undergoing phase III human clinical trials for the treatment of malignant mesothelioma.[1] A lot of work has been performed during the past years trying to understand the molecular basis underlying the cytotoxic activity of RNases and switch non-naturally cytotoxic RNases to potent toxins. As a proof, RNase A itself and human RNase 1 (human pancreatic ribonuclease, HP-RNase) are not toxic to mammalian cells, but properly engineered variants have acquired this ability.[2−5]

Human RNase 3 (eosinophil cationic protein, ECP) is a RNase A family member with reported Gram-positive and

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(1) Mikulski, S. M.; Costanzi, J. J.; Vogelzang, N. J.; McCachren, S.; Taub, R. N.; Chun, H.; Mittelman, A.; Panella, T.; Puccio, C.; Fine, R.; Shogen, K. Phase II trial of a single weekly intravenous dose of ranpirnase in patients with unresectable malignant mesothelioma. *J. Clin. Oncol.* **2002**, *20* (1), 274–81.

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Gram-negative antimicrobial activity[6,7] and cytotoxic properties on host epithelial tissues such as tracheal epithelium.[8,9] ECP is, together with human RNase 2 (eosinphil derived neurotoxin, EDN), a human host defense RNase involved in inflammatory processes mediated by eosinophils.[10] The ribonucleolytic activity of ECP with commonly used RNA substrates is low and does not appear to be necessary for the antibacterial capacity.[7,11] The contribution of aromatic and cationic surface-exposed residues of ECP to the membrane-lytic and bactericidal activity has been described recently.[12] ECP can bind and partially insert into the lipid bilayers, promoting its aggregation and final lysis, following a carpetlike mechanism.[13,14] Nevertheless, the same authors concluded that the membrane destabilizing activity cannot solely explain the ECP antimicrobial action.[12,15]

It has also been reported that these bactericidal determinants are involved in the inhibition of mammalian cell proliferation mediated by ECP.[15,16] In particular, the combination of Trp-Arg at positions 35−36 at the $\alpha 3-\beta 1$ loop of ECP, which is a unique feature among the members of the pancreatic-type RNase family, play a critical role in both the antimicrobial and cytotoxic activities displayed by ECP.

Although there is abundant information on the mechanism of action of antimicrobial peptides, much less is known on the larger polypeptides. The studies on the mammalian antimicrobial proteins that participate in the innate immune system are of great interest for the treatment of infection and inflammatory disorders.[17] Among them, antimicrobial RNases can also be regarded as potential therapeutic tools. Antimicrobial proteins and peptides are an emerging field of interest for the pharmaceutical industry both for the development of new antibiotics to fight the resistant strains and for an applied therapy for inflammatory disorders and cancer treatment.

In this study, we have used two nonbactericidal RNases, HP-RNase and ONC, as scaffolds onto which to engineer bactericidal activity. We reasoned that introducing different combinations of Trp-Arg residues into diverse positions of these RNases would endow them with toxic activity against bacterial cells. We describe the specific amino acid changes made to HP-RNase and ONC, the contribution of these substitutions to the conformational stability and ribonucleolytic activity, and how this approach ultimately results in ONC variants with bactericidal activity additional to preexisting cytotoxicity against eukaryotic cells and HP-RNase variants that are toxic only against bacterial cells. To the best of our knowledge these are the first engineered pancreatic-type RNases that have acquired bactericidal activity.

Experimental Section

Material. The *Escherichia coli* strain BL21(DE3) and the ribonuclease substrate 6-FAM-dArUdAdA-6-TAMRA were obtained from Novagen (Madison, WI). Oligonucleotides used for site-directed mutagenesis and molecular biology enzymes were from Roche (Basel, Switzerland). Human ribonuclease inhibitor (RI) was from Promega (Madison, WI). Other chemicals were from Sigma (ST. Louis, MO).

(2) Leland, P. A.; Schultz, L. W.; Kim, B. M.; Raines, R. T. Ribonuclease A variants with potent cytotoxic activity. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95* (18), 10407–12.

(3) Leland, P. A.; Staniszewski, K. E.; Kim, B. M.; Raines, R. T. Endowing human pancreatic ribonuclease with toxicity for cancer cells. *J. Biol. Chem.* **2001**, *276* (46), 43095–102.

(4) Bosch, M.; Benito, A.; Ribo, M.; Puig, T.; Beaumelle, B.; Vilanova, M. A nuclear localization sequence endows human pancreatic ribonuclease with cytotoxic activity. *Biochemistry* **2004**, *43* (8), 2167–77.

(5) Rutkoski, T. J.; Kurten, E. L.; Mitchell, J. C.; Raines, R. T. Disruption of shape-complementarity markers to create cytotoxic variants of ribonuclease A. *J. Mol. Biol.* **2005**, *354* (1), 41–54.

(6) Lehrer, R. I.; Szklarek, D.; Barton, A.; Ganz, T.; Hamann, K. J.; Gleich, G. J. Antibacterial properties of eosinophil major basic protein and eosinophil cationic protein. *J. Immunol.* **1989**, *142* (12), 4428–34.

(7) Rosenberg, H. F. Recombinant human eosinophil cationic protein. Ribonuclease activity is not essential for cytotoxicity. *J. Biol. Chem.* **1995**, *270* (14), 7876–81.

(8) Tai, P. C.; Ackerman, S. J.; Spry, C. J.; Dunnette, S.; Olsen, E. G.; Gleich, G. J. Deposits of eosinophil granule proteins in cardiac tissues of patients with eosinophilic endomyocardial disease. *Lancet* **1987**, *1* (8534), 643–7.

(9) Motojima, S.; Frigas, E.; Loegering, D. A.; Gleich, G. J. Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium in vitro. *Am. Rev. Respir. Dis.* **1989**, *139* (3), 801–5.

(10) Slifman, N. R.; Loegering, D. A.; McKean, D. J.; Gleich, G. J. Ribonuclease activity associated with human eosinophil-derived neurotoxin and eosinophil cationic protein. *J. Immunol.* **1986**, *137* (9), 2913–7.

(11) Boix, E.; Nikolovski, Z.; Moiseyev, G. P.; Rosenberg, H. F.; Cuchillo, C. M.; Nogues, M. V. Kinetic and product distribution analysis of human eosinophil cationic protein indicates a subsite arrangement that favors exonuclease-type activity. *J. Biol. Chem.* **1999**, *274* (22), 15605–14.

(12) Carreras, E.; Boix, E.; Rosenberg, H. F.; Cuchillo, C. M.; Nogues, M. V. Both aromatic and cationic residues contribute to the membrane-lytic and bactericidal activity of eosinophil cationic protein. *Biochemistry* **2003**, *42* (22), 6636–44.

(13) Torrent, M.; Cuyas, E.; Carreras, E.; Navarro, S.; Lopez, O.; de la Maza, A.; Nogues, M. V.; Reshetnyak, Y. K.; Boix, E. Topography studies on the membrane interaction mechanism of the eosinophil cationic protein. *Biochemistry* **2007**, *46* (3), 720–33.

(14) Torrent, M.; Navarro, S.; Moussaoui, M.; Nogues, M. V.; Boix, E. Eosinophil cationic protein high-affinity binding to bacteria-wall lipopolysaccharides and peptidoglycans. *Biochemistry* **2008**, *47* (11), 3544–55.

(15) Carreras, E.; Boix, E.; Navarro, S.; Rosenberg, H. F.; Cuchillo, C. M.; Nogues, M. V. Surface-exposed amino acids of eosinophil cationic protein play a critical role in the inhibition of mammalian cell proliferation. *Mol. Cell. Biochem.* **2005**, *272* (1−2), 1–7.

(16) Navarro, S.; Aleu, J.; Jimenez, M.; Boix, E.; Cuchillo, C. M.; Nogues, M. V. The cytotoxicity of eosinophil cationic protein/ribonuclease 3 on eukaryotic cell lines takes place through its aggregation on the cell membrane. *Cell. Mol. Life Sci.* **2008**, *65* (2), 324–37.

(17) Boix, E.; Nogues, M. V. Mammalian antimicrobial proteins and peptides: overview on the RNase A superfamily members involved in innate host defence. *Mol. Biosyst.* **2007**, *3* (5), 317–35.

Synthetic peptide YRWRYRWR was purchased from GeneScript (Piscataway, NJ).

The Mono-S HR 5/5 column was from Amersham Biosciences (Piscataway, NJ). Cell lines were provided by American type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) and RPMI medium as well as penicillin and streptomycin were provided by Gibco (Invitrogen Life Sciences, Carlsbad, CA), fetal clone II serum (FBS) was provided by Hyclone (Logan, UT), and glutamine and sodium pyruvate were purchased from Biochrom AG (Berlin, Germany).

Plasmid Construction for Onconase and HP-RNase (PM5) Variant Expression. All the plasmids coding for HP-RNase and ONC variants were obtained using pM5[18] and pONC[19] as templates, respectively. pM5 has a pET17 vector backbone while pONC was constructed on a pET22b(+) backbone. pM5 codes for a HP-RNase variant with mutations R4A, K6A, Q9E, D16G and S17N at the N-terminus of the protein, and it was chosen because it is more thermostable than the wild-type HP-RNase.[18,20]

PM5YRWR and ONCRWR where obtained using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Oligos 5′-gcgccgaaatatg*taccgttgg*agatgtaaacctg-3′ and 5′cag-gtttacatct*ccaacggta*catatttcggcgc-3′ were used to substitute the T36Q37G38 amino acid sequence for Y36R37W38 (in italics) in the HP-RNase variant, PM5. To introduce the R26W27R28 coding sequence (in italics) in onconase the oligos 5′-caacatcatgagt*cgttggcgt*acgtttccattgcaaag-3′ and 5′-ctttgcaatggaa*acgccaacg*actcatgatgttg-3′ were used. For the construction of the variants with the bactericidal determinants tagged at the C-terminus, ONC and PM5 genes were amplified from pONC and pM5 with a T7 promoter primer and a reverse primer that removed the stop codon and inserted a *Bam*HI site (in italics): 5′-ccccccccccc*ggatcc*agtagaatcttcaacgc-3′ for PM5, and 5′-ccccccccccc*ggatcc*gcaagaaccaacaccaac-3′ for ONC. The polymerase chain reaction products were digested with *Nde*I and *Bam*HI and ligated into pET22b(+). The double-stranded DNA coding for YRWRYRWR (5′-gatcctaccgttggcgttaccgttggcgttagc-3′ and 5′-ggccgctaacgccaacggtaacgccaacggtag-3′), GSYRWR (5′-gatcctaccgttggcgttagc-3′ and 5′-ggccgctaacgccaacggtag-3′) or GSGSYRWR (5′- gatccggatcctaccgttggcgttagc-3′and ggccgctaacgccaacggtaggatccg-3′) was inserted using *Bam*HI and *Ecl*XI (underlined) by cassette mutagenesis after both PM5 and ONC genes.

To generate the plasmid coding for ONC with the two active-site histidines (H10 and H97) mutated to alanine, pONCGSYRWR was used as a template for two consecutive rounds of site-directed mutagenesis using QuikChange (Stratagene, La Jolla, CA). All the constructions were confirmed by DNA sequencing analysis.

Expression and Purification of Wild-Type ONC, PM5 and the Bactericidal Variants. All the recombinant proteins were produced and purified essentially as described previously.[21,22] Briefly, BL21 (DE3) cells transformed with the corresponding plasmid were grown until an OD_{550} of 1.5 was reached. Protein expression was induced by adding isopropylthiogalactoside to 1 mM. After 3−4 h, the cells were collected by centrifugation, lysed with a French press set at 1100 psi, and inclusion bodies were harvested by centrifugation. The pellets were then resuspended in 10 mL of 6 M guanidinium-HCl, 2 mM EDTA, 100 mM Tris-acetate, pH 8.5, to assist protein solubilization. Samples were reduced by the addition of reduced glutathione (GSH^{red}) to a final concentration of 0.1 M, pH adjusted to 8.5 with solid Tris, and incubated at room temperature for 2 h under nitrogen atmosphere. Insoluble material was then removed by centrifugation (12000*g*, 30 min, 4 °C). Solubilized and reduced proteins were diluted dropwise (≈100-fold), to a final concentration of 50−100 $\mu g \cdot mL^{-1}$, into 0.5 M L-arginine, 1 mM oxidized glutathione ($GSSG^{ox}$), 2 mMEDTA, 0.1 M Tris-acetate, pH 8.5, and then incubated at 10 °C for at least 24 h. To stop oxidation, the pH was adjusted to 5.0 with acetic acid. Refolded samples were then concentrated by ultrafiltration using a Prep/scale TFF cartridge (Millipore, Bedford, MA). Wild-type ONC and ONC variants were dialyzed against 200 mM sodium phosphate, pH 7.2, for 48 h at room temperature, to allow for the cyclization of N-terminal glutamine to pyroglutamic acid. Precipitated or insoluble material was eliminated by centrifugation (12000*g*, 10 min, 4 °C). Refolded samples were then loaded onto a Mono-S HR 5/5 FPLC column (Amersham Biosciences, Uppsala, Sweden) and eluted with a linear gradient of 0−600 mM NaCl in 30 min.

Protein purity and homogeneity were confirmed by SDS−PAGE. Molecular masses were confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using Bruker-Biflex equipment at the Servei de Proteómica de la UCTS de l'Institut de Recerca de l'Hospital Universitari Vall d'Hebron, Barcelona (Spain).

(18) Canals, A.; Ribo, M.; Benito, A.; Bosch, M.; Mombelli, E.; Vilanova, M. Production of engineered human pancreatic ribonucleases, solving expression and purification problems, and enhancing thermostability. *Protein Expression Purif.* **1999**, *17* (1), 169–81.

(19) Leland, P. A.; Staniszewski, K. E.; Kim, B.; Raines, R. T. A synapomorphic disulfide bond is critical for the conformational stability and cytotoxicity of an amphibian ribonuclease. *FEBS Lett.* **2000**, *477* (3), 203–7.

(20) Benito, A.; Bosch, M.; Torrent, G.; Ribo, M.; Vilanova, M. Stabilization of human pancreatic ribonuclease through mutation at its N-terminal edge. *Protein Eng.* **2002**, *15* (11), 887–93.

(21) Ribo, M.; Benito, A.; Canals, A.; Nogues, M. V.; Cuchillo, C. M.; Vilanova, M. Purification of engineered human pancreatic ribonuclease. *Methods Enzymol.* **2001**, *341*, 221–34.

(22) Rodriguez, M.; Torrent, G.; Bosch, M.; Rayne, F.; Dubremetz, J. F.; Ribo, M.; Benito, A.; Vilanova, M.; Beaumelle, B. Intracellular pathway of Onconase that enables its delivery to the cytosol. *J. Cell Sci.* **2007**, *120* (Part 8), 1405–11.

Stability Determination. Temperature-unfolding studies were carried out essentially as previously described.[23] Proteins were dissolved to a concentration of 0.8 mg/mL in 50 mM sodium acetate buffer at pH 5.0. The decrease in absorbance at 287 nm (1 nm bandpath) was recorded as a function of temperature using a Lambda Bio20 (Perkin-Elmer, Waltham, MA) absorption spectrometer equipped with a thermostatted cell holder. The temperature was increased from 40 to 98 °C in 2−3 °C steps. Before each measurement samples were equilibrated for 5 min. Temperature-unfolding transition curves were fitted to a two-state thermodynamic model combined with sloping linear functions for the native and denatured states, and the thermodynamic parameters were calculated as reported previously.[24,25]

Ribonuclease Inhibitor Binding Assay. Onconases were tested for ribonucleolytic activity in the presence of RI using an agarose gel-based assay as described previously.[4] Fifteen nanograms (64 nM) in the standard assay of each ribonuclease in 20 μL of 20 mM Hepes, 12 mM NaCl, 10 mM DTT^{red}, 1 mM EDTA, pH 7.0, were incubated for 5 min at 37 °C with 0 or 40 units of RI (where 1 unit is the amount of RI required to inhibit the activity of 5 ng of RNase A by 50%). Afterward, 4 μg of 16S- and 23S-rRNA were added and the samples were incubated for a further 30 min at 37 °C. Reactions were stopped by the addition of 3 μL of loading buffer (40% (w/v) sucrose, 0.2% (v/v) diethyl pyrocarbonate, and 0.25% (w/v) bromophenol blue), and the mixtures subjected to electrophoresis in an agarose gel (1.5% (w/v)) containing ethidium bromide.

Assay of Ribonucleolytic Activity. The catalytic activity of all the ribonucleases used in this work was measured with the fluorogenic substrate 6-FAM-dArUdAdA-6-TAMRA[26,27] using a thermostatted Lambda LS50 fluorescence spectrometer (Perkin-Elmer, Waltham, MA) equipped with sample stirring. Cleavage of this substrate results in an increase in fluorescence intensity (excitation at 492 nm; emission at 515 nm). Assays were performed at 25 °C in 1.5 mL of 0.1 M (2-[*N*-morpholine] ethanesulfonic acid−NaOH buffer, pH 6.0, containing 0.1 M NaCl, 50 nM 6-FAM-dArUdAdA-6-TAMRA and 15 μM enzyme. According to ref 27 data were fitted to the equation

$$k_{cat}/K_m = (\Delta F/\Delta t)\{(F_{max} - F_0)[E]\} \quad (1)$$

where $\Delta F/\Delta t$ is the initial rate of the reaction, F_0 is the initial fluorescence intensity prior to the addition of the enzyme, F_{max} is the fluorescence intensity after complete cleavage of the substrate, accomplished with the addition of excess of wild-type RNase A, and [E] is the ribonuclease concentration. Each measurement was repeated 3 times.

Cytotoxic Activity. Cells were grown in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% glutamine 200 mM and 1% sodium pyruvate 100 mM. The cytotoxicity of ONC, PM5 and the corresponding bactericidal variants was assayed on the tumor human cell line HeLa (cervix carcinoma) which was seeded into 96-well plates at the appropriate density (2500 cells/mL). Cells were incubated with different concentrations of the proteins ranging from 0.2 μM to 60 μM. After three days, the cytotoxicity of onconases was measured using an assay that monitors the reduction of MTT to formazan (Celltiter 96 Aqueous, Promega, Madison, WI) essentially as described by the manufacturer instructions. The medium was removed and replaced with 100 μL of fresh medium with 10 μL of MTT. After 3 h the absorbance was read at 570 nm using an Elx800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VE). The results for a single experiment are the average of four determinations, and the experiments were repeated three independent times. The IC_{50} values represent the concentration of the assayed enzyme required to inhibit cell proliferation by 50%.

Antimicrobial Activity Assay. Antibacterial activity of the recombinant proteins was performed essentially as described previously.[6,12] Overnight cultures of Gram-negative *Escherichia coli* DH5α strain and Gram-positive *Staphylococcus aureus* 502A strain were used to inoculate fresh cultures in Luria−Bertani (LB) broth at 37 °C with agitation at 180 rpm until $OD_{550} = 1$. Cells were washed twice and suspended at 1:10000 in PBS buffer. 100 μL aliquots of the cell suspension were incubated for 4 h at 37 °C with different concentrations of the recombinant PM5 or ONC variants (0.5, 1 and 5 μM) in PBS buffer or the same volume of buffer as a control. Treated bacterial suspension were plated on LB-agar, incubated overnight at 37°, and the colony forming units (CFU)/mL for each treatment was determined. Each experiment, was carried out in triplicate and final results are the average of three independent experiments.

The antifungal activity was assayed using *Candida albicans*. The assay was the same described previously for the antibacterial activity but using Saboroud medium for the overnight cultures and Saboroud-agar to plate treated suspensions. The synthetic peptide YRWRYRWR was assayed at the same concentrations used for all proteins. Statistical analysis was performed by using the Student's *t* test. A p value <0.05 was considered statistically significant.

Assays of antibacterial activity of $PM5(YRWR)_2$ and $ONC(GS)_2YRWR$ on additional bacterial strains were per-

(23) Font, J.; Benito, A.; Torrent, J.; Lange, R.; Ribo, M.; Vilanova, M. Pressure- and temperature-induced unfolding studies: thermodynamics of core hydrophobicity and packing of ribonuclease A. *Biol. Chem.* **2006**, *387* (3), 285–96.

(24) Mozhaev, V. V.; Heremans, K.; Frank, J.; Masson, P.; Balny, C. High pressure effects on protein structure and function. *Proteins* **1996**, *24* (1), 81–91.

(25) Torrent, J.; Connelly, J. P.; Coll, M. G.; Ribo, M.; Lange, R.; Vilanova, M. Pressure versus heat-induced unfolding of ribonuclease A: the case of hydrophobic interactions within a chain-folding initiation site. *Biochemistry* **1999**, *38* (48), 15952–61.

(26) Kelemen, B. R.; Klink, T. A.; Behlke, M. A.; Eubanks, S. R.; Leland, P. A.; Raines, R. T. Hypersensitive substrate for ribonucleases. *Nucleic Acids Res.* **1999**, *27* (18), 3696–701.

(27) Park, C.; Kelemen, B. R.; Klink, T. A.; Sweeney, R. Y.; Behlke, M. A.; Eubanks, S. R.; Raines, R. T. Fast, facile, hypersensitive assays for ribonucleolytic activity. *Methods Enzymol.* **2001**, *341*, 81–94.

formed as described above for *E. coli* and *S. aureus*. Gram-negative *Klebsiella pneumoniae* ssp. *pneumoniae* (CECT 143, ATCC 13883), *Shigella sonnei* (CECT 457, ATCC 11060) and *Pseudomonas aeruginosa* (CECT 532, ATCC 19582) strains were grown in LB medium while Gram-positive *Streptococcus pyogens* (CECT 598, ATCC 8668), *Micrococcus luteus* (CECT 245, ATCC 10240) were grown in BHI (brain−heart infusion). All of them were obtained from the Spanish type Culture Collection (CECT).

Flow Cytometry. Flow cytometric analyses were performed to assay the permeabilization effect on bacterial cell membranes after treatment with PM5$(YRWR)_2$ and the corresponding wild-type protein. Briefly, cultured bacteria (*E. coli*, *S. sonnei* and *M. luteus*) were grown to OD_{550nm} of 1.0 (concentration range of 10^5 to 10^6 bacteria/mL) washed twice with PBS buffer, and diluted 1:100 in PBS pH 7.4 with 0.01% Tween-20 buffer. Bacterial suspensions were incubated at 37 °C with the recombinant proteins at different concentrations (0, 0.5, 1 and 5 μM) for 4 h, and at different times (0, 30 min, 1 and 3 h) with 5 μM for the time-course experiments. For staining viable and dead cells, 5 μL of each dye solution, thiazole orange (TO) and propidium iodide (PI) solutions from the BD Cell Viability kit (BD Biosciences, San Jose, CA), were added to 200 μL of the treated bacterial suspension. Final concentrations were 420 nM for TO and 48 μM for PI. Cells were incubated 5 min at room temperature prior to flow cytometric analysis. A minimum of 10,000 cells within the gated region were analyzed on a BD FACSCalibur flow cytometer equipped with 488 nm laser excitation (BD Biosciences, San Jose, CA). Data were analyzed using the BD CellQuest Pro software. Each experiment was conducted in triplicate.

Flow cytometry was also used to evaluate whether there could be an additive effect between PM5 and the peptide YRWRYRWR. Therefore, *K. pneumoniae* ssp. *pneumoniae* cells were incubated with 5 μM PM5$(YRWR)_2$, PM5, YRWRYRWR peptide or combining PM5 and the peptide equimolarly, and cells were stained with PI and TO and analyzed as described above to calculate the percentage of live/dead cells.

Results

Design and Production of PM5 and ONC Variants. The contribution of several ECP amino acid residues to the cell membrane interaction and to the cytotoxic activity against eukaryotic and bacterial cells has been previously described.[12,15] Among all the studied positions, W35 and R36 appeared as the most relevant hydrophobic-positive pair involved in the toxic effect exerted by ECP.

The aim of the present work was to engineer antimicrobial activity on two other ribonucleases: ONC and HP-RNase. The former with an extensively demonstrated cytotoxicity against tumor cells and the latter without this ability.

When designing the variants and selecting the positions into which introduce the substitutions, several factors were considered: (i) introduce the substitutions in the loop of HP-RNase and onconase homologous to the $\alpha3-\beta1$ loop of ECP that contains the W35R36 determinant, and at the C-terminus of HP-RNase and onconase to distinguish between sequential and conformational effects; (ii) the W residue should be centered in the most exposed position of the homologous loops in HP-RNase and onconase as it happens in the $\alpha3-\beta1$ loop of ECP; (iii) in ECP sequence, positions W35R36 are preceded by Y33R34; since the Y33R34 is also a hydrophobic-basic pair we decided to include these two residues as a part of the bactericidal determinant together with W35R36; (iv) the substitutions should not alter the length of the loops to avoid conformational strain; and (v) use PM5 (see Experimental Section), a HP-RNase variant more thermostable than wild-type HP-RNase.[18,20]

Based on these considerations, mutations have been performed in pM5 so that the nucleotide sequence coding for residues T36Q37G38R39 would code for the Y36R37W38R39 sequence to render PM5YRWR. Similarly, in ONC the wild-type coding region for T25N26L27 was substituted for the sequence coding for R25W26R27 (ONCRWR). No additional modifications were made in ONC because its homologous $\alpha1-\beta1$ loop is shorter than the $\alpha3-\beta1$ loop of ECP or the $\alpha1-\beta1$ loop of HP-RNase and the extra residues could compromise the conformational stability of the region.

To evaluate the role of the modifications in a context not associated with a local conformation, the same determinant, YRWR, was introduced at the C-terminus of PM5 and onconase as a double repeat connected to the C terminus (PM5$(YRWR)_2$ and ONC$(YRWR)_2$) or as a single determinant with a GS (PM5GSYRWR and ONCGSYRWR) or $(GS)_2$ (PM5$(GS)_2$YRWR and ONC$(GS)_2$YRWR) spacer (see Figure 1 for schematic drawing of all the variants).

All the PM5 and onconase variants except ONC$(YRWR)_2$, which could not be properly refolded, were purified to homogeneity, and the molecular masses were confirmed by MALDI-TOF mass spectrometry (Table 1 and Table 2).

Stability and Ribonuclelytic Activity of PM5 and ONC Variants. To correlate the conformational stability and the ribonucleolytic activity with the bactericidal or cytotoxic activity, the midpoint transition temperature of unfolding and the catalytic efficiency using the fluorogenic substrate 6-FAM-dArUdAdA-6-TAMRA have been determined for PM5, ONC and the corresponding variants. Results are listed in Table 1 and Table 2, respectively.

Wild-type ONC has an unusually high denaturation temperature with a $T_{1/2}$ of 87.2 °C[19,28] whereas PM5 has a much lower conformational stability $T_{1/2}$ of 58.1 °C.[18] Because of the much lower thermal stability of PM5 we focused on the analysis of the stability of PM5 variants. Although the variants with the antimicrobial determinants added at the C-terminus are only slightly destabilized ($\Delta T_{1/2}$

(28) Notomista, E.; Catanzano, F.; Graziano, G.; Dal Piaz, F.; Barone, G.; D'Alessio, G.; Di Donato, A. Onconase: an unusually stable protein. *Biochemistry* **2000**, *39* (30), 8711–8.

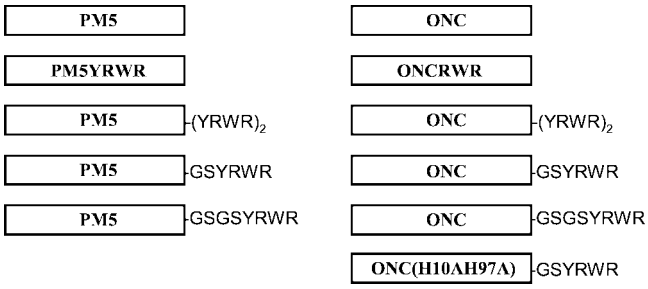

Figure 1. Schematic representation of the PM5 and ONC variants used in this work. PM5, a HP-RNase variant with mutations R4A, K6A, Q9E, D16G and S17N at the N-terminus of the protein; PM5YRWR, PM5 with T36YQ37RG38WR39 substitutions at the $\alpha1-\beta1$ loop; PM5$(YRWR)_2$, PM5 with a YRWRYRWR amino acid sequence extension at the C-terminus; PM5GSYRWR and PM5GSGSYRWR, PM5 with the C-terminal extension GSYRWR or GSGSYRWR, respectively; ONC, wild-type onconase; ONCRWR, ONC with T25RN26WL27R substitutions at the $\alpha1-\beta1$ loop; ONC$(YRWR)_2$, ONC with a YRWRYRWR amino acid sequence extension at the C-terminus; ONCGSYRWR and ONCGSGSYRWR, ONC with the C-terminal extension GSYRWR or GSGSYRWR, respectively; ONC(H10AH97A)GSYRWR, ONCGSYRWR with the active site histidine residues mutated to alanine.

oscillate between 0.7 and 4.4 °C) when the determinant is inserted in the $\alpha1-\beta1$ loop of PM5, the $T_{1/2}$ markedly decreases to 41.9 °C (Table 1).

When the PM5 variants are compared to wild-type in terms of catalytic efficiency (Table 1), a decrease of 55−75% in the k_{cat}/K_m value for 6-FAM- dArUdAdA-6-TAMRA cleavage is observed between them. Likewise, when comparing the catalytic efficiency of ONC variants, it is observed that all of them exhibit a reduction of the k_{cat}/K_m value between 80% and 93% related to wild-type ONC (Table 2). It is worth mentioning that the drop-off is much more pronounced for the ONC variants than for the PM5 variants related to their respective wild-type proteins.

The different variants were also screened for ribonucleolytic activity in the presence of RI by using an agarose gel-based assay. As shown in Table 1 wild-type PM5 and PM5 variants were sensitive to the effects of RI. On the other hand, substitutions introduced on ONC did not alter the ability of ONC to evade RI interaction, as shown in Table 2.

Citotoxicity on Tumor Cells. It is well-known that ONC is a ribonuclease with natural cytotoxic activity on a great diversity of cell lines. Conversely, PM5 or HP-RNase do not possess this biological activity, although it can be engineered, as previously reported. In order to evaluate the effect of the mutations introduced in PM5- and ONC-bactericidal variants, the cytotoxicity of all the variants was assayed on HeLa, a human cervix carcinoma cell line.

Interestingly, as shown in Table 3, none of the PM5 variants acquired cytotoxic activity against eukaryotic HeLa cells due to the engineered substitutions. When measuring the growth inhibitory effect of ONC and the corresponding variants, it is revealed that the substitutions introduced in ONC make these variants 4- to 7-fold less cytotoxic. Neither the ribonucleolytically inactive ONC variant ONC(H10AH97A)GSYRWR nor the peptide YRWRYRWR was cytotoxic on HeLa cells.

Antimicrobial Activity. The antimicrobial activity of wild-type PM5, wild-type ONC and their corresponding variants was evaluated against Gram-negative (*E. coli* DH5α) and Gram-positive (*S. aureus* 502A) strains and against the fungus *C. albicans*. The % of CFU remaining after incubation with different protein concentrations is shown in panels A and B of Figure 2, and Table 3. It is clearly observed that *E. coli* cells are sensitive to the different PM5 and ONC variants (Figure 2A,B), whereas no toxicity is exerted on *S. aureus* 502A cells (Table 3), except for the effect observed for PM5$(YRWR)_2$ variant at 5 μM concentration.

For a rapid comparison of the antimicrobial activities, the % of CFU remaining after 4 h of incubation with 1 μM of protein (for *E. coli*) or with 5 μM of protein (for *S. aureus* 502A and *C. albicans*) are listed in Table 3. It is revealed that the variants with a more pronounced bactericidal activity against *E. coli* DH5α cells are PM5$(YRWR)_2$, ONCGSYRWR and ONC$(GS)_2$YRWR with a % of remaining CFU at 1 μM of protein, of 5.7 ± 1.1 ($p < 0.001$), 18.2 ± 3.9 ($p < 0.001$) and 7.83 ± 2.56 ($p < 0.001$), respectively. All of them share in common that the % of remaining CFU is markedly dwindled below 50% at protein concentrations as low as 0.5 μM (Figure 2A,B). When these most bactericidal variants of PM5 and ONC were tested for antifungal activity against *C. albicans*, no toxic effect was observed at concentrations of 5 μM.

For both PM5 and ONC, the variants with the substitutions in an internal position, PM5YRWR and ONCRWR, yielded around 50% of *E. coli* cell survival at 1 μM of protein concentration, which is indicative of a modest bactericidal effect. Whereas bactericidal activity at protein concentrations of 5 μM is comparable among PM5YRWR, PM5GSYRWR and PM5$(GS)_2$YRWR variants, large differences are observed between the bactericidal activities of ONC variants depending on the position of the substitutions even at lower protein concentrations (Figure 2A,B). Hence, all the ONC variants with the C-terminal appended bactericidal determinants show a greater toxic effect on *E. coli* than ONCRWR. The peptide YRWRYRWR showed slight bactericidal activity only against *E. coli* cells (Table 3).

In an effort to evaluate the contribution of the ribonucleolytic activity to the bactericidal action, a variant was created in which histidines H10 and H97 of the active site of ONCGSYRWR were replaced with alanines. As shown in Figure 2B and in Table 3, the variant ONC(H10AH97A)GSYRWR exhibited bactericidal activity in spite of being null as a ribonuclease.

To corroborate the bactericidal activity observed against *E. coli* and *S. aureus*, the most active PM5 and ONC engineered variants, specifically PM5$(YRWR)_2$ and ONC$(GS)_2$YRWR, were assayed on additional Gram-negative (*K. pneumoniae* ssp. *pneumoniae*, *S. sonnei* and *P. aeruginosa*) and Gram-positive (*S. pyogens* and *M. luteus*)

Table 1. Characterization of PM5 and PM5-Bactericidal Variants

protein	mass (Da) theor	mass (Da) exptl	k_{cat}/K_m[a] (10^4 M^{-1} s^{-1})	catalytic efficiency[b] (%)	$T_{1/2}$[c] (°C)	RI evasion[d]
PM5	14533.2	14535.6	2.23 (0.40)	100	58.1	−
PM5YRWR	14752.5	14754.1	0.66 (0.01)	29.6	41.9	−
PM5$(YRWR)_2$	15856.8	15858.1	0.57 (0.04)	25.5	53.7	−
PM5GSYRWR	15339.1	15340.4	0.95 (0.02)	42.6	57.4	−
PM5$(GS)_2$YRWR	15483.3	15488.0	0.99 (0.02)	44.4	54.6	−

[a] Values of k_{cat}/K_m (±SD) are for catalysis of 6-FAM- dArUdAdA-6-TAMRA (50 nM) cleavage in 0.1 MES−NaOH, 0.1 M NaCl pH 6.0 and 25 °C. [b] Percentage (%) of catalytic efficiency was calculated setting k_{cat}/K_m of PM5 as 100%. [c] Values of $T_{1/2}$ (±1 °C) were determined by monitoring the decrease in absorbance at 287 nm as a function of the temperature in 50 mM sodium acetate buffer at pH 5.0. [d] RI evasion was determined by detection of ribonucleolytic activity of 15 ng of the different proteins incubated with 40 U of RI on rRNA substrate using an agarose-gel based assay. The minus symbol indicates that no ribonucleolytic activity was detected.

Table 2. Characterization of the ONC and ONC-Bactericidal Variants

protein	mass (Da) theor	mass (Da) exptl	k_{cat}/K_m[a] (10^2 M^{-1} s^{-1})	catalytic efficiency[b] (%)	RI evasion[c]
ONC	11819.6	11819.2	3.10 (0.62)	100	+
ONCRWR	11989.9	11990.5	0.26 (0.06)	8.4	+
ONCGSYRWR	12569.5	12562.7	0.21 (0.02)	6.8	+
ONC$(GS)_2$YRWR	12726.7	12725.1	0.60 (0.06)	19.4	+

[a] Values of k_{cat}/K_m (±SD) are for catalysis of 6-FAM-dArUdAdA-6-TAMRA (50 nM) cleavage in 0.1 MES−NaOH, 0.1 M NaCl pH 6.0 and 25 °C. [b] Percentage (%) of catalytic efficiency was calculated setting k_{cat}/K_m of ONC as 100%. [c] RI evasion was determined by detection of ribonucleolytic activity of 15 ng of the different proteins incubated with 40 U of RI on rRNA substrate using an agarose-gel based assay. The plus symbol indicates that ribonucleolytic activity was detected and therefore that the assayed proteins are not inhibited by the RI.

strains. As shown in Figure 3, both proteins exhibit a significant bactericidal activity against all examined bacterial strains, especially to *K. pneumoniae* ssp. *pneumoniae*, *S. sonnei* and *M. luteus*. Also, it is worth mentioning that Gram-negative strains are in general more susceptible to the toxic action of these ribonucleases since the decrease in the percentage of cell viability is already observed at low concentrations.

Analysis of Membrane Permeabilization by Flow Cytometry. In order get a deeper insight into the mode of action of the toxic variants, *E. coli*, *S. sonnei* and *M. luteus* cells were stained with propidium iodide (PI) and thiazole orange (TO) after incubation with PM5$(YRWR)_2$ and PM5 and analyzed by flow cytometry. While TO is a permeant dye and enters all cells, live and dead, to varying degrees, live cells that have intact membranes are impermeable to PI, which only leaks into cells with compromised membranes. Time-course results (Figure 4) show that PI quickly internalizes into bacterial cells treated with PM5$(YRWR)_2$, indicating that membrane permeabilization is an early event and suggesting that the toxic activity displayed by this variant is exercised by altering the bacterial membrane's stability. Results also indicate that Gram-negative bacteria are more quickly permeabilized than Gram-positive bacteria.

In order to evaluate if there was an additive effect due to a combination of determinants between PM5 and the engineered residues, *K. pneumoniae* ssp. *pneumoniae* cells were incubated with PM5 and YRWRYRWR peptide, either separately or combined at equimolar concentrations, and with the bactericidal PM5$(YRWR)_2$ variant. As shown in Figure 4, no differences in the percentage of dead cells are observed after incubation either with YRWRYRWR peptide alone or combined with PM5. As a consequence, it can be stated that no additive effect exists due to the mixture of the peptide and PM5.

Discussion

RNases have much potential as chemotherapeutics.[29−32] For example, ONC, a member of the RNase A superfamily, presently is undergoing phase III human clinical trials for the treatment of malignant mesothelioma.[1] A lot of work has been performed during the past years trying to understand the molecular basis underlying the cytotoxic activity of RNases and to switch non-naturally cytotoxic RNases to potent toxins. As a proof, RNase A itself and HP-RNase are not toxic to mammalian cells, but properly engineered variants have acquired this ability. However, neither HP-RNase nor ONC possesses a toxic activity against bacterial cells. Conversely, human RNase 3 (eosinophil cationic protein, ECP) is a RNase A family member with reported antimicrobial and cytotoxic properties.[6,7,15,16]

Our premise was that installing some of the determinants described as responsible for the ECP bactericidal activity on nonbactericidal RNases such as HP-RNase and ONC would endow these RNases with toxicity against bacterial cells. In addition, we envisioned that some of the designed variants could be capable of becoming bactericidal but not cytotoxic against mammalian cells.

In spite of the extraordinary efforts invested in studying the molecular basis underpinning the cytotoxicity of different

(29) Newton, D. L.; Futami, J.; Ruby, D.; Rybak, S. M. Construction and characterization of RNase-based targeted therapeutics. *Methods Mol. Biol.* **2003**, *207*, 283–304.

(30) Makarov, A. A.; Ilinskaya, O. N. Cytotoxic ribonucleases: molecular weapons and their targets. *FEBS Lett.* **2003**, *540* (1−3), 15–20.

(31) Benito, A.; Ribo, M.; Vilanova, M. On the track of antitumour ribonucleases. *Mol. Biosyst.* **2005**, *1* (4), 294–302.

(32) Lee, J. E.; Raines, R. T. Ribonucleases as novel chemotherapeutics: the ranpirnase example. *BioDrugs* **2008**, *22* (1), 53–8.

Table 3. Cytotoxic Activity against Eukaryotic HeLa Cells and Antimicrobial Activity of PM5- and ONC-Bactericidal Variants against *E. coli*, *S. aureus* and *C. albicans*

protein	net[a] charge	*E. coli*, 1 μM[b]	*S. aureus*, 5 μM[b]	*C. albicans*, 5 μM[b]	HeLa IC_{50} (μM)[d]
PM5	+4	101.3 (5.1)	92.7 (1.8)	na[c]	NC[e]
PM5YRWR	+5	51.2 (6.4)	92.0 (5.7)	na	NC
PM5$(YRWR)_2$	+8	5.70 (1.10)	61.4 (0.3)	107.3 (5.5)	NC
PM5GSYRWR	+6	86.5 (2.6)	87.3 (7.8)	na	NC
PM5$(GS)_2$YRWR	+6	72.1 (7.7)	96.2 (7.3)	na	NC
ONC	+6	92.0 (7.6)	92.7 (6.3)	na	0.38 (0.02)
ONCRWR	+8	52.4 (3.6)	90.4 (7.9)	na	1.7 (0.1)
ONCGSYRWR	+8	18.2 (3.9)	95.1 (4.4)	100.2 (3.3)	1.7 (0.1)
ONC$(GS)_2$YRWR	+8	7.83 (2.56)	89.0 (7.6)	102.7 (4.8)	2.6 (0.2)
ONC(H10AH97A)GSYRWR	+8	55.9 (3.4)	na	na	NC
YRWRYRWR peptide	+4	75.3 (2.5)	92.7 (9.6)	na	NC

[a] Expressed as the difference between Arg + Lys and Asp + Glu. [b] Percentage (% ± SD) of cell survival after incubation for 4 h at 37 °C with either 1 or 5 μM of recombinant PM5 or ONC variants in PBS buffer. CFUs observed after treatment with PBS buffer as a control were set as a 100%. [c] Not assayed. [d] IC_{50} (±SD) values were determined using the CellTiter96 cell viability assay as described in the *Experimental Section*. [e] NC: When IC_{50} values greater than 50 μM were observed, the corresponding protein was considered not cytotoxic.

RNases, a complete understanding of this process is still wanting. Nevertheless, from the knowledge gained so far, a multistep model has been generally accepted. The model postulates that cytotoxicity requires that RNases initially interact with the cell membrane and internalization proceeds via endocytosis. At some point in the endocytic pathway, cytotoxic RNases are translocated to the cytoplasm where they cleave cellular RNA(s), inhibiting protein biosynthesis and inducing apoptosis.[31,33]

Therefore, one of the key determinants of cytotoxicity is the efficiency of internalization which primarily depends on the Coulombic interactions between cationic residues and anionic cell-surface molecules.[34,35] Cationization has been previously used as an strategy to improve protein affinity for the cell surface and hence cellular internalization.[36] We are aware that the modifications engineered in the different variants increase between +1 and +4 the net molecular charge of the molecule (*Z*) (Table 3). Therefore, this acquisition should increase the cytotoxicity of the variants related to the wild-type enzymes.

Two additional attributes of a particular RNase that are directly related to the cytotoxic potency against mammalian cells are the conformational stability and the ribonuclolytic activity.[19,37] Comparison of the $T_{1/2}$ values of the different PM5 variants with the wild-type protein indicates that the

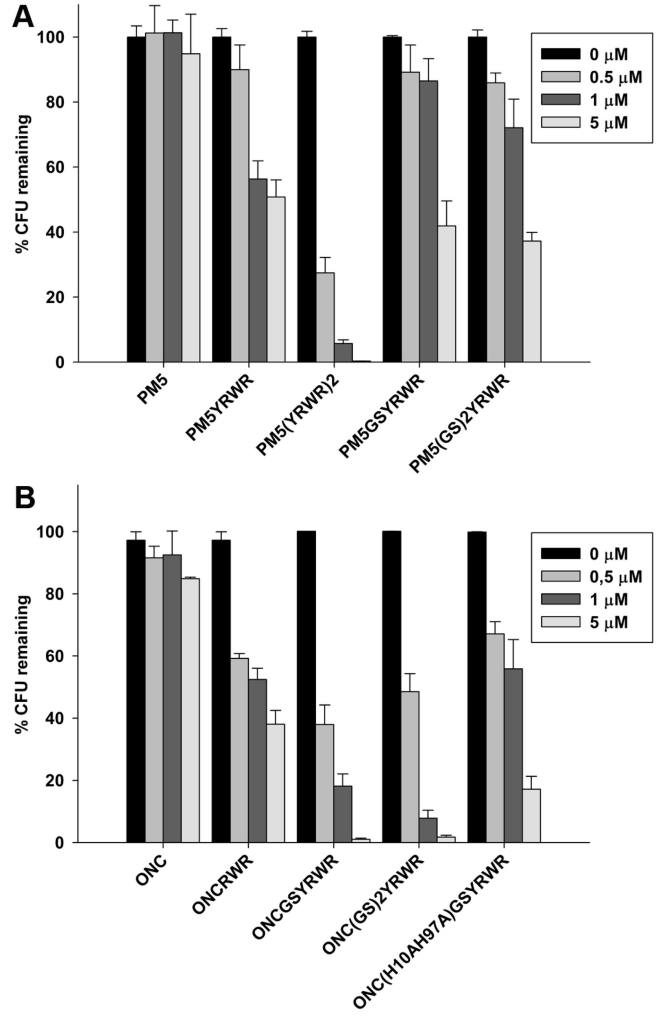

Figure 2. Percentage of CFUs remaining after exposure to engineered bactericidal ribonucleases. Percentage of *E. coli* DH5α survival after 4 h of incubation with increasing concentrations of the different PM5 (A) and ONC (B) variants. The percentage of *E. coli* cell survival after exposure to 5 μM of PM5$(YRWR)_2$, ONCGSYRWR and ONC$(GS)_2$YRWR is lower than 2%.

(33) Leland, P. A.; Raines, R. T. Cancer chemotherapy−ribonucleases to the rescue. *Chem. Biol.* **2001**, *8* (5), 405–13.
(34) Fuchs, S. M.; Rutkoski, T. J.; Kung, V. M.; Groeschl, R. T.; Raines, R. T. Increasing the potency of a cytotoxin with an arginine graft. *Protein Eng., Des. Sel.* **2007**, *20* (10), 505–9.
(35) Johnson, R. J.; Chao, T. Y.; Lavis, L. D.; Raines, R. T. Cytotoxic ribonucleases: the dichotomy of Coulombic forces. *Biochemistry* **2007**, *46* (36), 10308–16.
(36) Ilinskaya, O. N.; Koschinski, A.; Mitkevich, V. A.; Repp, H.; Dreyer, F.; Pace, C. N.; Makarov, A. A. Cytotoxicity of RNases is increased by cationization and counteracted by K(Ca) channels. *Biochem. Biophys. Res. Commun.* **2004**, *314* (2), 550–4.
(37) Klink, T. A.; Raines, R. T. Conformational stability is a determinant of ribonuclease A cytotoxicity. *J. Biol. Chem.* **2000**, *275* (23), 17463–7.

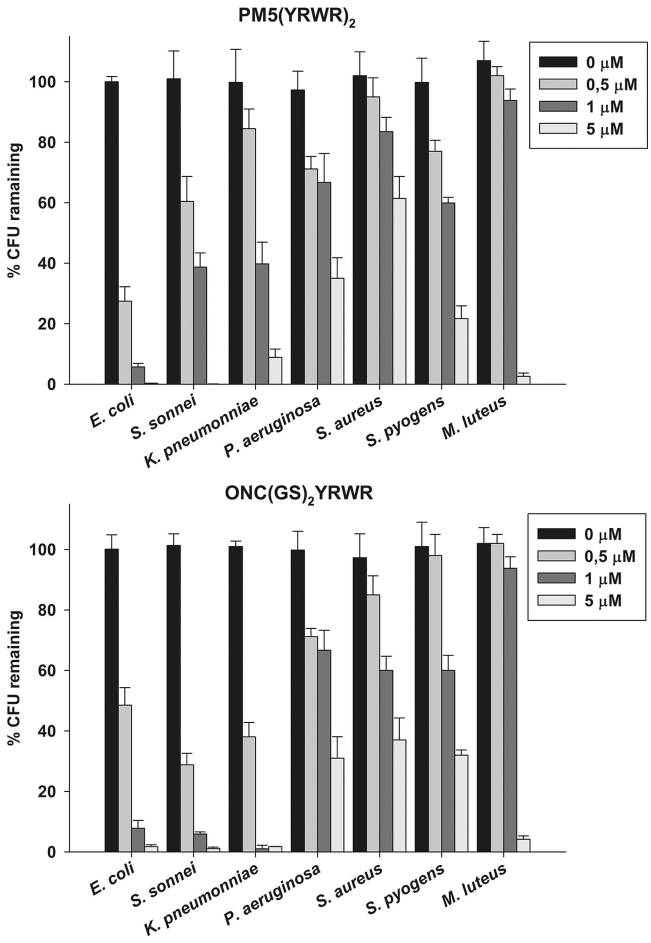

Figure 3. Antibacterial activity of PM5(YRWR)$_2$ and ONC(GS)$_2$YRWR on different bacterial strains. Percentage of survival of different Gram-negative (*Klebsiella pneumoniae* ssp. *pneumoniae*, *Shigella sonnei* and *Pseudomonas aeruginosa*) and Gram-positive (*Streptococcus pyogens* and *Micrococcus luteus*) strains, after 4 h of incubation with increasing concentrations of PM5(YRWR)$_2$ (A) and ONC(GS)$_2$YRWR (B) variants. The percentage of cells survival at 5 μM of PM5(YRWR)$_2$ or ONC(GS)$_2$YRWR is lower than 2% on *E. coli*, *S. Sonnei*, *K. pneumoniae* and *M. luteus*.

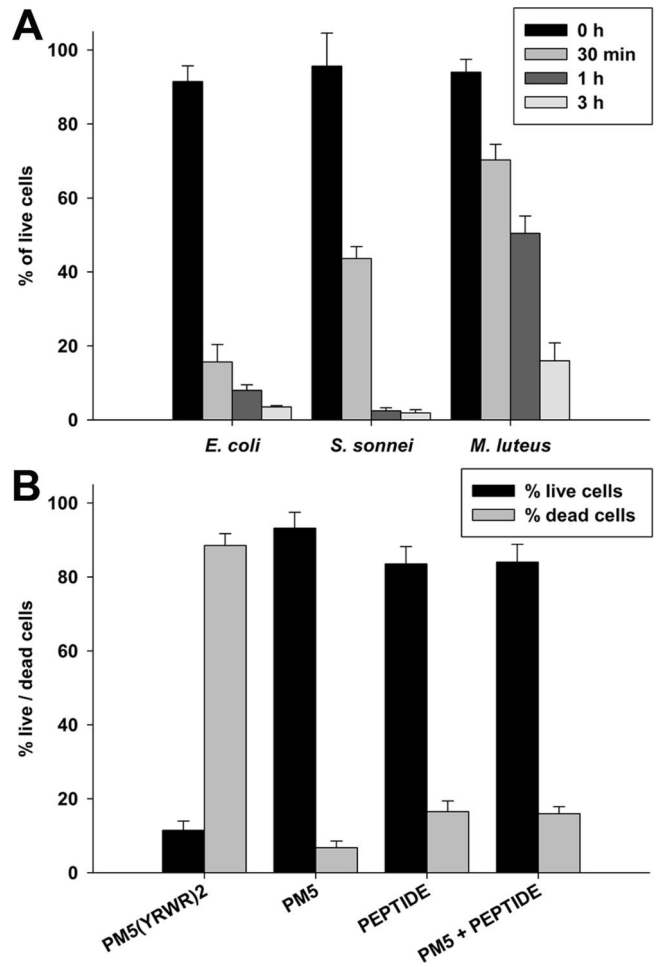

Figure 4. Flow cytometry analysis of membrane permeabilization and the combined effects of PM5 and the YRWRYRWR peptide. (A) For the time-course experiments to monitor propidium iodide internalization, cultured bacteria (*E. coli*, *S. sonnei*, and *M. luteus*) were diluted 1:100 in PBS pH 7.4 with 0.01% Tween-20 buffer, incubated at 37 °C with 5 μM of the recombinant proteins and samples were withdrawn at 0 h, 30 min, 1 and 3 h. Prior to flow cytometric analysis cells were stained with PI and TO. (B) To evaluate the combined effect of PM5 and YRWRYRWR peptide, *K. pneumoniae* ssp. *pneumoniae* cells were incubated with 5 μM of PM5, PM5(YRWR)$_2$, YRWRYRWR peptide and PM5 + YRWRYRWR peptide. After staining with PI and TO, the percentage of live/dead cells was determined by counting a minimum of 10,000 cells within the gated region by means of flow cytometry.

introduction of the YRWR determinant in the loop $\alpha1-\beta1$ of PM5 promotes a more pronounced decrease in the stability than the addition of the bactericidal determinant at the C-terminus of PM5 in any of the variants. These results are not surprising because the substitutions incorporated in an internal position are susceptible to inducing conformational strains that are not present at the C-terminal of the polypeptide chain, likely compromising the global stability.

When analyzing the consequences of the substitutions on the catalytic efficiency it is shown that all the variants show a decrease in the ribonucleolytic activity. Nevertheless, this reduction does not depend on the position to which the substitutions are confined. The decrease in the catalytic efficiency of those variants engineered at internal positions, PM5YRWR and ONCRWR, can be explained because the substitutions are in close proximity to K41 and K31, respectively. The lysine residue at this position has been postulated to interact with the negatively charged pentacoordinate phosphate group in the transition state of the reaction catalyzed by the RNases.[38−40] Therefore, the introduced modifications could change the orientation and the flexibility of the lysine residue that stabilizes the transition state. When

(38) Witzel, H.; Barnard, E. A. Mechanism and binding sites in the ribonuclease reaction. II. Kinetic studies on the first step of the reaction. *Biochem. Biophys. Res. Commun.* **1962**, *7*, 295–9.

(39) Witzel, H.; Barnard, E. A. Mechanism and binding sites in the ribonuclease reaction. I. Kinetic studies on the second step of the reaction. *Biochem. Biophys. Res. Commun.* **1962**, *7*, 289–94.

the determinants are engineered at the C-terminus of either PM5 or ONC, there is also a decrease in the catalytic efficiency. It is unlikely, though, that these additional residues would interfere with the active-site residues. The basicity of the polypeptide extension would make electrostatic repulsion interactions with the positively charged active-site cleft that would maintain the added residues apart from the main protein body. Although the cationic character of the extended C-terminus could initially favor the electrostatic interactions with the RNA substrate, it could also interfere with a proper alignment of the substrate molecule with the subsite recognition pattern needed for an efficient catalysis and, thus, be at the basis of the lessening of catalytic efficiency.

The cytotoxic action of different RNases may also be hampered by the action of the ribonuclease inhibitor (RI), a potent inhibitor which is found in the cytoplasm of mammalian cells.[41] As a result, the ability to evade the RI has been claimed as an essential requirement for a ribonuclease to be cytotoxic.[2,3] The RI-evasion assay used to measure the ability of RNases to evade the RI indicates that the changes introduced either on PM5 or on ONC do not modify the ability of binding or evading the RI, respectively.

When measuring the cytotoxicity of PM5 variants against HeLa cells, it is observed that all of them have IC_{50} values greater than 50 μM and, accordingly, are considered as noncytotoxic (Table 3).

From all the features evaluated and discussed in this work that could modulate the cytotoxic activity of PM5 variants, only the cationization could have enhanced the toxicity of PM5 variants against HeLa cells. However, the contribution of this attribute is not enough to compensate a decrease in conformational stability, a reduction of ribonucleolytic activity and the binding to RI, and consequently confer this ability to any of the variants of PM5.

As shown in Table 3 the growth inhibitory effect of ONC variants is reduced as a consequence of the changes introduced either in the $\alpha1-\beta1$ loop or at the C-terminus of ONC. Compared to the wild-type ONC, the IC_{50} values of ONC variants are between 4- and 7-fold higher. Regarding the attributes that could alter the cytotoxicity, ONC variants did not lose their ability to evade the RI and the cationization due to the incorporation of the bactericidal determinants simply would have improved their ability to interact with the cell surface and, thus, the internalization process. Consequently, the loss in cytotoxic potency can be mainly attributed to the decrease in catalytic efficiency observed for all the ONC variants.

The bactericidal activity of proteins may be due to a particular binding at the surface of the bacterial cell and to a straightforward transportation through the components of the cell wall, depending on whether it is a Gram-positive or Gram-negative species.[42] The polypeptides may bind to Gram-negative bacteria through electrostatic interactions with the negatively charged lipopolysaccharide, the major component of the outer leflet of the outer membrane, followed by insertion into the lipid matrix. After crossing the thin peptidoglycan layer, proteins could bind to the negatively charged groups of the cytoplasmic membrane, bring about its destabilization through hydrophobic interactions, and translocate across the bilayer.[43,44] In the case of Gram-positive bacteria, the negative charge on the surface is provided by the teichoic acids of the cell wall. Before interacting with the negatively charged cytoplasmic membrane, the protein should have to cross previously the thick peptidoglycan barrier,[45,46] and then, the effects on the cytoplasmic membrane might be similar to those described for Gram-negative bacteria. In both cases, two types of specific interactions are necessary: on one hand, the electrostatic binding between the negative groups on the membrane and basic amino acids of the protein and, on the other hand, specific hydrophobic interactions involved in membrane disruption.[47,48]

ECP has been shown to be toxic to both *S. aureus* and *E. coli* cells. Nogués and co-workers substituted cationic and hydrophobic residues from the surface of ECP and studied the role of these residues on the antibacterial and cytotoxic activity.[12,15,16] Their results suggest that distinct and specific structural features of the protein could be involved in the selective binding and disruption capacities of ECP to particular bacterial cell walls and membranes. Among all the ECP variants analyzed, W35AR36A showed the major decrease in cytotoxicity on mammalian cells and the greatest fold increase in *E. coli* and *S. aureus* survival. Remarkably, the fold increase in *E. coli* survival was 4 times higher (89 versus 26) than the fold increase in *S. aureus* cell survival due to the effect of W35AR36A mutations,[15] which suggests that these residues are critical for ECP-mediated antibacterial

(40) Cuchillo, C. M.; Pares, X.; Guasch, A.; Barman, T.; Travers, F.; Nogues, M. V. The role of 2′,3′-cyclic phosphodiesters in the bovine pancreatic ribonuclease A catalysed cleavage of RNA: intermediates or products. *FEBS Lett.* **1993**, *333* (3), 207–10.

(41) Hofsteenge, J. Ribonuclease Inhibitor. In *Ribonucleases: Structures and Functions*; D'Alessio, G., Riordan, J. F., Eds; Academic Press Inc.: New York, 1997; pp 621−658.

(42) Nikaido, H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **2003**, *67* (4), 593–656.

(43) Caroff, M.; Karibian, D. Structure of bacterial lipopolysaccharides. *Carbohydr. Res.* **2003**, *338* (23), 2431–47.

(44) Rosenfeld, Y.; Papo, N.; Shai, Y. Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides. Peptide properties and plausible modes of action. *J. Biol. Chem.* **2006**, *281* (3), 1636–43.

(45) Fournier, B.; Philpott, D. J. Recognition of Staphylococcus aureus by the innate immune system. *Clin. Microbiol. Rev.* **2005**, *18* (3), 521–40.

(46) Dmitriev, B. A.; Toukach, F. V.; Holst, O.; Rietschel, E. T.; Ehlers, S. Tertiary structure of Staphylococcus aureus cell wall murein. *J. Bacteriol.* **2004**, *186* (21), 7141–8.

(47) Ferguson, A. D.; Welte, W.; Hofmann, E.; Lindner, B.; Holst, O.; Coulton, J. W.; Diederichs, K. A conserved structural motif for lipopolysaccharide recognition by procaryotic and eucaryotic proteins. *Structure* **2000**, *8* (6), 585–92.

(48) Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415* (6870), 389–95.

activity, particularly on Gram-negative bacteria. In our work we have shown that the incorporation of the hydrophobic-basic amino acid repeats is enough to confer bactericidal activity to PM5 and ONC. Interestingly, all the engineered PM5 and ONC variants produced in this work have acquired bactericidal activity more pronounced to Gram-negative strains (Table 3, Figures 2 and 3). These results corroborate the significant contribution of these residues to the ECP bactericidal activity against Gram-negative bacteria reported previously. The differences in the antibacterial activity exhibited by these proteins against the assayed strains might rely on the structural differences that exist among their cell walls.

The skeletal inner layer of the *C. albicans* cell wall is mainly formed by chitin and β-1,3- and β-1,6-glucans, whereas the outer cell wall is enriched with proteins that are modified with both long-chain and highly branched *N*-linked mannosyl residues as well as short linear chains of *O*-linked mannosyl residues.[49,50] It has been reported that *C. albicans* cell wall may act as an ion exchanger due to the phosphodiester bridges of the mannan residues of cell wall proteins and, as a consequence, be capable of effectively binding positively charged proteins.[51] Although the variants assayed in this work are cationic proteins, it might happen that the inner layer, formed by glucans and chitin, constitutes a barrier for these variants to reach the cell membrane and impede the cytotoxic action on fungal cells.

It has been reported previously that ribonucleolytic activity is not required for ECP-mediated antibacterial activity.[7] On the other hand, the catalytic activity is necessary for the cytotoxicity of RNases to malignant cell lines.[52−54] In order to evaluate the correlation between the ribonucleolytic activity and the cytotoxic and bactericidal activities, His 10 and His 97 were substituted by alanine in ONCGSYRWR variant. These changes abolished the ribonucleolytic activity and, as expected, the cytotoxic activity of this ONC variant on HeLa cells (Table 3). However, the removal of the ribonucleolytic activity did not eliminate the bactericidal activity entirely (Table 3 and Figure 2B). The 3-fold decrease in bactericidal activity of ONC(H10AH97A)GSYRWR variant in comparison to ONCGSYRWR variant suggests that ribonucleolytic activity plays a significant role in the toxicity of these engineered variants against the bacterial strains tested.

The most bactericidal ONC variants are ONCGSYRWR and ONC$(GS)_2$YRWR. In both cases there is an increase of +2 in the net molecular charge and both show a similar toxic effect. Although the length of the spacer does not seem to have an effect on the bactericidal activity, the impossibility to properly refold ONC$(YRWR_2)$ suggests that the linker could provide the C-terminal $(YRWR)_2$ residues with additional conformational freedom to avoid undesired interactions with the main body of ONC all along the folding process. This is of special relevance in ONC since the amphibian RNase presents a synapomorphic C87/C104 disulfide bond that tethers the C-terminal Cys residue to a central β-strand.

PM5$(YRWR)_2$ is the most bactericidal PM5 variant. There are several factors that might explain this acquired activity. Although it is the PM5 variant with the lower ribonucleolytic activity, it is not excessively destabilized, and due to the duplication of the YRWR determinant, this variant possesses the highest increase in net molecular charge ($\Delta Z = +4$). The length of the extension might contribute not only to the overall basicity but also to establish a higher number of interactions with the cell wall or the cell membrane components and, thus, enhance the antibacterial activity. Likewise, it cannot be discarded that the length of the $(YRWR)_2$ determinant could allow the attainment of secondary structure in this particular region which would hold up for the bactericidal activity of the variant. A wide spectrum of antimicrobial peptides that have been identified in animals and plants or chemically synthesized follow an amphipathic structure composed mainly of hydrophobic and cationic residues.[55−58] These cationic peptides are composed of 12−50 residues with 2−9 positive residues and up to 50% of hydrophobic amino acids, although very efficient small synthetic peptides only 8 residues long have been produced

(49) Poulain, D.; Jouault, T. Candida albicans cell wall glycans, host receptors and responses: elements for a decisive crosstalk. *Curr. Opin. Microbiol.* **2004**, *7* (4), 342–9.

(50) Netea, M. G.; Gow, N. A.; Munro, C. A.; Bates, S.; Collins, C.; Ferwerda, G.; Hobson, R. P.; Bertram, G.; Hughes, H. B.; Jansen, T.; Jacobs, L.; Buurman, E. T.; Gijzen, K.; Williams, D. L.; Torensma, R.; McKinnon, A.; MacCallum, D. M.; Odds, F. C.; Van der Meer, J. W.; Brown, A. J.; Kullberg, B. J. Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest.* **2006**, *116* (6), 1642–50.

(51) Klis, F. M.; de Groot, P.; Hellingwerf, K. Molecular organization of the cell wall of Candida albicans. *Med. Mycol.* **2001**, *39* (Suppl. 1), 1–8.

(52) Ardelt, W.; Mikulski, S. M.; Shogen, K. Amino acid sequence of an anti-tumor protein from Rana pipiens oocytes and early embryos. Homology to pancreatic ribonucleases. *J. Biol. Chem.* **1991**, *266* (1), 245–51.

(53) Kim, J. S.; Soucek, J.; Matousek, J.; Raines, R. T. Catalytic activity of bovine seminal ribonuclease is essential for its immunosuppressive and other biological activities. *Biochem. J.* **1995**, *308* (Part 2), 547–50.

(54) Leu, Y. J.; Chern, S. S.; Wang, S. C.; Hsiao, Y. Y.; Amiraslanov, I.; Liaw, Y. C.; Liao, Y. D. Residues involved in the catalysis, base specificity, and cytotoxicity of ribonuclease from Rana catesbeiana based upon mutagenesis and X-ray crystallography. *J. Biol. Chem.* **2003**, *278* (9), 7300–9.

(55) Andreu, D.; Rivas, L. Animal antimicrobial peptides: an overview. *Biopolymers* **1998**, *47* (6), 415–33.

(56) Oren, Z.; Shai, Y. Mode of action of linear amphipathic α-helical antimicrobial peptides. *Biopolymers* **1998**, *47* (6), 451–63.

(57) Powers, J. P.; Hancock, R. E. The relationship between peptide structure and antibacterial activity. *Peptides* **2003**, *24* (11), 1681–91.

(58) Bulet, P.; Stocklin, R.; Menin, L. Anti-microbial peptides: from invertebrates to vertebrates. *Immunol. Rev.* **2004**, *198*, 169–84.

by high-throughput screening methods.[59] The helical wheel diagram of the sequence $(YRWR)_2$, provided that it could adopt an α-helix conformation, indicates that two pairs of Arg residues would be positioned at opposite sides of the helix while the two Trp and Tyr residues would be also at opposite sides, orthogonal to the position of Arg residues. Nonetheless, the most relevant feature of the bactericidal PM5$(YRWR)_2$ variant is that it is dispossessed of cytotoxicity on malignant Hela cells. This makes this variant a highly selective toxin directed toward bacterial cells. In addition, results obtained from membrane permeabilization studies using PM5$(YRWR)_2$ suggest that the toxic effect is mediated by a membrane permeabilization. This event takes place early as indicated by the results of the time-course experiments (Figure 4A).

In conclusion, the modifications engineered on PM5 and ONC have endowed both RNases with antibacterial activity. This activity is of the same order of magnitude as that described for ECP using similar experimental conditions.[7] The potency of this activity depends on the localization and the number of repeats constituted by a combination of hydrophobic and basic residues. As shown for the bactericidal and cytotoxic variant, ONCGSYRWR, while the ribonucleolytic activity is essential for the cytotoxic activity on malignant cells, the antibacterial activity is supplied by a combination of both ribonucleolytic activity and the singular effect of the engineered modifications on membrane destabilization. Finally, we have developed a variant, PM5-$(YRWR_2)$, with *de novo* acquired selective antibacterial activity and which is not cytotoxic against mammalian cells. Work is in progress to characterize at the molecular level the mechanism underlying the toxic action of the antibacterial RNases engineered in the course of this work. The understanding of the molecular basis of the antimicrobial activity might help in the design of alternative antibiotics that specifically target the microbial cell wall and, therefore, discriminate between the host and the pathogen cells. This knowledge will help in the development of useful RNase-based weapons that could be used for therapeutical purpose.

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(59) Hilpert, K.; Volkmer-Engert, R.; Walter, T.; Hancock, R. E. High-throughput generation of small antibacterial peptides with improved activity. *Nat. Biotechnol.* **2005**, *23* (8), 1008–12.