# Short Cationic Antimicrobial Peptides Interact with ATP[∇]

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**The mode of action of short, nonhelical antimicrobial peptides is still not well understood. Here we show that these peptides interact with ATP and directly inhibit the actions of certain ATP-dependent enzymes, such as firefly luciferase, DnaK, and DNA polymerase. α-Helical and planar or circular antimicrobial peptides did not show such interaction with ATP.**

Despite 2 decades of intensive research, we are still deciphering the complexity of cationic antimicrobial peptides. In recent years, it has become clear that these peptides not only are able to kill Gram-positive and Gram-negative bacteria, fungi, parasites, and enveloped viruses but also can modulate the immune response in mammals (8). However, the mode of action of these peptides is not yet well understood (8). Antibacterial activity is the best-studied peptide function, with the major focus to date being a peptide's interaction with model membranes. Various models for peptide interactions with membranes, such as the barrel-stave, aggregate, carpet, and toroidal-pore models, have been developed (4). However, recent studies indicate that many cationic peptides do not act primarily on the membrane but rather have internal targets in bacteria (4). For example, certain peptides inhibit RNA, DNA, and/or protein synthesis or inhibit enzyme activities, such as the ATP-dependent chaperone DnaK (1, 7, 12). It is becoming increasingly clear that many peptides have complex mechanisms of action involving several targets (3), leading to the term "dirty drugs" to describe their actions (10). Furthermore, at their MICs, some peptides inhibit macromolecular synthesis without depolarizing cells (9). Since most of the proposed cytoplasmic targets or synthetic processes inhibited by these peptides are ATP dependent, we aimed here to analyze whether cationic antimicrobial peptides can interact with anionic ATP, a cytoplasmic, highly negatively charged molecule, the enzyme-mediated hydrolysis of which drives many critical metabolic processes in bacteria. Here we report on the ability of a group of cationic antimicrobial peptides to interact with ATP and ATP-dependent enzymes.

We screened 18 representative peptides and gentamicin for their ability to affect ATP-dependent enzymes. Gentamicin, a cationic trisaccharide, was chosen as a control to study whether a small nonpeptidic molecule containing several $NH_2$ groups would show activities similar to those of the small cationic peptides. The most commonly used assay to determine available ATP concentrations is the ATP-dependent luciferase assay. Light (luminescence) production by the firefly luciferase from the beetle *Photinus pyralis* is directly ATP dependent, and by using a standard curve, the degree of luminescence can be directly correlated to the available ATP concentration. An ATP bioluminescence assay kit (Sigma-Aldrich) was used according to the protocol provided by the manufacturer. The kit contained lyophilized luciferase powder, luciferin, $MgSO_4$, dithiothreitol (DTT), EDTA, bovine serum albumin (BSA), and tricine buffer salts. After a standard curve using different ATP concentrations was developed, cationic peptides were mixed with the kit solution, ATP was added to begin the luminescence reaction, and light production was measured using a luminescence reader (Tecan U.S., Inc., Durham, NC). In this assay, cationic antimicrobial peptides were tested at 1, 3, and 10 times their MICs against the wild-type *Pseudomonas aeruginosa* PAO1 strain H103. It is worth noting that this assay can detect the overall effect of peptides on ATP-dependent light production but does not discriminate between the interactions of the peptide with either the enzyme luciferase, the added luminescence substrates, or ATP. Interestingly, out of 18 of the tested peptides, 9 demonstrated a reduction in light production. Surprisingly, none of the tested α-helical cationic antimicrobial peptides reduced the luminescence signal. All peptides were tested at concentrations related to their MICs in order to derive potentially important functional information. While the actual MICs and thus the concentrations tested varied substantially, peptides with both low MICs (2 μg/ml) and high MICs (16 μg/ml) were included among the peptides that did or did not inhibit luciferase, indicating that there was no particular relationship between MIC and the ability to inhibit luciferase

TABLE 1. Characterization of enzyme inhibition

| Peptide (reference) | Sequence[a] | Length (amino acids) | Charge | Structure[d] | MIC ($\mu$g/ml) | Inhibition of[b]: | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Luciferase | DnaK | qPCR |
| Indolicidin (13) | ILPWKWPWWPWRR-NH$_2$ | 13 | +4 | Open boat | 64 | +++ | + | + |
| Bac2A (5) | RLARIVVIRVAR-NH$_2$ | 12 | +5 | Unknown | 50 | ++ | + | + |
| W3 (5) | RLWRIVVIRVAR-NH$_2$ | 12 | +5 | Unknown | 8 | +++ | + | (+) |
| P7 (5) | RLARIVPIRVAR-NH$_2$ | 12 | +5 | Unknown | >250 | − | ND | ND |
| Sub5 (5) | RRWKIVVIRWRR-NH$_2$ | 12 | +7 | Unknown | 2 | +++ | + | + |
| Sub3 (5) | RRWRIVVIRVRR-NH$_2$ | 12 | +7 | Unknown | 2 | +++ | ND | ND |
| Bac014 (6) | RAVAVIIRLRRV-NH$_2$ | 12 | +5 | Unknown | 33 | ++ | ND | ND |
| Bac020 (6) | RRAAVVLIVIRR-NH$_2$ | 12 | +5 | Unknown | 7 | ++ | ND | ND |
| Bac034 (6) | VRLRIRVAVIRA-NH$_2$ | 12 | +5 | Unknown | 10 | + | ND | ND |
| CP27 | KWKLFKKIGIGAVLKVLTTGLPALIS | 26 | +5 | $\alpha$-Helical | 2 | − | ND | ND |
| CP26 (2) | KWKSFIKKLTSAAKKVVTTAKPLISS | 26 | +7 | $\alpha$-Helical | 2 | − | ND | ND |
| CP29 (2) | KWKSFIKKLTTAVKKVLTTGLPALIS | 26 | +6 | $\alpha$-Helical | 2 | − | − | − |
| V681 (13) | KWKSFLKTFKSAVKTVLHTALKAISS | 26 | +6 | $\alpha$-Helical | 2 | − | − | − |
| V25 (13) | KWKSFLRTLKSPAKTVFHTALKAISS | 26 | +6 | $\alpha$-Helical | 16 | − | ND | ND |
| V8 (13) | KWKSFLRTFKSPVRTVFHTALKPISS | 26 | +6 | $\alpha$-Helical | 16 | − | ND | ND |
| Gentamicin[c] | Cationic trisaccharide | | | | 25 | − | ND | ND |
| Gramicidin S (13) | cyclo[VOL$^D$FP]$_2$ | 10 | +2 | Cyclic | 0.2 | − | ND | − |
| Polyphemusin I (13) | RRWCFRVCYRGFCYRKCR-NH$_2$ | 18 | +8 | $\beta$-Hairpin | 2 | + | ND | ND |
| Polymyxin B (13) | (S)-6-Methyloctanoyl-BTB-cyclo[BB$^D$FLBBT] | 10 | +5 | Planar ring | 0.5 | − | − | − |

[a] Amino acid sequences are in the one-letter code, with the following exceptions: B indicates diaminobutyric acid and superscript D represents the D-enantiomeric amino acid (all other amino acids are of the L-form).

[b] In the luciferase assay, the influence of the peptides on this ATP-dependent enzyme was determined by measuring light production. Strong inhibition was labeled "+++" and indicates that at a 1× MIC, a peptide was able to inhibit light production by 75%. A peptide labeled with "++" inhibited light production by ≥75% at a 3× MIC, and "+" indicated inhibition by ≥75% at a 10× MIC. When 75% inhibition of light production was not achieved at a 10× MIC, the peptide was labeled "−." All studies were repeated at least three times independently with similar results. The interaction with DnaK was categorized as "+" (for inhibition of ATP turnover) and "−" (for no effect on ATP turnover). ND, not determined. In addition, we measured the effect of the peptides (1× MIC for *P. aeruginosa*) on a quantitative PCR. If no PCR product was obtained, a "+" was used; if the PCR was uninfluenced, "−" was used. For the peptide W3, inhibition was observed only at a 3× MIC and was labeled "(+)." All DnaK studies were repeated at least twice and qPCRs at least three times, with similar results.

[c] Gentamicin was obtained from Sigma-Aldrich, and according to the product sheet, it contains a mixture of gentamicin C1 (C$_{21}$H$_{43}$N$_5$O$_7$), gentamicin C2 (C$_{20}$H$_{41}$N$_5$O$_7$), and gentamicin C1a (C$_{19}$H$_{39}$N$_5$O$_7$). The ratio of C1 to C2 to C1a is 45:35:30.

[d] Circular-dichroism spectroscopy indicates an unordered structure in aqueous buffer. Upon contact with negatively charged lipid vesicles, the structure changes, but only a minor $\alpha$-helical signal occurs (here indicated by "unknown").

activity. In addition, peptide P7, which is very similar in sequence to W3 and Bac2A but is not antimicrobial, did not show an inhibition of the luminescence signal (Table 1). Similarly, the conventional antibiotic controls, including the cationic lipopeptide polymyxin B, the cyclic decameric peptide gramicidin, and the nonpeptidic polycation gentamicin, also failed to inhibit luminescence. Overall, the ability to inhibit ATP-dependent luciferase was highly dependent on peptide structure, and we also note that most of the inhibitory peptides contained a greater number of arginine residues than other noninhibitory peptides, which contained more lysine; however, the result with arginine-rich P7 tends to indicate that this parameter was not a definitive descriptor for inhibition.

A decrease in ATP turnover by luciferase, as caused by certain peptides, was confirmed by ATPase assays using radiolabeled ATP. ATPase assays were performed with 20 mM HEPES buffer, pH 8, with 10 mM Mg acetate and 100 mM NaCl. Luciferase was added to reaction mixtures containing 200 $\mu$M [$\alpha$-$^{32}$P]ATP (~8 Ci mmol$^{-1}$) and either a 1× MIC concentration of the indicated peptide or, as a control, no peptide and incubated at 37°C for 15 min. Two microliters of the reaction mixture was removed and the reaction stopped by addition of EDTA and SDS. Samples were spotted on dry polyethylenimine (PEI) cellulose thin-layer chromatography (TLC) plates (Sigma-Aldrich), which were developed in 1 M formic acid and 0.5 M LiCl. TLC plates were dried, exposed to a phosphorimager intensifying screen, and scanned, and exposed pixels were quantified using ImageQuant software v.5.2

(GE Healthcare). ATP hydrolysis was calculated by dividing the pixels in the phosphorimager spot corresponding to AMP by the sum of the pixels in the spots corresponding to ATP and AMP. All values were corrected by subtracting background [$\alpha$-$^{32}$P]AMP levels of control reaction mixtures with no protein addition and were the averages of results from two independent assays. These data showed the same basic result as the luciferase assays for the tested peptides, namely, that Bac2A, W3, and Sub5 inhibited the ATP turnover but that polymyxin B, CP29, and V681 did not inhibit or only weakly inhibited turnover.

We were interested in determining whether these nonhelical peptides would undergo a conformational change when binding to ATP. Circular-dichroism spectroscopy showed only very slight differences in the observed spectra of peptides from those of peptides in combination with ATP. Therefore, we conclude that an ability to undergo conformational changes in association with ATP cannot explain the ability of nonhelical peptides to inhibit ATP-dependent luciferase activity.

There were at least three different possibilities to explain the reduction in luminescence signal. First, the peptides might interact with or mask the ATP; second, they might interact directly with the luciferase; or third, they might interact with the substrate. To exclude the possibility of an interaction of peptides with the substrate, two additional enzymes with different nucleotide triphosphate (NTP) binding motifs and substrates were chosen.

It has already been shown that the ATP-dependent enzyme DnaK is inhibited by certain cationic antimicrobial peptides

Indolicidin [mM]

| ATP [mM] | 0,5 | 0,25 | 0,125 | 0,063 | 0,031 | 0,016 | 0,008 | 0,004 | 0,002 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | | | | | | | | | | |
| 0,008 | | | | | | | | | | |
| 0,016 | | | | | | | | | | |
| 0,031 | | | | | | | | | | |
| 0,063 | | | | | | | | | | |
| 0,125 | | | | | | | | | | |
| 0,25 | | | | | | | | | | |
| 0,5 | | | | | | | | | | |

Sub3 [mM]

| ATP [mM] | 0,5 | 0,25 | 0,125 | 0,063 | 0,031 | 0,016 | 0,008 | 0,004 | 0,002 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | | | | | | | | | | |
| 0,008 | | | | | | | | | | |
| 0,016 | | | | | | | | | | |
| 0,031 | | | | | | | | | | |
| 0,063 | | | | | | | | | | |
| 0,125 | | | | | | | | | | |
| 0,25 | | | | | | | | | | |
| 0,5 | | | | | | | | | | |

P7 [mM]

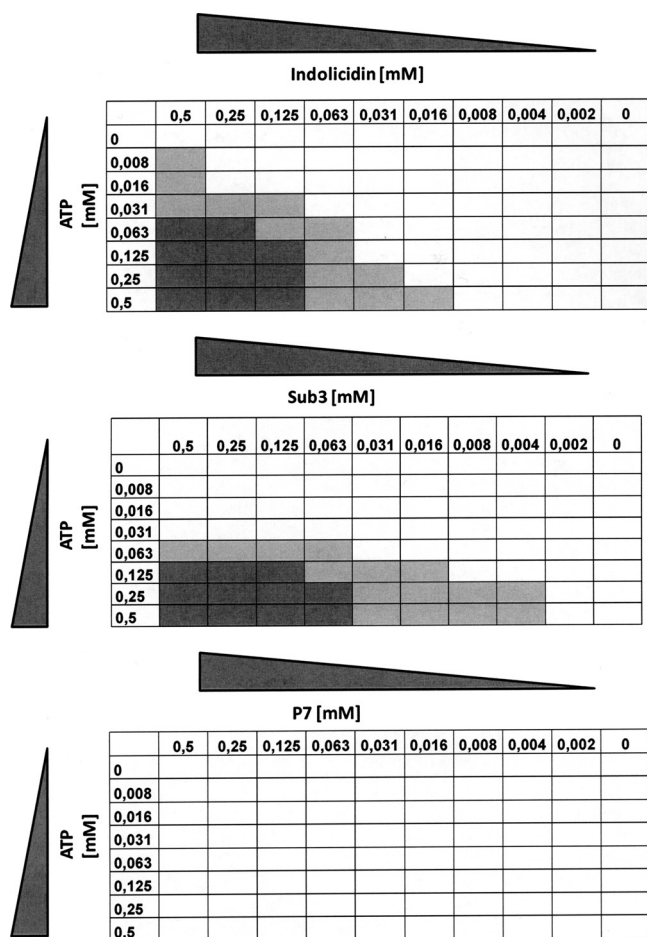| ATP [mM] | 0,5 | 0,25 | 0,125 | 0,063 | 0,031 | 0,016 | 0,008 | 0,004 | 0,002 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | | | | | | | | | | |
| 0,008 | | | | | | | | | | |
| 0,016 | | | | | | | | | | |
| 0,031 | | | | | | | | | | |
| 0,063 | | | | | | | | | | |
| 0,125 | | | | | | | | | | |
| 0,25 | | | | | | | | | | |
| 0,5 | | | | | | | | | | |

FIG. 1. ATP and peptides (indolicidin, Sub3, and P7) were separately mixed in 50 mM Tris buffer at pH 7.4. The optical density at 620 nm was assessed as a measure of coprecipitation. The results are color coded. White, OD value of <0.01; light gray, OD value of 0.01 to 0.05; dark gray, OD value of >0.05 (visible with the unaided eye).

FIG. 2. (A) $^1$H spectra of 20 $\mu$M indolicidin in the absence (bottom) or presence (top) of 2 mM ATP. The spectra were acquired by accumulation of 256 scans at 20°C on a Bruker Avance I 600-MHz spectrometer. The pH was set to 7.0 in 50 mM NH$_4$HCO$_3$ buffer. (B) A difference spectrum is shown where the spectrum of 2 mM ATP acquired under identical conditions is subtracted from the indolicidin/ATP spectrum. (C) $^{31}$P spectra of 0.5 mM ATP in the absence (bottom, 4,048 scans) or presence (top, 12,288 scans) of 0.5 mM indolicidin at 20°C. Phosphate (1 mM) buffer was added as an internal reference for quantification. Note that the intensity of the ATP phosphorous signals is severely reduced after addition of the indolicidin. Buffer was 10 mM Tris-HCl, pH 7.4, 50 mM NaCl.

(7); therefore, we tested several peptides for the turnover of radiolabeled ATP to ADP by DnaK. The ATPase assay and analysis were carried out as described for luciferase under identical buffer conditions, except that ADP production was measured to quantify ATP turnover. For DnaK, the controls were the quantification of spontaneous background ATP hydrolysis in a blank reaction mixture (no AMP, no DnaK) and quantification of background ATPase in an AMP-only reaction mixture. The former control was subtracted from DnaK-alone ATPase values, and the latter was subtracted from DnaK-plus-AMP ATPase values. The ATPase values under all conditions, including control conditions, were the averages of results from two independent assays. Indolicidin, Bac2a, W3, and Sub5 were able to inhibit the ATP turnover of DnaK; in contrast, the peptides V681, Cp29, and polymyxin B showed no influence on DnaK (Table 1). These results were identical to the profiles observed with the luciferase assay but in contrast to previous findings with proline-rich antimicrobial peptides, for which reduced ATP turnover was not observed but rather a 2-fold increase in steady-state ATPase activity was measured (11).

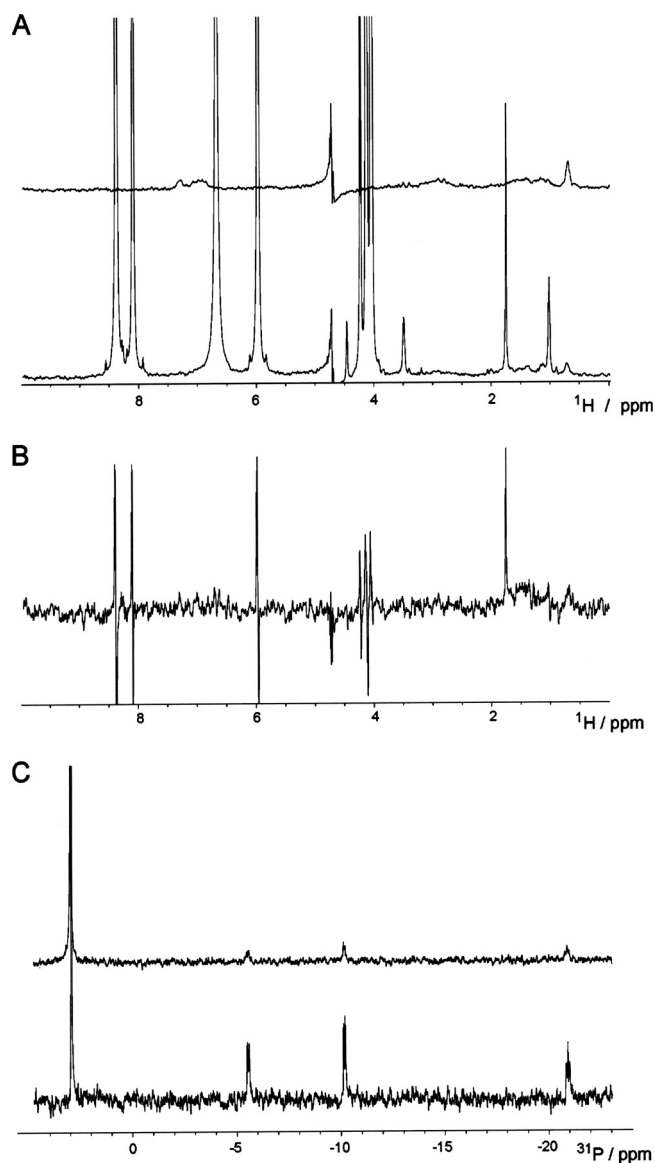To check whether the cationic antimicrobial peptides had any effect on ATP-dependent DNA polymerase in a PCR, selected peptides were also tested for their ability to interfere with a quantitative real-time PCR (qPCR). The effect of peptides on DNA polymerase was tested by qPCR, using a SuperScript III Platinum two-step kit with SYBR green (Invitrogen Life Technologies) in an ABI Prism 7000 sequence detection system (Applied Biosystems). A 1× MIC or 3× MIC of each peptide was added into a 12.5-$\mu$l PCR mixture (including 6.25

μl of qPCR super mix, 0.25 μl of Rox reference dye, 0.5 μl of GAPDH [glyceraldehyde-3-phosphate dehydrogenase] primers, and 2.5 μl of cDNA). As a negative control, master mix without cDNA was run, while as a positive control, the mixture described above without any peptide was used. An undetermined threshold cycle value from the qPCR after peptide treatment indicated that the qPCR was inhibited. Polymyxin B, CP29, and gentamicin did not influence the qPCR assay, whereas Bac2A, indolicidin, W3, and Sub5 inhibited the DNA polymerase driving this reaction.

Overall, these experiments showed that the peptides did not apparently alter the activities of ATP-dependent enzymes by interacting with the substrate, since each of these enzymes has very different substrates but a common requirement for ATP. However, an unspecific interaction of peptides with the substrates of enzymes, like DNA, needs to be studied and the contribution of this to the overall inhibition of the enzymes determined.

Although the interaction of the peptides with such disparate enzymes cannot be entirely ruled out, simply separately combining indolicidin or Sub3 with ATP in 50 mM Tris buffer, pH 7.4, led to immediate precipitation, which was dependent on the concentration of the peptide and ATP (Fig. 1). Control peptide P7, which is also highly positively charged, did not show any precipitation with ATP. This is consistent with the observation that this peptide was not antimicrobial and did not inhibit the luciferase reaction. Electrospray mass spectrometry indicated that ATP formed adducts with indolicidin under low-energy conditions. At higher concentrations of ATP and indolicidin (1 mM high-purity ATP and 1 mM indolicidin in $D_2O$, in the absence of buffer and other salts), small crystals formed but were not stable and dissolved after a few days. In addition, a $^1H$ one-dimensional nuclear magnetic resonance (NMR) spectrum of 20 μM indolicidin contained no peptide signals after addition of ATP, presumably due to precipitation of the complex from solution (Fig. 2).

We have thus demonstrated that small cationic antimicrobial peptides can interact with ATP and thereby reduce the enzymatic activities of luciferase, DnaK, and DNA polymerase. More-detailed investigations are necessary to determine the contribution of this interaction to peptide mechanisms of action, as are studies of the interactions of the peptides with other NTPs, nucleotide diphosphates (NDPs), and nucleotide monophosphates (NMPs). However, as the concentration of ATP in the luciferase assay was 200 μM and the peptide concentrations added were often lower than this, if the peptide-to-ATP binding stoichiometry is 1:1 (which we have not determined), we assume that the peptide interacts not only with ATP but also with the enzymes. It is becoming increasingly clear that antimicrobial peptides do not kill by a single, simple mechanism but rather have very complex multifaceted mechanisms of action. This study has defined an ability to inhibit ATP-dependent enzymes that could contribute to peptide killing of microbes.

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