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Prior Antibacterial Peptide-Mediated Inhibition of Protein Folding in Bacteria Mutes Resistance Enzymes

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The antimicrobial activity of amoxicillin against TEM-1-expressing strains could be fully recovered when bacteria were preincubated with sublethal doses of an antibacterial peptide derivative. Assays with the simultaneous administration of antibiotics or synergy assays with kanamycin or ciprofloxacin, where resistance development does not involve properly folded proteins, failed to yield similar results.

Resistance to antibiotics is developing at an alarming rate (22), and this trend involves all antimicrobial drug families (15). While resistance to fluoroquinolones or aminoglycosides is a major public health concern (2, 8), the inactivity of β-lactam antibiotics poses an economic burden to low-cost prescription patterns (9). Resistance to β-lactams is primarily due to hydrolysis of the ring by β-lactamases (16), of which the TEM-1 enzyme is the most commonly encountered resistance marker in members of the family *Enterobacteriaceae* (24).

Designer analogs of the small, proline-rich antimicrobial peptide pyrrhocoricin kill bacteria that are resistant to β-lactams (5), aminoglycosides (6), or fluoroquinolones (21). The mode of action of these peptide derivatives is inhibition of the 70-kDa heat shock protein (Hsp) DnaK and, thus, protein folding (14) combined with membrane disintegration (20). The peptides fail to bind to mammalian Hsp70 and are nontoxic to mammalian cells or experimental animals (6, 13, 21). Due to the renal clearance of peptide drugs (17) and the low-salt environment of the kidneys (10), we initially concentrated on *Escherichia coli* and *Klebsiella pneumoniae* strains, bacteria dominant in urinary tract infections (UTIs) (26). Bacterial Hsps are targets of natural antibacterial products, including xylitol (12), and inhibition of heat-induced Hsp expression leads to cytotoxic effects to bacteria (1).

β-Lactamases can be inactivated by compounds that bind to their active site (4, 23). Proline-rich antibacterial peptides inactivate test bacterial enzymes (14), and our idea was to use them to deplete all therapeutically relevant enzyme populations. In the study described in this report, we studied how sequential or simultaneous addition of the most active peptide derivative, A3-APO, and amoxicillin to clinical *E. coli* cultures of UTI origin expressing the TEM-1 β-lactamase influences the MIC. The control antibiotic was the aminoglycoside kanamycin, the main mechanism of resistance to which proceeds through mutation in chromosomal genes (18). Fluoroquinolones target DNA gyrase and topoisomerase II, and the resistance pathway involves alterations in the target sequences (11). Yet, we also included ciprofloxacin because fluoroquinolones induce DnaK production in *E. coli* (19).

Overnight cultures of amoxicillin-resistant, pyrrhocoricin-sensitive *E. coli* strains SEQ102 and BF1023 (6), grown in tryptic soy broth (TSB), were diluted 10-fold in TSB-ampicillin and grown for 3 h at 37°C. The cells were harvested and lysed, and the clear lysates were loaded onto Ampholine PAG plates 3.5-9.5 (GE Healthcare). An isoelectric focusing gel was run at 1,000 V for 1.5 h, and β-lactamases were visualized by 0.5 mg/ml nitrocefin. Figure 1 shows that both *E. coli* test strains expressed high levels of the TEM-1 enzyme. The other two strains were *E. coli* S5081, which is resistant to kanamycin (6), and ciprofloxacin-resistant strain *E. coli* 045-849 from the SENTRY collection (21).

The MICs and 50% inhibitory concentrations (IC_{50}s) of A3-APO and the small-molecule antibiotics were determined by the broth microdilution liquid growth inhibition method (3). Growth inhibition assays were performed with

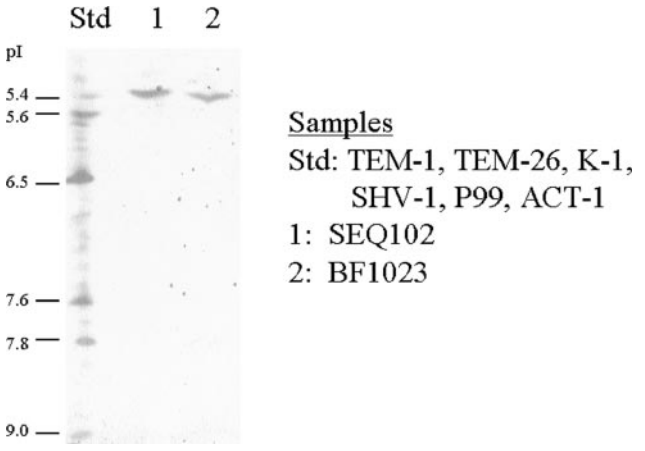

FIG. 1. β-Lactamase expression in the amoxicillin-resistant test bacterial strains. Isoelectric focusing of a standard containing a mixture of potential β-lactam resistance markers (lane Std) and *Escherichia coli* SEQ102 (lane 1) or BF1023 (lane 2) indicates intense TEM-1 production by the amoxicillin-resistant strains used in the current study.

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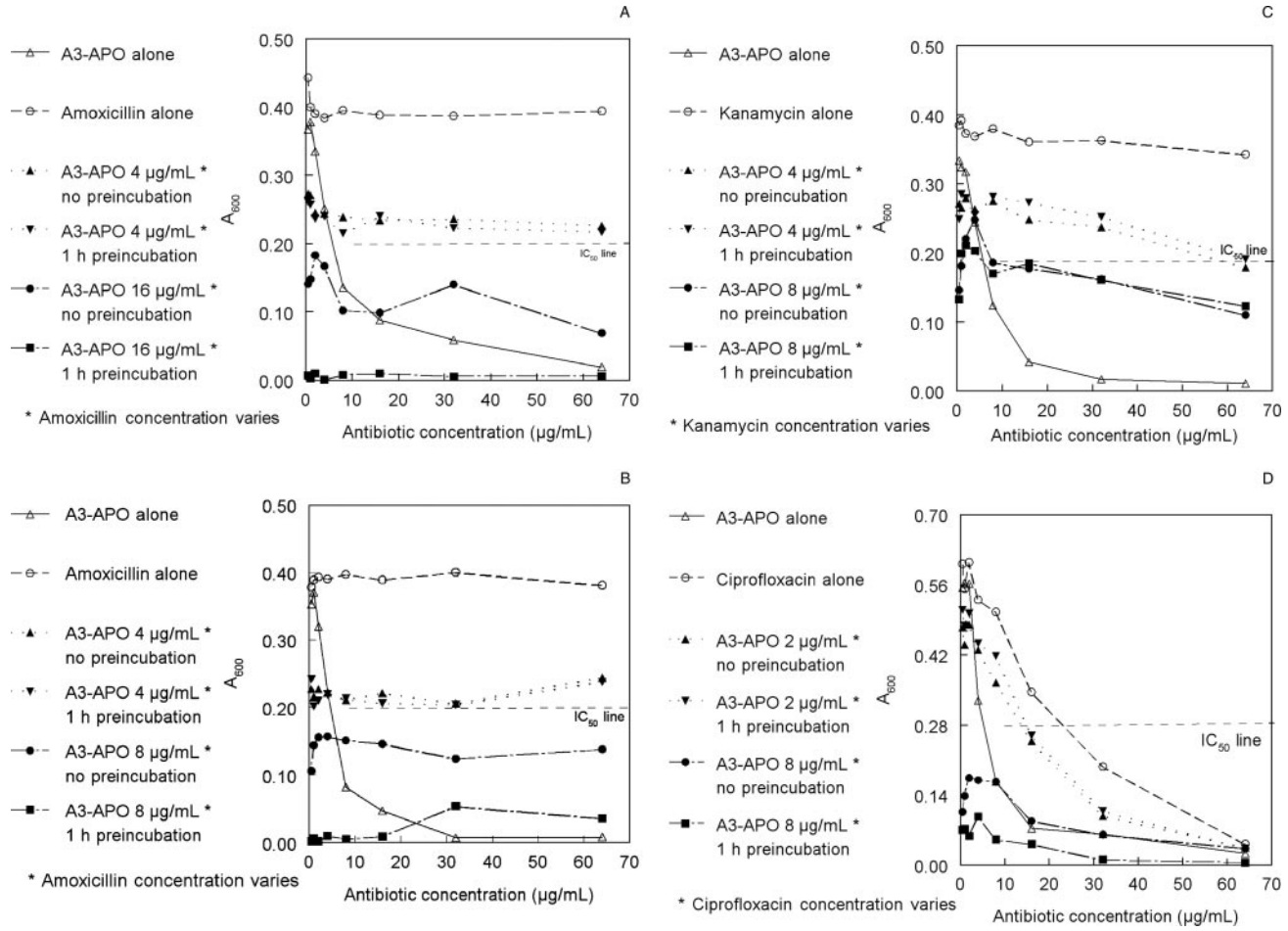

FIG. 2. Killing of *Escherichia coli* by antibiotics, as represented by a broth microdilution liquid growth inhibition assay. Peptide A3-APO (A to D) or the small-molecule antibiotics were added to bacterial cultures alone in the 0.5- to 64-μg/ml concentration range. (A) *E. coli* BF1023–amoxicillin; (B) *E. coli* SEQ102–amoxicillin; (C) *E. coli* S5081–kanamycin; (D) *E. coli* 045-849–ciprofloxacin. Synergy assays were run on the same plates, where peptide A3-APO was added at two fixed concentrations either 1 h prior to small-molecule antibiotic addition or together with various concentrations of the conventional antibiotics.

sterile Nunc 96-well plates in a final volume of 100 μl (5). The cell concentrations were estimated from the absorbance at 600 nm with the formula CFU/ml = $A_{600}(3.8 \times 10^8)$, where CFU is the number of CFU. Ninety microliters of mid-logarithmic-phase bacterial cultures (4×10^5 CFU/ml) in Mueller-Hinton broth was added to 10 μl of serially diluted antibiotics (0.5 to 64 μg/ml) dissolved in sterilized water. The cultures were incubated at 37°C for 16 to 20 h, and growth inhibition was measured by determination of the absorbance at 600 nm. The MIC was identified as the lowest antimicrobial dose when the absorbance readings did not exceed the values obtained in medium only. The IC_{50} data were calculated from the growth inhibition curves (Fig. 2) to identify sublethal doses of A3-APO for the subsequent synergy assay. Table 1 shows the sequence of the A3-APO peptide and the MIC and IC_{50} efficacy data. A3-APO exhibited MICs between 32 and 64 μg/ml and IC_{50} values between 4 and 8 μg/ml. Thus, in the synergy assay, the peptide derivative was added as low and high concentrations of 2 to 4 and 8 to 16 μg/ml, respectively. It is relevant here that the subcutaneous administration of 5 mg/kg pyrrhocoricin-based dimers to mice yields 21 μg/ml peptide in blood over a 2-h window, representing a therapeutic index of at least 100 (21). The conventional antibiotics were mostly inactive against their respective strains.

In the synergy assay, the bacteria were preincubated with the

TABLE 1. Activities of peptide and small-molecule antibiotics against clinical urinary tract *Escherichia coli* isolates[a]

Antimicrobial	Bacterial strain	MIC (μg/ml)	IC_{50} (μg/ml)
A3-APO	SEQ102	32	4–8
	BF1023	64	4–8
	S5081	32	4–8
	045–849	64	4–8
Amoxicillin	SEQ102	>64	>64
	BF1023	>64	>64
Kanamycin	S5081	>64	>64
Ciprofloxacin	045–849	>64	16–32

[a] The amino acid sequence of the A3-APO peptide is (Chex-Arg-Pro-Asp-Lys-Pro-Arg-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro-Arg-Pro-Val-Arg)$_2$-Dab (molecular mass, 5,034 Da). The synthesis details of the peptide derivative were published earlier (21).

A3-APO peptide for 1 h to inhibit protein folding. After 1 h the antibiotics were added and growth inhibition was measured. Figure 2A documents that amoxicillin alone was inactive against *E. coli* BF1023 but that its activity could be fully recovered when the culture was preincubated with 16 μg/ml A3-APO. The synergy was not observed when the peptide and the small-molecule antibiotic were added simultaneously. In the latter case, the number of remaining bacterial colonies hovered around the value obtained with the antibacterial peptide alone (25 to 30%), indicating that β-lactamase activity was manifested before the enzyme's folding could be inhibited. Likewise, no synergistic effect was observed at the lower peptide concentration (approximately 40% killing). To compensate for the minor variations in the MIC determinations, all growth inhibition assays for which the results are shown in Fig. 2A to D were run on single plates. The assays were repeated twice, with nearly identical results each time, although all individual assays showed slight experimental errors at extreme antibiotic concentrations.

The results of the experiments with *E. coli* SEQ102 mirrored the results with the other β-lactam-resistant isolate (Fig. 2B). The antimicrobial activity of amoxicillin could be fully recovered in a peptide dose- and preincubation regimen-dependent manner with 8- and 4-μg/ml active and inactive peptide concentrations, respectively. The results were completely different with kanamycin and *E. coli* S5081 (Fig. 2C), with which no activity of the small-molecule antibiotic could be recovered under any circumstance. The bacterial growth data in the synergy lanes reflect the efficacy of A3-APO alone at both concentrations and were indistinguishable whether the bacterial culture was preincubated with the peptide or not.

The results obtained with ciprofloxacin and *E. coli* 045-849 indicated a trend that fell between the results obtained with the β-lactam and aminoglycoside antibiotics (Fig. 2D). Preincubation of bacteria with 8 μg/ml peptide A3-APO in the synergy lane did result in absorbance readings lower than those observed after ciprofloxacin or A3-APO administration alone, and the effect was not seen with coadministration of the two antibiotics. A lower peptide concentration (2 μg/ml) did not result in a dramatic recovery of ciprofloxacin activity, although some synergy was seen with or without preincubation, suggesting that the peptide derivative potentiated the activity of ciprofloxacin by eliminating a fraction of the induced DnaK population.

Taken together, addition of a pyrrhocoricin-based dimer recovered the activity of amoxicillin, a β-lactam antibiotic, against resistant strains. The synergy mode of action is likely inhibition of protein folding because no such effect could be observed with kanamycin, to which resistance involves changes in the target protein sequence. Peptide activity on TEM-1 inactivation appears to be a transient phenomenon. Upon A3-APO treatment of *E. coli* BF1023 or SEQ102, the cell pellet weights showed a time-dependent reduction, consistent with the kinetics of killing (7), but without a noticeable change in TEM-1 activity on nitrocefin after peptide removal and cell resuspension. This hypothesis is supported by reports of a reversible unfolding process (25) and nonpeptide inactivation of TEM-1 (27). As opposed to current β-lactamase inhibitors and combination therapies that work against a limited number of strains, inhibition of all protein folding in bacteria is a universal treatment option. Elimination of resistance to β-lactams by proline-rich peptide derivatives may give renewed life to these antibiotics, large stockpiles of which are currently available.

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REFERENCES

1. **Akagawa, H., Y. Takano, A. Ishii, S. Mizuno, R. Izui, T. Sameshima, M. Kawamura, K. Dobashi, and T. Yoshioka.** 1999. Stresgenin B, an inhibitor of heat induced heat shock protein gene expression, produced by *Streptomyces* sp. AS-9. J. Antibiot. **52:**960–970.
2. **Alos, J. I., M. G. Serrano, J. L. Gomez-Garces, and J. Perianes.** 2005. Antibiotic resistance of *Escherichia coli* from community-acquired urinary tract infections in relation to demographic and clinical data. Clin. Microbiol. Infect. **11:**199–203.
3. **Amsterdam, D.** 1996. Susceptibility testing of antimicrobials in liquid media, p. 52–111. *In* V. Loman (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Philadelphia, Pa.
4. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation to molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
5. **Cudic, M., B. A. Condie, D. J. Weiner, E. S. Lysenko, Z. Q. Xiang, O. Insug, P. Bulet, and L. Otvos, Jr.** 2002. Development of novel antibacterial peptides that kill resistant clinical isolates. Peptides **23:**2071–2083.
6. **Cudic, M., C. V. Lockatell, D. E. Johnson, and L. Otvos, Jr.** 2003. *In vitro* and *in vivo* activity of a designed antibacterial peptide analog against uropathogens. Peptides **24:**807–820.
7. **Cudic, M., P. Bulet, R. Hoffmann, D. J. Craik, and L. Otvos, Jr.** 1999. Chemical synthesis, antibacterial activity and conformation of diptericin, an 82-mer peptide originally isolated from insects. Eur. J. Biochem. **266:**549–558.
8. **Doi, Y., K. Yokoyama, K. Yamane, J. Wachino, N. Shibata, T. Yagi, K. Shibayama, H. Kato, and Y. Arakawa.** 2004. Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides. Antimicrob. Agents Chemother. **48:**491–496.
9. **Haas, D. W., and T. Bonczar.** 1996. Effect of replacing cefotaxime with ceftizoxime in a hospital where penicillin-resistant pneumococcal disease is prevalent. J. Antimicrob. Chemother. **38:**293–299.
10. **Haversen, L. A., I. Engberg, L. Baltzer, G. Dolphin, L. A. Hanson, and I. Mattsby-Baltzer.** 2000. Human lactoferricin and peptides derived from a surface-exposed helical region reduce experimental *Escherichia coli* urinary tract infection in mice. Infect. Immun. **68:**5816–5823.
11. **Hooper, D. C.** 1999. Mechanisms of fluoroquinolone resistance. Drug Resist. Update **2:**38–55.
12. **Hrimech, M., D. Mayrand, D. Grenier, and L. Trahan.** 2000. Xylitol disturbs protein synthesis, including the expression of HSP-70 and HSP-60, in *Streptococcus* mutants. Oral Microbiol. Immunol. **15:**249–257.
13. **Kragol, G., R. Hoffmann, M. A. Chattergoon, S. Lovas, M. Cudic, P. Bulet, B. A. Condie, K. J. Rosengren, L. J. Montaner, and L. Otvos, Jr.** 2002. Identification of crucial residues for the antibacterial activity of the proline-rich peptide, pyrrhocoricin. Eur. J. Biochem. **269:**4226–4237.
14. **Kragol, G., S. Lovas, G. Varadi, B. A. Condie, R. Hoffmann, and L. Otvos, Jr.** 2001. The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. Biochemistry **40:**3016–3026.
15. **Levy, S. B., and B. Marshall.** 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. **10:**S122–S129.
16. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
17. **Lupetti, A., M. M. Welling, E. K. J. Pauwels, and P. H. Nibbering.** 2003. Radiolabeled antimicrobial peptides for infection detection. Lancet Infect. Dis. **3:**233–239.
18. **Mingeot-Leclercq, M. P., Y. Glupczynski, and P. M. Tulkens.** 1999. Aminoglycosides: activity and resistance. Antimicrob. Agents Chemother. **43:**727–737.
19. **Mizushima, T., M. Matsuo, and K. Sekimizu.** 1997. Induction of DnaK and GroEL heat shock proteins by fluoroquinolones in *Escherichia coli*. Antimicrob. Agents Chemother. **41:**193–195.
20. **Otvos, L., Jr., C. Snyder, B. A. Condie, P. Bulet, and J. D. Wade.** 2005. Chimeric antimicrobial peptides exhibit multiple modes of action. Int. J. Pept. Res. Ther. **11:**29–42.

21. **Otvos, L., Jr., J. D. Wade, J.-Q. Feng, B. A. Condie, J. Hanrieder, and R. Hoffmann.** 2005. Designer antibacterial peptides kill fluoroquinolone-resistant clinical isolates. J. Med. Chem. **48:**5349–5359.
22. **Overbye, K. M., and J. F. Barrett.** 2005. Antibiotics: where did we go wrong? Drug Discov. Today **10:**45–52.
23. **Payne, D. J., W. Du, and J. H. Bateson.** 2000. β-Lactamase epidemiology and the utility of established and novel β-lactamase inhibitors. Expert Opin. Investig. Drugs **9:**247–261.
24. **Thomson, C. J., P. M. Shanahan, and S. G. B. Amyes.** 1994. TEM-1 plasmids in the community. Lancet **343:**921.
25. **Vanhove, M., G. Guillaume, P. Ledent, J. H. Richards, R. H. Pain, and J. M. Frere.** 1997. Kinetic and thermodynamic consequences of the removal of the Cys77-Cys123 disulphide bond for the folding of TEM-1 β-lactamase. Biochem. J. **321:**413–417.
26. **Virella, G.** 1997. Urinary tract infections, p. 449–454. *In* G. Virella (ed.), Microbiology and infectious diseases. The Williams & Wilkins Co., Philadelphia, Pa.
27. **Wang, X., G. Minasov, and B. K. Stoichet.** 2002. Noncovalent interaction energies in covalent complexes: TEM-1 β-lactamase and β-lactams. Proteins **47:**86–96.