Journal of Medicinal Chemistry

Article

Designer Antibacterial Peptides Kill Fluoroquinolone-Resistant Clinical Isolates

Laszlo Otvos,, John D. Wade, Feng Lin, Barry A. Condie, Joerg Hanrieder, and Ralf Hoffmann

J. Med. Chem., **2005**, 48 (16), 5349-5359• DOI: 10.1021/jm050347i • Publication Date (Web): 16 July 2005

ZRPDKPRPYLPRPRPPRPVRX
(Chex-Arg-Pro-Asp-Lys-Pro-Arg-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro-Arg-Pro-Val-Arg)$_2$-Dab

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Designer Antibacterial Peptides Kill Fluoroquinolone-Resistant Clinical Isolates

Laszlo Otvos, Jr.,*,†,‡ John D. Wade,§ Feng Lin,§ Barry A. Condie,‡ Joerg Hanrieder,|| and Ralf Hoffmann||

OLPE, LLC, 801 Mockingbird Lane, Audubon, Pennsylvania 19403, The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104, Howard Florey Institute, Melbourne, Victoria 3010, Australia, and Center for Biotechnology and Biomedicine, Faculty of Chemistry and Mineralogy, University of Leipzig, Leipzig D-04103, Germany

Received April 13, 2005

A significant number of *Escherichia coli* and *Klebsiella pneumoniae* bacterial strains in urinary tract infections are resistant to fluoroquinolones. Peptide antibiotics are viable alternatives although these are usually either toxic or insufficiently active. By applying multiple alignment and sequence optimization steps, we designed multifunctional proline-rich antibacterial peptides that maintained their DnaK-binding ability in bacteria and low toxicity in eukaryotes, but entered bacterial cells much more avidly than earlier peptide derivatives. The resulting chimeric and statistical analogues exhibited 8−32 μg/mL minimal inhibitory concentration efficacies in Muller−Hinton broth against a series of clinical pathogens. Significantly, the best peptide, compound **5**, A3-APO, retained full antibacterial activity in the presence of mouse serum. Across a set of eight fluoroquinolone-resistant clinical isolates, peptide **5** was 4 times more potent than ciprofloxacin. On the basis of the in vitro efficacy, toxicity, and pharmacokinetics data, we estimate that peptide **5** will be suitable for treating infections in the 3−5 mg/kg dose range.

Introduction

Bacterial resistance is increasing in an alarming rate,[1] presenting a major global public health threat that involves all major microbial pathogens and currently used antimicrobial drugs.[2] Especially worrying is the recent surge in resistance to fluoroquinolones, once powerful antibiotics that remain the drugs of choice against many bacterial infections.[3] Fluoroquinolones remain the major weapons against urinary tract infections (UTI), although the current resistance rate to norfloxacin and ciprofloxacin has increased to 14% and 19% in uncomplicated or complicated UTI, respectively.[4] Fluoroquinolone resistance is accompanied by resistance to the trimethoprim−sulfamethoxazole combination (TMP/SMX), the second most frequently subscribed drug in UTI, with only approximately half of the strains still sensitive to ciprofloxacin responding to TMP/SMX.[5] The general sentiment is that the pharmaceutical industry has to respond to these clinical challenges by bringing forward a stream of new-generation antimicrobials with activity against resistant isolates.[6]

New antimicrobials regularly reach the clinical trial stage. These novel molecules belong to many antimicrobial classes and interfere with various prokaryotic cellular processes.[7] In addition to quinolones, major antibacterial classes are new generation β-lactams, oxazolidinones, tetracyclines, dihydrofolate reductase inhibitors, peptide deformylase inhibitors, nonclassical glycopeptides, and peptide antibiotics. Peptide-based antimicrobials offer decreased potential for resistance induction,[8] but their parenteral use is occasionally hampered by inadequate safety margins and frequently by rapid clearance leaving them suitable only for topical applications.[7] Some of the toxicity concerns stem from the mode of action: similar polycationic molecules evaluated in the clinics kill bacteria by disintegrating the bacterial membrane structure. Peptide selectivity to bacteria is theoretically possible due to the increased negative transmembrane potential of bacterial membranes compared to mammalian counterparts and their lack of cholesterol that is present in eukaryotic cells.[9] However, even suboptimal antibacterial peptide derivatives are able to interact with mammalian cell membranes, indeed frequently turning antibacterial peptides acting solely on bacterial membranes toxic to eukaryotes.[10] Recent studies have focused on improving the therapeutic index of membrane-active antimicrobial peptides.[11] Having said this, optimally we should be able to harness the highly specific activity of peptide therapeutics, their most attractive feature for drug development,[12] in antimicrobial drug therapy.

To be comparable with conventional antibiotics, antibacterial peptides are expected to preferentially target intracellular biopolymers.[13] Two major peptide families that can do just that are the buforins that bind to bacterial DNA[14] and the short, proline-rich peptide family that kills bacteria by selectively binding to the 70 kDa bacterial heat shock protein DnaK.[15] DnaK provides protein folding assistance to cells with the help of its ATPase activity, two biological processes that are equally inhibited by pyrrhocoricin and drosocin.[15] The molecular architecture of the proline-rich peptide family consists of an N-terminal DnaK-binding domain and a C-terminal cell-penetrating, delivery fragment.[16] From the three insect-derived proline-rich peptides, pyrrhocoricin exhibits the most powerful DnaK-binding unit and drosocin has the most potent cell-penetrating

* To whom correspondence should be addressed: Laszlo Otvos, Ph.D., OLPE, LLC, 801 Mockingbird Lane, Audubon, PA 19403. Tel/fax: 1-610-666-7110; E-mail: lotvos@comcast.net.
† OLPE, LLC.
‡ The Wistar Institute.
§ Howard Florey Institute.
|| University of Leipzig.

10.1021/jm050347i CCC: $30.25
Published on Web 07/16/2005

fragment.[17] The various forms of apidaecin kill different bacteria,[18] most likely due to membrane-selectivity of the slightly variable C-terminal halves. Designer pyrrhocoricin-based peptides show protective properties to experimental animals and lack of toxicity in vitro and in vivo.[19−21] However the in vitro bacterial killing activity is restricted to diluted growth media, and the in vivo efficacy is noticeable only in mild local or systemic infections.[22] The lack of more powerful antimicrobial activity stems from an inefficient membrane-penetrating domain of native pyrrhocoricin.

We decided to modify the sequences of the short, proline-rich antibacterial peptide family to develop antimicrobial compounds that can compete with conventional, non-peptide-based antibiotics and retain the attractive features of peptide drugs, specificity, and low toxicity. The readout of the successful design was (i) activity in full-strength media, preferably Muller−Hinton broth, a solvent broadly used in the microbiology literature; (ii) activity in the presence of serum and acceptable stability in biological fluids in vitro and in vivo; (iii) lack of toxicity to eukaryotes; and (iv) improved activity compared to ciprofloxacin against fluoroquinolone-resistant clinical isolates. To achieve these goals, first we prepared pyrrhocoricin−drosocin chimeras, equipped with additional modifications geared to improve the pharmacological properties.[22] These peptides fulfilled requirements i and iii, but lost activity when the growth inhibition assay was run in the presence of serum and were no match for ciprofloxacin under any condition. In the current report we further expanded the statistical approach, and based on the proven molecular architecture of the pyrrhocoricin−drosocin chimeras, made pyrrhocoricin−apideacin chimeras and fully statistical proline-rich peptide derivatives. One of the new peptides, compound **5**, showed a cumulative 4-fold increased activity compared to ciprofloxacin against a panel of fluoroquinolone-resistant *Escherichia coli* and *Klebsiella pneumoniae* clinical UTI isolates and represents a serious alternative to nonpeptide antibiotics for human and veterinary clinical development.

Results

Peptide Design. Proline-rich antibacterial peptides are major weapons against infection in insects and other lower animals.[23] The three major representatives are pyrrhocoricin from the European sap-sucking bug,[24] drosocin from the fruitfly,[25] and the apidaecins from bees.[26] Both native pyrrhocoricin and drosocin show some selectivity toward Gram-negative organisms, mostly from the Enterobacteriaceae family killing *E. coli*, *Salmonella typhimurium*, *K. pneumoniae*, and *Agrobacterium tumefaciens*.[27−28] Additional Gram-negative drosocin-susceptible strains include *Enterobacter cloacae* and *Erwinia carotovora carotovora*. Pyrrhocoricin is active against *Haemophilus influenzae*.[20] The differences in the activity spectra between pyrrhocoricin and drosocin are either due to evolutionary changes, or more likely, to the identity of the invading microorganisms. The strain specificity of the apidaecins supports this notion. These are 18−20 residue-long peptides with highly conserved carboxy termini. The N-terminal substitutions feature both conservative and nonconservative residue changes. The constant domains are considered responsible for the general antibacterial activity, and the variable domains for the bacteria-specific activity spectrum. Indeed, huge activity differences can be found among the apidaecin peptides, with the peptides from the honeybee killing *Yersinia enterocolitica* but not affecting *Campylobacter jejuni*, and the analogues isolated from the wasps acting in exactly the opposite way.[18] As we are trying to develop proline-rich peptides for mammalian therapy, we introduce a second layer of complexity by screening the efficacy against human and veterinary pathogens, unlikely to equal to the threats to insects. Our best option is to combine all the sequence features of known native proline-rich antibacterial peptides in the hope that the resulting chimeric or statistical peptides will reflect the diversity of Nature and will provide peptide analogues with increased activity against selected clinical isolates and perhaps with increased activity spectrum. Nevertheless, we have to avoid dominant features of drosocin because of its lack of stability in mammalian sera.

Table 1 aligns the sequences of all known native proline-rich antibacterial peptides in insects. The first nine of these represent the full sequences; the bottom four come from larger peptides and proteins. On the basis of successful experiments with pyrrhocoricin−drosocin chimeras in peptide ApiCd−Pyrr-ApiCd, we designed a pyrrhocoricin−apidaecin chimera in the hope of avoiding the proteolytic sensitivity of drosocin-based peptides.[19] From the three apidaecin alleles we selected apidaecin Cd 3+ because of its higher coverage to our target pathogens. Drosocin, formaecin, and pyrrhocoricin are naturally glycosylated, although the carbohydrate on Thr11 (numbering based on pyrrhocoricin) has no major influence on the antibacterial activity.[28] In the designer sequence Glyco combi, the most dominant features of the three peptide sequences were selected, with some bias to the most active nonglycosylated family member pyrrhocoricin (retaining Asp2 and Pro14) and with two additional changes (Arg13 and His16) to improve membrane penetration and serum stability. In the sequence Top peptide combi, the most abundant residues from the nine full-length peptides were chosen without bias to any of them. Finally in All peptide combi the four partial sequences were also added with the resulting peptide being a cross-section of all 13 known insect-derived proline-rich antibacterial peptides. However, a closer examination of this sequence reveals a few inconsistencies. For example, Val3 probably would not be present if the sequences of pyrrhocoricin and metalnikowin did not start with valine, an amino-terminal residue providing stability to proteins (just like glycine).[29] Likewise, Thr14 is present in the native glycopeptides to carry the disaccharide moiety, but is clearly not needed for the nonglycosylated family members. In the All peptide opt(imized) sequence the nonproductive residues were eliminated or replaced with residues more common in the proline-rich peptides. Basically in All peptide opt, the sequences were optimized not only vertically, but also horizontally.

In a second step the designer peptides were incorporated into a molecular framework that is required for good antibacterial activity and desired pharmaceutical properties. The features of this architecture are the

Table 1. Alignment of Native Proline-Rich Antibacterial Peptide Sequences, Design of New Analogues, and Sequences of Synthetic Peptides[a,b]

Native peptides (- indicates the lack of that particular residue):

```
Drosocin                 G K - P R P Y S P R P T S H P R P - I - - R V
Formaecin 1              G R - P N P V N N K P T P Y P H - - L
Formaecin 2              G R - R N P - N N K P T P H P R - - L
Pyrrhocoricin          V D K - G S - Y L P R P T - P P R P I - Y N R N
Metalnikowin 1         V D K - P D - Y R P R P - R P P - - - - - N - M
Apidaecin 1a       G N - N R - P - V Y I P Q P - R P P H P - - - - R I
Apidaecin 1b       G N - N R - P - V Y I P Q P - R P P H P - - - - R L
Apidaecin Cd 3+     G K P S K - P R P A P I K P - R P P H P - - - - R L
Heliocin           Z R F I H - P T - Y - - R P P P Q P R R P V I M R A

Dros pro attC      Z R P Y T Q P L I Y Y P P P P T P P R - - I Y - R A
Diptericin Nter          D - D M T - - - M K P T P P P - Q - - Y P - L
Sarcotoxin Nter          Q K - - L P V P I - P P P T N - P P V
Pro attA                     L P S - - L P - T T G P I R - - V - R R
```

Designed analogs of this report:

```
ApiCd-Pyrr-ApiCd   G K V D K   G S   Y L P R P   R P P H P         R L
Glyco combi            V D K   P R P Y L P R P T R P P H P   I     R L
Top peptide combi  G N V D K   P N P Y I P R P T R P P R P   L Y N R L
All peptide combi  G R V D K   P S P Y L P K P T P P P R P   V Y N R L
All peptide opt    G R P D K   P R P Y L P R P   R P P R P   V     R L
```

[a] In this report the synthetic peptide based on ApiCd-Pyrr-ApiCd is referred to as compound **1**, LO2C, the peptide based on Glyco combi as compound **2**, WI-517, the peptide based on Top peptide combi as compound **3**, A1-TPC, the peptide based on All peptide combi as compound **4**, A2-APC, and the peptide based on All peptide opt(imized) as compound **5**, A3-APO. [b] Sequences of synthetic analogues (all peptides had free N-termini and were C-terminal amides): **1**: ZKVDKGSYLPRPRPPHPRX. (Chex-Lys-Val-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro-His-Pro-Arg)$_2$-Dab. **2**: ZDKPRPYLPRPTRPPHPIRX. (Chex-Asp-Lys-Pro-Arg-Pro-Tyr-Leu-Pro-Arg-Pro-Thr-Arg-Pro-Pro-His-Pro-Ile-Arg)$_2$-Dab. **3**: ZNVDKPNPYIPRPTRPPRPLYNRX. (Chex-Asn-Val-Asp-Lys-Pro-Asn-Pro-Tyr-Ile-Pro-Arg-Pro-Thr-Arg-Pro-Pro-Arg-Pro-Lys-Tyr-Asn-Arg)$_2$-Dab. **4**: ZRVDKPSPYLPKPTPPPRPVYNRX. (Chex-Arg-Val-Asp-Lys-Pro-Ser-Pro-Tyr-Leu-Pro-Lys-Pro-Thr-Pro-Pro-Pro-Arg-Pro-Val-Tyr-Asn-Arg)$_2$-Dab. **5**: ZRPDKPRPYLPRPRPPRPVRX. (Chex-Arg-Pro-Asp-Lys-Pro-Arg-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro-Arg-Pro-Val-Arg)$_2$-Dab. Control peptide (ref 21): Compound **6**, Pip-pyrr-MeArg dimer: *Z*DKGSYLPRPTPPRPIYN*R*NX. (Pip-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-MeArg-Asn)$_2$-Dab where Chex is 1-amino-1-cyclohexanecarboxylic acid, Dab is 2,4-diaminobutyric acid, Pip is 4-amino-4-carboxypiperidine, and MeArg is *N*-methylarginine.

C-terminally linked dimeric structure, proven to increase the interaction with bacterial membranes as well as to prevent carboxypeptidase degradation.[20] As a carboxy-terminal scaffolding agent, we use 2,4-diaminobutyric acid (Dab), an appropriate mimic of the natural leucine of the monomers in this position.[21] The N-terminal amino-cyclohexyl carboxylic acid moiety (Chex) is uniformly employed to avoid aminopeptidase cleavage[28] and improve the activity spectrum[20] as well as to eliminate nonspecific binding to the conventional substrate-binding pocket of mammalian Hsp70 and thus to reduce peptide toxicity to infected animals.[16] Chex is hydrophobic and can be considered a cyclic mimic of the amino-terminal glycine or valine.[21] The synthetic peptide based on ApiCd−Pyrr-ApiCd is compound **1**, the peptide based on Glyco combi is compound **2**, the peptide based on Top peptide combi is compound **3**, the peptide based on All peptide combi is compound **4**, and the peptide based on All peptide opt(imized) is compound **5** (Table 1). For comparison with existing peptide antimicrobials the in vitro efficacy assays included compound **6**, the most active designer analogue until the current research started with proven serum stability and in vivo efficacy.[21]

Peptide Stability and Bioavailability. The first test of the success of the design strategy was the evaluation of peptide accessibility in biological media. This was accomplished by two different assays and using the two optimized sequences. First we studied the stability of peptide **5** in mouse serum. Second, we investigated the bioavailability of peptide **2** after subcutaneous administration into mice. Peptide therapeutics are administered intravenously or subcutanously, and their in vivo stability in blood is generally modeled by in vitro stability in serum or plasma.[30] The main concerns here are to use a completely sterile serum preparation for the ensuing antimicrobial assays with fully active proteolytic enzymes and the selective precipitation of all serum proteins without eliminating large antimicrobial peptides or their fragments. To remove all serum proteins we increase the customarily used 6% trichloroacetic acid (TCA) content to 15% although this exercise may reduce the detected amount of antimicrobial peptide dimers.[21] Compound **5**, at high concentration (1.28 mg/mL), was incubated at 25 °C with a commercially available, proteolytically active sterile mouse serum for 1 h. We selected this time period because in 25% pooled mouse serum more than 80% of the original amount of drosocin decomposes as detected by reversed-phase high performance liquid chromatography (RP-HPLC) with comparable levels of degradation products observed by mass spectroscopy.[28] As Figure 1 indicates, after precipitation with TCA, most of the mass spectrally detected peptide species was the intact peptide **5** with a molecular weight of 5034 Da. The main degradation products exhibited molecular weights at 2576 and 2321 Da, corresponding to one full chain (from Chex1 to Dab21) and a C-terminally truncated Chex1−Val19 fragment, respectively. The main proteolytic cleavage site was found around the C-terminal Arg residue, in agreement with earlier studies on proline-rich peptides.[28] With the replacement of the Ser5-Tyr6 dipeptide fragment, we managed to eliminate the second major endopeptidase cleavage site in native pyrrhocoricin. It is likely that peptide fragments others than those listed above were also present in the digest, but that these evaded isolation or mass spectral surveillance. Proline-rich antibacterial peptide derivatives,

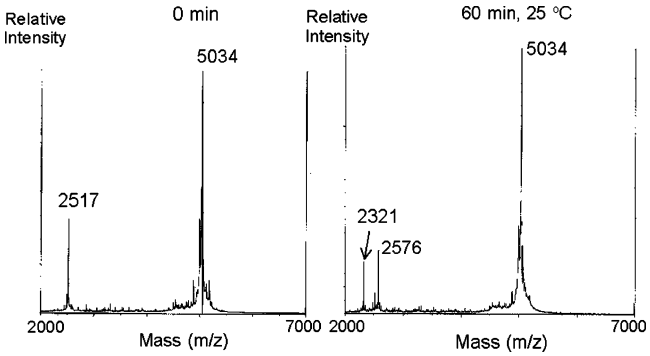

Figure 1. MALDI mass spectra of the peptide **5** before (left image) and after (right image) 1-h incubation with pooled mouse serum at 25 °C. The peaks at 5034 and 2517 *m/z* represent the intact molecule. Peaks at 2321 and 2576 correspond to degradation products cleaved around the C-terminal arginine residue.

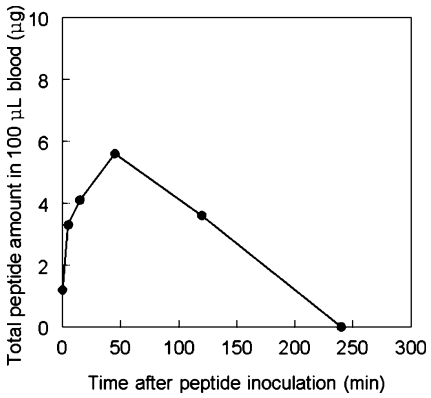

Figure 2. Antibacterial peptide present in the blood after subcutaneous inoculation of 100 μg of peptide **2** into Balb/c mice. Peptide amounts coeluting with intact compound **2** on a narrow-bore reversed-phase HPLC column were estimated based on a calibration curve. The data points are averages of peptides in the blood drawn from the eye of four mice per time-point.

especially the dimers, are naturally amphipathic and stick to amphipathic surfaces found in cells, biological media, or chemical instrumentation, making their analysis and quantitation extremely difficult.[22] Despite all the variables present in the assay and detection methodology, the most important finding was the improved serum stability of peptide **5** compared to drosocin. When the assay was repeated at 37 °C at a significantly lower peptide concentration (40 μg/mL), identical mass spectrometry peaks were obtained, but the recovery (even after 0 min incubation) of the small amount of large molecular weight species was significantly reduced, most likely due to permanent attachment of the construct to amphipathic surfaces present in the serum, vials, or instrumentation, or were precipitated by TCA. It is worth noting that the five designer peptides of this report showed very similar serum stability and mass spectroscopy behavior throughout the study.

In an ensuing assay, peptide **2** was injected subcutaneously into Balb/c mice at a single dose of 5 mg/kg (100 μg), and the peptide amount in the blood was estimated after 5, 15, 45, 120, and 240 min. For these experiments, we drew blood from the eye because this is the cleanest blood available in mice. We needed as clear a sample as was available because of the above-detailed sticky nature of proline-rich peptide antibiotic dimers. However, since the blood drawing could not be repeated within the assay period, for every time point we used different animals. To compensate for the interindividual alterations in peptide availability and processing, four mice were used for each time-point. After precipitation of cells and serum proteins, the available peptide amount was estimated by RP-HPLC using a narrow-bore column.[31] Analyte coeluting with intact compound **2** was used to generate Figure 2. The pharmacokinetics followed regular peptide bioavailability schemes.[32] No peptide was detected at 4 h, and the small amount at 0 min represents the lag time between peptide inoculation and our ability to find the eye vein and draw blood afterward. The detected peptide amount peaked at 45 min reaching 80% of the injected amount. Even if this value was overestimated due to the variability of the assay, a 2-h window can safely be identified between 5 and 125 min where approximately 40% of the injected peptide was present in the blood (Figure 2). Although the killing kinetic of proline-rich peptides is longer than that of peptide antibiotics without an intracellular target,[33] a 2-h time period is sufficient for full antibacterial activity of pyrrhocoricin and its derivatives.[10] Mass spectral analysis of peptides recovered from the reversed-phase column in a 5-min window flanking the retention time of peptide **2** indicated an array of degradation products. The intact peptide was represented by the doubly charged peak at 2361 *m/z*. Dominant singly charged peaks were observed at 2930, 3215, and 3531 *m/z*, with an intensity ratio independent of the incubation period, suggesting that, just like during the in vitro serum stability assay, mass spectrometrically detected peptide recovery was biased toward smaller, more hydrophilic or less adsorptive peptide fragments. Apparently the dimeric arrangement of the C-terminal intracellular delivery unit, needed for full activity, remained intact during peptide inoculation, adsorption, and distribution.

Antibacterial Activity. On the basis of previous studies on proline-rich antibacterial peptides,[20,21] we speculated that the designer peptides will be most suitable to treat infections caused by Enterobactericeae, preferably resistant UTI pathogens. *E. coli* accounts for 75−90% of the UTI cases,[34] and *K. pneumoniae* is responsible for another 8−13%.[35] To test the efficacy of the designer proline-rich peptides, we obtained eight fluoroquinolone-resistant UTI strains, six *E. coli* and two *K. pneumoniae*, from the collection of the SENTRY Antimicrobial Surveillance Program. These clinical isolates were collected from various locations in the world and included urine samples as well as pathogens from catheter-induced infections. The activity of the peptide antibiotics was compared with ciprofloxacin and the TMP/SMX drug combination using the liquid broth microdilution assay[36] in full-strength Muller−Hinton medium. All strains, except one *E. coli* isolate, were completely resistant to TMP/SMX with minimal inhibitory concentration (MIC) values above the highest tested antimicrobial concentration of 128 μg/mL (Table 2). Ciprofloxacin exhibited a somewhat better activity, with one *E. coli* and one *K. pneumoniae* strain being almost entirely resistant (MIC = 128 μg/mL, IC_{50} = 40−60 μg/mL), one *E. coli* strain showing midrange sensitivity (MIC = 32 μg/mL, IC_{50} = 12 μg/mL), and the rest

Table 2. Activity of Antibacterial Peptides and Control Nonpeptide Antibiotics against Fluoroquinolone-Resistant Clinical Urinary Tract Isolates

		MIC (in μg/mL) in full-strength Muller−Hinton broth IC_{50} (in μg/mL) in full-strength Muller−Hinton broth							
bacterial strain	origin	**6***	2	1	3	4	5	Cipro	TMP/SMX[a]
E. coli 045-849	Mexico, urine	>128[a]	>128	128		>128	16	128	>128*
		90[a]	30	20		>128	7	40	>128[a]
E. coli 002-2605	US, catheter	>128[a]	>128	32		>128	8	32	>128[a]
		60[a]	40	12		>128	6	12	>128[a]
E. coli 069-3171	Turkey, urine	>128	>128	64	>128	>128	8	8	>128[a]
		90	40	20	>128	>128	3	5	>128[a]
E. coli 095-2488	Germany, catheter	>128	>128	64	>128	>128	8	16	>128[a]
		100	60	40	>128	>128	5	8	>128[a]
E. coli 014-2258	US, urine	>128	>128	128	>128	>128	8	8	2[a]
		>128	50	50	>128	>128	5	4	1[a]
E. coli 043-686	Chile, catheter	>128	>128	64	>128	>128	16	8	>128[a]
		100	60	30	>128	>128	7	6	>128[a]
K. pneumoniae 012-3132	US, catheter	>128[a]	>128	32		>128	16	128	>128[a]
		>128[a]	>128	15		>128	7	60	>128[a]
K. pneumoniae 025-2753[b]	US, catheter	>128[a]	>128	32		>128	16	4	>128[a]
		100[a]	50	10		>128	6	2	40[a]

antibiotic	**5**	ciprofloxacin	**1**	**2**	**6**
mean efficacy MIC (in μg/mL)	12	42	68	>128	>128
IC_{50} (in μg/mL)	6	17	25	60	106
approximate relative efficacy to compound **5** (%)	100	30	20	<10	<6

[a] Marked activities measured on different plates. [b] Not truly fluoroquinolone-resistant.

of the strains are being sensitive (MIC = 4−16 μg/mL, IC_{50} = 2−8 μg/mL). Taken together, in our hands the majority of these strains were more sensitive to fluoroquinolonones than their SENTRY designation would indicate. The two completely statistical peptides, **3** and **4** were fully inactive against the strains tested showing not only MIC but even IC_{50} values higher than 128 μg/mL (Figure 3, panel A and Table 2). Although no MIC could be detected, the previously most active, entirely pyrrhocoricin-based analogue **6** and the slightly optimized glycopeptide-based statistical derivative **2** exhibited some activity against six and seven of the eight strains, respectively. The new peptide, compound **2**, was more active with IC_{50} figures between 30 and 60 μg/mL. In contrast, peptides **1** and especially **5** showed significant activity against the clinical UTI isolates (Figure 3, panel A and Table 2). While compound **1** was more active than ciprofloxacin against two strains, peptide **5** exhibited superior activity against five isolates. Remarkably, compound **5** had a uniform 8−16 μg/mL MIC activity, suggesting that very similar efficacies can be expected against any other *E. coli* or *K. pneumoniae* clinical UTI pathogen significantly simplifying prescription and dosing patterns. The designer peptide **5** is far the most potent antimicrobial peptide our laboratory has ever tested killing the eight test strains with mean MIC and IC_{50} values of 12 and 6 μg/mL, respectively (Table 2). On average, this optimized peptide was 3−4-fold more active than ciprofloxacin on a weight basis customary in the microbiology literature; in molarity terms the peptide was more active than ciprofloxacin by 50-fold. From the rest of the peptide antibiotics, compound **5** was 5-fold more active than the pyrrhocoricin−apidaecin chimera compound **1**, more than 10-fold more active than the other optimized designer derivative compound **2**, and showed almost 20-fold better activity than the purely pyrrhocoricin-based compound **6**.

As it could be predicted from the results of the serum stability assay, peptide **5** did not lose any efficacy in the presence of sterile pooled mouse serum. We tested the effect of serum on antibacterial activity with two independent assays by using yet another *K. pneumoniae* UTI isolate from the SENTRY collection (Figure 3, panel B). First the activity of the peptide with and without serum treatment was compared using the supernatants from the serum stability assay described above. The bacterial killing curves without preincubation and after the 60-min serum treatment could be perfectly overlaid. In the next step the broth microdilution assay was run on single plates with 90 μL medium and 10 μL peptide solution in one halves, and with 65 μL medium, 10 μL peptide, and 25 μL mouse serum (25% serum) in the others. Once again, the bacterial killing curves were very similar, except the higher background and less aggressive bacterial growth in the wells containing serum.

Compound **5** was tested for its ability to kill resistant Enterobactericeae other than those from the SENTRY collection. Against all *E. coli*, *K. pneumoniae*, and *S. typhimurium* strains tested the peptide retained a 16−32 μg/mL MIC efficacy, and the composition of the test medium did not influence the activity either (Table 3). The designer analogue was equally active in full-strength Muller−Hinton broth and 1% tryptic soy broth, two media preferred in the microbiology literature. In line with the inhibition of protein folding only in phylogenetically related strains (i.e similar DnaK sequence), no full bacterial killing (MIC) could be detected against the more distant Gram-positive bacterium *Staphylococcus aureus*. *S. aureus* was killed only in 1/4 strength Muller−Hinton broth with an MIC of 64 μg/mL (data not shown).

Toxicity to Normal Cells. Toxicity to eukaryotic cells is always a concern when working with membrane-active polycationic compounds. Many research groups

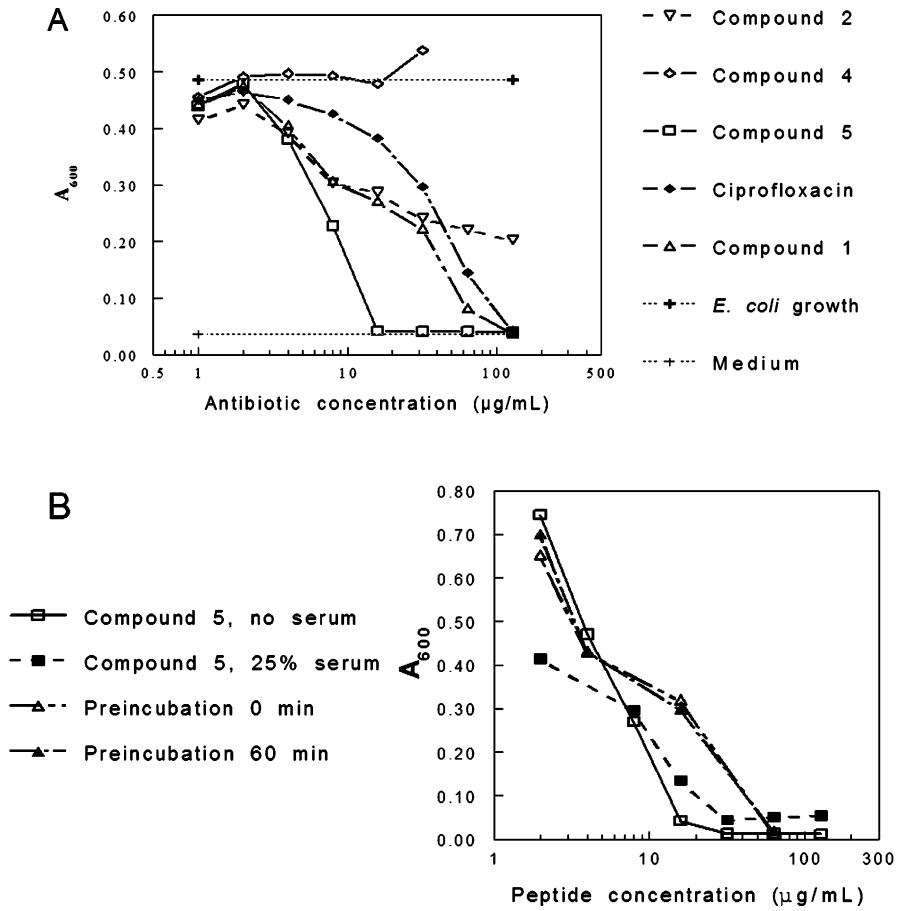

Figure 3. Antibacterial activity of designer proline-rich peptides. Panel A: Representative growth inhibition liquid broth microdilution assay using *Escherichia coli* 045−849 as target microorganism. The assay was run overnight at 37 °C in full-strength Muller−Hinton broth. Compound **5** showed superior antibacterial activity compared to other peptide analogues or the control fluoroquinolone antibiotic ciprofloxacin. Panel B: Effect of serum treatment on the antibacterial activities of peptide **5**. This figure corresponds to a composite of two independent assays, both using *Klebsiella pneumoniae* 048 as test microorganism. In one assay the medium was either full-strength Muller−Hinton broth (open squares) or 25% of medium was replaced with pooled mouse serum (closed squares). Serum addition had no effect on the MIC value (16 μg/mL). Please note the higher background and slower bacterial growth in the presence of serum. In the second assay the standard protocol was run in Muller−Hinton broth, but the antimicrobial peptide was preincubated with pooled mouse serum (from Figure 1, final serum concentration 50%) for 0 min (open triangles) or 60 min (closed triangles). Once again, serum treatment did not alter the antibacterial activities. The higher MIC value during the preincubation assay (32 μg/mL) can be explained with inaccurate peptide concentration input due to the numerous dilution and transfer cycles of the peptide antibiotic.

Table 3. Minimal Inhibitory Concentrations of Compound **5** against Bacterial Strains Sensitive to Fluoroquinolones, but Resistant to Other Antibiotics

	MIC (in μg/mL) in	
bacterial strain	full-strength Muller−Hinton broth	1% tryptic soy broth
E. coli JC7623	16	
K. pneumoniae K6	16	
K. pneumoniae 048	16	
S. typhimurium S5	32	
E. coli DC2		8
E. coli B221A1		8
E. coli ATCC 25922		16
K. pneumoniae ATCC 10031		32

test the toxic properties against red blood cells, but others prefer cells with regular nuclei. Indeed, the hemocidin family of antimicrobial peptides is not hemolytic at all but shows dose-dependent cytotoxicity to fibroblasts and hepatoma cells.[37] To maintain the theme of treatment of UTI, our control cell line of choice is COS-7, immortalized monkey kidney cells.[21] In general, our toxicity figures to COS-7 cells and erythrocytes of the control antibiotic magainin 2 are almost identical.[22] The activity of the two more-or-less optimized peptides, compounds **2** and **5** on COS-7 cells was studied on a 24-hour cell proliferation assay. In a preliminary assay none of the peptides were toxic up to 1.2 mg/mL. The experiments were repeated with compound **5** in the 1.2−2.5 mg/mL concentration range (Figure 4). Untreated cells grew overnight from 10000 to 57000 cells/well. When the toxicity control 10% dimethyl sulfoxide (DMSO) was added to the cells, only 9000 cells survived. Peptide **5** at 1.2 and 1.5 mg/mL concentration did not influence the cell count beyond the experimental errors of the assay. Increasing the peptide concentration to 2.0 mg/mL reduced the cell count to 32000, indicating a possible toxic effect. However, further increase of the peptide load to 2.5 mg/mL partially restored the number of surviving cells (40000). Taken together no toxicity to COS cells was detected up to 1.5 mg/mL, and even higher peptide concentrations were significantly less toxic to the cells than the control 10% DMSO solution.

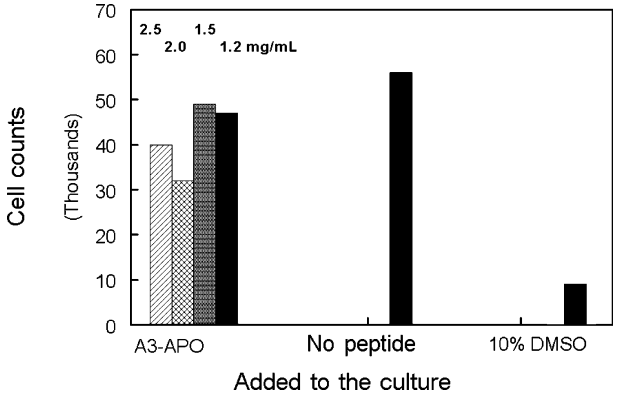

Figure 4. Effect of peptide **5** on the growth of COS-7 cells. Growing cells were incubated with various concentrations of the peptide or the toxicity control 10% dimethyl sulfoxide for 24 h, and the number of live cells was counted. No eukaryotic cell toxicity of the peptide construct could be observed at least up to 1.5 mg/mL.

Discussion

In the past 15 years, native antibacterial peptides were constantly considered suitable for human or veterinary applications. However frustrations grew with every preclinical or clinical trial along the way. As a first drawback, most peptide antibiotics are insufficiently active in media used in the microbiology literature.[20] Solvents with high salt content or dielectric constant deactivate peptide antibiotics,[37] likely because of neutralization of the positive side-chains, needed for the initial interaction with bacterial membranes. The question is still open whether the currently used media represent well the in vivo environment in which antibacterial peptides operate, or just used because bacteria grow in these media without restriction. It is worth examining Figure 3, panel B, which illustrates that addition of serum actually reduces the rate of bacterial growth, questioning the validity of these growth conditions. In support, antibacterial peptides in general are more active in vivo than the in vitro bacterial killing data would indicate. Alternatively, the unexpected in vivo properties can be due to the immunostimulatory activity of native antibacterial peptides.[38] Nevertheless, systemic efficacy of the control compound **6** cannot be observed when the 10^6 cfu (colony forming units) bacterial inoculum resulting in morbidity in mice is increased to 10^7 cfu, rapidly killing untreated animals.[22] As difficult as it is to admit, the original calculations suggesting a required therapeutic dose 130% of the MIC measured in full-strength media[7] may indeed be needed for protecting animals in a bacteremia model. With the designer compound **5**, we managed to develop an antibacterial peptide derivative that kills bacteria in various full-strength media. Calculating with 16 μg/mL MIC, our dose would be 21 μg/mL, or 36 μg peptide per mouse, or 2 mg/kg. This dose range is comparable with that of nonpeptide antibiotics on the market.[39]

The systemic applicability of peptide drugs is further hampered by the rapid degradation in mammalian body fluids. The in vivo instability of the previous generation pyrrhocoricin−drosocin chimeras made them nonstarters for clinical development despite their relatively good MIC in full-strength Muller−Hinton broth.[22] The in vitro serum stability is a good indication of the potential utility of these peptides in mammalian therapy, as the complete in vivo inefficacy of the in vitro highly unstable native drosocin indicates.[19] In addition, the proline-rich peptide family interferes with an intracellular bacterial housekeeping mechanism and such requires an extended duration of action. Our bioavailability (preliminary pharmacokinetics) studies indicate that a 2-h period exists, enough to kill bacteria, when 40% of the subcutaneously injected peptide (or a coeluting active metabolite) is present in body fluids. The partial decomposition of the combinatorial proline-rich peptides in vivo will push the dose up to 5 mg/kg for systemic applications against bacteremia. As antibacterial peptides are concentrated in the urinary tract (and to a smaller degree at the site of infection),[40] this figure will certainly be lower against UTI.

A third, and sometimes detrimental drawback of antibacterial peptide drug development, is the toxicity of the molecules in vitro and in vivo exhibiting poor therapeutic indices,[41] indeed rendering peptide antibiotics too toxic for systemic treatment.[42] As a result, antibacterial peptides end up in the treatment of local or topical infections,[43−44] although none of these applications have obtained regulatory approval. To make peptide antibiotics more attractive for the pharmaceutical industry, current research focuses on the development of nontoxic analogues,[45] but the true solution would be the development of antibacterial peptide drugs acting primarily on intracellular target molecules, such as the buforins or the proline-rich peptides. Since the peptides have to easily enter bacteria, a compromise has to be struck between efficacious membrane penetration and lack of toxicity to eukaryotes.[10] Compound **5** may be a solution to this problem. It has good membrane activity but ultimately bacteria are killed by DnaK inhibition, as indicated by the comparison of the activities against *E. coli* and *S. aureus* in full-strength and diluted media. Indeed, as we show here, peptide **5** lacks considerable toxicity to COS cells. Even with conservative data interpretation no toxicity was found at 1.5 mg/mL, and with the 16 μg/mL efficacy figure we are talking about a therapeutic index of at least 100. Clearly toxicity concerns will not limit the dose in vivo. Our COS-7 cell toxicity assay appears to mimic well the effects of antibacterial peptides on mammalian membranes as in our hands magainin 2 exhibits the expected middle-level toxicity, the lowest toxic concentration being approximately 500 μg/mL,[10] a value very close to its hemolytic concentration.[22] It needs to be added that due to the improved bacterial membrane-penetrating properties of the dimeric proline-rich peptide constructs, resistance induction cannot be observed as we documented with compound **6**[21] or the pyrrhocoricin−drosocin mixed chimera.[22]

An intriguing basic research question is what structural features make some of these very similar peptides active and others inactive. The dimeric peptide derivatives of this report are designed to kill bacteria by multiple modes of action. First they have to bind the DnaK D−E helix region of the target organisms, and second they must have a potent membrane-penetrating domain.[22] As Table 4 shows, the sequences of all active peptides contain the YLPRP fragment, the hypothetical *E. coli* and *S. typhimurium* DnaK-binding motif.[10] In two of the three inactives, the YLPRP pentapeptide is

Table 4. Structure−Activity Relationships of Proline-Rich Antibacterial Peptides

peptide	activity	sequence of the hypothetical DnaK-binding domain	binding to *E. coli* DnaK D−E helix	sequence of C-terminal delivery unit	total number of Arg, His, or Pro residues
5	+++	YLPRP	+	RPPRPVR	7/6
1	++	YLPRP	n.t.[a]	RPPHPR	6/6
2	+	YLPRP	n.t.	TRPPHPIR	8/6
6	−	YLPRP	n.t.	TPPRPIYN*R*N	10/5
3	−	YIPRP	+/−	TRPPRPLYNR	10/6
4	−	YLPKP	+	TPPPRPVYNR	10/6

[a] n.t., not tested.

altered; in compound **3** isoleucine is substituted for the leucine, and in peptide **4** lysine is substituted for the arginine. This suggests that full bacterial killing requires the intact DnaK-binding domain. Indeed, even the best peptide of this series, compound **5** fails to kill *S. aureus* in full-strength Muller−Hinton broth, a strain with a modified DnaK sequence at the peptide-binding D−E helix region.[46] At the time of the writing of this report, the DnaK sequence of *K. pneumoniae* has not yet been published, but based on the phylogenic proximity to *E. coli* and *S. typhimurium*, we expect very little sequence alterations. It needs to be mentioned that compound **4** does bind a fluorescein-labeled *E. coli* DnaK D−E helix preparation, despite the Arg to Lys change (for compound **3**, the fluorescence polarization method used did not provide clear-cut results). Apparently membrane penetration is equally important. Examination of the C-terminal delivery halves indicate that the active peptides are significantly more compact and lack residues that do not promote entry into cells. Positively charged residues, especially arginine,[47] as well as prolines are known to facilitate peptide entry into cells.[48] The delivery domains of compounds **5**, **1**, and **2** do not have to carry unproductive, filler residues into the cytoplasm (Table 4). While the function of residues N-terminal to the YLPRP active site other than the Asp-Lys dipeptide fragment is unclear,[10] it is tempting to speculate that the improved antibacterial activity of peptide **5** compared to peptide **1** is due to its cell-entry focused N-terminal heptapeptide fragment. In support, another pyrrhocoricin−apidaecin construct, without the N-terminal Lys-Val dipeptide fragment failed to kill the test bacterial strains (data not shown). The argument presented above questions the need for the valine, the only nonterminal residue left in compound **5** without DnaK-binding or cell-penetrating functions. However, as it was shown earlier, a limited number of hydrophobic residues are needed for the initial interaction of antimicrobial peptides with the outer layer of bacterial membranes,[49] valine being a very attractive option.[50] The hydrophobic residues may improve the interaction of the peptides with the lipidic headgroups of bacterial surface structures or induce conformational orientation on the membrane surface resulting in more direct interaction of the negatively charged bacterial membrane with the cationic cell-penetrating residues.[13] In either event, the midchain tyrosine, leucine, and valine residues as well as the two nonproteinogenic terminal moieties in peptide **5** will serve just that purpose.

The correct alignment of the proline-rich antibacterial peptides for their structural similarities and differences is a subject of constant debate. Different research groups follow different algorithms even if they collaborate (compare this alignment with refs 51−53). The alteration in the interpretations stem from the presence of multiple Pro-Arg-Pro repeats that make not only the sequence-related speculations but also peak assignment in nuclear magnetic resonance spectroscopy troublesome.[52] Naturally alternative alignment protocols would result in vastly different combinatorial sequences. Nevertheless, while this can be truly confusing in regard to the location of potential glycosylation sites when the threonine is not present in the sequences,[51] in our case different alignments would not significantly change the outcome of iterations leading to the best sequence represented by compound **5**. The second dimensional optimization changes included the introduction of additional proline and arginine residues, and thus the All peptide opt(imized) sequence with its abundance of PRP repeats (exactly two more than the All peptide combi analogue) can be generally aligned with any proline-arginine-rich native antimicrobial peptide. As outlined in the previous paragraph, peptide **5** encompasses only the minimally required delivery, DnaK binding; delivery triad motifs and alternative alignments would not further simplify the final structure.

The dose calculations above suggest that the peptide **5** will be suitable for the treatment of systemic infections that manifest in complete bacteremia in the experimental animals. While we believe that this is the case, the utility of the peptides in the treatment of local infections, such as gastrointestinal or urinary tract infections, can be predicted with even stronger confidence. Peptide antibiotics preferentially target the urinary tract,[40] kill resistant uropathogens as documented here, and with the low ionic strength environment of the renal tubules in conditions where antibacterial peptides are most active,[54] appear to be ideal drugs to combat UTI. UTI are among the most common bacterial infections in humans causing more than 8 million physician visits in the United States each year and affecting 50% of woman in their lifetime.[55] Mouse models of upper UTI, pyelonephritis, exist,[39] but as we noticed, the bacterial regimens used today result in only partial infection of the animals.[21] However recurrent and resistant UTI are as devastating in companion animals such as dogs and cats as in humans. A retrospective study of bacterial urine cultures collected over a 26-year period at a Californian veterinary hospital identified 383 dogs (more than one new case monthly) with resistant UTI, representing 0.3% of all dogs seen by veterinarians.[56] The major UTI pathogens in dogs, just like in humans, are *E. coli* and *K. pneumoniae*,[57] and a large number of these strains became recently nonsusceptible to ciprofloxacin and enrofloxacin, the fluoroquinolone variation in veterinary medicine.[58−59] On one hand, this discomforting trend provides plenty of naturally infected dogs for the in vivo efficacy studies; on the other hand, the

large veterinary market can help progress the product early into animal therapeutics, providing financial resources and safety data for ensuing human clinical trials.

Experimental Section

Peptides and Conventional Antibiotics. Amino acids protected with the 9-fluorenyl-methoxy-carbonyl group (Fmoc)[60] were used for the synthesis of the peptides. The peptide chain assembly was carried out on a Rainin PS3 automated synthesizer. After trifluoroacetic acid cleavage, peptides were purified by RP-HPLC in a water/acetonitrile/trifluoroacetic acid elution system, until matrix-assisted laser-desorption/ionization mass spectra (MALDI-MS) revealed only single species. Table 1 lists the synthetic peptides. Purity data for compounds **1**−**6** are found in Supporting Information.

The TMP/SMX combination (1:5, w/w) and ciprofloxacin were diluted from their intravenous formulations prepared by Elkins-Sinn (Cherry Hill, NJ) and Bayer (West Haven, CT), respectively.

Antibacterial Assay. Antibacterial growth inhibition assays were performed using sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 μL as described previously.[20] The cell concentrations were estimated by measuring the ultraviolet absorbance at 600 nm and applying the formula cfu/mL = $A_{600}(3.8 \times 10^8)$, where cfu is the number of colony-forming units. Briefly, 90 μL of a suspension of mid-logarithmic phase bacterial cultures at an initial absorbance at 600 nm of 0.001 (4×10^5 cfu/mL) in full-strength Muller−Hinton broth was added to 10 μL of serially diluted peptides dissolved in sterilized water. The highest final peptide concentration was 128 μg/mL. Cultures were then incubated at 37 °C for 16−20 h without shaking, and growth inhibition was measured by recording the absorbance at 600 nm using a microplate reader. MIC were identified as the lowest antimicrobial doses when the 600 nm absorbance did not exceed of the negative control medium only values. The IC_{50} data were calculated by averaging the absorbance figures of no growth and full bacterial growth and identifying the antibiotic concentration where the growth curves crossed this imaginary line.

Toxicity to COS Cells. Toxicity to mammalian kidney cells was studied similar to the cell toxicity assay of our earlier report.[22] Briefly, COS-7 cells were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37 °C in an atmosphere of 10% CO_2. The cells were plated in 24-well tissue culture dishes (5×10^3 cells/well) and incubated for 24 h prior to peptide addition. Peptides, dissolved in 0.5 mL water, were inoculated into 1 mL of medium, in final concentrations of 0.6−2.5 mg/mL in duplicates. The plates were incubated for an additional 24 h at 37 °C in an atmosphere of 10% CO_2, the medium was aspirated, and the assay was terminated by adding 100 μL of trypsin ethylenediamine tetraacetate (EDTA) (0.25% trypsin/0.1% EDTA in Hank's balanced salt solution; Cellgro). One milliliter of fresh medium was added to the detached cells, and 50 μL of this solution was mixed with 50 μL of trypan blue solution (0.4%, Sigma) to stain dead cells. The remaining live cells were counted using a hemacytometer.

Serum Stability Studies. (1) Peptide stability in mouse serum. Fifty microliters of sterile mouse serum (Equitech-Bio, Kerrville, TX) was added to compound **5** dissolved in distilled water at 1.28 mg/mL concentration. The peptide was incubated with the serum for 1 h at 25 °C, 20 μL 15% TCA was added, and the mixture was centrifuged. The supernatant was submitted to MALDI-MS analysis. In a separate assay the experiment was repeated at 37 °C with 40 μg/mL original peptide concentration. (2) Effect of serum addition to antibacterial activity. To evaluate the antibacterial activity of the peptides in the presence of mammalian serum, the antibacterial assay was repeated as described above, with the only difference being that instead of 90 μL of medium each well contained 65 μL of medium and 25 μL of mouse serum. The controls on each plate were peptides and control antibiotics without serum addition, the bacterial inoculum with serum but without antimicrobials added for determining the bacterial growth, and serum plus medium for sterility control. Additionally, the antibacterial activity of compound **5** was determined from the supernatants of the serum stability assay above.

Pharmacokinetics in Mice. One hundred micrograms of peptide **2** (5 mg/kg) dissolved in 200 μL of sterile phosphate buffered saline (PBS) pH 7.2 was injected subcutaneously (dorsally, around the shoulder blade) into healthy Balb/c mice (Charles River Laboratories) using four mice for each time-point. About 100 μL of blood was taken from the eye at 0 (right after peptide administration), 5, 15, 45, 120, and 240 min. Each animal was used only once. Cells were centrifuged, and 20 μL of 15% TCA was added per 100 μL of plasma. After repeated centrifugation, 10 μL of supernatant was loaded to a C18 narrowbore HPLC column (2.1 mm/20 cm) that had previously been calibrated with 10 ng, 30 ng, and 100 ng peptide **2** dissolved in PBS. Absorbance was measured at 214 nm, and the amount of peptide coeluting with compound **2** was estimated based on the peak areas relative to the calibration curve.

Acknowledgment. The authors acknowledge the financial support from the Commonwealth Universal Research Enhancement Program, Pennsylvania Department of Health. We also thank Dr. Ronald N. Jones and Mr. Douglas J. Biedenbach from the JMI Laboratories for providing us with the fluoroquinolone-resistant bacterial strains, Professor Peter Zipfel and Dr. Ute Mollmann from the University of Jena for the 1% tryptic soy broth assay, and Ms. Wynetta Giles-Davis for the COS cell toxicity experiments.

Appendix

Abbreviations: cfu, colony forming units; DMSO, dimethyl sulfoxide; EDTA, ethylene-diamine tetraacetate; HPLC, high performance liquid chromatography; MALDI-MS, matrix-assisted laser desorption/ionization mass spectroscopy; MIC, minimal inhibitory concentration; PBS, phosphate-buffered saline; RP, reversed-phase; TCA, trichloroacetic acid; TMP/SMX, trimethoprim/sulfametoxazole drug combination, 1:5 w/w; UTI, urinary tract infections.

Supporting Information Available: Summary of MS-MALDI data of new peptides **1**−**5**, HPLC conditions, MS and HPLC spectra of compounds **1**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Overbye, K. M.; Barrett JF. Antibiotics: where did we go wrong? *Drug Discovery Today* **2005**, *10*, 45−52.
(2) Levy, S. B.; Marshall, B. Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med*. **2004**, *10*, S122−129.
(3) Dan, M. The use of fluoroquinolones in gonorrhea: the increasing problem of resistance. *Expert Opin. Pharmacother*. **2004**, *5*, 829−854.
(4) Alos, J. I.; Serrano, M. G.; Gomez-Garces, J. L.; Perianes, J. Antibiotic resistance of *Escherichia coli* from community-acquired urinary tract infections in relation to demographic and clinical data. *Clin. Microbiol. Infect*. **2005**, *11*, 199−203.
(5) Hooton, T. M.; Besser, R.; Foxman, B.; Fritsche, T. R.; Nicolle, L. E. Acute uncomplicated cystitis in an era of increasing antibiotic resistance: a proposed approach to empirical therapy. *Clin. Infect. Dis*. **2004**, *39*, 75−80.
(6) Bush, K. Antibacterial drug discovery in the 21st century. *Clin. Microbiol. Infect*. **2004**, *10* Suppl. 4, 10−17.
(7) Bush, K.; Macielag, M.; Weidner-Wells, M. Taking inventory: antibacterial agents currently at or beyond Phase I. *Curr. Opin. Microbiol*. **2004**, *7*, 466−476.
(8) Ge, Y.; MacDonald, D. L.; Holroyd, K. J.; Thornsberry, C.; Wexler, H.; Zasloff, M. *In vitro* properties of pexiganan, an analog of magainin. *Antimicrob. Agents Chemother*. **1999**, *43*, 782−788.

(9) Hancock, R. E. W.; Scott, M. G. The role of antimicrobial peptides in animal defenses. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8856−8861.
(10) Bower, M. A.; Cudic, M.; Campbell, W.; Wade, J. D.; Otvos, L., Jr. Walking the fine line between intracellular and membrane activities of antibacterial peptides. *Lett. Pept. Sci.* **2003**, *10*, 463−473.
(11) Chen, Y.; Mant, C. T.; Farmer, S. W.; Hancock, R. E.; Vasil, M. L.; Hodges, R. S. Rational design of α-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index. *J. Biol. Chem*. **2005**, *280*, 12316−12329.
(12) Loffet, A. Peptides as drugs: is there a market? *J. Pept. Sci*. **2002**, *8*, 1−7.
(13) Cudic, M.; Otvos, L., Jr. Intracellular targets of antibacterial peptides. *Curr. Drug Targets* **2002**, *3*, 101−106.
(14) Park, C. B.; Kim, H. S.; Kim, S. C. Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem. Biophys. Res. Commun*. **1998**, *244*, 253−257.
(15) Kragol, G.; Lovas, S.; Varadi, G.; Condie, B. A.; Hoffmann, R.; Otvos, L., Jr. The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. *Biochemistry* **2001**, *40*, 3016−3026.
(16) Kragol, G.; Hoffmann, R.; Chattergoon, M. A.; Lovas, S.; Cudic, M.; Bulet, P.; Condie, B. A.; Rosengren, K. J.; Montaner, L. J.; Otvos, L., Jr. Identification of crucial residues for the antibacterial activity of the proline-rich peptide, pyrrhocoricin. *Eur. J. Biochem.* **2002**, *269*, 4226−4237.
(17) Bencivengo, A.-M.; Cudic, M.; Hoffmann, R.; Otvos, L., Jr. The efficacy of the antibacterial peptide, pyrrhocoricin, is finely regulated by its amino acid residues and active domains. *Lett. Pept. Sci*. **2001**, *8*, 201−209.
(18) Casteels, P.; Romagnolo, J.; Castle, M.; Casteels-Josson, K.; Erdjument-Bromage, H.; Tempst, P. Biodiversity of apidaecin-type peptide antibiotics. Prospects of manipulating the antibacterial spectrum and combating acquired resistance. *J. Biol. Chem*. **1994**, *269*, 26107−26115.
(19) Otvos, L. Jr.; Bokonyi, K.; Varga, I.; Otvos, B. I.; Hoffmann, R.; Ertl, H. C. J.; Wade, J. D.; McManus, A. M.; Craik, D. J.; Bulet, P. Insect peptides with improved protease-resistance protect mice against bacterial infection. *Protein Sci*. **2000**, *9*, 742−749.
(20) Cudic, M.; Condie, B. A.; Weiner, D. J.; Lysenko, E. S.; Xiang, Z. Q.; O, I.; Bulet, P.; Otvos, L., Jr. Development of novel antibacterial peptides that kill resistant clinical isolates. *Peptides* **2002**, *23*, 271−283.
(21) Cudic, M.; Lockatell, C. V.; Johnson, D. E.; Otvos, L., Jr. In vitro and in vivo activity of a designed antibacterial peptide analogue against uropathogens. *Peptides* **2003**, *24*, 807−820.
(22) Otvos, L., Jr.; Snyder, C.; Condie, B.; Bulet, P.; Wade, J. D. Chimeric antimicrobial peptides exhibit multiple modes of action. *Int. J. Pept. Res. Ther*. **2005**, *11*, 29−42.
(23) Otvos, L. The short, proline-rich antibacterial peptide family. *Cell. Mol. Life Sci*. **2002**, *59*, 1138−1150.
(24) Cociancich, S.; Dupont, A.; Hegy, G.; Lanot, R.; Holder, F.; Hetru, C.; Hoffmann, J. A.; Bulet, P. Novel inducible antibacterial peptides from a hemipteran insect, the sap sucking-bug *Pyrrhocoris apterus*. *Biochem. J*. **1994**, *300*, 567−575.
(25) Bulet, P.; Dimarcq, J.-L.; Hetru, C.; Lagueux, M.; Charlet, M.; Hegy, G.; van Dorsselaer, A.; Hoffmann, J. A. A novel inducible antibacterial peptide from Drosophila carries an O-glycosylated substitution. *J. Biol. Chem*. **1993**, *268*, 14893−14897.
(26) Casteels, P.; Ampe, C.; Jacobs, F.; Vaeck, M.; Tempst, P. Apidaecins: antibacterial peptides from honeybees. *EMBO J*. **1989**, *8*, 2387−2391.
(27) Bulet, P.; Urge, L.; Ohresser, S.; Hetru, C.; Otvos, L., Jr. Enlarged scale chemical synthesis and range of activity of drosocin, an O-glycosylated antibacterial peptide from *Drosophila*. *Eur. J. Biochem*. **1996**, *238*, 64−69.
(28) Hoffmann, R.; Bulet, P.; Urge, L.; Otvos, L., Jr. Range of activity and metabolic stability of synthetic antibacterial glycopeptides from insects. *Biochim. Biophys. Acta* **1999**, *1426*, 459−467.
(29) DiMarchi, R. D.; Neireiter, G. W.; Heath, W. F.; Gurd, F. R. Structural significance of the amino-terminal residues of sperm whale myoglobin. *Biochemistry* **1980**, *19*, 2454−2465.
(30) Powell, M. F.; Grey, H.; Gaeta, F.; Sette, A.; Colon, S. Peptide stability in drug development: a comparison of peptide reactivity in different biological media. *J. Pharm. Sci.* **1992**, *81*, 731−735.
(31) Wong, H.; Jia, L.; Camden, J. B.; Weitman, S. D. Liquid chromatography−mass spectrometry assay of a thiadiazole derivative in mice: application to pharmacokinetic studies. *J. Chromatogr.* **2001**, *765*, 55−62.
(32) Weijzen, S.; Meredith, S. C.; Velders, M. P.; Elmishad, A. G.; Schreiber, H.; Kast, W. M. Pharmacokinetic differences between a T cell-tolerizing and a T cell-activating peptide. *J. Immunol*. **2001**, *166*, 7151−7157.
(33) Cudic, M.; Bulet, P.; Hoffmann, R.; Craik, D. J.; Otvos, L. Jr. Chemical synthesis, antibacterial activity and conformation of diptericin, an 82-mer peptide originally isolated from insects. *Eur. J. Biochem*. **2002**, *266*, 549−558.
(34) Johnson, D. E.; Lockatell, C. V.; Russell, R. G.; Hebel, J. R.; Island, M. D.; Stapleton, A.; Stamm, W. E.; Warren, J. W. Comparison of *Escherichia coli* strains recovered from human cystitis and pyelonephritis infections in transurethrally challenged mice. *Infect. Immun*. **1998**, *66*, 3059−3065.
(35) Virella, G. *Microbiology and infectious diseases*; Williams and Wilkins: Philadelphia, 1997; pp 449−454.
(36) Amsterdam, D. Susceptibility testing of antimicrobials in liquid media. In *Antibiotics in Laboratory Medicine*; Loman, V., Ed; Williams and Wilkins: Philadelphia, 1996; pp 52−111.
(37) Mak, P.; Wojcik, K.; Silberring, J.; Dubin, A. Antimicrobial peptides derived from heme-containing proteins: hemocidins. *Antonie Van Leeuwenhoek* **2000**, *77*, 197−207.
(38) Scott, M. G.; Davidson, D. J.; Gold, M. R.; Bowdish, D.; Hancock, R. E. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J. Immunol*. **2002**, *169*, 3883−3891.
(39) Hvidberg, H.; Struve, C.; Krogfelt, K. A.; Christensen, N.; Rasmussen, S. N.; Frimodt-Moller, N. Development of a long-term ascending urinary tract infection mouse model for antibiotic treatment studies. *Antimicrob. Agents Chemother*. **2000**, *44*, 156−163.
(40) Lupetti, A.; Welling, M. M.; Pauwels, E. K. J.; Nibbering, P. H. Radiolabeled antimicrobial peptides for infection detection. *Lancet Infect. Dis*. **2003**, *3*, 233−239.
(41) Hui, L.; Leung, K.; Chen, H. M. The combined effects of antibacterial peptide cecropin A and anti-cancer agents on leukemia cells. *Anticancer Res*. **2002**, *22*, 2811−2816.
(42) Gura, T. Ancient system gets new respect. *Science* **2001**, *291*, 2068−2071.
(43) Chen, J.; Falla, T. J.; Liu, H.; Hurst, M. A.; Fujii, C. A.; Mosca, D. A.; Embree, J. R.; Loury, D. J.; Radel, P. A.; Chang, C. C.; Gu, L.,; Fiddes, J. C. Development of protegrins for the treatment and prevention of oral mucositis: structure−activity relationships of synthetic protegrin analogues. *Biopolymers* **2000**, *55*, 88−98.
(44) Ge, Y.; MacDonald, D.; Henry, M. M.; Hait, H. I.; Nelson, K. A.; Lipsky, B. A.; Zasloff, M. A.; Holroyd, K. J. In vitro susceptibility to pexiganan of bacteria isolated from infected diabetic foot ulcers. *Diagn. Microbiol. Infect. Dis*. **1999**, *35*, 45−53.
(45) Navon-Venezia, S.; Feder, R.; Gaidukov, L.; Carmeli, Y.; Mor, A. Antibacterial properties of dermaseptin S4 derivatives with in vivo activity. *Antimicrob. Agents Chemother*. **2002**, *46*, 689−694.
(46) Otvos, L. Jr.; O, I.; Rogers, M. E.; Consolvo, P. J.; Condie, B. A.; Lovas, S.; Bulet, P.; Blaszczyk-Thurin, M. Interaction between heat shock proteins and antimicrobial peptides. *Biochemistry* **2000**, *39*, 14150−14159.
(47) Rothbard, J. B.; Kreider, E.; VanDeusen, C. L.; Wright, L.; Wylie, B. L.; Wender, P. A. Arginine-rich molecular transporters for drug delivery: role of backbone spacing in cellular uptake. *J. Med. Chem*. **2002**, *45*, 3612−3618.
(48) Fernandez-Carneado, J.; Kogan, M. J.; Castel, S.; Giralt, E. Potential peptide carriers: amphipathic proline-rich peptides derived from the N-terminal domain of γ-zein. *Angew. Chem., Int. Ed.* **2004**, *43*, 1811−1814.
(49) Chapple, D. S.; Hussain, R.; Joannou, C. L.; Hancock, R. E.; Odell, E.; Evans, R. W.; Siligardi, G. Structure and association of human lactoferrin peptides with *Escherichia coli* lipopolysaccharide. *Antimicrob. Agents Chemother*. **2004**, *48*, 2190−2198.
(50) Deslouches, B.; Phadke, S. M.; Lazarevic, V.; Cascio, M.; Islam, K.; Montelaro, R. C.; Mietzner, T. A. *De novo* generation of cationic antimicrobial peptides: influence of length and tryptophan substitution on antimicrobial activity. *Antimicrob. Agents Chemother*. **2005**, *49*, 316−322.
(51) Gobbo, M.; Biondi, L.; Filira, F.; Gennaro, R.; Benincasa, M.; Scolaro, B.; Rocchi, R. Antimicrobial peptides: synthesis and antibacterial activity of linear and cyclic drosocin and apidaecin 1b analogues. *J. Med. Chem*. **2002**, *45*, 4494−4504.
(52) McManus, A. M.; Otvos, L. Jr.; Hoffmann, R.; Craik, D. J. Conformational studies by NMR of the antimicrobial peptide, drosocin, and its non-glycosylated derivative: effects of glycosylation on solution conformation. *Biochemistry* **1999**, *38*, 705−714.
(53) Rabel, D.; Charlet, M.; Ehret-Sabatier, L.; Cavicchioli, L.; Cudic, M.; Otvos, L., Jr.; Bulet P. Primary structure and in vitro antibacterial properties of the *Drosophila melanogaster* attacin C Pro-domain. *J. Biol. Chem*. **2004**, *279*, 14853−14859.
(54) Haversen, L. A.; Engberg, I.; Baltzer, L.; Dolphin, G.; Hanson, L. A.; Mattsby-Baltzer, I. Human lactoferricin and peptides derived from a surface-exposed helical region reduce experimental *Escherichia coli* urinary tract infection in mice. *Infect. Immun.* **2000**, *68*, 5816−5823.

(55) Henry, D. C., Jr.; Bettis, R. B.; Riffer, E.; Haverstock, D. C.; Kowalsky, S. F.; Manning, K.; Hamed, K. A.; Church, D. A. Comparison of once-daily extended-release ciprofloxacin and conventional twice-daily ciprofloxacin for the treatment of uncomplicated urinary tract infection in women. *Clin. Ther*. **2002**, *24*, 2088−2104.
(56) Norris, C. R.; Williams, B. J.; Ling, G. V.; Franti, C. E.; Johnson, D. L.; Ruby, A. L. Recurrent and persistent urinary tract infections in dogs: 383 cases (1969−1995). *J. Am. Anim. Hosp. Assoc.* **2000**, *36*, 484−492.
(57) Ling, G. V.; Ruby, A. L. Gentamicin for treatment of resistant urinary tract infections in dogs. *J. Am. Vet. Med. Assoc*. **1979**, *175*, 480−481.
(58) Cohn, L. A.; Gary, A. T.; Fales, W. H.; Madsen, R. W. Trends in fluoroquinolone resistance of bacteria isolated from canine urinary tracts. *J. Vet. Diagn. Invest*. **2003**, *15*, 338−343.
(59) Cooke, C. L.; Singer R. S.; Jang, S. S.; Hirsh, D. C. Enrofloxacin resistance in *Escherichia coli* isolated from dogs with urinary tract infections. *J. Am. Vet. Med. Assoc.* **2002**, *220*, 190−192.
(60) Fields, G. B.; Noble, R. L. Solid-phase peptide synthesis using 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Pept. Protein Res*. **1990**, *35*, 161−214.

JM050347I