Insect peptides with improved protease-resistance protect mice against bacterial infection

LASZLO OTVOS, JR., KRISZTINA BOKONYI, ISTVAN VARGA, BALINT I. OTVOS, RALF HOFFMANN, HILDEGUND C.J. ERTL, JOHN D. WADE, AILSA M. McMANUS, DAVID J. CRAIK, AND PHILIPPE BULET

1The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104
2Unité Propre du CNRS No. 9022, “Réponse Immunitaire et Développement chez les Insectes,” Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg Cedex, France
3Biologisch-Medizinisches Forschungszentrum, Heinrich-Heine-Universität, Moorstrasse 5, 40225 Düsseldorf, Germany
4Howard Florey Institute, Parkville 3052, Victoria, Australia
5Centre for Drug Design and Development, University of Queensland, Brisbane 4072, Queensland, Australia

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Abstract

At a time of the emergence of drug-resistant bacterial strains, the development of antimicrobial compounds with novel mechanisms of action is of considerable interest. Perhaps the most promising among these is a family of antibacterial peptides originally isolated from insects. These were shown to act in a stereospecific manner on an as-yet unidentified target bacterial protein. One of these peptides, drosocin, is inactive in vivo due to the rapid decomposition in mammalian sera. However, another family member, pyrrhocoricin, is significantly more stable, has increased in vitro efficacy against Gram-negative bacterial strains, and if administered alone, as we show here, is devoid of in vitro or in vivo toxicity. At low doses, pyrrhocoricin protected mice against Escherichia coli infection, but at a higher dose augmented the infection of compromised animals. Analogs of pyrrhocoricin were, therefore, synthesized to further improve protease resistance and reduce toxicity. A linear derivative containing unnatural amino acids at both termini showed high potency and lack of toxicity in vivo and an expanded cyclic analog displayed broad activity spectrum in vitro. The bioactive conformation of native pyrrhocoricin was determined by nuclear magnetic resonance spectroscopy, and similar to drosocin, reverse turns were identified as pharmacologically important elements at the termini, bridged by an extended peptide domain. Knowledge of the primary and secondary structural requirements for in vivo activity of these peptides allows the design of novel antibacterial drug leads.

Keywords: antibacterial peptides; in vivo anti-infection assay; metabolite; nuclear magnetic resonance spectroscopy; serum stability; stereospecific target protein

Antibacterial peptides and glycopeptides isolated from insects (Gillespie et al., 1997; Bulet et al., 1999) are promising candidates for drug development due to their unusual mode of action. While many antibacterial peptides from other origins kill bacteria by disrupting the cell membrane, some of the insect peptides bind to an as-yet unidentified stereospecific target molecule, presumably a protein or glycoprotein (Bulet et al., 1996). This unusual mechanism of action will likely attract the attention of the pharmaceutical industry, which is anxiously awaiting novel antimicrobial compounds that can break drug tolerance in nosocomial infections. Significantly, no drug resistance other than increased proteolytic activity could be attributed to these molecules (Boman, 1995). The three most studied groups of the insect antibacterial peptides are the apidaecins (Casteels & Tempst, 1994) (18 amino acid residues), the drosocins (19 residues), and the pyrrhocoricins (20 residues). The last two groups contain a disaccharide in midchain position (Bulet et al., 1993; Cociancich et al., 1994). The presence of the sugar increases the in vitro antibacterial activity of drosocin, but decreases the activity of pyrrhocoricin and lebocin, a somewhat more distant family member (Hara & Yamakawa, 1995; Bulet et al., 1996; Hoffmann et al., 1999). Pyrrhocoricin is more active against Gram-negative bacteria than drosocin in vitro, but the peptide is almost completely inactive against Gram-positive strains. Drosocin is moderately active against Gram-positive bacteria.

When glycosylated drosocin alone is injected into mice, the glycopeptide shows no toxicity (Hoffmann et al., 1999). However, the glycopeptide cannot protect mice against Escherichia coli infection. This is probably due to the peptide’s rapid decomposition in mammalian sera (Hoffmann et al., 1999). While drosocin needs up to 12 h to kill bacteria in vitro (Cudic et al., 1999), it is...
completely degraded in diluted human and mouse sera within a 4-h period (Hoffmann et al., 1999). Both aminopeptidase and carboxypeptidase cleavage pathways are observed. Pyrrhocoricin appears to be more resistant to degradation in mouse serum than drosocin, but decomposes relatively quickly in some batches of human serum (Hoffmann et al., 1999). The first metabolites from serum stability assays were identified, and it was shown that the metabolites of drosocin and pyrrhocoricin, lacking as few as five amino terminal or two carboxy terminal amino acids become inactive in vitro (Bulet et al., 1996; Hoffmann et al., 1999). Although experiments measuring the rate of peptide degradation are fairly straightforward, there are several serum batch-related factors that may produce highly variable results (Powell et al., 1993). The use of pooled human sera somewhat improves the reproducibility of the stability assays, although even commercial sera with the same catalog number are notoriously different. Therefore, in the current study, we investigated the stability of the pyrrhocoricin-related peptides in two different batches of pooled human serum (approximately 80 donors in each batch).

The hypothesis concerning the two independent active sites is further supported by a recent model of the bioactive secondary structure of drosocin that identifies two reverse turns, one at each terminal region, as binding sites to the target molecule (McManus et al., 1999). The situation is further complicated by the fact that the degradation speed and pathway of a given peptide in diluted mouse serum is somewhat different from those observed in diluted human serum. The stability of the peptides is markedly increased in insect hemolymph where they manifest their biological function (Hoffmann et al., 1999).

We decided to design modified peptides based on pyrrhocoricin. Our goal was to retain the peptide’s high antibacterial potency in vitro and obtain good metabolic stability and lack of toxicity for ensuing in vivo efficacy assays and drug development. We selected pyrrhocoricin over drosocin because (1) it is more potent against some strains of Gram-negative bacteria, (2) glycosylation is not needed for full biological activity, and (3) the peptide is more stable in mouse serum. Importantly, we wanted to obtain peptides for which the degradation pathway in mouse serum (the most suitable test animal for in vivo efficacy assay) is similar to that in human serum (the modified peptides are targeted for human therapy). In addition, we wanted to determine the bioactive conformation of native pyrrhocoricin and compare it with that of the published structure of native drosocin to establish the secondary structural requirements for antibacterial activity.

Results and discussion

Design of peptides

Pyrrhocoricin has the amino acid sequence: VDKGSYLPRPT-PRP1YNRN, with Thr11 glycosylated in the native peptide. In our earlier studies, we synthesized the peptide with a free amino terminus and an amidated carboxy-terminus. In the current study, an extensive series of fragments and analogs was made for biochemical characterization of the peptide and for drug design purposes. The following findings were considered in the design of pyrrhocoricin analogs for biotechnological uses: (1) the main sites of the degradation of unmodified pyrrhocoricin are the N- and the C-termini, indicating mainly exopeptidase cleavage; (2) an endopeptidase cleavage site was also identified between Ser6 and Tyr7; (3) N-terminal acetylation usually protects peptides from aminopeptidase cleavage; (4) however, most antibacterial peptides need a positive charge at or near the native amino terminus (Vunnam et al., 1997); (5) unnatural, glycosylated, or D-amino acid residues are likely candidates to improve stability in serum (Powell et al., 1993); and (6) cyclic peptides cannot be cleaved by exopeptidases.

In this first round of studies, we did not consider backbone modifications to conserve the conformation of the peptide. As backbone-modified versions of a membrane-active antibacterial peptide were reported to improve proteolytic stability and yield analogs with slightly modified activity spectrum (Oh et al., 1999), backbone modifications will be considered in future steps. Table 1 lists the synthetic peptides and the in vitro antibacterial activities.

In vitro antibacterial activity

The in vitro antibacterial activity of the peptides was measured against two Gram-positive strains, Micrococcus luteus and Bacillus megaterium, and three Gram-negative strains, E. coli D22, Agrobacterium tumefaciens, and Salmonella typhimurium. Although these bacterial strains are not necessarily clinically relevant, we used these models to obtain a direct comparison with the antimicrobial activity of other related peptides, most importantly drosocin and its fragments (Hoffmann et al., 1999). The experiments were conducted over a seven-month period. To assess the variability of the assay, peptide 18 was reassayed three months after the original study. The differences in the IC50 values between 18/a and 18/b demonstrate this variability. Basically identical results were obtained against M. luteus, B. megaterium, E. coli D22, and S. typhimurium, indicating the high reproducibility of the assay. The fourfold difference in the IC50 value against A. tumefaciens is in line with the culture-to-culture variability of this strain. From the peptides made for biochemistry investigations, we learned that the two putative binding sites cannot be separated (peptides 2 and 3). In fact, an equimolar mixture of shorter peptides 2 and 3 remained inactive in all five bacterial strains studied. An analog made of only D-amino acids was similarly inactive (peptide 4), indicating that pyrrhocoricin, like drosocin, binds stereospecifically to a target protein. The N-terminus of the native glycopeptide (peptide 5) could not be blocked by acetylation without a major loss of antibacterial activity. Pyrrhocoricin analogs containing labels and an additional lysine at the N-terminus (peptides 6 and 7) retain biological activity and can later be used to isolate and characterize the bacterial target protein.

Turning to the drug candidates, modification at either termini reduced the potency of unmodified pyrrhocoricin. From the only N- or C-terminally modified peptides, 1-amino-cyclohexylcarboxylic acid at the amino-(peptide 11) and the acetylated 2,3-diamino-propionic acid at the carboxy-terminus (peptide 12) appeared to retain most of pyrrhocoricin’s antibacterial activity. For drug development, both termini have to be modified to block or at least slow down exopeptidase cleavage that was observed on the native and unglycosylated peptides. Peptides containing modifications at both termini featured acetylation together with positively charged amino acid addition, incorporation of unnatural amino acids, such as Chex or Dap(Ac), glycosylation, imide formation, and D-amino acid substitution or cyclization (peptides 13–23). Considering the requirement for submicromolar activity against at least one strain, peptide 19, Chex-pyrrhocoricin-Dap(Ac), and peptide 18, Ac-R-pyrrhocoricin-Dap(Ac), were selected for further studies, which included cell toxicity and serum stability as well as metabolism assays. These peptides showed remarkable high activ-
Cyclic peptide for killing both Gram-negative and Gram-positive bacteria was identified against Gram-negative bacteria. Significantly, the best analog corresponded to the middle domain of pyrrhocoricin and was in a residue spacer between the original N- and C-termini. The spacer amino terminus of the peptide, which is cyclized with an eight-residue gap, demonstrated a broad activity spectrum at low micromolar concentrations. In this peptide, a lysine residue was added to the C-terminal modification.

The synthesis of this fragment retained the native orientation of the bioactive domains (the original termini). The synthesis of this peptide in quantities large enough for toxicity and in vivo efficacy studies, as well as detailed conformational analysis, is currently in progress in our laboratory.

Cytotoxicity of pyrrhocoricin and its analogs

To test whether native and modified pyrrhocoricin peptides were toxic to mammalian cells, pyrrhocoricin, Chex-pyrrhocoricin-Dap(Ac), Ac-R-pyrrhocoricin-Dap(Ac), melittin (positive control), and peptide 31D, a T-cell epitope negative control, were tested at 20 μM concentration.

All amino acids were of the L-configuration, except for peptides 4 and 20, in which the indicated amino acids were of the D-configuration. All carbohydrates were of the D-configuration.

Table 1. In vitro antibacterial activity of pyrrhocoricin-based peptides

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Peptide composition</th>
<th>Micrococcus luteus</th>
<th>Bacillus megaterium</th>
<th>E. coli D22</th>
<th>Agrobacterium tumefaciens</th>
<th>Salmonella typhimurium</th>
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<tbody>
<tr>
<td>1</td>
<td>Pyrrhocoricin 1–20</td>
<td>10</td>
<td>5</td>
<td>&lt;0.075</td>
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<td>Analogs for biochemistry studies</td>
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<tr>
<td>2</td>
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<td>—</td>
<td>—</td>
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<td>3</td>
<td>Pyrrhocoricin 10–20</td>
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<td>—</td>
<td>—</td>
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<td>—</td>
</tr>
<tr>
<td>4</td>
<td>All n-pyrrhocoricin 1-20</td>
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<td>—</td>
<td>—</td>
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<td>5</td>
<td>Ac-pyrrhocoricin GalNAc (T11)</td>
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<td>10</td>
<td>—</td>
<td>—</td>
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<td>6</td>
<td>Biotin-K-pyrrhocoricin 1–20</td>
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<td>1.25</td>
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<td>Fluorescein-K-pyrrhocoricin 1–20</td>
<td>80</td>
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<td>Ac-K-VDK-pyrrhocoricin 1–20</td>
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<td>Chex-pyrrhocoricin 2–20</td>
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<td>C-terminal modification</td>
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<td>12</td>
<td>Pyrrhocoricin 1–19-Dap(Ac)</td>
<td>5</td>
<td>20</td>
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<td>&lt;0.30</td>
<td>&lt;0.30</td>
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<td>Dual modifications</td>
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<td>Ac-K-pyrrhocoricin 1–19-D20</td>
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<td>14</td>
<td>Ac-K-pyrrhocoricin 1–20 imide (N20)</td>
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<td>16</td>
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<td>18a</td>
<td>Ac-R-pyrrhocoricin 1–19-Dap(Ac)</td>
<td>10</td>
<td>80</td>
<td>0.3</td>
<td>0.6</td>
<td>2.5</td>
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<tr>
<td>18b</td>
<td>Ac-R-pyrrhocoricin 1–19-Dap(Ac)</td>
<td>10</td>
<td>40</td>
<td>0.3</td>
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<td>2.5</td>
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<td>19</td>
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<td>40</td>
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<td>10</td>
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<td>External and internal modification</td>
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<td>Chex-pyrrhocoricin 2–19-(A5F6)-Dap (Ac)</td>
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<td>40</td>
<td>20</td>
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<td>22</td>
<td>Cyclo-[K-pyrrhocoricin 1–19-D20]</td>
<td>—</td>
<td>—</td>
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<tr>
<td>23</td>
<td>Cyclo-[K-pyrrhocoricin 1–20-14-7]</td>
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<td>—</td>
<td>0.6</td>
<td>2.5</td>
<td>2.5</td>
</tr>
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</table>

*Abbreviations: V, valine; D, aspartic acid; K, lysine; G, glycine; S, serine; Y, tyrosine; L, leucine; P, proline; R, arginine; T, threonine; I, isoleucine; N, asparagine; A, alanine; F, phenylalanine; Ac, acetyl; Chex, 1-amino-cyclohexane-carboxylic acid; Dap(Ac), β-acetyl-2,3-diamino propionic acid; GlcNAc, 2-acetamido-2-deoxy-glucose; GalNAc, 2-acetamido-2-deoxy-galactose; Gal, galactose; D-val, D-valine; D-asn, D-asparagine; fluorescein, 5(6)carboxy-fluorescein.

The inhibitory concentration (IC50) is expressed as the concentration tested at which 50% growth inhibition is observed. Growth inhibition assays were performed at 30°C for 24 h in Luria–Bertani rich nutrient medium. No activity up to 80 μM concentration is indicated by —. Peptide 22 was tested up to 20 μM concentration.

All amino acids were of the L-configuration, except for peptides 4 and 20, in which the indicated amino acids were of the D-configuration. All carbohydrates were of the D-configuration.
In vivo stability of peptides in mammalian serum

In vivo stability of peptides in blood is currently modeled well by in vitro stability in serum or plasma (neglecting renal and hepatic clearance) (Powell et al., 1993). Serum stability studies represent one of the most important secondary screening assays in peptide drug development, largely because they eliminate peptides that have short half-lives and are, therefore, unlikely to be therapeutically effective (Powell et al., 1993). Because diluted serum increases peptide recovery as well as retards the reaction kinetics to a manageable rate, the experiments were performed using 25% aqueous sera. It has been demonstrated earlier that the degradation rates are linearly proportional to serum concentration (Powell et al., 1992). We used mouse serum and two different batches of pooled human sera. In our experience, reproducible results can be obtained by using a given serum preparation over a long time period, but batch-to-batch variations result in highly variable decomposition curves. The mouse serum was identical to the one that was used to characterize the metabolic behavior of drosocin (Hoffmann et al., 1999). When evaluating the first metabolites after 45 min digestion (Table 3), it was evident that the C-terminal asparagine is cleaved off the unmodified pyrrhocoricin (peptide 1). In contrast, the modified C-terminal residue stayed on the peptides containing a carboxy-terminal Dap(Ac) residue that replaced Asn20 (peptides 18 and 19). The Ac-R-N-terminal peptide (peptide 18) produced more internal cleavage products close to the amino terminus than the Chex-N-terminal peptide (peptide 19), most notably a fragment in which the VDK tripeptide fragment was missing. While for unmodified pyrrhocoricin (peptide 1) the degradation products were different in the two human sera, for the Chex-pyrrhocoricin-Dap(Ac) peptide (peptide 19) they were very similar. They were also closer to those observed after digestion with the

<table>
<thead>
<tr>
<th>Peptide composition</th>
<th>Serum</th>
<th>-C1</th>
<th>-C2</th>
<th>-C3</th>
<th>-C6</th>
<th>-N3</th>
<th>-N5</th>
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<td></td>
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<tr>
<td>1</td>
<td>Pyrrhocin</td>
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<tr>
<td></td>
<td></td>
<td>Human 2</td>
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<td>Chex-pyrrhocin 2–19-Dap(Ac)</td>
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<td>0</td>
<td>0</td>
<td>2</td>
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mouse serum. Mice being the species for ensuing in vivo efficacy studies, mouse serum appeared to be a good model for the study of the in vivo efficacy of peptide 19.

Figure 1 shows the kinetics of the degradation for peptides 1 and 19. The degradation curves for unmodified pyrrhocoricin (peptide 1) and the Chex-pyrrhocoricin-Dap(Ac) peptide (peptide 19) were similar. Unmodified pyrrhocoricin was somewhat more stable than peptide 19 in mouse serum and in the weaker human serum, but less stable in the more active human serum. Although the lack of exopeptidase cleavage sites reduced the degradation rate of peptide 19 compared to peptide 1, this loss of protease activity was compensated for by increased endopeptidase activity C-terminal to Ser5 and Asn18. In support of this, metabolism studies of the broad spectrum cyclo-[K-pyrrhocoricin 1-20–14-7] derivative (peptide 23) indicated a single endopeptidase cleavage site between Asn18 and Arg19 in both mouse and human sera. In accordance with the increased number and amount of internal degradation products near the amino termini, the Ac-R-pyrrhocoricin-Dap(Ac) peptide (peptide 18) degraded considerably faster than either peptide 1 or peptide 19 (data not shown). Remarkably, after 8 h of digestion, in mouse serum, where the in vivo experiments were planned, both peptides 1 and 19 had 20% of the initial amounts intact. Because we currently use the peptides well above the efficacious concentrations, this 20% remaining uncleaved peptide amount can successfully protect the experimental animals from live bacterial challenge.

In vivo antibacterial activity

Peptides 1 and 19 were submitted to in vivo toxicity and antibacterial efficacy assays (Fig. 2). Mice of the CD-1 strain were intravenously infected with E. coli strain ATCC 25922. Peptides were intravenously injected 1 h after infection at 10, 25, and 50 mg/kg doses, followed by a second injection after 5 h. The survival rate was compared with that of control mice who were submitted to the same peptide treatment, but received 5% dextrose (DSS) instead of the bacteria. Neither peptide alone showed any toxicity at the 50 mg/kg dose. To assess the success of the infection, five mice were infected with E. coli, but received DSS instead of the test peptides. Of these control mice, two died on the third day and the other three became clinically ill, which included decreased activity and head tilt. When the efficacy of the peptides was studied, the Chex-pyrrhocoricin-Dap(Ac) derivative (peptide 19) protected all 15 mice, regardless of the dose. For unmodified pyrrhocoricin (peptide 1), the mice that were injected with 10 and 25 mg/kg survived without any clinical signs of disease. However, from the 50 mg/kg group, one mouse died and the other four showed clinical signs of disease. This mirrors the drosocin studies in which the peptide was not toxic alone, but became toxic (or augmented the infection) when the mice were infected and became compromised (Hoffmann et al., 1999). Increased mortality is often detected pharmacologically when high doses of drugs are administered to challenged animals. Such an effect was not found for the Chex-pyrrhocoricin-Dap(Ac) peptide. All mice showed some enlargement of the abdominal region during the study; therefore, at termination, the mice were necropsied. Stomachs and intestines were observed to be enlarged due to the presence of food or stool. There were no other findings. These data indicate that the increase of the proteolytic stability of the pyrrhocoricin peptides compared to drosocin resulted in peptides capable of protecting mice against bacterial infection. The results also warrant further pharmaceutical development with peptides based on the Chex-pyrrhocoricin-Dap(Ac) lead. This analog showed good potency, complete lack of toxicity, and similarity in the degradation pathway between human and mouse sera. The 10–50 mg/kg dosage range was applied to obtain direct comparison with the drosocin study (Hoffmann et al., 1999). Our next experiments will include the identification of the lowest active dose, optimal mode of delivery, and determination of in vivo activity spectrum of peptide 19 and peptide 23.

Conformation of native pyrrhocoricin

Because native pyrrhocoricin could not be shortened nor could the amino acid composition be changed without a loss of in vitro antibacterial activity, we hypothesized that the peptide has to assume a certain secondary structure to bind stereospecifically to the target protein. Accordingly, we determined the bioactive conformation of native glycosylated pyrrhocoricin and its nonglycosylated analog (peptide 1) by two-dimensional nuclear magnetic resonance (NMR) spectroscopy. Here, the structures are described in comparison with the recently published conformation of drosocin (McManus et al., 1999). It appeared that the structure of pyrrhocoricin, like drosocin, was largely random coil, and there was little change in the backbone conformation upon glycosylation. For pyrrhocoricin, however, there was a subpopulation with organized structure at both the N- and C-termini. Figure 3A and 3B summarize the short- and medium-range nuclear Overhauser effects (NOEs) and show that there were a series of dNOE(i,i+1) NOEs present at both the N- and C-termini of the pyrrhocorcinics. Additional dNOE(i,i+2) NOEs were found in the spectra of the glycosylated derivative. Taken together, these data indicated the presence of reverse turns at the pharmacologically important terminal regions. The increase in the turn potential at the termini compared to drosocin (McManus et al., 1999) may explain the increased in vitro antibacterial activity of pyrrhocoricin (Hoffmann et al., 1999). A comparison of the αH NMR shifts with random coil values (Merutka et al., 1995)
is shown in Figure 3C. These results suggested that the middle domain of pyrrhocoricin, like drosocin, had an extended structure. Both the unordered-turn conformational equilibrium and the presence of extended regions were further verified by circular dichroism (CD) spectroscopy (data not shown). Nonglycosylated pyrrhocoricin (peptide 1) exhibited a linear unordered → reverse turn conformational transition upon going from water to trifluoroethanol as solvents. The CD spectrum of the native glycopeptide peptide 1.

Fig. 2. In vivo antibacterial activity of pyrrhocoricin (peptide 1) (open symbols) and Chex-pyrrhocoricin-Dap(Ac) (peptide 19) (closed symbols). Three mice per group were used for toxicity (broken lines), and five mice per group were used for efficacy studies (solid lines). Five additional mice were infected with E. coli ATCC 25922 for negative controls and received 5% dextrose (DS5) instead of test peptides (dots and dashes with crosses).

Fig. 3. Summary of NOE connectivities (A and B) and deviations of the aH chemical shifts from their “random coil” values (C) for nonglycosylated pyrrhocoricin (peptide 1), and its native counterpart, containing a Gal-GalNAc disaccharide moiety on Thr11. The random coil values (Merutka et al., 1995) are corrected for sequence-specific shift of 0.29 ppm for residues preceding Pro. For the NOE connectivities, the intensities are indicated by the thickness of the line.
recorded in water was more similar to those of unordered peptides (type U spectra). However, the type C spectrum, and therefore the final turn structure, was stabilized at a lower trifluoroethanol concentration (50%) for the glycopeptide compared to the nonglycosylated analog, fully supporting the NMR findings. The broadening of the negative band between 210 and 220 nm in the aqueous spectra identified the presence of extended structures for both peptides.

In summary, according to the NMR data, the bioactive conformation of native pyrrhocoricin involves two reverse turns at the termini bridged by an extended peptide segment in the middle domain. This hypothetical structure is supported by the antibacterial activity of the cyclic peptide analogs. Cyclization stabilizes the final turn structure, was stabilized at a lower trifluoroethanol concentration and was expected to improve potency, but the cyclic pyrrhocoricin derivative, without expanding the cycle (peptide 22), lost activity compared to the analog linear peptide (peptide 13), probably due to distortion of the extended domain in the middle of the peptide. When the ring size was increased by repeating an internal octapeptide fragment (peptide 23), the resulting cyclic analog became highly active against both Gram-negative and Gram-positive bacterial strains. This hypothesis will be tested experimentally when higher amounts of the cyclo-[K-pyrrhocoricin 1-20–14-7] peptide are available for detailed CD and NMR studies. High resolution conformational analysis of the drug lead Chex-pyrrhocoricin-Dap(Ac) peptide (peptide 19) is also currently in progress in our laboratories. This structure will be reported later, together with the complete pharmacological profile of this peptide analog.

Materials and methods

Peptide synthesis

Peptides were assembled by a Milligen 9050 continuous-flow automated synthesizer on an Fmoc-PAL-polyethylene-glycol-polystyrene copolymer resin with an initial load of 0.17 mmol/g (PerSeptive Biosystems, Warrington, United Kingdom). Standard Fmoc-chemistry was used throughout (Fields & Noble, 1990) with 4 mol excess of the acylating amino acids and HATU (1-hydroxy-7-azabenzotriazole uranium salt) activation, recommended for the synthesis of complex peptides (Angell et al., 1994). The side-chain protecting groups were trityl for Asn, tert-butyl ether for Tyr, Ser, and Thr, tert-butyl ester for Asp, 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl for Arg, and tert-butylxylo-carbonyl for Lys. Fmoc-1-amino-cyclohexane-carboxylic acid was purchased from Neosystem (Strasbourg, France). Fmoc-diamino-propionic acid from Bachem Biosciences (King of Prussia, Pennsylvania) was acetylated with equimolar amount of pentafluorophenyl acetate prior to peptide synthesis and was used for peptide assembly without any further purification. The glycopeptides were synthesized from commercially available glycoamino acid building blocks, including Fmoc-Thr[Gal(Ac5)-GalNAc(Ac5)]-OH and Fmoc-Asn[GlcNAc(Ac5)]-OH (Novabiochem, San Diego, California). Peptides were cleaved from the solid support by trifluoroacetic acid in the presence of m-cresol (2.5%), ethane–dithiol (2.5%), thioanisole (5%), and water (5%) as scavengers for 2–3 h. Deacetylation of the sugar hydroxyl groups was accomplished by a 2 min treatment with 0.1 M NaOH (Otvos et al., 1994). After cleavage, peptides were purified by reversed-phase high-performance liquid chromatography. The final products were characterized by matrix-assisted laser desorption/ionization mass spectrometry at the Wistar Institute Protein Microchemistry Laboratory on a Voyager Biospectrometry Workstation by standard methods. Mass spectra verified the anticipated composition of the peptides.

In vitro antibacterial and hemolytic assays

Antibacterial assays were performed in sterile 96-well plates (Nunc F96 microtiter plates) with a final volume of 100 μL as described earlier (Bulet et al., 1996). Briefly, 90 μL of a suspension of a midlogarithmic phase bacterial culture at an initial 600 nm ultraviolet (UV) absorbance of 0.001 in Luria-Bertani–rich nutrient medium was added to 10 μL of serially diluted peptides in sterilized water. The final peptide concentrations ranged between 0.15 and 80 μM. Plates were incubated at 30°C for 24 h with gentle shaking, and growth inhibition was measured by recording the increase of the UV absorbance at 600 nm on a SLT Labinstruments 400 ATC microplate reader. The higher temperature compared to traditional antibacterial assays (25°C) was applied to increase the sensitivity of the assay. Hemolytic activity was assayed with sheep erythrocytes suspended in Alsever’s solution (BioWhittaker, Walkersville, Maryland), and diluted with Dulbecco’s phosphate-buffered saline, pH 7.2. Thirty microliters of 1% suspension of the red blood cells was incubated with agitation at 39°C with 30 μL of 40–256 μM peptides dissolved in phosphate-buffered saline for 1 h and centrifuged at 1,000 g for 5 min. Fifty microliters of the supernatant was collected, and the release of hemoglobin was detected by measuring the UV absorbance at 405 nm.

In vivo toxicity and efficacy studies

In vivo toxicity and antibacterial activity of pyrrhocoricin and the Chex-pyrrhocoricin-Dap(Ac) derivative were evaluated at Chrysalis Preclinical Services Corporation (Olyphant, Pennsylvania). The conditions were designed to duplicate those during the similar assay of drosocin (Hoffmann et al., 1999). According to this protocol, male mice of CD-1 strain (Harlan Sprague Dawley, Inc., Stamford, Connecticut) were intravenously infected in the tail with 1,000,000 colony forming units (0.2 mL) of E. coli strain ATCC 25922. To obtain better infection, mice were also fed with E. coli. With this infection strategy, one of five control mice died on day 1, and two of five mice died on day 2. All the control mice remaining alive became clinically sick by day 2. The pyrrhocoricin peptides were intravenously injected 1 h after infection at doses of 10, 25, and 50 mg/kg, followed by a booster injection of the peptides after 5 h of infection. Mice were observed at 1 and 5 h, and 1 and 2 days postinfection for clinical signs or mortality, and were compared with control mice who received 5% dextrose (DS5) instead of peptides (negative controls), or were submitted to the same peptide treatment, but received 50 mg/kg of DS5 instead of the bacteria (toxicity). Clinical signs included decreased activity and head tilt.

NMR spectroscopy

1H NMR spectra were recorded on a Bruker DMX 750 MHz spectrometer at temperatures in the range 283–298 K. The sample consisted of 600 μg of native glycosylated or nonglycosylated pyrrhocoricin in 125 μL of 50% TFE-d5:50% H2O solution in a 2.5 mm NMR tube. Two-dimensional total correlation spectroscopy (TOCSY) and NOE spectroscopy (NOESY) spectra were recorded in the phase-sensitive mode using time-proportional phase incrementation for quadrature detection in the f1-dimension (Marion
The data were processed on a Silicon Graphics computer using the UXNMR software package. The f1-dimension was zero-filled to 4,096 real data points, with f1- and f2-dimensions being multiplied by a squared sine function and Gaussian function, respectively, prior to Fourier transformation.

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