MiniReview

Antibacterial properties of cationic steroid antibiotics

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Received 6 August 2002; received in revised form 25 September 2002; accepted 25 September 2002
First published online 22 October 2002

Abstract

Cationic steroid antibiotics have been developed that display broad-spectrum antibacterial activity. These compounds are comprised of steroids appended with amine groups arranged to yield facially amphiphilic morphology. Examples of these antibiotics are highly bactericidal, while related compounds effectively permeabilize the outer membranes of Gram-negative bacteria sensitizing these organisms to hydrophobic antibiotics. Cationic steroid antibiotics exhibit various levels of eukaryote vs. prokaryote cell selectivity, and cell selectivity can be increased via charge recognition of prokaryotic cells. Studies of the mechanism of action of these antibiotics suggest that they share mechanistic aspects with cationic peptide antibiotics.

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Keywords: Antibiotic; Drug-resistant bacterium; Amphiphile; Cationic peptide antibiotic; Membrane-active; Steroid; Sensitizer; Cell selectivity

1. Membrane-active antibiotics

With the emergence of strains of multidrug-resistant bacteria has come the call for new types of antibiotics [1,2]. Drug resistance of Gram-positive organisms has received significant attention; however, the threat from Gram-negative bacteria may be just as large. Due to their membrane structure, Gram-negative bacteria are inherently resistant to many antibiotics [3]. In addition, Gram-negative bacteria with specific resistant mechanisms are continuing to emerge [4–7]. The threat that these bacteria, both Gram-positive and Gram-negative, pose to human health has prompted investigation of new targets for bacterial action including: bacterial efflux pumps [8], lipopolysaccharide synthesis [9], bacterial mRNA [10] and bacterial membranes. Bacterial membranes constitute an appealing target because among Gram-positive and Gram-negative bacteria most structural elements of membranes are conserved, and resistance to membrane-active antibiotics requires major changes in membrane structure, which in turn influences the permeability barrier provided by the membranes [3].

Based upon a study of membrane-active compounds, we proposed that cationic, facially amphiphilic molecules could disrupt bacterial membranes [11,12]. Facial amphiphiles differ from typical amphiphilic compounds [13,14]. The latter, typified by sodium dodecyl sulfate, possess a hydrophilic head group and a hydrophobic tail, while facial amphiphiles display separate hydrophilic and hydrophobic faces (Fig. 1). Many membrane-active compounds are facial amphiphiles including most cationic peptide antibiotics [15], cationic antibiotics derived from L-peptide [16,17], and cationic steroid antibiotics.

Cationic peptide antibiotics (CPAs), also termed cationic antimicrobial peptides, have been isolated from organisms ranging from bacteria to mammals [18,19]. In general, CPAs may be considered facially amphiphilic [15,18]. Two of the major classes of CPAs are β-sheet-forming and

Fig. 1. Schematic representation of (A) a ‘typical’ amphiphile and (B) a facial amphiphile.
α-helix-forming peptides. In both classes of compounds side chains from hydrophobic residues are segregated on one face of the molecule, while cationic side chains are oriented on the other face. For example, cecropin P1, isolated from pig intestine [20], is believed to adopt an α-helical conformation in the presence of bacterial membranes. A helix wheel representation of cecropin P1 (Fig. 2) demonstrates the facial amphiphilicity of the molecule in an α-helix.

Recently, amide polymers derived from β-amino acids have been prepared that adopt facially amphiphilic conformations. These compounds, prepared by Liu and DeGrado [17] and Gellman and co-workers [16], are armed with multiple cationic groups on one face and hydrophobic groups on the opposite face and display antibacterial activities comparable to those of CPAs from biological sources. The fact that membrane-active antibiotics can be prepared from monomers other than β-amino acids suggests that the antibacterial properties of CPAs are not dependent upon specific components of the antibiotics but rather are dictated by the disposition of charged and hydrophobic groups in the peptide secondary structure yielding facial amphiphilic conformations. Furthermore, because other compounds can be prepared in a fashion that results in facial amphiphilicity, antibacterial membrane activity similar to that of CPAs should be possible using non-peptide-based compounds.

Kahne et al. first reported facial amphiphiles derived from cholic acid prepared for use as transfection agents [21]. These compounds, prepared from cholic acid with carbohydrates appended on one face of the molecule (1 in Fig. 3), proved to facilitate the movement of polar compounds across eukaryotic membranes. However, the antibacterial properties of these compounds have not been reported. In an effort to mimic the facial amphiphilicity of polymyxin B, a widely used CPA, we prepared multiple series of cholic acid derivatives with amine groups appended on one face of the steroid [11,12,22–24]. These cationic steroid antibiotics (CSAs) have proven to have antibacterial activities similar to those of many CPAs, including rapid bactericidal activity and the ability to permeabilize the outer membranes (OM) of Gram-negative bacteria [25,26]. Considering the similar cationic, facially amphiphilic morphologies of CPAs and CSAs (for example see 2 in Fig. 3), it is not surprising that they appear to share common mechanisms of action [24].

In this minireview the antibacterial properties of CSAs will be presented, including descriptions of the activities of CSAs designed to mimic polymyxin B and those designed to mimic squalamine. In addition, studies directly comparing CSAs to CPAs will be discussed.

2. Antibacterial properties of CSAs

2.1. Assessment criteria used in evaluating CSAs

CSAs display two types of antibacterial activity. The first is a lethal activity; many CSAs are broad-spectrum bactericidal agents active against both Gram-negative and Gram-positive organisms. This activity is characterized using minimum bactericidal concentrations (MBCs), although more commonly antibacterial activity is compared using minimum inhibitory concentrations (MICs). The second is a non-lethal activity that entails permeabilization of the OM of Gram-negative bacteria. Examples of CSAs do not display potent bactericidal activity against Gram-negative bacteria yet retain the ability to sensitize these organisms to hydrophobic antibiotics that alone are ineffective in traversing the OM. These two types of activity are also seen from polymyxin B and its derivatives. The ability of a compound to sensitize bacteria to another antibiotic can be quantified using a fractional inhibition

Fig. 2. Helix wheel drawing of a portion of cecropin P1 showing segregation of hydrophobic and cationic residues.

Fig. 3. Structures of facial amphiphiles 1 and 2 with a perspective drawing of 2 showing segregation of the hydrophobic surface and cationic groups.
concentration (FIC) [27]. FIC values are calculated as $FIC = \frac{[A]}{MIC_A} + \frac{[B]}{MIC_B}$, where $MIC_A$ and $MIC_B$ are the MICs of compounds A and B, respectively, and $[A]$ and $[B]$ are the concentrations at which compounds A and B, in combination, inhibit bacterial growth. Synergism is defined by $FIC < 0.5$. Hydrophobic antibiotics that are commonly used in combination with OM permeabilizers include erythromycin, fusidic acid, novobiocin and rifampicin. Because CSAs are membrane-active, a key criterion in evaluating their potential clinical utility is their membrane selectivity. A major impediment to the use of membrane-active antibiotics is the tendency of these compounds to disrupt eukaryotic membranes. This activity is typically measured using erythrocytes and is expressed as a minimum hemolytic concentration (MHC). A measure of membrane selectivity can be determined by comparing MIC and MHC values. High membrane selectivity is observed with high MHC values and low MIC values.

2.2. Structural classes of CSAs

CSAs can be separated into categories: (1) polymyxin mimics and (2) squalamine and its mimics. There have been many CSAs prepared in each category, and rather than providing an exhaustive list of all of the compounds prepared, only representative antibiotics and their activities will be presented. Compounds within the first category are distinguished by the attachment of three amine groups, via tethers, to a steroid nucleus (e.g., 2 in Fig. 4) [12,24]. Various groups that influence antibacterial activities have been attached at C24 (see Fig. 4 for steroid numbering) including hydrophobic chains and polyamines (compare 3 and 4 in Fig. 4). Squalamine (Fig. 4) is a CSA first isolated from the dogfish shark [28]. Also in the second category are multiple mimics of squalamine that have been prepared with the most extensive series prepared by Regen et al. [29,30]. This group reversed the position of the polyamine and sulfate groups and also deleted the sulfate group to give compounds 5 and 6 (Fig. 4). While there are significant structural differences between the polymyxin and squalamine mimics, it has been proposed that squalamine and its mimics can adopt facially amphiphilic conformations in the presence of membranes by passing the polyamine chain common to these compounds over one face of the steroid [29].

2.3. OM-permeabilizing properties of CSAs

The OM of Gram-negative bacteria forms an effective barrier to many hydrophobic molecules. Consequently, many antibiotics that are active against Gram-positive organisms are much less active against Gram-negative bacteria. The permeability barrier of the OM is formed from cross-bridging between lipid A molecules via divalent cations (magnesium and/or calcium) [3]. Compounds that bind either lipid A or divalent cations disrupt the organization of the OM, increasing its permeability, and sensitize bacteria to hydrophobic antibiotics that ineffectively traverse the OM. For example, at relatively high concentrations (millimolar) EDTA, a cation binder, sensitizes Gram-negative bacteria to hydrophobic antibiotics [3]. A truncated form of polymyxin B, termed polymyxin B non-

Fig. 4. Structures of CSAs 2-6 and squalamine.
apeptide [31], is not bactericidal alone yet retains the ability to bind lipid A and sensitizes Gram-negative bacteria at relatively low concentrations (micromolar).

CSAs display a range of abilities to permeabilize the OM. In some cases the lethal (bactericidal) activity of the antibiotic is so great that it is not possible to observe non-lethal (permeabilization) activity. For example, 3 displays MIC values of 2–3 μg ml⁻¹ against Gram-negative strains, and its FIC values with hydrophobic antibiotics are relatively high (∼0.5) [25]. In contrast, 2 is much less active against Gram-negative bacteria (MIC values of 21–47 μg ml⁻¹), yet it retains the ability to bind to lipid A and effectively permeabilize the OM giving very low FIC values (see Table 1). To illustrate further, 2 at a concentration of <1 μg ml⁻¹ lowers the MIC of erythromycin from 61 to 1 μg ml⁻¹ with Salmonella typhimurium [25]. Results from a study of series of CSAs with hydrophobic chains of various lengths attached at C24 revealed that a hydrophobic chain is required for bactericidal activity but is less important for permeabilizing the OM [12]. It was suggested that the hydrophobic chain facilitates movement of the CSA through the OM to the cytoplasmic membrane where it causes cell death. Of the squalamine mimics, only 6 was tested for the ability to sensitize Gram-negative bacteria to hydrophobic antibiotics [30]. It proved to be an active permeabilizer but less effective than 2 (Table 1).

The abilities of CSAs to permeabilize the OM of Gram-negative bacteria can make these organisms as susceptible to hydrophobic antibiotics as Gram-positive bacteria. Consequently, CSAs may prove to enlarge the arsenal of antibiotics that are useful in fighting Gram-negative bacterial infections.

### 2.4. Bactericidal/bacteriostatic activities of CSAs

Many of the CSAs reported display broad-spectrum antibacterial activity. CSAs with a hydrophobic chain, such as 3, are bactericidal at low concentrations and rival (and in some cases surpass) the antibacterial activity of polymyxin B against Gram-negative bacteria and are more active against Gram-positive bacteria (see Table 2). Interestingly, 2 is only weakly active against Gram-negative bacteria as compared to 3, yet is comparable in activity against Gram-positive organisms. This result exemplifies the need for a hydrophobic chain for transport across the OM: with Gram-negative bacteria the cytoplasmic

### Table 1

FIC values of CSAs in combination with hydrophobic antibiotics

<table>
<thead>
<tr>
<th>Compound</th>
<th>Gram-negative rods (ATCC number in parentheses)</th>
<th>E. coli (25922)</th>
<th>P. aeruginosa (27853)</th>
<th>K. pneumoniae (13883)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.085</td>
<td>0.083</td>
<td>0.030</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
<td>0.16</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.074</td>
<td>&lt;0.13</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Novobiocin</td>
<td></td>
<td>0.033</td>
<td>&lt;0.26</td>
<td></td>
</tr>
</tbody>
</table>

FIC values were taken from [25,30].

aMIC values of erythromycin and novobiocin were >100 μg ml⁻¹ with *P. aeruginosa*.

### Table 2

MIC and MHC values of CSAs and polymyxin B

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (MBC) values (μg ml⁻¹)</th>
<th>MIC values with Gram-negative rods</th>
<th>MIC values with Gram-positive cocci</th>
<th>MHC values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>E. coli</em> (25922)</td>
<td><em>P. aeruginosa</em> (27853)</td>
<td><em>K. pneumoniae</em> (13883)</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>1.8 (1.8)</td>
<td>0.20 (3.9)</td>
<td>5.3 (6.8)</td>
<td>40 (&gt;100)</td>
</tr>
<tr>
<td>2</td>
<td>36 (40)</td>
<td>21 (36)</td>
<td>47 (50)</td>
<td>3.3 (19)</td>
</tr>
<tr>
<td>3</td>
<td>3.0 (3.0)</td>
<td>2.0 (3.2)</td>
<td>2.6 (6.7)</td>
<td>3.1 (5.5)</td>
</tr>
<tr>
<td>4</td>
<td>7.3</td>
<td>nr</td>
<td>nr</td>
<td>nr</td>
</tr>
<tr>
<td>Squalamine</td>
<td>1–2</td>
<td>4–8</td>
<td>nr</td>
<td>1–2</td>
</tr>
<tr>
<td>5</td>
<td>12.5</td>
<td>1.56</td>
<td>nr</td>
<td>3.13</td>
</tr>
<tr>
<td>6</td>
<td>3.13</td>
<td>3.13</td>
<td>3.13</td>
<td>3.13</td>
</tr>
</tbody>
</table>

nr = not reported.

MIC values not given were not reported.

MIC values were taken from [24,25,30].

aATCC numbers are given in parentheses.

bATCC number 29213.
membrane is not accessible to 2, and with Gram-positive bacteria there is no such barrier so 2 and 3 display similar activities.

As indicated by their MBC values, CSAs not only inhibit bacterial growth, but they also actively kill bacteria. Interestingly, the MIC and MBC values reported for 2 and 3 are very similar. That is, a threshold concentration of a CSA is necessary for antibacterial activity, and once this concentration is reached, the CSA causes cell death. This behavior is consistent with the proposed mechanism of action of this series of CSAs (see below).

Prokaryotic membranes are generally made up of anionic lipids, while zwitterionic lipids form eukaryotic membranes. Consequently, charge recognition may be used to enhance cell selectivity (prokaryote vs. eukaryote). CSA 4 was prepared with polyamines extending from C24 with the expectation that this compound would retain antibacterial activity while interacting less strongly with eukaryotic membranes [24]. Even without a hydrophobic chain, 4 remains moderately active against Gram-negative bacteria and highly active against Gram-positive organisms, and as discussed below 4 does not disrupt eukaryotic membranes at concentrations below 200 µg ml⁻¹.

Squalamine was first characterized by its broad-spectrum antibacterial activities, displaying low MIC values against both Gram-negative and Gram-positive bacteria [28]. Due to the difficulty of preparing squalamine, simpler mimics of squalamine were prepared by Regen et al. [29,30]. These compounds incorporate functionality similar to that of squalamine but in an altered arrangement. Nevertheless, these squalamine mimics proved to have antibacterial activities similar to the parent compound (see Table 2). In addition, time-kill studies demonstrated that these compounds are bactericidal and fast-acting, causing dramatic drops in bacterial counts in less than 2 h.

2.5. Hemolytic properties of CSAs

The fact that CSAs are membrane-active raises the issue of membrane selectivity. Provided that CSAs can disrupt bacterial membranes, can they kill prokaryotes without disrupting the membranes of host cells? The answer to this question is that some CSAs display good membrane selectivity, while others disrupt both prokaryotic and eukaryotic membranes (similarly, some CPAs are membrane-selective while others are not). The MHCs of CSAs 2-6 are given in Table 2. Notably, the effects of the hydrophobic chain on 3 can be seen in that it gives a lower MHC than 2, while the additional cationic groups on the chain extending from C24 in 4 provide a greater level of cell selectivity. Of the CSAs reported, 4 appears to be the most cell-selective giving low MICs with many bacterial strains, while hemolytic activity was not observed up to concentrations of 200 µg ml⁻¹. Squalamine proved to be moderately hemolytic; less so than melittin, a non-selective CPA, and more so than magainin II amide, a cell-selective CPA. MHC values for the squalamine mimics roughly parallel their antibacterial activity; the most strongly bactericidal compounds are also the most hemolytic.

3. Mechanism of action of CSAs

The similarity in the amphiphilic morphology of CSAs and CPAs suggests that they may have similar mechanisms of action. As a means of directly comparing these two classes of antibiotics, comparative studies of CSAs 2 and 3 and CPA magainin were performed. These studies included measurement of rates of membrane depolarization and observation of the bacterial promoters activated in response to CSAs.

Membrane depolarization can be observed using the fluorescent dye 3,3'-diethylthiodicarbocyanine [32]. This lipophilic dye incorporates into polarized membranes and becomes only weakly fluorescent, and depolarization of the membrane results in large increases in its fluorescence. Magainin I at 50 µg ml⁻¹ (21 µM), 2 at 2 µg ml⁻¹ (3.5 µM), and 3 at 0.5 µg ml⁻¹ (0.74 µM) caused nearly identical increases in fluorescence of the cyanine dye in the same time frame (3–5 min) [24]. These results demonstrate that both types of antibiotic are membrane-active and that...
their activity occurs at the same rapid rate. Significantly, the CSAs exhibit their activity at concentrations much lower than that of the CPA magainin I.

Bacterial promoters for genes expressing proteins that respond to osmotic or oxidative stress have been coupled to a bacterial luminescence reporter operon (luxCDABE) on a plasmid introduced into *Escherichia coli*. Exposure of bacteria carrying these plasmids to osmotic or oxidative stress causes the bacteria to luminesce. CPAs, such as the magainins and cecropins, have been characterized by the stress they activate [33,34], and studies with CSAs demonstrate that they activate the same promoters [24]. This finding suggests that the bacteria respond in similar ways to CSAs and CPAs, and that these two types of antibiotic may share mechanistic aspects.

Two models for the mechanism of action of CPAs have been proposed: the ‘carpet’ and ‘barrel-stave’ models [15]. Briefly, the carpet model of action requires the antibiotic to associate with the cell surface (presumably via ionic interactions), and when sufficient local concentrations of the antibiotic are reached, whole patches of the membrane are removed (Fig. 5). In the barrel-stave model, the antibiotics aggregate into bundles in the membrane, like staves of a barrel, forming semi-stable pores in the membrane. A majority of the α-helix-forming CPAs, such as the magainins and cecropins, are believed to act via the carpet model. Because of the similarity in activity between the CSAs and these two CPAs, it is most likely that CSAs operate via the carpet model.

4. Conclusions and future directions

Considering membrane-active antibiotics, CSAs offer advantages over other types of compounds. They are relatively easy to prepare, have varied properties including bactericidal and/or sensitization activity, and their hemolytic activities can be controlled. Because they are membrane-active, they are unlikely to induce resistance formation (bacterial resistance requires major changes in membrane structure which in turn alters membrane permeability). Although the in vitro antibacterial activities of CSAs have been well characterized, their in vivo properties are not fully understood, especially potential toxicities. In view of clinical studies with CPAs [19], it is probable that CSAs can be used in topical applications, and as toxicity issues are better understood, their potential for use in systemic applications may be realized. The most likely impediment to the systemic use of CSAs is cell selectivity. Charge recognition provides a high degree of cell selectivity (prokaryote over eukaryote) [24]. However, increased recognition may be possible by targeting specific components of bacterial membranes (e.g., lipid A and lipid II). CSAs provide a useful means of killing bacteria, and ongoing efforts are designed to improve membrane selectivity.

Acknowledgements

Financial support from the National Institutes of Health (GM 54619) and the National Science Foundation (CAREER) is gratefully acknowledged.

References


