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Activities of cholic acid-derived antimicrobial agents against multidrug-resistant bacteria

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Cationic cholic acid derivatives displayed potent and broad-spectrum activity against multidrug-resistant Gram-negative and -positive bacteria. Specific examples were effective permeabilizers of the outer membranes of many strains of multidrug-resistant Gram-negative bacteria and sensitized these to hydrophobic antibiotics. We also prepared a new cholic acid derivative with improved apparent selectivity for prokaryote membranes.

Introduction

With the emergence of many strains of multidrug-resistant bacteria has come a renewed interest in new antimicrobial agents. Cationic peptide antibiotics, which have been isolated in organisms ranging from bacteria to animals, have received considerable attention in part because of their broad spectrum of activity.[1] Generally, the targets of these antibiotics are bacterial membranes.[2] We recently reported the activities of a series of membrane-active cationic cholic acid derivatives with potent antimicrobial activities that may share mechanistic aspects with cationic peptide antibiotics.[3,4] Examples of these compounds were shown to be bactericidal to a broad spectrum of Gram-negative and -positive organisms. Other cholic acid derivatives were only weakly active against Gram-negative organisms, but effectively permeabilized the outer membranes and sensitized the bacteria to hydrophobic antibiotics such as erythromycin and rifampicin. To better characterize the cholic acid derivatives, we have measured their antibacterial activities against multidrug-resistant bacteria, including both Gram-negative and -positive organisms. In addition, we have measured the abilities of the cholic acid derivatives to sensitize multidrug-resistant Gram-negative bacteria to hydrophobic antibiotics with the expectation that potent sensitizers will in effect increase the number of antibiotics that can be used against these organisms.

A feature of membrane-active antimicrobial agents that may be an impediment to their systemic use is that they often display haemolytic properties.[2] Indeed, the properties of the cholic acid derivatives we have reported have ranged from very haemolytic to very weakly haemolytic.[3] In this report, we describe a new compound with the weakest haemolytic activity of any potent antimicrobial cholic acid derivative reported to date.

Materials and methods

Antibacterial compounds

The syntheses of compounds **1** and **2** have been reported and compound **3** was prepared using similar synthetic techniques (synthetic details will be reported elsewhere).[4] Erythromycin was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA), and rifampicin and novobiocin were obtained from Sigma Chemical Co. (St Louis, MO, USA) and were used as received.

Microorganisms

The bacterial strains were recent clinical isolates. *Staphylococcus aureus* (MNUM 3, MNUM 16, MNUM 28) and *Streptococcus pneumoniae* (HIP68, HIP 1321, HIP 1396) strains were received from the Centers for Disease Control (Atlanta, GA, USA). These strains were resistant to multiple antibiotics, including clindamycin and erythromycin. *Enterococcus faecalis* (E33-8, E38-17, E80-8), *Escherichia coli* (32-7825, 45-2161A, 46-7157A), *Klebsiella pneumoniae* (21-2751C, 40-6564A), *Pseudomonas aeruginosa* (CF1,

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Table I. MICs (mg/L) of cholic acid derivatives **1**–**3** against multidrug resistant Gram-positive cocci and Gram-negative bacilli

	Gram-positive cocci								
	S. aureus			*S. pneumoniae*			*E. faecalis*		
Compounds	MNUM3	MNUM16	MNUM28	HIP68	HP1321	HIP1396	E33-8	E38-17	E80-8
1	0.3	0.2	0.2	3	3	4	1.3	3.0	2.3
2	0.7	1.0	0.8	8.8	9.0	15	20	25	25
3	0.3	0.5	0.5	6.3	15	15	9.7	15	20

	Gram-negative rods							
	E. coli		*K. pneumoniae*		*P. aeruginosa*		*S. typhimurium*	
	32-7825	45-2161A	21-2751C	40-6564A	CF1	CF132	19-3855A	45-10003A
1	2.0	3.7	1.0	1.0	2.0	2.0	0.8	0.4
2	53.3	53.3	43.3	21.3	20	30	20	15
3	8.0	16.7	13.5	5.3	25	15	5	6

CF132) and *Salmonella typhimurium* (19-3855A, 45-10003A) were kind gifts from Dr Ronald N. Jones (Department of Pathology, University of Iowa Medical School, Iowa City, IA, USA). The *E. faecalis* was resistant to multiple antibiotics, including erythromycin. The Gram-negative strains were resistant to multiple antibiotics including aminoglycosides and β-lactams. Bacterial strains were maintained on Mueller–Hinton agar plates (*S. pneumoniae* and *E. faecalis* were maintained on Mueller–Hinton agar supplemented with 5% sheep blood).

Determination of MICs

MICs were determined using a broth macrodilution method using Mueller–Hinton broth[5] (Mueller–Hinton broth for *S. pneumoniae* and *E. faecalis* was supplemented with lysed horse blood). Each compound initially was screened to determine its MIC range. Subsequently, concentration increments of the cholic acid derivatives were decreased to more accurately determine the MICs. Each MIC was measured a minimum of three times. Averaged results are shown in Table I.

Determination of FICs

Fractional inhibition concentration (FIC) values[6] were calculated as FIC = [A]/MIC_A + [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. Synergy is defined by FIC < 0.5. Synergy tests with erythromycin, novobiocin and rifampicin were performed using a broth macrodilution method with 1.0 mg/L concentrations of these antibiotics. A single concentration of erythromycin and small incrementally varied concentrations of compounds **2** and **3** were used to provide an effective means of observing small differences in the activities of these cholic acid derivatives. Each FIC was measured a minimum of three times, and averaged results are shown in Table II.

Determination of MHCs

For determination of the minimum haemolytic concentration (MHC) of compound **3**, the compound was dissolved in incrementally varied concentrations in 0.85% saline, diluted with Dulbecco's phosphate-buffered saline (PBX-1X) and added to a 1% suspension of sheep erythrocytes. The samples were incubated for 24 h and centrifuged. The MHC was determined by measuring absorbance of the supernatant at 540 nm. Sheep erythrocytes were used to allow direct comparison of the MHC of **3** with those of other antimicrobial cholic acid derivatives.

Results and discussion

Against Gram-positive bacteria, the compounds all displayed antimicrobial activity. In fact, the compounds were generally more active against the multidrug-resistant strains as compared with standard strains.[3] Compound **1**, with the hydrophobic octylamine group, was especially active. The *E. faecalis* strains were moderately resistant to compounds **2** and **3**, which lack a hydrophobic chain.

Table II. Concentrations of compounds **2** and **3** required to lower the MIC of erythromycin from >200–57 mg/L to 1 mg/L

	Concentrations (mg/L) (FIC)						
	E. coli	*K. pneumoniae*		*P. aeruginosa*		*S. typhimurium*	
Compounds	32-7825	21-2751C	40-6564A	CF1	CF132	19-3855A	45-10003A
2	0.8 (<0.025)	0.7 (0.027)	9.7 (<0.46)	18[a]	15 (<0.50)	1.3 (<0.075)	0.7 (<0.057)
3	1.0[a]	0.7 (0.063)	4.7[a]	15[a]	15[a]	0.6 (<0.13)	0.5 (<0.093)

[a] The lower limits of these FICs were >0.5.

Against the Gram-negative organisms, the compounds exhibited a range of activities. We have noted that a hydrophobic chain, such as the one found in **1**, is required for the compound to traverse the outer membranes of Gram-negative bacteria, gaining access to the cytoplasmic membrane and causing cell death.[4] Consequently, we expected **1** to be more active than **2** or **3** against Gram-negative organisms. Compound **1** was very active against each of the multidrug-resistant strains including the *P. aeruginosa* strains, while **2** and **3** were much less active alone. Nevertheless, **2** and **3** were potent sensitizers of Gram-negative bacteria to a hydrophobic antibiotic (erythromycin). With only a few exceptions, these compounds are able to permeabilize the outer membranes of drug-resistant bacteria. And, as noted among the MIC data, in many cases the compounds were more active against the drug-resistant strains.

Results with the resistant strains of *K. pneumoniae* were particularly interesting. Strain 40-6564A was more sensitive to **2** and **3** than strain 21-2751C. In contrast, the former strain was poorly sensitized to erythromycin as compared with the latter strain. To determine whether strain 40-6564 displayed a specific resistance mechanism for erythromycin, FICs were determined using **2** and **3** in combination with novobiocin and rifampicin. With novobiocin, FICs with **2** and **3** were 0.24 and 0.58, respectively, and with rifampicin the FIC values were 0.19 and 0.13, respectively. These FICs are much higher than those measured with other *K. pneumonia* strains.[3] The inherent resistance of strain 40-6564A may be due to a change in its membrane structure or the action of efflux pumps.

We have previously reported the MHC values for **1** and **2** (6 and 100 mg/L, respectively). In an effort to increase the selectivity of cholic acid derivatives for negatively charged prokaryotic membranes over their electrically neutral eukaryotic counterparts,[7] an additional positive charge was included in **3** (as compared with **2**). As expected, the MHC of **3** (170 mg/L) was much greater than those of **1** and **2**. The fact that the MHCs of the cholic acid derivatives increased upon addition of another positive charge may indicate a general means of increasing the prokaryote/eukaryote selectivity of this type of compound. We are currently investigating this hypothesis.

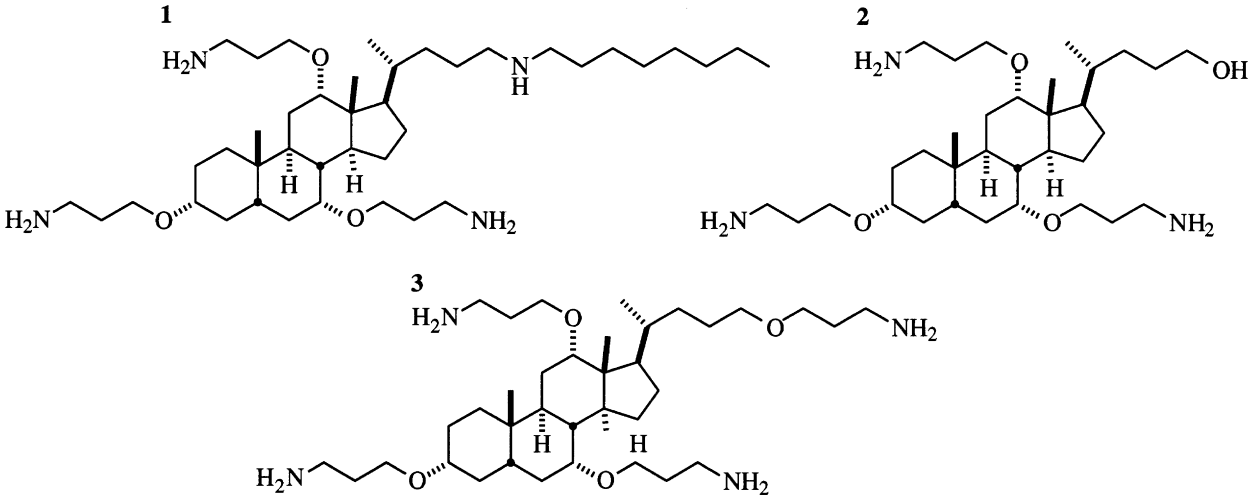

Figure. Structures of antibacterial compounds **1**, **2** and **3**.

As might be expected for membrane active antimicrobial agents, compounds **1**–**3** are active against multidrug-resistant organisms. This behaviour, coupled with the idea that haemolytic activity is markedly decreased in compound **3**, may make these compounds useful in treating resistant bacterial infections. In addition, because compounds **2** and **3** permeabilize bacterial membranes at low concentrations, they may prove valuable in enlarging the arsenal of antibiotics that can be used against Gram-negative organisms and allow use of hydrophobic antibiotics that alone ineffectively traverse the outer membrane.

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