Bioorganic & Medicinal Chemistry Letters 20 (2010) 975–978

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synergistic effects between aminoethyl-chitosans and β-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA)

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ARTICLE INFO

Article history:
Received 13 October 2009
Revised 25 November 2009
Accepted 14 December 2009
Available online 21 December 2009

Keywords:
MRSA
Antimicrobial agent
Aminoderivatized chitosans
Synegistic effect

ABSTRACT

Two kinds of aminoethyl-chitosans (AEC), AEC90 and AEC50, which had degrees of deacetylation of 90% and 50%, respectively, were prepared and their synergistic effects in combination with β-lactams including ampicillin, penicillin, and oxacillin against two standard methicillin-resistant *Staphylococcus aureus* (MRSA) strains and twelve clinical isolated MRSA strains were investigated. When AECs and β-lactams were combined, synergistic effects were observed with fractional inhibitory concentration (FIC) indices of 0.252–0.508, and the MICs of β-lactams in the presence of AECs were dramatically reduced.

Staphylococcus aureus is one of the most dangerous human pathogens and is responsible for not only severe infections of the skin and kin structures but also life-threatening diseases such as pneumonia, endocarditis, and bacteremia.[1] *S. aureus* was susceptible to the earliest antimicrobial substances, most notably penicillin. However, the overuse of antibiotics resulted in the spread of staphylococcal resistance[2] and multi-drug resistance *S. aureus* soon emerged. Therefore, the resistance of this pathogen to a number of antibiotics is of growing concern in the medical field, and has recently been recognized as a global nosocomial problem. Since its discovery in early 1961, methicillin-resistant *S. aureus* (MRSA) has now emerged as a predominant and serious pathogenic bacterium, leading to high morbidity and mortality.[3] Due to its multi-drug resistant genotype, MRSA is not sensitive to the β-lactams including penicillin, methicillin, oxacillin, and flucloxacillin and it is also resistant to aminoglycosides, fluoroquinolones, chloramphenicol and macrolides, but not glycopeptides such as vacomycin and teicoplanin.[4] Therefore, glycopeptides have long been considered the last-resort antibiotics against MRSA and are widely used to treat MRSA infections.[5] However, with the increasing use of vancomycin, vancomycin-intermediate and -resistant *S. aureus* (VISA and VRSA) have emerged.[6,7] In view of these problems, there is an urgent need for the development of new anti-MRSA compounds.

Chitosan, the N-deacetylated derivative of chitin, is an important functional biomaterial because of its biodegradability, biocompatibility, non-toxicity, and adsorption properties. Chitosan exhibits a wide variety of physiological activities such as antitumor activity, immumo-stimulating effect, antimicrobial effect, and cholesterol-reducing effect.[8–11] Although chitosan has many beneficial functional properties, it is not soluble in water, which prevents its use in a wide range of different applications. Therefore, many studies have attempted to develop methods to improve the water-solubility and biological activity of chitosan. Furthermore, chemical modifications of chitosan could provide desired biological activities and physicochemical properties. Recently, our laboratory developed aminoderivatized chitosans, which exhibited versatile biological activities such as antioxidant, antihypertensive, enzyme inhibition, and antimicrobial characteristics.[12–15] In addition, aminoderivatized chitosans exhibited strong anti-MRSA activities against standard strains and clinical isolates.[16] Therefore, in this study, we evaluated the synergistic effects between aminoderivatized chitosans and β-lactams including penicillin, oxacillin and ampicillin against standard MRSA strains and clinical isolates.

The chemical structure of aminoderivatized chitosans used in this study is shown in Figure 1. Chitosans with different degrees of deacetylation (90% and 50%) were modified with aminoalkyl groups at the C-6 position rather than the C-2 position because the free amino group at the C-2 position is highly important to the biological activity of chitosan.[16] Aminoderivatized chitosans were designated as aminoethyl-chitosan (AEC90, degree of substitution: 0.88), dimethylaminoethyl-chitosan (DMAEC90, degree of

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doi:10.1016/j.bmcl.2009.12.049

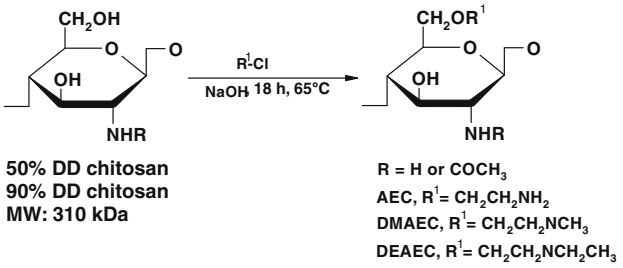

Figure 1. Chemical structure of aminoderivatized chitosans.

substitution: 0.75), and diethylaminoethyl-chitosan (DEAEC90, degree of substitution: 0.67) derived from 90% deacetylated chitosan, and AEC50 (degree of substitution: 0.92), DMAEC50 (degree of substitution: 0.69), and DEAEC50 (degree of substitution: 0.63) derived from 50% deacetylated chitosan.

The anti-MRSA activity of aminoderivatized chitosans was determined using a growth inhibition assay.[17] As shown in Figure 2, all aminoderivatized chitosans inhibited the growth of MRSA, and AEC50 and AEC90 showed the strongest anti-MRSA activity. The antibacterial activity of chitosans has been well documented and it showed a broad spectrum of antibacterial activity against Gram positive and Gram negative bacteria. Furthermore, the antibacterial activity was dependent on various factors such as the degree of depolymerization and deacetylation, and the type of bacterium. According to the literature, high molecular weight chitosans with high degrees of deacetylation showed more potent antibacterial activity.[18] These effects were attributed to the free amino groups in the chitosan backbone. Our group also demonstrated that chitosans with a high degree of deacetylation showed more potent antibacterial activity than chitosans with a low degree of deacetylation.[15] In this study, chitosans were modified with aminoalkyl group at the C-6 position in order to improve water-solubility and quantity of free amino groups. As a result of this modification, the aminoderivatized chitosans showed different levels of antibacterial activity, and AEC90 and AEC50 exhibited more potent antibacterial activity than DMAECs and DEAECs due to the newly introduced free amino groups. In our previous report, we evaluated the minimum inhibitory concentration (MIC) of AEC90 and AEC50 using a twofold serial dilution method against methicillin-susceptible *S. aureus* (MSSA) and against MRSA strains including two MRSA standard and 12 clinical isolates strains that were

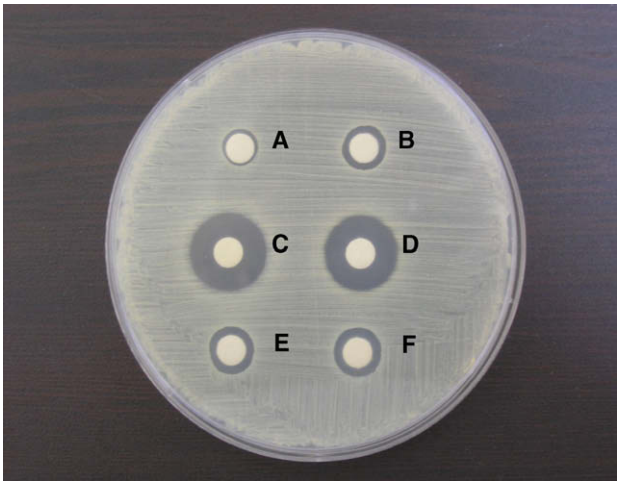

Figure 2. Photograph of the antimicrobial activity of aminoderivatized chitosans against MRSA. A, DMAEC50; B, DMAEC90; C, AEC50; D, AEC90; E, DEAEC50; F, DEAEC90.

*mec*A gene positive, and one standard MSSA strain that was *mec*A gene negative. Using this approach, the MICs of AEC90 and AEC50 were determined to range from 16 to 32 μg/mL.[16] However, β-lactam antibiotics used in this study exhibited lower MICs than those of AEC90 and AEC50 (Tables 1 and 2). These results occurred because of the presence of the *mec*A gene. The *mec*A gene is responsible for the specific penicillin-binding proteins (PBP2a or PBP2), which can reduce the affinity to β-lactam antibiotics.

It is well known that β-lactam antibiotics inhibit several enzymes associated with the final step of peptidoglycan synthesis.[19] However, the overuse of antibiotics led to the appearance of drug-resistant bacteria and the ever-increasing development of pathogenic microbial resistance to traditional antibiotics have already reached alarming levels. Therefore, the development of new drugs or alternative therapies is clearly a matter of urgent necessity.[20] Several reports have suggested that the restoration of antibiotic activity in combination with antibacterial material derived from natural bioresources against drug-resistant bacteria is a more effective strategy than the development of new drugs.[21,22] Therefore, in this study, we evaluated the synergistic effects of AEC90 and AEC50 in combination with β-lactam antibiotics against MRSA including clinical isolates.[23] As shown in Table 1, the MICs of ampicillin against two standard MRSA strains (40510 and 40511) were reduced dramatically from 512 to 1 and 0.5 μg/mL when administered in combination with 16 μg/mL of AEC90. This result indicates that AEC90 reversed the high-level ampicillin resistance of MRSA. We also evaluated the synergistic effect of AEC90 in combination with penicillin and oxacillin. The MICs of penicillin were also dramatically decreased from 512 and 256 μg/mL to 1 and 0.5 μg/mL when administered in combination with 16 μg/mL of AEC90. However, the MICs of oxacillin were different from the MICs of ampicillin and penicillin. Oxacillin showed lower MICs (128 μg/mL) than those of ampicillin and penicillin, but the MICs were higher (32 and 16 μg/mL) than those of ampicillin and penicillin (1 and 0.5 μg/mL) when administered in combination with 16 μg/mL of AEC90. As summarized in Table 2, the synergistic effect of AEC50 in combination with β-lactams also showed the same pattern as AEC90. The MICs of all β-lactams were dramatically reduced when treated in combination with AEC50, but the MICs of oxacillin showed higher values compared to the MICs of oxacillin when combined with AEC90. The synergy was evaluated in terms of fractional inhibitory concentration (FIC) index between AEC90, AEC50 and β-lactams. The FIC indices of ampicillin ranged from 0.252 to 0.508 when used in combination with AEC90 (8 and 16 μg/mL) against two standard MRSA and 12 clinical isolates, indicating that a synergistic effect existed when combined with AEC90. The FIC indices of penicillin also ranged from 0.254 to 0.516, indicating AEC90 and penicillin synergistically inhibited the growth of MRSA, but the FIC indices of oxacillin were higher than those of ampicillin and penicillin. When 8 μg/mL of AEC90 was used, a synergistic effect was observed with the exception of three clinical isolates; however, an additive effect was observed when 16 μg/mL of AEC90 was used. The FIC indices of ampicillin and penicillin also ranged from 0.252 to 0.508 when used in combination with AEC50 (8 and 16 μg/mL), indicating synergy between AEC50 and ampicillin and penicillin. Oxacillin in combination with AEC50 also showed similar FIC indices to oxacillin in combination with AEC90. The synergistic effect between dieckol and β-lactams against MRSA was reported, and differences in the synergistic effects of ampicillin, penicillin and oxacillin were observed.[20] No synergy was observed between dieckol and oxacillin against MRSA strains. Zhao et al.[21] also evaluated the synergistic effect between epigallocatechin gallate (EGCg) and β-lactams. EGCs displayed a synergistic effect against MRSA when used in combination with penicillin and oxacillin, but no synergy was observed when it was used in combination with ampicillin. Our results also demonstrated that

Table 1
Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration (FIC) indices of AEC90 in combination with β-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA)

Strain	Ampicillin					Penicillin					Oxacillin				
	MIC (μg/mL)			FIC index[a]		MIC (μg/mL)			FIC index		MIC (μg/mL)			FIC index	
	A	B	C	b	c	A	B	C	b	c	A	B	C	b	c
MRSA40510	512	2	1	0.254	0.502	512	2	1	0.254	0.502	128	32	16	0.500	0.625
MRSA40511	512	1	0.5	0.252	0.501	256	1	0.5	0.254	0.502	128	32	16	0.500	0.625
MRSA D-3	128	1	0.5	0.258	0.504	128	2	1	0.266	0.508	512	32	16	0.313	0.531
MRSA D-4	128	2	1	0.258	0.504	256	2	1	0.258	0.504	128	32	16	0.500	0.625
MRSA D-5	256	2	1	0.258	0.504	256	1	0.5	0.254	0.502	128	32	16	0.500	0.625
MRSA D-6	256	1	0.5	0.254	0.502	128	2	1	0.266	0.508	256	32	16	0.375	0.563
MRSA D-8	256	2	1	0.258	0.504	128	2	1	0.266	0.508	512	128	64	0.500	0.625
MRSA D-10	128	2	1	0.266	0.508	128	2	1	0.266	0.508	128	32	16	0.500	0.625
MRSA D-12	128	1	0.5	0.258	0.504	128	1	0.5	0.258	0.504	64	32	16	0.750	0.750
MRSA D-13	128	2	1	0.266	0.508	128	2	1	0.258	0.504	256	32	16	0.375	0.563
MRSA D-14	128	1	0.5	0.258	0.504	256	2	1	0.281	0.516	128	32	16	0.500	0.625
MRSA D-17	128	2	1	0.266	0.508	64	2	1	0.266	0.508	128	64	32	0.750	0.750
MRSA D-18	128	1	0.5	0.258	0.504	128	2	1	0.266	0.508	512	32	16	0.313	0.531
MRSA D-19	128	1	0.5	0.258	0.504	128	1	0.5	0.258	0.504	64	32	16	0.750	0.750

A, without AEC90; B to C and b to c, AEC90 at 8 and 16 μg/mL, respectively.
[a] The FIC index indicated synergy: ⩽0.5, synergic; >0.5 to ⩽1, additive; >1 to ⩽2 independent; >2, antagonistic.

Table 2
Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration (FIC) indices of AEC50 in combination with β-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA)

Strain	Ampicillin					Penicillin					Oxacillin				
	MIC (μg/mL)			FIC index[a]		MIC (μg/mL)			FIC index		MIC (μg/mL)			FIC index	
	A	B	C	b	c	A	B	C	b	c	A	B	C	b	c
MRSA40510	512	2	1	0.254	0.502	512	2	1	0.254	0.502	128	64	32	0.750	0.750
MRSA40511	512	1	0.5	0.252	0.501	256	1	0.5	0.254	0.502	128	32	16	0.500	0.625
MRSA D-3	128	1	0.5	0.258	0.504	128	2	1	0.266	0.508	512	32	16	0.313	0.531
MRSA D-4	128	1	0.5	0.258	0.504	256	2	1	0.258	0.504	128	32	16	0.500	0.625
MRSA D-5	256	2	1	0.258	0.504	256	1	0.5	0.254	0.502	128	32	16	0.500	0.625
MRSA D-6	256	1	0.5	0.254	0.502	128	2	1	0.266	0.508	256	64	32	0.500	0.625
MRSA D-8	256	2	1	0.258	0.504	128	2	1	0.266	0.508	512	32	16	0.313	0.531
MRSA D-10	128	2	1	0.266	0.508	128	2	1	0.266	0.508	128	32	16	0.500	0.625
MRSA D-12	128	1	0.5	0.258	0.504	128	1	0.5	0.258	0.504	64	32	16	0.750	0.750
MRSA D-13	128	2	1	0.266	0.508	256	2	1	0.258	0.504	256	64	32	0.500	0.625
MRSA D-14	128	1	0.5	0.258	0.504	64	1	0.5	0.266	0.508	128	32	16	0.500	0.625
MRSA D-17	128	2	1	0.266	0.508	128	2	1	0.266	0.508	128	32	16	0.500	0.625
MRSA D-18	128	1	0.5	0.258	0.504	128	2	1	0.266	0.508	512	64	32	0.375	0.563
MRSA D-19	128	1	0.5	0.258	0.504	128	1	0.5	0.258	0.504	64	32	16	0.750	0.750

A, without AEC50; B to C and b to c, AEC50 at 8 and 16 μg/mL, respectively.
[a] The FIC index indicated synergy: ⩽0.5, synergic; >0.5 to ⩽1, additive; >1 to ⩽2, independent; >2, antagonistic.

difference in the synergistic effects between AEC90, AEC50, and β-lactams existed, and this discrepancy in susceptibilities was attributed to their different structure.

In our previous report, AEC90 and AEC50 showed less cytotoxicity (over 90% cell viability) against human lung fibroblast cells (MRC-5) and human endothelial cells (ECV304).[24] From a clinical standpoint, the results presented in this study indicate that AEC90 and/or AEC50 may be possibly used together with β-lactams to treat MRSA-infected patients. However, it is hard to estimate the in vivo synergistic effects just on the basis of the presented in vitro data, because it is difficult to determine the efficiency concentration of AEC90 and AEC50. Furthermore, an in vivo toxicity study regarding AEC90 and AEC50 was not conducted; thus, these studies would need to be conducted to further examine the possibility of using AEC90 and/or AEC50 in combination with β-lactams.

Acknowledgment

This study was supported by research funds from Chosun University, 2008.

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FIC Index = $FIC_A + FIC_B = MIC_A$ in combination/$MIC_A + MIC_B$ in combination/MIC_B
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