ORIGINAL ARTICLE

Methicillin/per-6-(4-methoxybenzyl)-amino-6-deoxy- β -cyclodextrin 1:1 complex and its potentiation *in vitro* against methicillin-resistant *Staphylococcus aureus*

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Some of β -cyclodextrin (β -CD) derivatives as host compounds can form specific inclusion complexes with guest drugs in defined shapes and conformations. In observing one of the known mechanisms of action of β -lactam antibiotic-resistances, a unique β -CD derivative/antibiotic complex may act in accommodating the antibiotic to increase affinity for the resistant target such as the altered penicillin-binding protein PBP2a, which results in recovering and potentiating its antibacterial activities against resistant strains. A designed β -CD derivative, per-6-(4-methoxybenzyl)-amino-6-deoxy- β -CD HCl salt (1), was synthesized from starting material β -CD. The formation and the conformation of 1:1 (molar ratio) methicillin/compound 1 complex was determined through ^1H NMR and NOESY experiments. The *in vitro* MIC of the methicillin in combination of 1, along with methicillin alone, and 1:1 hydroxypropyl- β -CD (HP- β -CD, a commercially available product)/methicillin were assayed against two methicillin-resistant *Staphylococcus aureus* (MRSA) strains. The MIC values of methicillin combined with 1 against MRSA COL and MRSA USA300 were significantly decreased with 30- to 60-fold, compared with those for the antibiotic alone, and 1:1 HP- β -CD/methicillin in this assay. This revealed that compound 1 recovered/potentiated methicillin against MRSA COL and MRSA USA300 *in vitro* combined with methicillin at 1:1 compound 1/methicillin in the aqueous solution.

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INTRODUCTION

As host compounds, β -CD derivatives have been designed for the improvement of guest compound solubility, formulation or specific permeability through complexation properties and functionalities.^{1–3} Based on the outer surfaces of doughnut-shaped molecules, which show both a wider rim (hydrophilic) and narrower rim (hydrophobic) sides,⁴ β -CD may be developed to be specific derivatives for formation of complexes in certain accommodations for guest drugs to access targets. Utilizing the axial open cavity with a hydrophobic character and capability to form a geometric inclusion complex, the structural modification of β -CD could be focused on the primary hydroxyl of 6-position for certain specific molecules or moieties to be inserted into the cavity. Monofacially alternative substitutes for per-6-substituted-deoxy- β -CD should have an appropriate 'length' and hydrophobic compatibility in which a suitable corona can be formed from the narrower rim so that a guest molecule or moiety intends to be a particular form, which would accommodate a certain target.

Methicillin, an antibiotic first developed in 1959, contains a β -lactam nucleus in its molecular structure. However, only 2 years

later, the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) was reported in England.⁵ In this case, methicillin was chosen to study on potentiation though it is no longer used to treat patients. Methicillin is insensitive to β -lactamase (also known as penicillinase) enzymes⁶ secreted by many penicillin-resistant bacteria. The presence of the *ortho*-dimethoxyphenyl group in the structure directly attached to the side-chain carbonyl group of the penicillin nucleus facilitates the β -lactamase resistance, as those enzymes are relatively intolerant of side-chain steric hindrance.⁷ The effectiveness of β -lactam antibiotics against bacteria relies on their ability to reach and bind the penicillin-binding proteins (PBPs). The β -lactam antibiotics are analogs of D-alanyl-D-alanine—the C-terminal amino-acid residues on the precursor N-acetylmuramic acid- and N-acetylglucosamine-peptide subunits of the nascent peptidoglycan layer. The structural similarity between the β -lactam moiety and D-alanyl-D-alanine facilitates their binding to the active site of PBPs. The β -lactam nucleus of the molecule irreversibly binds to the Ser₄₀₃ residue of the PBP active site. This irreversible inhibition of the PBPs prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer,

disrupting cell wall synthesis.⁸ However, MRSA strains alternate to generate other PBPs such as PBP2a, and PBPA, which show structurally conformational changes of the active sites and poor affinities of β -lactam antibiotics for acylation.^{9–11} Vancomycin, a glycopeptide antibiotic with relatively large molecules, is used to treat the infection caused by MRSA.^{12–13} Its mechanism of action against MRSA is similar to those of β -lactam antibiotics, to target the D-alanyl-D-alanine—the terminal amino-acid residues on the precursor N-acetylmuramic acid- and N-acetylglucosamine-peptide subunits.^{14–16} Likewise, specific β -CD derivative/methicillin complex is presumed to have suitable conformation to accommodate the active site of the PBP2a target.

NMR spectral techniques are very useful tools to determine the formation of CD-guest complexes and provide structure futures such as conformations, and physicochemical properties of the resulting complexes and chemical environments around guest compounds.^{17–20}

In attempts reported herein, per-6-(4-methoxybenzyl)-amino-6-deoxy- β -CD HCl salt (**1**) was synthesized from starting material β -CD. The formation of compound **1**/methicillin complex was determined through ¹H NMR analysis while the conformation of the complex was also investigated by an NOESY experiment. Antimicrobial activities of methicillin alone and in combination with **1** at a molar ratio of 1:1 compound **1**-methicillin were measured *in vitro* against two MRSA strains (USA300 and COL).

MATERIALS AND METHODS

Material and instruments

All chemicals/reagents were purchased by Shanghai Medicilon Inc., Shanghai, China, and used without purification. Starting material β -cyclodextrin (β -CD)

was recrystallized twice from water and dried under vacuum, over P₂O₅, before use. Methicillin, linezolid, vancomycin and hydroxypropyl- β -CD (HP- β -CD) were obtained from Sigma-Aldrich Co. LLC (Shanghai, China). LC/MS/MS data were obtained from API 4000 Q TRAP with Shimadzu L20 HPLC. ¹H NMR spectra were recorded on an Inova-500 spectrometer in 0.9% NaCl D₂O at room temperature. Antibacterial assay was performed by WuXi AppTec (Shanghai) Co., Ltd, Shanghai, China.

Preparation of compound **1**/methicillin inclusion complex

For the NMR measurements, complexes were prepared by mixing methicillin with compound **1** at molar ratios of 0.5:1, 1:1 and 2:1 (methicillin/compound **1**), dissolving in 0.9% saline D₂O, and staying at room temperature for 1 h. The preparation of 1:1 (molar ratio) methicillin/compound **1** for the assay was indicated as the procedure.

NMR spectra

¹H NMR data of all samples were collected in 0.9% NaCl D₂O at room temperature; test concentrations were: methicillin + compound **1**, 0.5:1 methicillin (0.008 mmol)-compound **1** (0.016 mmol); 1:1 methicillin (0.016 mmol)-compound **1** (0.016 mmol); 2:1 methicillin (0.016 mmol)-compound **1** (0.008 mmol); All ¹H NMR data were recorded in δ p.p.m. and tetramethylsilane (TMS) as standard. A default NOESY program on the Inova-500 spectrometer was used for the NOESY experiment (1:1 methicillin-compound **1**, 0.016 mmol for each).

Assay for antibacterial activities

The stock solutions of 20 $\times$ assay concentrations were prepared for methicillin, positive control linezolid, vancomycin, and 10 mM for **1**. All stock solutions were in the Mueller-Hinton II Broth (cation-adjusted) on the day of experiment. The starting concentrations were serially diluted by a factor of 2 for 10 times to generate the final concentration of 0.125 mg l⁻¹. Compound **1**

Figure 1 Synthesis of compound **1** starting from β -cyclodextrin.

and HP- β -CD were mixed with methicillin, respectively, both at concentration of molar ratio of 1:1. Each bacterial strain was streaked out one day before the MIC test. Fresh bacterial cultures were prepared by suspending bacterial colonies in the Mueller-Hinton II Broth and adjusted to the final counts of $\sim 5 \times 10^5$ CFU ml⁻¹—the actual CFU ml⁻¹ of each culture was determined. The assay was performed on 96-well microtiter plates. The resulting plates (two for each strain) were kept at 37 °C and 85% humidity for 20 h, after which MIC was recorded as the low concentration that completely inhibits the bacterial growth by visual inspection.

RESULTS

Synthesis of compound 1

Per-6-(4-methoxybenzyl)-amino-6-deoxy- β -CD was synthesized under argon by treating per-6-iodo- β -CD²¹ (prepared from β -CD) with 4-methoxybenzylamine at 80 °C for 48 h. Applying per-6-iodo- β -CD was evaluated to be better than per-6-bromo- β -CD, the former resulting in less side products and higher yield. In corresponding to negative charge of methicillin structure, compound 1 was converted into HCl salts (1, water soluble).

The detail procedure of preparation of 1 (Figure 1) is described as follows: per-6-iodo- β -CD (571 mg, 0.30 mmol) was added to 4-methoxybenzylamine (3.21 mL, 24.60 mmol). The mixture was stirred under argon at 80 °C for 48 h then cooled to room temperature. Ter-butyl methyl ether (5 mL) was added and the precipitated product was filtered, washed with acetone (5 $\times$ 10 mL) and dried under vacuum. Per-6-(4-methoxybenzyl)-amino-6-deoxy- β -CD was obtained as a white solid, yield 90%. The crude product (512 mg) was partially dissolved in water (15 mL) with stirring and treated with 1 N NaOH (2.35 mL). The mixture was stirred for 1 h then filtered. The solid was washed with water (5 $\times$ 10 mL), acetone (5 $\times$ 10 mL) and dried under vacuum. The free base was obtained, and yielded 425 mg. The free base was suspended in water (2.0 mL) and treated with 1 N HCl ($\sim$ 1.25 mL) with stirring until pH reached 2.5. The mixture was left stirring at room temperature for 30 min and filtered to remove any insoluble particles. The filtrate was diluted with acetone (30 mL) upon which a milky suspension formed. The precipitated product was collected by centrifugation, washed with acetone (5 $\times$ 5 mL) and dried under vacuum. Per-6-(4-methoxybenzyl)-amino-6-deoxy- β -CD hydrochloride salt (1) was obtained as a white solid: yield 390 mg. LC-electrospray ionization mass spectrometry (ESIMS) m/z (rel. %): 1969.5 [M + 1]⁺ (10), 985.8 [M + 2H]²⁺/2 (100), and 617.2 [M + 3H]³⁺/3 (20); ¹H NMR (500 Hz, 0.9% NaCl D₂O) δ ppm: 7.32 (d, J = 7.5 Hz, 14 H, H-2', 6'), 6.75 (d, J = 7.5 Hz, 14 H, H-3', 5'), 5.21 (d, J = 1.0 Hz, 7 H, H-1), 4.45 (brs, 7 H, H-5), 4.06 (t, J = 9.0 Hz, 7 H, H-3), 3.78 (d, J = 13.0 Hz, 7 H, H- α), 3.73 (brd, J = 9.0 Hz, 7 H, H-2), 3.65 (d, J = 13.0 Hz, 7 H, H- α'), 3.60 (dd, J = 9.0, 5.5 Hz, 7 H, H-4), 3.52 (s, 21 H, -OCH₃), 3.36 (m, 7 H, H-6 α), and 3.32 (m, 7 H, H-6 β).

¹H NMR analyses of the complex

The formation of an inclusion complex between the β -CD derivative (1) and methicillin was determined based on the changes in their ¹H chemical shifts (δ p.p.m.) at molar ratios of 0.5:1, 1:1, 2:1 methicillin-compound 1 in 0.9% NaCl D₂O solution. As summarized in Table 1 (also Supplementary Figures 1 and 2), proton chemical shift changes ($\Delta\delta$ values in p.p.m.) for methicillin and 1 in the combination were in a range of 0.02–0.13 p.p.m. compared with those for them alone. Particularly, the $\Delta\delta$ values for the protons of the antibiotic or compound 1 in the complexes were observed in increase or decrease order with the ratios of methicillin/compound 1, which implied that

Table 1 ¹H Chemical shifts (δ p.p.m.)^a for methicillin, 1, and methicillin + 1

Methicillin					1				
+ 1					+ Methicillin				
H	Alone (δ)	Molar ratio ^b	δ	$\Delta\delta$	H	Alone (δ)	Molar ratio ^b	δ	$\Delta\delta$
A	1.58	0.5:1.0	1.57	0.03	OCH ₃	3.52	0.5:1.0	3.49	0.03
		1.0:1.0	1.56	0.02			1.0:1.0	3.46	0.06
		2.0:1.0	1.55	0.01			2.0:1.0	3.45	0.07
B	1.68	0.5:1.0	1.68	0.03	3', 5'	6.75	0.5:1.0	6.73	0.02
		1.0:1.0	1.65	0.03			1.0:1.0	6.70	0.05
		2.0:1.0	1.67	0.01			2.0:1.0	6.69	0.06
C	4.30	0.5:1.0	4.23	0.07	2', 6'	7.32	0.5:1.0	7.27	0.05
		1.0:1.0	4.24	0.06			1.0:1.0	7.24	0.08
		2.0:1.0	4.26	0.04			2.0:1.0	7.23	0.09
D	5.68	0.5:1.0	5.60	0.08	a	3.78	0.5:1.0	3.77	0.01
		1.0:1.0	5.61	0.07			1.0:1.0	3.76	0.02
		2.0:1.0	5.64	0.04			2.0:1.0	3.76	0.02
E	5.72	0.5:1.0	5.59	0.13	a'	3.65	0.5:1.0	3.63	0.02
		1.0:1.0	5.61	0.11			1.0:1.0	3.60	0.05
		2.0:1.0	5.64	0.08			2.0:1.0	3.60	0.05
F, H	6.81	0.5:1.0	6.79	0.04	6-a	3.36	0.5:1.0	3.34	0.02
		1.0:1.0	6.78	0.03			1.0:1.0	3.32	0.04
		2.0:1.0	6.80	0.01			2.0:1.0	3.32	0.04
G	7.49	0.5:1.0	7.47	0.02	6-b	3.32	0.5:1.0	3.31	0.01
		1.0:1.0	7.47	0.02			1.0:1.0	3.29	0.03
		2.0:1.0	7.50	0.01			2.0:1.0	3.28	0.04
I, J	3.87	0.5:1.0	3.82	0.05	1	5.20	0.5:1.0	5.19	0.01
		1.0:1.0	3.82	0.05			1.0:1.0	5.18	0.02
		2.0:1.0	3.85	0.02			2.0:1.0	5.18	0.02
					2	3.73	0.5:1.0	3.72	0.01
							1.0:1.0	3.71	0.02
							2.0:1.0	3.71	0.02
					3	4.06	0.5:1.0	4.05	0.01
							1.0:1.0	4.03	0.03
							2.0:1.0	4.03	0.03
					4	3.60	0.5:1.0	3.58	0.02
							1.0:1.0	3.57	0.03
							2.0:1.0	3.57	0.03
					5	4.45	0.5:1.0	4.43	0.02
							1.0:1.0	4.40	0.05
							2.0:1.0	4.39	0.06

^aIn 0.9% NaCl D₂O at room temperature; Molar ratio: methicillin: compound 1.

^bMolar ratio: methicillin: compound 1.

an inclusion complex was formed in the in 0.9% NaCl D₂O solution. Furthermore, at 1:1 methicillin/compound 1, relatively intense changes of $\Delta\delta$ 0.05 p.p.m. assigned for the protons of the methoxyl groups (labeled as I and J) of methicillin and $\Delta\delta$ 0.03, 0.04, 0.05 p.p.m. in 1 for H-4, H-6 α and H-5, respectively, indicated that the molecule of methicillin was inserted into the hydrophobic cavity of 1 with the methoxyl-substituted benzene located near the bottom of the cavity. Additionally, most large $\Delta\delta$ values of 0.06, 0.07 and 0.11 p.p.m. arranged for the protons (labeled as C, D, and E) on the β -lactam of methicillin, and $\Delta\delta$ 0.05, 0.06 and 0.08 p.p.m. for H-3', 5', -OCH₃, and H-2' 6' in 1 revealed that the β -lactam of the antibiotic was placed close to the corona surface of the cavity. This conformation of the

Table 2 Antibacterial activities for methicillin and 1 alone, and in combination (MIC, mg l⁻¹)

Test article	Bacteria	<i>S. aureus</i>	<i>S. aureus</i>
	Strain (ATCC)	MRSA USA300	MRSA COL
1		50–100	50–100
Linezolid		2.0–4.0	4.0–8.0
Vancomycin		2.0–4.0	1.0–2.0
Methicillin		>128	>128
Methicillin + HP- β -CD		>64	>64
Methicillin + 1		2.0–4.0	2.0–4.0

inclusion complex would keep the β -lactam moiety in active functionality to access to target. Also, the ¹H NMR spectrum showed that the compound 1/methicillin complex was stable in 0.9% NaCl D₂O solution in 12 h.

NOESY experiment of the complex

The non-covalent interaction between compound 1 and methicillin in the inclusion complex was also investigated by an NOESY experiment at the molar ratio of 1:1 methicillin/compound 1. A clear correlation of the peak at δ_{H} 5.61 p.p.m. for proton(s) D or E or both on the β -lactam of methicillin with the signal at δ_{H} 7.24 p.p.m. assigned for H-2' 6' in 1 was observed for a molar ratios of 1:1 methicillin-compound 1 in 0.9% NaCl D₂O solution in an NOESY experiment (Supplementary Figure 3). This indicated that the position of the β -lactam was set in the cavity close to the region of methoxyl-substituted benzenes of 1. This observation was consistent with ¹H NMR studies for the inclusion complex. Notably, this experiment confirmed the conformation of the antibiotic molecule in the inclusion complex with the β -lactam moiety toward the surface of the corona of the cavity.

Antibacterial potentiation of methicillin/compound 1 complex

The antimicrobial activities of methicillin alone and in combination with 1 (the inclusion complex) were measured *in vitro* against bacterial strains MRSA COL and MRSA USA300. As the results indicated in Table 2, the notable MIC values of 2.0~4.0 mg l⁻¹ for methicillin combined with 1 against MRSA COL and MRSA USA300 at a molar ratio of 1:1 methicillin-compound 1, compared with >128.0 mg l⁻¹ for methicillin alone, as well as >64 mg l⁻¹ for a commercially available HP- β -CD combined with methicillin at the same ratio of 1:1, suggested that compound 1 was a strong antimicrobial potentiator for methicillin against resistant bacterial strains MRSA. The antimicrobial potency of the combination of compound 1/methicillin against MRSA was comparable to those of anti-MRSA antibiotics vancomycin and linezolid in this experiment.

DISCUSSION

In summary, in this method for preparation of per-6-(4-methoxybenzyl)-amino-6-deoxy- β -CD provided the final product in higher yield. There were only two steps that followed from an inexpensively starting material β -CD to the product. In corresponding to negative charge of the β -lactam structure, compound 1 was converted into HCl salts (1) with the positive ion nitrogen, which can increase water-solubility and enhance complexation with the antibiotic. Formation and conformation of compound 1/methicillin inclusion complex in

0.9% saline solution were confirmed through the ¹H NMR and NOESY experiments. The antimicrobial tests showed compound 1 had strong potentiation of methicillin *in vitro* against MRSA. Recently, Lim and Strynadka⁹ reported for the first time that structural analysis of the PBP2a active site and complexes of PBP2a with nitrocefin, penicillin G and methicillin reveal that the poor affinities is due to a distorted active site, which must undergo novel conformational changes at strand β 3 and at the helix α 2 N-terminus for acylation of β -lactam with PBP2a to occur. Based on the investigation of the detail NMR data, the specific conformation of 1:1 compound 1/methicillin complex in 0.9% saline solution was presumed to provide better affinity for active β -lactam moiety to accommodate to the narrow active site groove of MRSA PBP2a, and allow stabilization of the interaction for the acylation.^{22,23} This report is an attempt to explore β -CD derivatives as novel potentiators of β -lactam antibiotics against MRSA.

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