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Studying antibiotic–membrane interactions via X-ray diffraction and fluorescence microscopy

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ABSTRACT

Antibiotic drug resistance is a serious issue for the treatment of bacterial infection. Understanding the resistance to antibiotics is a key issue for developing new drugs. We used penicillin and sulbactam as model antibiotics to study their interaction with model membranes. Cholesterol was used to target the membrane for comparison with the well-known insertion model. Lamellar X-ray diffraction (LXD) was used to determine membrane thickness using successive drug-to-lipid molar ratios. The aspiration method for a single giant unilamellar vesicle (GUV) was used to monitor the kinetic binding process of antibiotic–membrane interactions in an aqueous solution. Both penicillin and sulbactam are found positioned outside the model membrane, while cholesterol inserts perpendicularly into the hydrophobic region of the membrane in aqueous solution. This result provides structural insights for understanding the antibiotic–membrane interaction and the mechanism of antibiotics.

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1. Introduction

Acinetobacter baumannii (*A. baumannii*) is an aerobic Gram-negative bacterium that is resistant to most antibiotics [1,2]. Since 1990s, it has been recognized as an important infectious bacterium within hospitals, especially in ICUs [3,4]. A survey conducted in 2007 by the Center for Disease Control in Taiwan indicated that *A. baumannii* ranked the first among common pathogens for nosocomial infections in ICUs. Developing a new type of antibiotic is a priority for the treatment of *A. baumannii* infections. There are no known antibiotics to kill *A. baumannii* effectively. However, it has recently been reported that a compound, sulbactam, killed *A. baumannii* 19606 effectively [5], but the detail mechanism of the bactericidal effect of sulbactam remains unclear. In that report the authors found that when treated with sulbactam the bacterial ATP-binding cascade (ABC) was down-regulated which inhibited the ability of bacteria to uptake nutrients and expel toxic material and subsequently caused bacterial death. We compared the interactions of penicillin, sulbactam, and cholesterol with a model membrane, giant unilamellar vesicle (GUV), by measuring the changes of membrane thickness and surface area of GUV, as well as drug-membrane kinetic binding behavior, to elucidate a possible structural insight for understanding the sulbactam-membrane interaction and the mechanism of bactericidal effect of sulbactam.

Penicillin is the most important antibacterial compound used clinically [6,7] and is historically significant because it was the first antibiotic that was efficient for treating many bacterial infections. The interaction between penicillin and its targeted penicillin binding proteins (PBPs) is the most typical example for elucidating the molecular mechanism of disrupting bacterial growth by antibiotics [8,9]. Various forms of PBP exist and bacteria usually contain many types of PBPs within them. The most common forms of PBPs are grouped into classes 1, 2, 3, and 4 [10,11]. Very early studies showed that higher concentration of antibiotics caused the death of bacteria [12] and interfered with the cell membrane synthesis [13]. Further extensive and laborious studies in 1970s and 1980s then established our present knowledge for the bacteria-killing mechanism of β-lactam antibiotics. In this mechanism, the β-lactam moiety of penicillin binds covalently with the catalytic serine residue on the active site of PBPs [8,14,15]. PBPs are constituent proteins on bacterial membranes and are involved in the synthesis of cross-linked peptidoglycan, which is the major component of bacterial cell walls. Penicillin binds with the

Abbreviations: *A. baumannii*, *Acinetobacter baumannii*; LXD, lamellar X-ray diffraction; GUV, giant unilamellar vesicle

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transpeptidase C-terminal domain of PBPs and inhibits the cross-linking of peptidoglycan [16,17] and eventually leads to bacteria death. A recent crystal structure proved that a native form of PBP (PBP3) becomes covalently linked to antibiotics (carbenicillin and ceftazidime) [18]. In addition, bacteria always have higher internal osmotic pressure than the external one [19]. It has been reported that resisting the osmotic stress is crucial for cell growth and division, as well as maintaining the normal cell shapes. It was found that penicillin could be involved in the osmotic barrier and lying close to the bacteria cell surface [20,21]. Structure defects of peptidoglycan on bacterial cell wall would disrupt the integrity of cell membrane and resulted in the rupture of peptidoglycan [19]. Thus, β-lactam antibiotics such as penicillin would disrupt the osmotic pressure and expedite bacteria death. Hence, penicillin and derivatives have been widely used for treating hospital infections [22]. However, most bacteria soon developed antibiotic-resistance. Sulbactam is a β-lactam analog that is typically given in combination with another antibiotics ampicillin to form a β-lactam–β-lactamase inhibitor. Ampicillin/sulbactam is a common penicillin-derived antibiotic that is used to treat infections caused by bacteria that are resistant to β-lactam antibiotics. In recent years, researchers evaluated the clinical efficacy of ampicillin/sulbactam in the treatment of infections caused by multidrug-resistant *A. baumannii* [23,24]. Currently, ampicillin/sulbactam is the first-line therapy for diverse respiratory and skin infections [25].

Here, we focused on the direct interaction between penicillin, sulbactam and model membranes to clarify this mechanism of inhibition of bacterial growth [5]. Cholesterol is known to interact with membranes by inserting into their hydrophobic region to cause membrane thickening [26–28]. Therefore, cholesterol is used as a good model molecule for comparison with antibiotics.

In this study, we used penicillin and sulbactam as model antibiotics and studied their interactions with model membranes. Cholesterol was used in the same condition for comparison. Lamellar X-ray diffraction (LXD) was used to determine membrane thickness. Thickness changes induced by antibiotics or cholesterol binding to the membrane were extracted from the X-ray data. The giant unilamellar vesicle (GUV) experiment was used to examine the area change of a single GUV in an aqueous solution during the entire binding process.

The structural changes induced by cholesterol partitioning into the membrane were observed not only by LXD but also by GUV measurements. Furthermore, sulbactam and penicillin caused structural changes that were observed by LXD but not GUV measurements. The results of this study show that cholesterol was found perpendicularly inserted to model membrane whereas penicillin and sulbactam were found positioned outside the surface of model membrane in aqueous solution.

2. Materials and methods

2.1. Materials

2.1.1. 1,2-Dierucoyl-sn-glycero-3-phosphocholine (Di22:1PC),

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rhod PE), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Penicillin was purchased from YF Chemical Corp., Taiwan. Sulbactam was purchased from TTY Biopharm, Taiwan.

2.2. Lamellar X-ray diffraction (LXD)

Sample preparation was performed as described in previous reports [29–31]. In brief, antibiotics or cholesterol and lipids of a chosen molar ratio were co-dissolved in methanol and chloroform 1:1 (v/v). A suitable amount of solution was deposited onto a clean glass surface. The sample was vacuumed to remove the remaining solvent and then incubated under high humidity for hydration and rearrangement.

For lamellar X-ray diffraction measurement [30,31], the sample was held in a temperature-humidity controlled chamber (Fig. S1 Supplementary data, schematic of chamber). The machine collected at least five diffraction peaks using the θ–2θ reflectivity mode. The 12 keV X-ray light source of BL13A at the National Synchrotron Radiation Research Center (NSRRC) was used for the measurements. Each θ–2θ scan was measured from $\theta = 0.5^\circ$ to 7.5° with a step angle of $\Delta\theta = 0.001^\circ$ and a 1-s exposure time per step. Every sample was measured at near full hydration and two lower levels of humidity for the purpose of phase determination by the swelling method [32]. Each sample was measured twice to confirm that it was in equilibrium and to prove that it was not damaged by X-ray radiation. X-ray diffraction was used to construct the relative electron density profile along the direction normal to the bilayer surface to determine membrane thickness.

2.3. Giant unilamellar vesicle (GUV) aspiration method

Di22:1PC and a 1% molar ratio of the dye-labeled lipid Rhod PE were co-dissolved in chloroform. The proper amount of solution was deposited on an indium tin oxide coating glass (ITO glass) in the production chamber. The steps of the GUV experiment were described in our previous report [30]. Briefly, GUVs were produced in 100 mM sucrose solution by the electroformation method [33–35]. Then, the isotonic glucose solution was added to the production chamber (Fig. S2(A)) so that the molar ratio of glucose to sucrose outside the GUVs was 3:4. A glass micropipette of 5–10 μm in diameter was used to slightly suck one selected GUV to transfer it to the solution containing antibiotics or cholesterol in the observation chamber (Fig. S2(B)), which was separated from the production chamber. Because cholesterol is insoluble in water, we dissolved cholesterol in methanol first [36]. The protrusion of the GUV in the glass micropipette was monitored during the entire process of the binding of the antibiotics or cholesterol to the GUV [30].

3. Results and discussion

3.1. Membrane-thinning effect examined by LXD

The diffraction patterns of all samples measured at 30 °C and near full hydration are shown in Fig. 1. Penicillin/sulbactam and lipids were mixed to form solutions with P/L molar ratios of 0, 1/50, 1/15, and 1/5. Cholesterol and lipids were mixed to form solutions with P/L molar ratios of 0, 1/50, 1/25, 1/15, 1/10, and 1/5. At least six peaks were recorded for each diffraction pattern. No peak broadening was observed, indicating that fluctuations in the measurements were negligible. To determine the phases of the diffraction peaks, each sample was measured in a series of lowering hydration levels to produce patterns at different repeating distance D values. They were normalized relative to each other by the Blaurock method [32] and plotted as a function of scattering vector Q to determine the phases on the basis of the swelling method [37–39]. A representative example is shown in Fig. 2. After phase determination, the amplitudes of the diffraction patterns were used to remodel the transbilayer electron density profiles. The electron density profiles constructed from the diffraction data are shown in Fig. 3. The peak-to-peak distance (PtP) of the trans-bilayer electron density profile was defined as the membrane thickness. The previous study shows that the repeating distances of

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