Effects of Combined Treatment with Sansanmycin and Macrolides on **Pseudomonas aeruginosa** and Formation of Biofilm

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**Objective** To observe the effects of combined treatment with sansanmycin and macrolides on **Pseudomonas aeruginosa** and formation of biofilm. **Methods** Micro-dilution method was used to determine the minimal inhibitory concentrations (MICs) of sansanmycin, gentamycin, carbenicillin, polymyxin B, roxithromycin, piperacillin, and tazobactam. PA1 and PA27853 biofilms were observed under optical microscope after staining and under SEM after treatment with sansanmycin at different dosages and combined treatment with sansanmycin and roxithromycin. **Results** The MIC of sansanmycin was lower than that of gentamycin and polymyxin B, but was higher than that of carbenicillin. Roxithromycin enhanced the penetration of sansanmycin to PA1 and PA27853 strains through biofilms. PA1 and PA27853 biofilms were gradually cleared with the increased dosages of sansanmycin or with the combined sansanmycin and roxithromycin. **Conclusion** Sub-MIC levels of roxithromycin and sansanmycin substantially inhibit the generation of biofilms and proliferation of bacteria. Therefore, combined antibiotics can be used in treatment of intractable bacterial infection.

**Key words:** Sansanmycin; Macrolides; **Pseudomonas aeruginosa**; Biofilm

**INTRODUCTION**

Sansanmycin, a novel antibiotic, was found from an unidentified Streptomyces sp SS (CGMCC No. 1764) which was isolated from a soil sample in Guizhou province of China[1]. As the previous research has shown, it is a narrow spectrum antibiotic and its effect is focused on **Pseudomonas aeruginosa** and Mycobacterium tuberculosis. Chemical and spectroscopic analyses revealed that it belongs to the class of nucleosidyl-peptide antibiotics[11]. Unlike other antibiotics, it rarely causes dysbacteriosis, and can thus be extensively used in clinical practice. **Pseudomonas aeruginosa** is a clinically important opportunistic pathogen and often causes respiratory infection, burn infection and complications of cystic fibrosis infection[2,3]. During the infection period, **Pseudomonas aeruginosa** may survive in another state to form biofilm which is one of the main mechanisms of antibiotic resistance and occurs in corpore detaining medical treatments, like prosthetic replacement of joints, heart valve replacement, artery and venous duct, trachea cannula and infection of mechanical ventilation. This kind of infection revokes the aggravation of diseases and makes them intractable[4,5].

The purpose of the study was to identify the effective approach to cure the infection with **Pseudomonas aeruginosa** and its biofilm using the novel antibiotic sansanmycin in combination with macrolides.

**MATERIALS AND METHODS**

**Antibiotics**

Sansanmycin was prepared by the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences. Gentamycin, carbenicillin, polymyxin B, and roxithromycin are commercially

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available antibiotics.

**Bacteria**

Clinical segregation strains of *P. aeruginosa* PA1, PA3, PA5, PA2059 were presented as a gift from the General Hospital of Tianjin Medical University. Type strain ATCC27853 was purchased from the National Institute for the Control of Pharmaceutical and Biological Products.

*Comparison of Cell Adhesion Ability of Different Pseudomonas aeruginosa Strains*

A cover glass was put into a 6-well plate and 1x10^4/mL Hela cells were added into each well. The plate was incubated at 37 °C in 5% CO_2 for 18 h. The single clone of clinical strain PA1 and the type strain ATCC27853 were inoculated in 50 mL pancreatin soy broth respectively. The bacteria were then collected after shaking at 150 rpm overnight at 37 °C. The suspension was centrifuged at 2000 rpm for 30 min and washed three times with phosphate buffered saline (PBS). The concentration of bacteria was adjusted with Dulbecco’s minimum essential medium (DMEM) to 1.5x10^7/mL. Wells were washed twice with PBS, and 2 mL of bacterial suspension was added into each well. The plate was shaken at 80 rpm for 1 h at 37 °C. After washed three times with PBS, the cover slip was taken out. After dried in open-air, the cover slip was fixed with methanol for 15 min, washed successively with double-distilled water, and then stained with safranine and observed under a microscope.

*Comparison of Effects of Sansanmycin and Other Antibacterials against Pseudomonas aeruginosa and Synergistic Actions of Combined Antibiotics*

Diluents of sansanmycin, polymycin B, gentamycin, carbenicillin, roxithromycin, combined pipercillin and tazobactam, and combined sansanmycin and roxithromycin at multiple proportions were prepared with their final concentrations at 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 μg/mL, respectively. A 96-well plate was used for each drug. Blank control and positive control were set accordingly. Each kind of drug at various concentrations was added to each well at the dosage of 100 μL followed by the same dosage of bacteria. The original density of bacteria was 0.5 Mcb turbidimetric, and the working concentration was diluted with Muller-Hinton broth at 1:1000. The plate was incubated at 37 °C for 16-20 h. The lowest concentration of drugs without bacteria in the well was defined as the minimal inhibitory concentration (MIC). The chessboard microdilution method was used to combine the drugs and each drug was added at the dosage of 50 μL, and two of the drugs were added to the micro-well plate by cross combination. The rest experiment was the same as previously described.

*Establishment of in vitro Biofilm Model*

The cultured clone of *P. aeruginosa* was inoculated in 20 mL TSB and shaken at 170 rpm for 4.5 h. The concentration of bacteria was adjusted to 0.5 Mcb turbidimetric. The gel silica film sterilized with anticyclone was put into a 24-well plate. Two mL TSB and 50 μL bacterial suspension were put into each well, cultured at 37 °C for 7 days. The broth was changed every two days. The growth of biofilm was observed under a phase-contrast microscope.

*Effect of Combined Sansanmycin and Roxithromycin on Biofilm*

The bacterial suspension (0.5 Mcb turbidimetric) was diluted to 4.5x10^6, 2.25x10^6, 1.13x10^6, 5.6x10^5, 2.8x10^5, 1.4x10^5, 7.0x10^5, 3.5x10^5, 1.75x10^5, 8.7x10^5, 4.3x10^5, and 2.1x10^5 CFU/mL, respectively. Each concentration of the bacterial suspension was added into a 96-well plate at the dosage of 190 μL and MTT (5 mg/mL) was added at the dosage of 20 μL, cultured at 37 °C for 2 h. After misce bene, 90 μL

\[
\text{The survival rate of cells} = \frac{\text{Mean OD value of the experiment group} - \text{mean OD value of the blank group}}{\text{Mean OD value of the cell control group} - \text{mean OD value of the blank group}}
\]
lysate was added into each well and the optical density (OD) at 595 nm was determined after complete schizolysis to make a standard curve to determine the number of bacteria in the biofilms by the OD value. The degree of precision was under strict control.

The chessboard microdilution method was used to combine sansanmycin and roxithromycin. The concentration of sansanmycin was 0.1/4, 1/2 and 1MIC, respectively, and the concentration of roxithromycin was 0.1/16 and 1/4MIC, respectively. The combination of these two drugs was put into a 24-well plate, then the silica gel film adhered to the *Pseudomonas aeruginosa* with formation of biofilms was put into each well. The 24-well plate was incubated at 37 °C for 24 h in an incubator containing 5% CO₂. The film was taken out and washed several times with normal sodium (NS) to remove the floating bacteria and then put into a tube containing 1 mL MH broth. Ultrasonic and whirlpool were used to wash the bacteria off to the broth completely. The rest experiment was the same as the description of the standard curve. The number of bacteria was calculated by the standard curve according to the OD value.

**Morphological Change of Pseudomonas aeruginosa after Combined Treatment with Sansanmycin and Roxithromycin**

Observation of Biofilm under Microscope after Silver Staining  The experiment was performed in PA1 blank group and Sansanmycin treatment group at the dosage of 32 μg/mL, 16 μg/mL, 8 μg/mL, and 4 μg/mL respectively. The division of groups was the same for type strain PA27853. The dosage of sansanmycin and roxithromycin was 4, 1, and 0.25 μg/mL, respectively. The chessboard microdilution method was used to combine sansanmycin and roxithromycin. The establishment of biofilm model was described previously. During cultivation of the biofilm, drugs at different concentrations were put into each well respectively. The gel silica film was taken out after 7 days. After washed several times with NS to remove the floating bacteria, the gel silica film was fixed with 2.5% glutaral for 1 h and washed once with distilled water. Saturated calcium chloride was added to each well and removed 15 min later. The film was washed with distilled water for 1 min, treated with 5% argent nitrate for 15 min, stained with 1% hydroquinone for 2 min, washed with distilled water for 1 min, and fixed with 5% sodium thiosulfate for 2 min. Finally, the film was washed with distilled water for 1 min and observed under a microscope at 1000×. Pictures of biofilm were taken and grey scale of the gel silica film was analyzed using the computer image analysis system (MediaImages Advanced 3.1). Statistical analysis was performed using ANOVA single factor analysis with SPSS.

Observation of Biofilm under Scanning Electron Microscope  A biofilm model was established as described above. Drugs and groups were divided as described in silver staining preparation. Carriers of the biofilm were taken out, washed several times with NS to remove the floating bacteria, fixed with 2.5% phosphoric acid glutaraldehyde buffer for 3 h, washed three times with PBS for 15 min, then dehydrated for 15 min in 50%, 70%, 95%, and 100% alcohol, respectively. The step at 100% concentration was performed in triplicate. The carriers of biofilm were put into a container with hexamethyldisilane for 2-3 min and taken out to be dried, put into a container with self-indicating silica gels and dried. The silica gels were put on a glass slide in order and gilded at the circumstance of roughing vacuum by ion sputtering and a picture was taken under a scanning electron microscope.

**RESULTS**

Adhesion Difference  The adhesive differences of PA1 and PA27853 in Hela cells are shown in Table 1. SPSS was used to do *t* test and the *P* value was 0.275 (*P*<0.05). The results indicate that the adhesion differences in cells of PA1 and PA27853 were statistically significant. PA1 was easier to adhere to Hela cells than PA27853.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Adhesiveness (Bacteria/Cells)</th>
<th>Group1</th>
<th>Group2</th>
<th>Group3</th>
<th>Group4</th>
<th>Group5</th>
<th>Total (X ± s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA1</td>
<td></td>
<td>358</td>
<td>373</td>
<td>386</td>
<td>397</td>
<td>412</td>
<td>385.2±20.9</td>
</tr>
<tr>
<td>PA27853</td>
<td></td>
<td>239</td>
<td>190</td>
<td>233</td>
<td>277</td>
<td>280</td>
<td>243.8±36.9</td>
</tr>
</tbody>
</table>
**Results of Cytotoxicity Experiment**

The cell survival rate at different concentrations of sansanmycin. As detected using EXCEL They was 151%, 138%, 122%, 115%, 107%, 108%, and 109%, respectively. Our finding suggests that sansanmycin might have no cytotoxicity to Vero cells but might have a certain effect on cell proliferation, which needs to be further confirmed.

**MIC Values**

The MIC values for antibiotics against *Pseudomonas aeruginosa* are shown in Tables 2 and 3. From Table 2, we could see that the MIC value for gentamycin was the lowest and gentamycin was the best antibiotics against *Pseudomonas aeruginosa*. The effect of polymycin was better than sansanmycin and carbenicillin. From Table 3, we could find that roxithromycin itself had no effect on antipseudomonas. However, roxithromycin in combination with sansanmycin could substantially enhance the effect on *pseudomonas*. The combined effect was better than that of a single drug. The effect of combined piperacillin and tazobactam was promising.

**TABLE 2**

<table>
<thead>
<tr>
<th>Sansanmycin</th>
<th>Roxithromycin</th>
<th>Gentamycin</th>
<th>Carbenicillin</th>
<th>Polymycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA1</td>
<td>32</td>
<td>256</td>
<td>0.5</td>
<td>64</td>
</tr>
<tr>
<td>PA3</td>
<td>32</td>
<td>256</td>
<td>0.5</td>
<td>64</td>
</tr>
<tr>
<td>PA5</td>
<td>32</td>
<td>256</td>
<td>0.5</td>
<td>128</td>
</tr>
<tr>
<td>PA2059</td>
<td>64</td>
<td>256</td>
<td>0.5</td>
<td>256</td>
</tr>
<tr>
<td>PA27853</td>
<td>256</td>
<td>256</td>
<td>0.5</td>
<td>64</td>
</tr>
</tbody>
</table>

**TABLE 3**

<table>
<thead>
<tr>
<th>Sansanmycin and Roxithromycin</th>
<th>Piperacillin and Tazobactam</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA1</td>
<td>16+16</td>
</tr>
<tr>
<td>PA3</td>
<td>16+16</td>
</tr>
<tr>
<td>PA5</td>
<td>16+16</td>
</tr>
<tr>
<td>PA2059</td>
<td>16+16</td>
</tr>
<tr>
<td>PA27853</td>
<td>16+16</td>
</tr>
</tbody>
</table>

The effects of sansanmycin and combined sansanmycin and roxithromycin at different concentrations on biofilm formation are shown in Table 4. From Table 4, we could see that after combined treatment with sansanmycin and roxithromycin, the number of bacteria in the biofilm was dropped dramatically. The difference was statistically significant ($P<0.05$).

**TABLE 4**

<table>
<thead>
<tr>
<th>Group</th>
<th>Experiments ($n$)</th>
<th>Switching Values for Bacteria ($\bar{x} \pm s$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>PA1: 5.817 ± 1.893, PA27853: 6.868 ± 4.384E-02</td>
</tr>
<tr>
<td>Group without Roxithromycin</td>
<td>6</td>
<td>PA1: 7.638 ± 1.134, PA27853: 8.441 ± 0.111</td>
</tr>
<tr>
<td>Group with Roxithromycin at the Dose of 16 μg/mL</td>
<td>8</td>
<td>PA1: 6.098 ± 1.269, PA27853: 6.921 ± 1.012</td>
</tr>
<tr>
<td>Group with Roxithromycin at the Dose of 64 μg/mL</td>
<td>8</td>
<td>PA1: 7.431 ± 0.983, PA27853: 9.327 ± 0.541</td>
</tr>
</tbody>
</table>

$F$: 3.093, $p$: 0.050*; $q(1)$: (2) = 1.883, $q(1)$: (3) = 0.3, $q(1)$: (4) = 1.724, $q(2)$: (3) = 2.408*, $q(2)$: (4) = 0.324, $q(3)$: (4) = 0.251*, $q(2)$: (4) = 5.217*

**Note.** *$P<0.05$.**
Biofilm Formation Observed after Silver Staining

The carrier after argentation was observed under an immersion objective microscope (×1000) and pictures were taken under Motic digital and biological microscope. The results are shown in Fig. 1. The graphs of the models were observed under a microscope after argentation. A blackly stained thickening substance interlaced in the silica gel film and rod bodies were observed at the interspace, suggesting that the model was successfully established.

After treatment with sansanmycin at an increasing concentration, the density of the blackly stained thickening substance decreased and the results of combined treatment were the same. Especially when the concentrations of sansanmycin and roxithromycin were the highest, the density of the blackly stained thickening substance was the lowest. The results of PA27853 were the same as those of PA1.

The gray scale of graphs and the data were analyzed with MediaImages Advanced 3.1 and SPSS and ANOVA, respectively (Table 5).

![Biofilm of PA1](image1)

![Biofilm treated with 4μg/mL sansanmycin](image2)

![PA1 biofilm treated with 4μg/mL sansanmycin](image3)

![PA1 biofilm treated with combined treated with sansanmycin and roxithromycin (32μg/mL)](image4)

**Fig. 1. Silver-stained biofilm of PA1.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Experiments (n)</th>
<th>Mean Value for Gray Scale (X ± s)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>133.5299±0.70916</td>
<td>119.9701±5.55439</td>
</tr>
<tr>
<td>Sansanmycin-treated Group</td>
<td>4</td>
<td>114.5652±2.80957</td>
<td>112.4047±3.23785</td>
</tr>
<tr>
<td>Combined Sansanmycin and Roxithromycin</td>
<td>5</td>
<td>115.9585±3.46511</td>
<td>112.7244±1.24432</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>30.233</td>
<td>5.229</td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>0.000</td>
<td>0.035</td>
</tr>
<tr>
<td>q(1): (2)</td>
<td>-</td>
<td>7.289*</td>
<td>2.985*</td>
</tr>
<tr>
<td>(1): (3)</td>
<td>-</td>
<td>6.990*</td>
<td>2.96*</td>
</tr>
<tr>
<td>(2): (3)</td>
<td>-</td>
<td>0.691</td>
<td>0.163</td>
</tr>
</tbody>
</table>

*Note.* *P*≤0.05.
**Biofilm Formation Observed under Scanning Electron Microscope**

The observations under scanning electron microscope (SEM) are shown in Fig. 2. Bacterial PA1 in a short rod shape was gathered, agglomerated, and surrounded by thick mucosal fluid under SEM. Thalline was stuck to one another and the biofilm was hence constructed. When treated with increasing concentrations of sansanmycin, the number of bacteria decreased, the extent of aggregation was lower, and the mucoid substance in bacteria dropped significantly. Combined sansanmycin and roxithromycin at the highest dose had the best curative effect. Its curative effect on PA27853 and PA1 was similar.

**DISCUSSION**

**Antibiotic Resistance Mechanisms**

*P. aeruginosa* has a strong ability to adjust itself to the environment and its drug resistance is a big problem in clinical practice. *P. aeruginosa* has almost the biggest genome among all bacteria. Nowadays, the function of its many genes is still unknown. No matter how hard we try to design the treatment protocol, the therapeutic efficacy remains discouraging. The drug resistance mechanism of *P. aeruginosa* is mainly due to its genome regulation which adjusts itself to the environment. It has the genetic ability to express a wide repertoire of resistance mechanism. Its general resistance is due to a combination of the following factors:

1) Alginate and formation of biofilm constitute a barrier which can hamper the drug infiltration;

2) It is intrinsically resistant to antimicrobial agents due to the low permeability of its cell wall;

3) The efflux pump plays an important role in the drug resistance. All antibiotics except for polymyxin are sensitive to one or more efflux systems. Up-regulation of MexA-MexB-OprM arises at a high frequency as a coregulation of nalB mutation at the mexR locus;

4) The passive effect of antibiotics, which can acquire additional resistance genes from other organisms via plasmids, transposons, and bacteriophages;

5) The alternation of drug targets.

**Virulence Factors of *P. aeruginosa***

The virulence factors of *P. aeruginosa* are surface factors including flagellae, pilus, LPS, and secretion factors including exoproducts, type III secretion protein, quorum-sensing molecular, and alginate. *P. aeruginosa* has two kinds of strains, one is the non-mucoid strain, the other is the mucoid
strain. Mathee et al.\textsuperscript{[13]} performed an experiment using a low concentration of H$_2$O$_2$ to mimic the \textit{in vivo} polymorphonuclear neutrophilic leukocytes (PMN) which release H$_2$O$_2$ to induce PAO1, a typical non-mucoid strain to convert to mucoid variants, and found that a gene mutation in mucA produces mucA22 variant which can produce 2-6 times of alginate that of non-mucoid strain. Alginate, the main ingredient of biofilm, can make the treatment more difficult.\textsuperscript{[14]}

Treating Strategy for \textit{P. aeruginosa} Biofilms and mechanisms of Macrolides

\textit{P. aeruginosa} biofilms are formed in individual bacterial cells embedded in an extracellular polysaccharide matrix. Quorum-sensing (QS) allowing bacteria to “sense” the density of bacterial population plays a very important role in the formation of biofilms. QS \textit{P. aeruginosa} consists of three systems. The first and second systems are directly interrelated with N-acylhomoserine lactone (AHL)-dependent systems las and rhl, while the third system employs 2-heptyl-3-hydroxy-4-quinolone, the Pseudomonas QS (PQS)\textsuperscript{[15]}. There are three possibilities to target and inhibit QS, and in this way, formation of biofilms can be prevented by blocking AHL production, inactivating signal molecules, and jamming the signal receptor which is most extensively studied in context of drug development\textsuperscript{[16]}. One of the potential compounds of interest is the macrolide azithromycin, which is normally used against gram-positive bacteria. Recently, its impact on the transcription and protein expression level of \textit{P. aeruginosa} has been tested under infection-related conditions. Azithromycin inhibits the QS regulator as well as formation of biofilms\textsuperscript{[17]}, and affects the polymerization of alginate\textsuperscript{[18]}. Its QS inhibitory properties have also been confirmed \textit{in vivo} in a CF mouse model\textsuperscript{[18]}. It was reported that macrolides themselves have no effects on \textit{P. aeruginosa}, but their combination with other drugs has a synergistic effect on biofilm formation\textsuperscript{[10]}. In the present study, we discovered a new antibiotic and tried to combine it with macrolides to enhance its effect on \textit{P. aeruginosa}, especially on the formation of biofilms. Our results are consistent with the reported data\textsuperscript{[19]}.

Our Findings and Future Steps

Sansamycin, a fermentation product of Streptomyces sp SS, possesses antipseudomonous effects\textsuperscript{[1]}. The effect is significantly enhanced by combined piperacillin and tazobactam. This is why we intended to enhance the effect of sansamycin on \textit{P. aeruginosa}.

In this study, the effects of sansamycin and its combination with roxithromycin on \textit{P. aeruginosa} and formation of biofilms were observed. The initial results provide a new strategy for the treatment of \textit{P. aeruginosa} infection and are of great value for its extensive application in clinical practice. It is hypothesized that combined antibiotics can effectively overcome the resistance of bacteria to antibiotics. More evidence can be identified. For instance, \textit{in vitro} studies have shown that the number of biofilm-growing bacteria can be reduced to 20% after treatment with high doses of combined antibiotics (piperacillin+tobramycin). Furthermore, it was reported that sub-MIC concentrations of antibiotics can suppress the production of exoproducts such as protease, phospholipase C, alginate of \textit{P. aeruginosa} and colistin that binds to LPS of \textit{P. aeruginosa}\textsuperscript{[19]}.

Experiments have proved that combined macrolide antibiotics and β-lactam antibiotics can reduce the quantity and vigor of \textit{P. aeruginosa} infection in CF and DPB patients. The chronic inhibitory effect of such treatment is related to the amelioration of pulmonary function and can last one to two months after treatment\textsuperscript{[20]}. Chronic suppressive therapy with long-term daily erythromycin can significantly reduce symptoms and inflammatory parameters and increases 10-year survival rate from 12% to over 90% in diffuse panbronchiolitis (DPB) patients\textsuperscript{[20]}. Similar results have been obtained after treatment with macrolides and fluoroquinolones. A similar effect of chronic suppressive therapy has been reported in CF patients with chronic \textit{P. aeruginosa} infection\textsuperscript{[21]}. The efficacy of macrolides lacking of bacteriostatic or bactericidal effect against \textit{P. aeruginosa} was studied \textit{in vitro} and in animal models, which may be due to a sub-MIC inhibitory effect on the production of proteins such as the exoproteases of \textit{P. aeruginosa}, interference with biofilm matrix\textsuperscript{[22]}, anti-inflammatory activity\textsuperscript{[23]} and interference with the QS systems\textsuperscript{[17]}. As the next step, we will establish an \textit{in vivo} mouse model of \textit{P. aeruginosato} observe the effect of sansamycin and its combination with azithromycin by intramuscular injection. Moreover, we will try to explore the mechanism of sansamycin and its synergistic effects with other drugs by proteo-metabolic and immunological approaches.

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