SYNERGISTIC ACTIVITY OF ASTROMICIN AND β-LACTAM ANTIBIOTICS AGAINST PSEUDOMONAS AERUGINOSA
IN VITRO AND IN VIVO

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Synergistic activity of astromicin and an antipseudomonal β-lactam antibiotic such as piperacillin, cefsulodin or carbenicillin against Pseudomonas aeruginosa was demonstrated in vitro and in vivo.

Synergy in vitro was observed more often when astromicin was combined with piperacillin or cefsulodin than when it was combined with carbenicillin. The combination of astromicin with piperacillin showed a bactericidal activity against Pseudomonas aeruginosa at a bacteriostatic concentration of each antibiotic alone.

The synergy observed in vitro was reproduced against experimental mouse infections, and the astromicin-piperacillin or cefsulodin combination produced significantly greater protective effects than the single use of individual antibiotics.

The incidence of Pseudomonas aeruginosa infections has shown an increase in recent years, and it is well recognized that P. aeruginosa is among the most resistant microorganisms to commonly used antibiotics1).

Serious infections due to P. aeruginosa are often treated with a combination of an aminoglycoside antibiotic and an antipseudomonal β-lactam antibiotic such as carbenicillin or sulbenicillin to achieve additive or synergistic antibacterial activity.

Astromicin (ASTM)*, an aminoglycoside antibiotic containing the novel aminocyclitol and fortamine, is highly active in vitro and in vivo against most Gram-positive and Gram-negative bacteria2-4). This antibiotic is characterized by stability to aminoglycoside-inactivating enzymes except AAC(III)-15), and low-grade oto- and nephro-toxicity5). It shows low activity against P. aeruginosa however, and it is marketed as fortimicin A.

The present report describes a synergistic relationship of astromicin with β-lactam antibiotics against P. aeruginosa strains both in vitro and in vivo.

Materials and Methods

Antibiotics
Piperacillin (PIPC) was purchased from Toyama Chemical Co., Ltd.; cefsulodin (CFS) and carbenicillin (CBPC) were from Takeda Pharmaceuticals Co., Ltd. ASTM was prepared in Kyowa Hakko Kogyo Co., Ltd.

Organisms
The laboratory strains were maintained on heart infusion agar (HIA, Eiken Chemicals, Tokyo).

* Previously, astromicin was called fortimicin A.
Clinical isolates were kindly supplied by several clinical laboratories and maintained in cooked meat medium (Nissui Seiyaku Co., Ltd., Tokyo).

**Antibiotic Susceptibility Testing**

MICs of antibiotics were measured by the two-fold serial dilution method using HIA plates with an inoculum of $10^8$ colony forming units (cfu). The MIC values ($\mu$g/ml) were read after 18 hours of incubation at 37°C.

**Synergy In Vitro**

The MICs of ASTM in combination with each β-lactam antibiotic against 27 strains of *P. aeruginosa* were determined by the checkerboard method using HIA at an inoculum of $10^8$ cfu. After 18 hours of incubation at 37°C, the MICs of the β-lactam antibiotic and ASTM used alone or in combination were defined by no visible growth on plates.

The fractional inhibitory concentration (FIC) index was calculated by dividing the MIC obtained for each of the components in the combination by that for each component alone and adding the two quotients. Criteria for the effects of combination were defined as follows:

1) Synergy — the index was $\leq 0.5$
2) Partial synergy — the index was $>0.5 - 0.75$
3) Indifferent — the index was $>0.75 - 1.0$
4) Antagonism — the index was $>1.0$

**Bactericidal Activity**

Rates of killing of two *P. aeruginosa* strains by ASTM alone and in combination were measured in heart infusion broth (Difco Laboratories, U.S.A.) using a fresh dilution of organisms from an overnight culture in Trypticase soy broth (Nissui Seiyaku Co., Ltd., Tokyo). After incubation for 2 hours at 37°C with shaking, serial two-fold dilutions of ASTM and PIPC were added and samples of culture were taken at selected intervals, immediately diluted in broth, and plated on HIA. The cfu was counted after overnight incubation.

**Synergy In Vivo**

Male ddY mice, weighing 18 to 20 g, were infected intraperitoneally with suspensions of test organism in 5% hog gastric mucin (ICN Pharmaceuticals, Inc., U.S.A.) in a volume of 0.5 ml. The challenge dose was approximately minimal lethal doses (inoculum size: $0.7 \times 10^4 - 0.5 \times 10^5$ cells/mouse). The antibiotics were administered subcutaneously to a group of 10 mice 2 hours after infection. Based on the survival rate on the 7th day of infection, the 50% effective dose (ED$_{50}$) and its confidence limits were calculated by the LITCHFIELD-WILCOXON method. The extent of synergy of a combination was expressed as the synergistic ratio (SR). The SR, which corresponds to the FIC index, is a ratio of experimentally determined potency of the combined drugs over a hypothetical potency in which additive effect of both drugs is assumed. Synergy *in vivo* was considered to have occurred if the lowest 95% confidence limit of the SR was greater than 1.0.

**Results**

**Antibacterial Activity In Vitro**

*In vitro* susceptibilities of 27 strains of *P. aeruginosa* to ASTM, PIPC, CFS and CBPC are shown in Fig. 1.

ASTM inhibited 15 of 27 strains of *P. aeruginosa* at a concentration of 25 $\mu$g/ml. It was 2 to 4 times more active than CBPC, slightly less active than PIPC, but much less active than CFS. CFS was the most active of the four antibiotics against strains of *P. aeruginosa*, with an MIC$_{50} \leq 6.25$ $\mu$g/ml.

**Synergy In Vitro**

The FIC indices of the combination of ASTM and β-lactam antibiotics using the checkerboard titration method on 27 strains of *P. aeruginosa* are summarized in Table 1.
The ASTM-PIPC combination was most synergistic. A synergistic effect was observed more often when ASTM was combined with PIPC (17 strains, 63.0%) or CFS (15 strains, 55.6%) than when it was combined with CBPC (8 strains, 29.6%). Synergy plus partial synergy were produced against all strains by the ASTM-PIPC combination, against 24 (88.9%) and 23 (85.2%) by the ASTM-CFS and ASTM-CBPC combinations, respectively.

**Table 1. Combinations of ASTM with PIPC, CFS or CBPC against *P. aeruginosa* (27 strains).**

<table>
<thead>
<tr>
<th>Combination</th>
<th>Synergy</th>
<th>Partial synergy</th>
<th>Indifferent</th>
<th>Antagonism</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASTM+PIPC</td>
<td>17</td>
<td>10 (63.0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ASTM+CFS</td>
<td>15</td>
<td>9 (33.3)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>ASTM+CBPC*</td>
<td>8</td>
<td>15 (55.6)</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

* FIC index of one strain was not determined.

The ASTM-PIPC combination was most synergistic. A synergistic effect was observed more often when ASTM was combined with PIPC (17 strains, 63.0%) or CFS (15 strains, 55.6%) than when it was combined with CBPC (8 strains, 29.6%). Synergy plus partial synergy were produced against all strains by the ASTM-PIPC combination, against 24 (88.9%) and 23 (85.2%) by the ASTM-CFS and ASTM-CBPC combinations, respectively.

**Bactericidal Effect**

The comparative growth of *P. aeruginosa* F-1997 and E-2 in the presence of ASTM or PIPC alone and in combination are shown in Fig. 2.

Against *P. aeruginosa* strain F-1997, the addition of a sub-lethal concentration of PIPC resulted in a marked increase in the rate of bacterial killing of ASTM. Against *P. aeruginosa* strain E-2, the rate of bacterial killing was also clearly enhanced by the drug combination.
Fig. 2. Comparative growth curves of *P. aeruginosa* strain F-1997 (A) and strain E-2 (B) in the presence of ASTM or PIPC alone and in combination.

- Control, ○ astromicin 3.13 µg/ml, △ piperacillin 3.13 µg/ml, ■ astromicin 3.13 µg/ml + piperacillin 3.13 µg/ml.

Table 2. Synergistic effect of ASTM, PIPC or CFS against intraperitoneal *P. aeruginosa* injection in mice.

<table>
<thead>
<tr>
<th>Strain* &amp; inoculum (cells/mouse)</th>
<th>FIC index</th>
<th>Antibiotic** (MIC, µg/ml)</th>
<th>Combination ratio</th>
<th>ED$_{50}$ (mg/kg)</th>
<th>Synergistic ratio</th>
<th>Confidence limits (P=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-2043 0.5 x 10$^9$</td>
<td>0.19</td>
<td>ASTM (100)</td>
<td>1:5</td>
<td>29.3</td>
<td>2.1</td>
<td>1.4~3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PIPC (200)</td>
<td></td>
<td>7,060</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASTM + PIPC</td>
<td></td>
<td>80.3</td>
<td>3.2</td>
<td>2.1~5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:10</td>
<td>95.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-1997 0.7 x 10$^4$</td>
<td>0.16</td>
<td>ASTM (100)</td>
<td>1:5</td>
<td>90.3</td>
<td>2.2</td>
<td>1.5~3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PIPC (25)</td>
<td></td>
<td>639</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASTM + PIPC</td>
<td></td>
<td>143</td>
<td>2.0</td>
<td>1.4~2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:10</td>
<td>208</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-2 0.8 x 10$^6$</td>
<td>0.38</td>
<td>ASTM (25)</td>
<td>1:5</td>
<td>208</td>
<td>2.1</td>
<td>1.2~3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PIPC (6.25)</td>
<td></td>
<td>2,868</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASTM + PIPC</td>
<td></td>
<td>446</td>
<td>1.8</td>
<td>1.0~3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:10</td>
<td>740</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KY-8512 0.5 x 10$^6$</td>
<td>0.38</td>
<td>ASTM (12.5)</td>
<td>1:1</td>
<td>137</td>
<td>3.7</td>
<td>1.4~9.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CFS (6.25)</td>
<td></td>
<td>297</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASTM + CFS</td>
<td>1:5</td>
<td>50.5</td>
<td>3.7</td>
<td>0.6~3.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:10</td>
<td>167</td>
<td>1.5</td>
<td>0.8~4.7</td>
</tr>
</tbody>
</table>

* *P. aeruginosa* was inoculated with 5% mucin.
** Antibiotic was administered subcutaneously 2 hours after injection.
Synergy In Vivo

To ascertain further the effect of combinations in vivo, the efficacy of ASTM, PIPC or CFS alone and in combination, was measured against murine peritoneal infections due to P. aeruginosa. In Table 2 the results of in vivo tests performed with 4 strains of P. aeruginosa are summarized. The ED₅₀ values of the ASTM-PIPC combination at a ratio of 1:5 or 1:10 were definitely decreased as compared with that of each antibiotic alone.

The combination showed synergistic activity with SR values of 1.8 to 3.2 against all 3 strains of P. aeruginosa tested.

On the other hand, the ASTM-CFS combination at a ratio of 1:5 or 1:10 resulted in an additive effect against infection due to strain KY-8512. A synergistic effect was observed at a ratio of 1:1 with this combination however.

These results support the view that the synergistic effect in fact occurs in vivo as well as in vitro.

Discussion

Various combinations of antibiotics and chemotherapeutic agents are now used to obtain an extended antibacterial spectrum, to increase antibacterial activity and to prevent the appearance of resistant bacteria. ASTM, a new aminoglycoside antibiotic, has antimicrobial activity similar to that of amikacin, but lacks significant activity against P. aeruginos₃₄₁₀). It has been found previously however that there is often a synergistic antimicrobial effect between aminoglycosides and β-lactams₃₁₄).

The results of experiments reported here show that the combination of ASTM with antipseudomonal β-lactam antibiotics exhibited partially synergistic or synergistic activity against most strains of P. aeruginosa tested. In particular, the ASTM-PIPC combination showed partially synergistic or synergistic activity against all strains tested as well as strong bactericidal activity at a bacteriostatic concentration of each antibiotic alone. The synergy observed in vitro was reproduced against experimental mouse infections, and ASTM-PIPC or ASTM-CFS combinations produced a significantly greater protective effect than the individual antibiotics. Recently, YAMASHITA et al.) reported that the combination of ASTM with β-lactam antibiotics such as CBPC or PIPC may act synergistically in vitro against P. aeruginosa including ASTM- and GM-resistant strains.

The present studies report the first demonstration of ASTM-PIPC and ASTM-CFS synergy in vivo for infections caused by isolates of P. aeruginosa, against which these antibiotics are synergistic in vitro, and provide experimental support for further clinical trials. Taking into consideration that peak serum levels achieved after 200 mg intramuscular injection of ASTM in patients were around 12 μg/ml, this antibiotic combination therapy seems promising.

References


