Cefroxadine (CGP-9000), an Orally Active Cephalosporin

K. YASUDA,1 S. KURASHIGE,2 AND S. MITSUHASHI*2

Department of Microbiology, School of Medicine, Gunma University, Maebashi, Gunma,2 and Medical Department, Pharmaceuticals Division, CHIBA-GEIGY Limited, Takarazu,1 Japan

Cefroxadine (CGP-9000; CXD), 7β-p-2-amino-2-(1,4-cyclohexadienyl)-acetamido]-3-methoxy-ceph-3-em-carboxylic acid, is a new orally active cephalosporin derivative. The spectrum of antibacterial activity of CXD is identical with that of cephalaxin (CEX), but CXD was twofold more effective than CEX against Escherichia coli and Klebsiella pneumoniae. CXD was as stable to penicillinase as CEX, but it was hydrolyzed by cephalosporinase, with a relative rate of hydrolysis similar to that of CEX. The affinities of CXD and CEX to penicillin-binding proteins of E. coli were estimated; the affinity of CXD to penicillin-binding protein 1Bs was higher than that of CEX. Consistent with this, CXD had more intensive lytic activity than CEX. In vivo antibacterial activities of CXD and CEX were compared using systemic infections of mice with E. coli and K. pneumoniae; CXD was consistently more active than CEX.

Although there are many cephalosporins available that are suitable for parenteral administration, orally active cephalosporins are less common. Among oral cephalosporins introduced on the market, cephalaxin (CEX) is now in widespread use in the treatment of a variety of infectious diseases. Cefroxadine (CXD) is the dihydrophenylglycin derivative of a structurally modified 7-amino-cephalosporanic acid and is structurally related to CEX, but possesses antibacterial properties that distinguish it from CEX. This paper presents the in vitro and in vivo microbiological evaluation of CXD.

MATERIALS AND METHODS

Antibiotics. CXD, 7β-p-2-amino-2-(1,4-cyclohexadienyl)-acetamido]-3-methoxy-ceph-3-em-carboxylic acid, is an orally active cephalosporin derivative (Fig. 1) which was synthesized in the Research Laboratories, Pharmaceuticals Division, CIBA-GEIGY Limited, Basel, Switzerland. Other antibiotics were commercial products.

Test strains. Strains stocked in this laboratory were used as standard stock cultures and are from the Reference Laboratory of Drug-Resistant Bacteria, Gunma University. They were originally isolated from clinical materials.

Media. Heart infusion (HI) agar (Eiken), HI broth (Eiken), and antibiotic medium no. 3 (Difco) were used. Other media were peptone water and medium B. The former consisted of 10 g of peptone, 5 g of NaCl, and 1 liter of distilled water; the latter contained 2 g of yeast extract, 10 g of peptone, 7 g of Na2HPO4·12H2O, 2 g of KH2PO4, 1.2 g of glucose, and 0.4 g of MgSO4·7H2O in 1 liter of distilled water.

In vitro antibacterial activity. Minimal inhibitory concentration (MIC) of a drug was determined by an agar dilution method. Overnight culture in peptone water was diluted to 10⁶ cells per ml with fresh peptone water. A loopful (about 0.005 ml) of diluted culture was inoculated by Micro-planter (Kubota, Japan) onto agar plates containing a series of serial twofold dilutions of a drug. MIC values were scored after overnight incubation at 37°C.

The effect of inoculum size was determined by means of a twofold serial agar dilution method on HI agar. The inocula were overnight cultures diluted in physiological saline to 10⁻⁴, 10⁻⁵, and 10⁻⁶.

The 50% Infective dose. Values for 50% infective dose were determined by the method of Rado et al. (2). A bacterial culture in peptone water was diluted to 2 x 10³ to 3 x 10⁷ cells per ml with fresh peptone water. HI agar plates containing various concentrations of a drug were prepared, and a 0.1-ml sample of diluted bacterial suspension was spread on each plate. After overnight incubation at 37°C the number of colonies that had grown on the plate were counted. The mean growth inhibition was calculated from a mean number of colonies on five plates at each drug concentration and on five drug-free agar plates.

Bactericidal activity. An overnight culture of each strain in antibiotic medium no. 3 (Difco) was diluted to a final concentration of about 10⁵ cells per ml with antibiotic medium no. 3 containing a series of serial twofold dilutions of a drug. MICs were read after incubation at 37°C for 18 h. One loopful of each culture in the MIC test series was spotted onto drug-free HI agar plates, and after incubation at 37°C for 18 h, the minimal bactericidal concentrations of antibiotic were determined as the lower concentration of drug that prevented visible growth on HI agar plates. Another method consisted of counting the number of viable cells at appropriate time intervals after addition of drugs.

Stability to β-lactamase. The enzyme samples were prepared as follows. A 1-ml brain heart infusion culture of each strain was diluted 10-fold with medium B and incubated at 37°C. The cells were harvested by centrifugation, washed with 0.05 M phosphate buffer (pH 7.0), and resuspended in the same buffer. The
cells were disrupted in an ultrasonicator. Enzyme activity was photometrically measured (6). All the measurements were made at a substrate concentration of 0.1 mM. The figures in Table 3 are relative values, taking the absolute rate of cephaloridine hydrolysis as 100 for cephalosporinase and that of penicillin G hydrolysis as 100 for penicillinase.

*Pseudomonas aeruginosa* ML4259 Rms139* (7) and *Klebsiella pneumoniae* GN49 were used as the standard strains capable of producing the known types of penicillinase, and *Escherichia coli* GN5482, *P. aeruginosa* GN918 (12), *Enterobacter cloacae* GN7471, and *Proteus morganii* GN5407 were used for cephalosporinase.

PBPs. The affinity of CXD and the comparative compound, CEX, to the penicillin-binding proteins (PBPs) was examined. Seven PBPs were detected in *E. coli* JG1011 by the modified method (11) described by Spratt (8). CXD and CEX were used as competitors for 14C-labeled penicillin G binding to *E. coli* PBPs. The concentrations of competitor added to the reaction mixture were 1-, 5-, and 25-fold greater than the concentration of 14C-labeled penicillin G (34 μg/ml). The binding reaction was terminated, and the proteins of the inner membrane were selectively solubilized and fractionated on a sodium dodecyl sulfate-polyacrylamide slab gel. The PBPs were detected by fluorography with X-ray film (Fuji Rxs). The level of [14C] penicillin G binding to 1A and 1B at each competitor concentration was quantitated by densitometry of the X-ray film.

In vivo antibacterial activity. The in vivo antibacterial activity of CXD was determined by experimental infection of mice with gram-negative bacteria. Twenty male mice (ICR strain) weighing 18 to 22 g were used for each dose level. The mice were challenged intraperitoneally with sufficient microorganisms to kill all nontreated mice within 48 h. The microorganisms were grown on an HI agar plate and suspended in physiological saline solution. Mice infected with *E. coli* ML4707 and *K. pneumoniae* GN6445 were treated orally immediately after infection and 3 h later. The total number of surviving mice was recorded, usually 1 week after infection, and the amount of a single dose (milligrams per kilogram) that gave protection to 50% of the infected mice was estimated by means of a log-probit plot (3).

RESULTS

Antibacterial spectrum. The spectrum of antibacterial activity of CXD against gram-positive and gram-negative bacteria is shown in Table 1. CXD was active against both gram-positive and gram-negative organisms susceptible to CEX. Both antibiotics exhibited almost similar effectiveness against gram-positive organisms, but CXD was more active against CEX-susceptible gram-negative organisms.

### Table 1. Antibacterial activity of CXD against standard strains of bacteria

<table>
<thead>
<tr>
<th>Test organism</th>
<th>MIC (μg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CXD</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> FDA209IPJC-1</td>
<td>1.56</td>
</tr>
<tr>
<td><em>S. aureus</em> E-46</td>
<td>3.13</td>
</tr>
<tr>
<td><em>S. aureus</em> Terajima</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Escherichia coli</em> NIHJ-JC-2</td>
<td>3.13</td>
</tr>
<tr>
<td><em>Salmonella typhi</em> 901</td>
<td>1.56</td>
</tr>
<tr>
<td><em>S. paratyphi</em> 1015</td>
<td>1.56</td>
</tr>
<tr>
<td><em>S. schottmuelleri</em> 8006</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> PCI-602</td>
<td>1.56</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> IAM1 184</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> Ox-19</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>P. rettgeri</em> IFO3850</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>P. mirabilis</em> IFO3849</td>
<td>6.25</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> IFO3445</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

* The tested strains are the standard strains stocked at the Laboratory of Bacterial Resistance, School of Medicine, Gunma University.

* Overnight HI broth culture was diluted with physiological saline, and one loopful (0.005 ml) of 10^6 or 10^6 cells per ml was inoculated.
Both CXD and CEX acted equally poorly against Serratia marcescens, Proteus species, and P. aeruginosa.

Antibacterial activity. The antibacterial activity of CXD against gram-positive and gram-negative bacteria was compared in about 50 to 300 clinical isolates of each species of bacteria including Staphylococcus aureus, E. coli, K. pneumoniae, and Proteus mirabilis (Fig. 2). The percentage of isolates of gram-negative bacteria inhibited by CXD was somewhat larger than that of CEX. In the case of S. aureus, the percentage of isolates inhibited by CXD was almost identical to that for CEX. The concentration of CXD required to inhibit the growth of 50% of the total number of tested E. coli strains (MIC<sub>50</sub>) was 3.13 to 6.25 µg/ml, whereas that of CEX was 6.25 to 12.5 µg/ml. The MIC<sub>50</sub> of CXD against K. pneumoniae strains was 3.13 to 6.25 µg/ml, and that of CEX was 6.25 to 12.5 µg/ml. Therefore, CXD was approximately twofold more active than CEX against E. coli and K. pneumoniae strains. CXD and CEX exhibited almost identical MIC<sub>50</sub> values against P. mirabilis strains.

The determination of 50% infective dose with CXD and CEX was carried out against E. coli ML4707 and K. pneumoniae GN6445 (Fig. 3).

**Fig. 2.** Susceptibility of several species of gram-positive and gram-negative clinical isolates to CXD (●) and CEX (○). Inoculum size, one loopful of 10<sup>6</sup> cells per ml.

**Fig. 3.** Antibacterial activity of CXD and CEX against E. coli ML4707 and K. pneumoniae GN6445. Each point indicates an arithmetic mean of five determinations. Each bar indicates a standard deviation. (a) E. coli ML4707; (b) K. pneumoniae GN6445.
Linearity was observed over a drug concentration range that inhibited from 5 to 95% of the bacterial growth. The 50% infective doses for CXD and CEX against \textit{E. coli} ML4707 were 1.2 and 3.9 \( \mu \text{g/ml} \), respectively. Against \textit{K. pneumoniae} GN6445, the 50% infective doses of CXD and CEX were 1.45 and 2.15 \( \mu \text{g/ml} \), respectively. Therefore, CXD is more effective in vitro against \textit{E. coli} ML4707 and \textit{K. pneumoniae} GN6445 than is CEX.

The effect of inoculum size on antibacterial activity of CXD and CEX against clinical isolates of \textit{E. coli} is shown in Table 2. The size of the inoculum in the test had a significant effect on MICs: the in vitro activity of CXD increased when the size of the inoculum was decreased, as compared to only a slight increase for that with CEX.

**Bactericidal activity.** The MICs and minimal bactericidal concentrations obtained with CXD and CEX against each of 25 clinical isolates of \textit{E. coli} and \textit{K. pneumoniae} are illustrated in Fig. 4. Ninety-eight percent of the \textit{E. coli} strains were inhibited by CXD at a concentration of 6.25 \( \mu \text{g/ml} \), as compared to 50% of the strains inhibited by CEX at the same antibiotic concentration. The bactericidal effectiveness of CXD was also more pronounced than that of CEX; CXD affected 80% of the strains at the same concentration, as compared to only 20% affected by CEX. In the case of \textit{K. pneumoniae}, the percentage of isolates inhibited by CXD was twofold larger than that by CEX, and the percentage of isolates killed by CXD was also about twofold larger than that by CEX.

Bactericidal activity of CXD against \textit{E. coli} ML4707 was examined by counting viable cells (Fig. 5). When the culture reached a density of approximately \( 10^4 \) cells per ml, from one-fourth- to fourfold the MICs of CXD and CEX were added, and incubation was continued. After 4 \( \text{h} \) of incubation, both drugs reduced the number of viable cells to \( 10^2 \) at concentrations of one- to fourfold the MIC. CXD had more bactericidal activity than CEX against \textit{E. coli} ML4707 (Fig. 5).

**Susceptibility to \( \beta \)-lactamases.** The relative rate of hydrolysis of five cephalosporins and

---

**Table 2. Effect of inoculum size on antibacterial activity of CXD and CEX against 100 \textit{E. coli} strains**

<table>
<thead>
<tr>
<th>Inoculum (cells per ml)*</th>
<th>MIC(_{50})</th>
<th>MIC(_{70})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CXD</td>
<td>CEX</td>
</tr>
<tr>
<td>( 10^8 )</td>
<td>11.5</td>
<td>16.5</td>
</tr>
<tr>
<td>( 10^6 )</td>
<td>5.4</td>
<td>10.5</td>
</tr>
<tr>
<td>( 10^4 )</td>
<td>4.8</td>
<td>9.8</td>
</tr>
</tbody>
</table>

* Overnight culture was diluted with physiological saline. One loopful of the diluted culture was inoculated.

---

**Fig. 4**. MICs and minimal bactericidal concentrations of CXD (●) and CEX (○). Twenty-five strains of \textit{E. coli} and \textit{K. pneumoniae} were used. (a) \textit{E. coli}; (b) \textit{K. pneumoniae}.

**Fig. 5**. Bactericidal effects of CXD and CEX on \textit{E. coli} ML4707. Number of viable cells was counted on drug-free agar plates at 2-h intervals after addition of drugs (arrow).
penicillin G by R plasmid-mediated penicilllnase and cephalsporinase is shown in Table 3. CXD, CEX, and other cephalsporins that were stable against a type IV penicilllnase produced by a P. aeruginosa strain carrying an R plasmid and against the penicilllnase produced by K. pneumoniae GN69. On the other hand, CXD, CEX, and the other cephalsporins tested were susceptible to four cephalsporinases. CXD was more stable than other cephalsporins except for CEX and was slightly less stable than CEX against hydrolysis by cephalsporinases from P. aeruginosa GN918, E. cloacae GN7471, and P. morganii GN5407, but slightly more stable than CEX against hydrolysis by the cephalsporinase from E. coli GN5482.

Affinity of CXD to PBPs of E. coli. The affinities of CXD and the comparative compound, CEX, to PBPs were estimated by measuring the competition of unlabeled CXD and CEX with [14C]penicillin G for binding to PBPs. The PBPs separated from E. coli by sodium dodecyl sulfate-acrylamide slab gel electrophoresis were detected by fluorography on X-ray film (11). The pattern of competition of unlabeled CXD and CEX with [14C]penicillin G for binding to PBPs of E. coli is shown in Fig. 6, and the quantitation of the remaining radioactivity of [14C]penicillin G is shown in Fig. 7. Both CXD and CEX compete for the binding to PBP-1A and PBP-1B. The affinity of CXD to PBP-1A and -1B was higher than that of CEX. There

### Table 3. Substrate profiles of various β-lactamases

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Type of β-lactamase</th>
<th>Sp act (U/mg of protein)</th>
<th>Relative rate of hydrolysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli GN5482</td>
<td>Cephalosporinase</td>
<td>0.24</td>
<td>100 33 41 135 691 29</td>
</tr>
<tr>
<td>P. aeruginosa GN918</td>
<td>Cephalosporinase</td>
<td>0.24</td>
<td>100 43 32 160 480 25</td>
</tr>
<tr>
<td>E. cloacae GN7471</td>
<td>Cephalosporinase</td>
<td>3.58</td>
<td>100 62 56 50 402 83</td>
</tr>
<tr>
<td>P. morganii GN5407</td>
<td>Cephalosporinase</td>
<td>5.22</td>
<td>100 58 31 74 242 100</td>
</tr>
<tr>
<td>P. aeruginosa E. coli PBPs</td>
<td>Penicilllnase IV</td>
<td>0.05</td>
<td>9 1 1 1 1 100</td>
</tr>
<tr>
<td>ML4259 Rmsl39*</td>
<td>Penicilllnase</td>
<td>0.11</td>
<td>15 1 1 3 3 100</td>
</tr>
<tr>
<td>K. pneumoniae GN69</td>
<td>Penicilllnase</td>
<td>0.24</td>
<td>100 43 32 160 480 25</td>
</tr>
</tbody>
</table>

* CER, cephaloridine; CEZ, cefazolin; CET, cephalothin; PC-G, penicillin G.
was no difference in the affinities to PBP-2, -3, -4, and -5/6 between CXD and CEX.

In vivo antibacterial activity. Chemotherapeutic effects of CXD on experimental infections of mice with E. coli ML4707 and K. pneumoniae GN6444 are shown in Table 4. With both strains, CXD was more effective. Although CXD had MICs almost equal to those of CEX, the protective activity of CXD in experimental infection in mice was greater than that of CEX.

DISCUSSION

PBP-1Bs of E. coli is reported to be involved in the cross-linking reaction of cell wall peptidoglycan (11). The lack of PBP-1A alone in E. coli does not affect cell growth, because this protein is supposed to be a detour enzyme-carrying function compensating for the lack of PBP-1Bs (11). PBP-2 is supposed to be involved in maintaining the cell shape (8, 9), and PBP-3 is supposed to be concerned in septum formation (8). PBP-4 is identical to n-alanine carboxypeptidase IB (1, 5), and PBP-5/6 corresponds to d-alanine carboxypeptidase IA (4, 10). Therefore, in view of the affinity of CXD to PBP-1Bs, it seems likely that CXD has more intensive lytic activity than CEX.

LITERATURE CITED