Mecillinam (FL 1060), a 6 β -Amidinopenicillanic Acid Derivative: In Vitro Evaluation

L. TYBRING

Bacteriological Research Department, Leo Pharmaceutical Products, DK-2750 Ballerup, Denmark

Received for publication 26 December 1974

Mecillinam (formerly called FL 1060), a novel 6 β -amidinopenicillanic acid derivative, showed markedly higher activity than ampicillin against clinical isolates of *Enterobacteriaceae*. The mode of action of mecillinam is different from that of ampicillin and consequently no intrinsic cross-resistance between the two antibiotics was observed. Mecillinam is inactivated by β -lactamases but is generally more stable than ampicillin; thus an apparent cross-resistance exists for strong β -lactamase producers. The protein binding was low and the activity was relatively independent of pH between 5.5 and 8.0. Slow-growing, insensitive variants of *Escherichia coli* could be selected but these were not stable. The frequency of insensitive variants was affected by the sodium chloride concentration in the medium.

The hitherto known antimicrobially active penicillins, whether of natural, biosynthetic or semisynthetic origin, are acyl derivatives of 6-aminopenicillanic acid. In a previous paper (2), however, a new group of penicillanic acid derivatives, the 6 β -amidinopenicillanic acids, was described. This group shows antimicrobial properties in vitro which differ fundamentally from those of benzylpenicillin and ampicillin. One member of the group, mecillinam (proposed international nonproprietary name), 6 β -[(hexahydro-1H-azepin-1-yl)-methyleneamino]-penicillanic acid (formerly called FL 1060), was selected for further study.

Mecillinam is 300 times more active in vitro against *Escherichia coli* than against penicillin-sensitive strains of *Staphylococcus aureus*, whereas ampicillin is 100 times less active against the *E. coli* strain than against the *S. aureus* strain. In addition to this difference in antimicrobial spectrum there is also a difference in the mode of action, as judged from the morphological response of *E. coli* to the compound (1, 2, 4) and from biochemical studies (3, 5).

In this paper the results of comparative studies of mecillinam and ampicillin will be presented.

MATERIALS AND METHODS

Antibiotics. Mecillinam was used as its hydrochloride dihydrate which forms colorless crystals and is readily soluble in water. The stability of aqueous solutions depends on the pH and on the buffer system. At 37 C a 0.05% mecillinam solution in citrate buffer

has a half-life of 120 h at pH 6.8 to 7.5, although it is less stable at lower pH. In phosphate buffer the half-life at pH 7.0 is only 10 h, whereas the optimum stability is at pH 5.3, with a half-life of 100 h. The substance is rapidly destroyed by sodium sulfite, bringing the half-life in Endo agar down to 45 min. Since mecillinam is a β -lactam antibiotic it is inactivated by various β -lactamases, although it is generally more resistant to them than ampicillin is. Mecillinam is not inactivated by amidase.

Ampicillin was used as the trihydrate and the concentrations of both compounds were calculated as the anhydrous zwitterion. Solutions of the compounds were freshly prepared.

Bacterial strains. Most of the cultures used in this study were recent clinical isolates of *Enterobacteriaceae* obtained from the Institute of Medical Microbiology, University of Copenhagen, or isolated from specimens submitted to our laboratory. Ten clinical isolates of *Yersinia enterocolitica* were obtained from Statens Seruminstitut, Copenhagen, and two reference strains (serotype 3 and serotype 9) were obtained for comparison.

Culture media. NIH agar medium or the corresponding fluid medium were used throughout the study. The medium contains 0.5% yeast extract (Difco), 1.5% casein hydrolysate (pancreatic), 0.1% dextrose, 0.25% sodium chloride, 0.005% L-cystine, and 1% agar (Oxoid no. 1) in distilled water, pH 7.1, after autoclaving.

Antibacterial activity. The antibacterial activity was determined by the conventional serial dilution technique in liquid medium. An inoculum of 10^4 cells per ml of medium was used. The amount of growth was read by the naked eye, after 18 h of incubation at 36 C (frequently controlled by optical density readings) and recorded as -, +, ++, +++, or +++++. The mean activity was expressed as IC_{50} , i.e., the

FIG. 1. The chemical structures of mecillinam and ampicillin.

concentration found by graphic interpolation to produce 50% inhibition of growth or ++ growth as compared to the control culture (++++ growth).

Frequency of insensitive variants. Overnight cultures of *E. coli* (Leo HA2) and *Salmonella typhimurium* (NCTC 5710) in NIH fluid medium were used. One-tenth milliliter of serial 10-fold dilutions in the same medium was plated in quadruplicate on NIH agar containing various concentrations of mecillinam or ampicillin. Since colonies developed slowly on plates containing higher concentrations of mecillinam the number of colony-forming units was determined after 72 h at 36 C.

Protein binding. The protein binding was measured in human serum by the ultrafiltration technique (Amicon CF50A), the ultrafiltrates being assayed on *E. coli* (Leo HA2) plates against standards prepared in ultrafiltrate of the same serum.

RESULTS AND DISCUSSION

Antibacterial activity. The in vitro activity, expressed as IC_{50} , of mecillinam and ampicillin against a number of clinical isolates belonging to the *Enterobacteriaceae* is shown in Fig. 2-7. It will be seen that mecillinam is markedly more active than ampicillin against *E. coli* (Fig. 2), 84% of the 236 isolates being inhibited by 0.1 $\mu\text{g/ml}$, whereas no isolates were inhibited by ampicillin at that concentration. An ampicillin concentration of 5 $\mu\text{g/ml}$ was required to inhibit 88% of the isolates.

The range of sensitivity of *Klebsiella pneumoniae* (144 isolates) and *Klebsiella oxytoca* (26 isolates) to mecillinam (Fig. 3) was wide and varied from 0.03 $\mu\text{g/ml}$ to more than 100 $\mu\text{g/ml}$. Although 50% of the isolates were inhibited by 0.3 μg of mecillinam per ml, 12% of the strains were not inhibited by 100 $\mu\text{g/ml}$. Only 5% of the strains of *K. pneumoniae* and none of the *K. oxytoca* isolates were inhibited by 10 μg of ampicillin per ml, and 100 μg of ampicillin per ml did not inhibit 36% and 54% of these isolates, respectively.

The sensitivities of 26 isolates of *Proteus inconstans* (*Providencia*) (Fig. 4) to mecillinam was distributed within narrow limits and 50% of them were inhibited by 0.1 $\mu\text{g/ml}$. In contrast, the same isolates showed a broader distribution of sensitivity to ampicillin and generally higher IC_{50} values, the median value being 16 $\mu\text{g/ml}$.

Of the *Proteus vulgaris* (56) and *Proteus morganii* (36) isolates, 21 and 47%, respectively, were not inhibited by 100 μg of mecillinam per ml, but of the rest about 70% were inhibited by 1 $\mu\text{g/ml}$ (Fig. 5 and 6). None was inhibited by 0.6 μg of ampicillin per ml and only about 40% was inhibited by 100 $\mu\text{g/ml}$.

Mecillinam was slightly more active than ampicillin against 201 *Proteus mirabilis* isolates (Fig. 7), but the difference was less marked than against the other *Proteus* species.

In contrast to the broad distribution of the sensitivities to mecillinam of isolates belonging to *Proteus* and *Klebsiella* species, 12 clinical strains of *Y. enterocolitica* were all inhibited by 0.03 to 0.05 μg of mecillinam per ml and by 16 μg of ampicillin per ml.

A comparison of the sensitivities of the 236 *Escherichia coli* isolates to mecillinam and ampicillin is shown in Fig. 8. The geometric mean of the IC_{50} values of mecillinam (0.07 $\mu\text{g/ml}$) was 40 times less than those of ampicillin (2.8 $\mu\text{g/ml}$). The straight line represents the expected regression line if all isolates were 40 times more sensitive to mecillinam than to ampicillin, i.e., if the difference between the two compounds was only quantitative and not qualitative. However, as the distribution of the sensitivity to ampicillin ranged from 0.2 $\mu\text{g/ml}$ to more than 100 $\mu\text{g/ml}$ for isolates that showed sensitivities to mecillinam about the mean IC_{50} value of 0.07 $\mu\text{g/ml}$, there was no evidence of cross-resistance between the two antibiotics. This is consistent with the difference in the mode of action of the two compounds (3, 4, 5). An apparent cross-resistance exists for strong β -lactamase producers as both antibiotics are inactivated by these enzymes. Preliminary experiments in our laboratory have suggested a difference in sensitivity to various β -lactamases. Generally mecillinam is more stable than ampicillin in cultures of β -lactamase producers (6).

Protein binding. The binding of mecillinam to human serum determined by the ultrafiltration technique was found to be about 5 to 10%, i.e., only one-quarter to one-half that of ampicillin.

Influence of pH. The activity of mecillinam against three species of bacteria tested in liquid media at varying initial pH's is shown in Table

FIG. 2 to 7. Cumulative percentage of isolates inhibited by mecillinam and ampicillin.

FIG. 8. Comparison of sensitivities to mecillinam and ampicillin of 236 *E. coli* isolates.

TABLE 1. Influence of initial pH on the antibacterial activity of mecillinam

pH	IC ₅₀ (µg/ml)		
	<i>E. coli</i> Leo HA2	<i>K. pneu-</i> <i>moniae</i> Leo HE	<i>S. typhi-</i> <i>murium</i> NCTC 5710
5.5	0.025	0.063	0.13
6.0	0.016	0.050	0.050
6.5	0.016	0.050	0.050
6.9	0.016	0.050	0.050
7.4	0.016	0.063	0.13
8.0	0.050	0.16	0.50
8.9	0.16	0.50	16

1. It will be seen that the activity was relatively independent of pH between 5.5 and 8.0.

Frequency and selection of insensitive variants. A population analysis of a strain of *S. typhimurium* and of *E. coli* was made by counting the number of colony-forming units on agar media containing various concentrations of mecillinam or ampicillin. After 72 h of incubation the two strains showed a heterogeneous distribution of sensitivity to mecillinam (Fig. 9 and 10). This heterogeneity was due to the occurrence of slow-growing, morphologically altered cells. Successive transfers of bacteria from plates containing 1, 10, and 100 µg of mecillinam per ml were made into liquid media containing no mecillinam. After an overnight incubation the cells had returned to normal shape. A repeated analysis, made after the first transfer in drug-free media, showed that

FIG. 9. Population analysis of *S. typhimurium* (NCTC 5710) on agar containing mecillinam or ampicillin (72-h readings).

FIG. 10. Population analysis of *E. coli* (Leo HA2) on agar containing mecillinam or ampicillin (72-h readings).

the viable counts were only affected by concentrations of more than 30 µg/ml (Fig. 11). The isolates from the 1- and 10-µg/ml plates

FIG. 11. Population analysis after transfers without mecillinam of *E. coli* (Leo HA2) isolates on agar containing mecillinam. Symbols: (●) culture originally isolated from 100-μg/ml plates; (○) same, from 10-μg/ml plates; and (■) same, from 1-μg/ml plates (72-h readings).

developed few normal-growing colonies on the control plates in 18 h. After the second transfer the number of resistant cells decreased 100- to 1,000-fold and after the fifth transfer the distributions were similar to that of the original strain. However, the strains isolated from 1- and 10-μg/ml plates contained fewer resistant bacteria than the original strain, but gradually became indistinguishable from the latter after further transfers.

It was thus possible to select resistant variants from the original strain. The sensitivity of

these variants to mecillinam was independent of the concentrations used for the selections. The original *E. coli* strain grew in fluid medium without mecillinam with a doubling time of approximately 20 min, whereas the resistant variants grew more slowly under the same conditions. Doubling times between 60 and 90 min have been measured for cultures evolved from resistant variants. The variants were not stable, as indicated by the fact that normal-growing, sensitive cells appeared during transfers without mecillinam, and these cells outgrew the slow-growing variants.

The frequency of resistant variants forming colonies on plates containing inhibitory concentrations of mecillinam was affected by the sodium chloride concentration in the medium. On the normal medium containing 0.25% sodium chloride and 0.1 to 10 μg of mecillinam per ml, the frequency of resistant *E. coli* variants was 10^5 to 1.4×10^5 (Fig. 10). The frequency increased by a factor of 6 when the medium contained 0.8% sodium chloride and decreased by a factor of 15 when sodium chloride was omitted.

LITERATURE CITED

1. Greenwood, D., and F. O'Grady. 1973. FL 1060: a new beta-lactam antibiotic with novel properties. *J. Clin. Pathol.* **26**:1-6.
2. Lund, F., and L. Tybring. 1972. 6β-amidinopenicillanic acids—a new group of antibiotics. *Nature (London) New Biol.* **236**:135-137.
3. Matsushashi, S., T. Kamiryo, P. M. Blumberg, P. Linnett, E. Willoughby, and J. L. Strominger. 1974. Mechanism of action and development of resistance to a new amidino penicillin. *J. Bacteriol.* **117**:578-587.
4. Melchior, N. H., J. Blom, L. Tybring, and A. Birch-Andersen. 1973. Light and electron microscopy of the early response of *Escherichia coli* to a 6β-amidinopenicillanic acid (FL 1060). *Acta Pathol. Microbiol. Scand. Sect. B* **81**:393-407.
5. Park, J. T., and L. Burman. 1973. FL 1060, a new penicillin with a unique mode of action. *Biochem. Biophys. Res. Commun.* **51**:863-868.
6. Tybring, L., and N. H. Melchior. 1975. Mecillinam (FL 1060), a 6β-amidinopenicillanic acid derivative: bactericidal action and synergy in vitro. *Antimicrob. Agents Chemother.* **8**:271-276.