Comparison of the Binding Properties of Two 6β-Amidinopenicillanic Acid Derivatives That Differ in Their Physiological Effects on Escherichia coli

BRIAN G. SPRATT

Department of Genetics, University of Leicester, Leicester LE1 7RH, England

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The 6-β-amidinopenicillanic acid derivative, mecillinam, was highly specific in its action on the growth of Escherichia coli. Concentrations from the minimal inhibitory concentration (0.05 μg/ml) up to at least 200 μg/ml resulted in the conversion of E. coli rods into osmotically stable spherical cells without significantly inhibiting cell growth or causing cell lysis. A second amidinopenicillanic acid derivative [6-(4-morpholinylmethylene) amino] penicillanic acid] showed identical effects on cell growth at concentrations from its minimal inhibitory concentration (0.2 μg/ml) up to at least 5 μg/ml but, at higher concentrations, increasing amounts of lysis occurred. Neither of these compounds showed the immediate inhibition of cell division that is observed with typical β-lactam antibiotics. We have compared the binding of these two amidinopenicillanic acids to the individual penicillin-binding proteins of E. coli. Both compounds showed a high specificity of binding to penicillin-binding protein 2 at low concentrations. At higher concentrations mecillinam still maintained its high specificity for protein 2 and very little binding of mecillinam to any of the other binding proteins was detected with concentrations up to 1 mg/ml. The morpholino compound, however, showed extensive binding to proteins 1 and 4, and slight binding to proteins 5 and 6 at high concentrations. The morpholino compound therefore combined both the physiological properties and the binding properties of mecillinam with some of those of typical penicillins and cephalosporins. Lysis probably occurs at high concentrations of morpholino compound because it binds to penicillin-binding protein 1, since this is believed to be the target with which β-lactams interact to inhibit cell elongation.

The amidinopenicillanic acid derivative, mecillinam (Fig. 1A), is the prototype of a new class of β-lactam antibiotics (6). The replacement of the usual 6-acylamino side chain of normal β-lactams by the 6-amido linkage in mecillinam results in a compound with properties strikingly different from those of typical penicillins and cephalosporins. Mecillinam (previously called FL1060) is relatively ineffective against gram-positive organisms but is about 100 times more active than other β-lactams against most gram-negative organisms (6, 9, 15). This antibiotic converts Escherichia coli cells into large osmotically stable spherical forms (6, 9). Although cell division is eventually inhibited (5) and cell lysis occurs after several hours of growth in the presence of mecillinam (7, 16), these effects are quite different from the rapid lysis and immediate inhibition of division observed with typical β-lactams (4, 11, 14).

The biochemical properties of mecillinam are also unusual. Penicillin-susceptible D-alanine carboxypeptidase 1, peptidoglycan transpeptidase, and endopeptidase activities are only slightly inhibited by this compound at concentrations far above those required to inhibit the growth of E. coli (7, 10). Mecillinam was originally reported not to compete for the binding of [14C]benzylpenicillin to membranes of E. coli (7), but recently the separation of the individual penicillin-binding proteins (PBPs) of this organism has shown that it competes with high affinity for one of the six proteins in the cytoplasmic membrane that bind [14C]benzylpenicillin (13). [14C]mecillinam was subsequently shown to bind exclusively to this protein (PBP 2), and mutants of E. coli have been isolated that fail to bind either [14C]benzylpenicillin or [14C]mecillinam to PBP 2 (12).

Mecillinam clearly exerts its effects on E. coli by interacting with PBP 2. The penicillin-susceptible enzyme that presumably corresponds to this binding protein has not been identified.
Typical β-lactams generally show low affinity for PBP 2 but bind with high affinity to other PBPs which are believed to be the targets at which these antibiotics inhibit cell division and cause cell lysis (12).

A new amidopenicillanic acid derivative, 6-[[4-morpholinylmethylene] amino] penicillanic acid (Fig. 1B), (referred to as morpholino compound) which combines the properties of mecillinam with some of those of typical β-lactams has been brought to our notice by F. Lund of Leo Pharmaceutical Laboratories. In this paper we compare the physiological and binding properties of mecillinam with the morpholino compound.

MATERIALS AND METHODS

Bacterial strains and conditions of growth. E. coli KN126 F′ tryE8929 tyr− ile− sup−126 was used in all experiments and was originally obtained from T. Nagata. Cells were grown in Penassay broth (Difco antibiotic medium number 3) at 37°C with vigorous aeration. Exponentially growing cells were used in all experiments.

Measurement of cell growth. Absorbance of the culture was monitored at 550 nm with a Gilford model 300 automatic sampling spectrophotometer using sterile broth as a blank. Total cell numbers were measured with a Coulter counter model B. Cells were diluted into ice-cold membrane-filtered (Millipore Corp.) 0.9% NaCl containing 0.37% formaldehyde and then counted with a 30-μm probe.

Preparation of membranes. Three-liter batches of exponentially growing cells were cooled on ice, harvested by centrifugation for 6 min at 6,000 × g at 4°C, and resuspended in 80 ml of ice-cold 10 mM sodium phosphate buffer, pH 7.0. An 0.8-ml amount of 2-mercaptoethanol was added and the cells were disrupted by three 30-s periods of sonication with intermittent periods of cooling. Cell debris was removed by centrifugation for 20 min at 8,000 × g, and the cell membranes were pelleted from the supernatant at 100,000 × g for 40 min at 4°C. The membranes were washed twice and finally resuspended in the above buffer at a protein concentration of approximately 40 mg/ml and stored at −80°C. These membrane preparations consist of both inner and outer membranes.

Binding of [14C]mecillinam. Two-hundred-microliter portions of membranes (5 to 10 mg of protein/ml in 50 mM sodium phosphate buffer, pH 7.0) were incubated for 10 min at 30°C with 10 μl of twofold increasing concentrations of [14C]mecillinam (53 mCi/mmol). The binding was terminated by the addition of 5 μl of 40-mg/ml nonradioactive mecillinam and 10 μl of 20% (wt/vol) Sarkosyl NL-97. After 20 min at room temperature, the Sarkosyl-insoluble peptidoglycan and outer membrane were pelleted at 100,000 × g for 40 min at 10°C and the Sarkosyl-soluble inner-membrane proteins were removed and fractionated on a 10% sodium dodecyl sulfate-polyacrylamide slab gel as described previously (12, 13). The dried gel was fluorographed by exposure to Kodak RP Royal X-omat X-ray film at −80°C (12, 13).

Binding of amidopenicillanic acids by competition for the binding of [14C]benzylpenicillin. Portions (200 μl) of membranes (as above) were preincubated for 10 min at 30°C with 10 μl of increasing concentrations of nonradioactive amidopenicillanic acids, and the binding proteins remaining accessible were measured by the addition of 20 μl of 50-μCi/ml [14C]benzylpenicillin (54 mCi/mmol) for a further 10 min at 30°C. This concentration of [14C]benzylpenicillin is sufficient to saturate all PBPs under these conditions (B. G. Spratt, manuscript in preparation). The binding was terminated by the addition of 8 μl of 120-mg/ml nonradioactive benzylpenicillin and 10 μl of 20% (wt/vol) Sarkosyl NL-97, and the binding proteins in the Sarkosyl-soluble fraction were detected as described above.

Binding proteins were quantitated by densitometry of their images on the X-ray films with a Joyce-Loebl Mk111c microdensitometer.

Measurement of minimal inhibitory concentrations. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the antibiotic that produced a clear alteration of the rod morphology of E. coli KN126 when added to a culture of 2 × 10^9 organisms/ml growing at 37°C in Penassay broth.

Chemicals. [14C]benzylpenicillin (54 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, England. [14C]mecillinam (53 mCi/mmol), nonradioactive mecillinam (batch GA 32138) and morpholino compound (batch GA 33088) were kindly provided by F. Lund of Leo Pharmaceutical Products, Ballerup, Denmark. Benzylpenicillin and cephalaxin were kindly provided by Glaxo Laboratories, Greenford, Middlesex, England. Solutions of all β-lactams were freshly prepared immediately before use. Sarkosyl NL-97 was purchased from Geigy Industrial Chemicals, New York.
RESULTS

Comparison of the effects of mecillinam and morpholino compound on the morphology, growth, and division of E. coli KN126. The MICs of mecillinam and morpholino compound were 0.05 μg/ml and 0.2 μg/ml, respectively. Addition of mecillinam at concentrations up to at least 200 μg/ml had little effect on the growth rate during a 90-min period at 37°C. During this period absorbance increased by 10.6-fold in the control and 10.1-fold in the presence of 200 μg of mecillinam per ml (Fig. 2), and the latter culture consisted entirely of large osmotically stable spherical cells. The morpholino compound also produced little inhibition of growth at concentrations up to 5 μg/ml, and the morphology of the cells was indistinguishable from that produced by mecillinam; at higher concentrations increasing amounts of lysis occurred (Fig. 2).

Mecillinam at 200 μg/ml (or 2 μg/ml) had no effect on cell division for 30 min at 37°C, and then division slowed and stopped. Total cell number increased by about 170% after the addition of mecillinam (Fig. 3). The morpholino compound (2 μg/ml) gave an identical increase in cell number and even 200 μg of this compound per ml allowed division to occur at the normal rate for 20 min at which time cell number could no longer be followed because cell lysis took place. At the time lysis began the increase in cell number was 72% and this should be compared with the 10 to 20% residual division that was obtained with 50 μg of cephalaxin per ml (a typical β-lactam antibiotic; MIC, 3 μg/ml). Benzylpenicillin (MIC, 6 μg/ml) at concentrations of 25 μg/ml and 50 μg/ml also inhibited cell division within 10 min and only allowed a residual increment in cell number of 21.8% and 19.1% respectively (data not shown).

Binding of [14C]mecillinam. Figure 4 shows the binding of increasing concentrations of [14C]mecillinam to the inner-membrane proteins of E. coli KN126. No binding to the Sarkosyl-insoluble outer-membrane proteins has been detected with this, or any other, [14C]-labeled β-lactam (12). Binding was observed with high affinity for one inner-membrane protein with an electrophoretic mobility identical to that of [14C]benzylpenicillin-binding protein 2 as previously observed (12). In this experiment 50% saturation of PBP 2 with mecillinam was obtained at 0.023 μg/ml for 10 min at 30°C. A trace of high-affinity binding was also seen to a protein with a mobility slightly faster than PBP 4. This is almost certainly a proteolytic product of PBP 2 since mutants that fail to bind [14C]mecillinam to PBP 2 also fail to show any binding to this minor protein (B. G. Spratt, unpublished observations).

Binding of amidopenicillanic acids by competition with [14C]benzylpenicillin. Mecillinam at concentrations up to at least 2 μg/ml binds exclusively to PBP 2 (see above and reference 12). Since morpholino compound was not available radioactively labeled, we have studied itsbinding by competition for [14C]benzylpenicillin binding. Figure 5 shows

![Fig. 2](http://aac.asm.org/)

**Fig. 2.** Growth of E. coli KN126 in the presence of various concentrations of mecillinam and morpholino compound. Mecillinam: (A) control, (B) 2 μg/ml, and (C) 200 μg/ml. Morpholino compound: (D) 2 μg/ml, and (E) 5 μg/ml, (F) 10 μg/ml, (G) 50 μg/ml, (H) 150 μg/ml, and (I) 200 μg/ml.

![Fig. 3](http://aac.asm.org/)

**Fig. 3.** Cell division of E. coli KN126 in the presence of mecillinam, morpholino compound, and cephalaxin. Mecillinam: (A) control, (B) 2 μg/ml, and (C) 200 μg/ml. Morpholino: (D) 2 μg/ml, and (E) 200 μg/ml. Cephalaxin: (F) 50 μg/ml. The cell number at zero time was 8.6 × 10^6 organisms/ml.
Fig. 4. Binding of $[^{14}C]$mecillinam and $[^{14}C]$benzylpenicillin to membranes of E. coli KN126. (A) 0.001 μg/ml and then twofold increasing concentrations up to (K) 1.0 μg of $[^{14}C]$mecillinam per ml; (L) 31.0 μg of $[^{14}C]$benzylpenicillin per ml. Electrophoresis was from top to bottom, and the gel was exposed to X-ray film for 92 days. PBPs 5 and 6 have not resolved into two proteins on this gel.

Fig. 5. The competition of low concentrations of morpholino compound for the binding of $[^{14}C]$benzylpenicillin to membranes of E. coli KN126. (A) Control, (B) 0.0024 μg/ml and then threefold increasing concentrations up to (K) 47.6 μg of morpholino compound per ml. Morpholino compound was prebound at the above concentrations, and the residual binding of $[^{14}C]$benzylpenicillin (31.0 μg/ml) was measured. Electrophoresis was from top to bottom and the gel was exposed to X-ray film for 60 days.
an X-ray film from an experiment in which the competition of a range of concentrations (up to 47.6 μg/ml) of morpholino compound was studied. At these concentrations the morpholino compound showed strong competition for PBP 2 but only very slight competition for any of the other PBPs. The minor protein with a mobility slightly faster than PBP 4 is probably the minor proteolytic product of PBP 2 discussed above. Two other binding proteins of low molecular weight (PBPs 7 and 8) were also seen on this gel. These proteins have been found in several experiments (although in this experiment PBP 8 is unusually prominent) and may also be proteolytic products of other PBPs as they are only detected in some membrane preparations and are highly variable in amount. Fifty percent inhibition of the binding of 14C benzylpenicillin to PBP 2 was obtained with 0.066 μg of the morpholino compound per ml.

The morpholino compound, in contrast to meccillinam, showed typical penicillin lysis at concentrations of 50 μg/ml and above (Fig. 2), and we therefore compared the competition of high concentrations (up to 1 mg/ml) of these compounds for 14C benzylpenicillin binding. Figure 6 and Table 1 shows that the morpholino compound competed extensively for the binding of 14C benzylpenicillin to PBPs 1 and 4 and to a lesser extent to PBPs 5 and 6, whereas meccillinam, even at 1 mg/ml, competed little for any of the PBPs (except PBP 2). Very little competition for PBP 3 was observed with either amidinopenicillanic acid.

**DISCUSSION**

The morphological and physiological effects of meccillinam and morpholino compound were identical at concentrations up to 5 μg/ml. Neither compound caused cell lysis or significantly inhibited cell mass increase at these concentrations. Cell division was also not inhibited during the first 30 min but then division slowed and finally ceased, and the cells were converted to large osmotically stable spherical forms. Similar effects of meccillinam have been reported by other investigators (2, 5–8).

Mecillinam inhibits the growth of E. coli by interacting with PBP 2 (12, 13), and as expected the morpholino compound bound exclusively to this protein at low concentrations. Fifty percent binding of both meccillinam and morpholino compound was attained at concentrations close to the MIC values of these antibiotics.

At concentrations above about 50 μg/ml these two amidinopenicillanic acid derivatives had distinctly different properties. Two hundred micrograms of meccillinam per ml had no more effect on the growth, division, and morphology than did 2 μg/ml, whereas increasing amounts of morpholino compound resulted in cell lysis which occurred earlier and to a greater extent as the concentration was raised further. At 200 μg of morpholino compound per ml lysis resulted within 20 min and was indistinguishable from that produced by typical penicillins and cephalosporins (4, 11). Even at this concentration cell division continued at the same rate as in the control culture until lysis

![Graph showing competition of high concentrations of meccillinam and morpholino compound for binding of 14C benzylpenicillin.](http://aac.asm.org/)

**Fig. 6.** The competition of high concentrations of meccillinam (●) and morpholino compound (▲) for the binding of 14C benzylpenicillin. The experiment was performed as described in the legend to Fig. 5 and the PBPs were quantitated by densitometry. (A) PBP 1; (B) PBP 3; (C) PBP 4; (D) PBP 5; (E) PBP 6.

<table>
<thead>
<tr>
<th>Amidinopenicillanic acid</th>
<th>MIC (μg/ml)</th>
<th>Concentration of amidinopenicillanic acids required to give 50% binding or competition (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mecillinam (direct binding)</td>
<td>0.05</td>
<td>ND°, 0.023, ND, ND</td>
</tr>
<tr>
<td>Mecillinam (competition)</td>
<td>0.05</td>
<td>&gt;1,000, 0.04, &gt;1,000, &gt;1,000, &gt;1,000, &gt;1,000, &gt;1,000</td>
</tr>
<tr>
<td>Morpholino compound (competition)</td>
<td>0.2</td>
<td>49.0, 0.066, 956, 25.7, 324, 468</td>
</tr>
</tbody>
</table>

° ND, None detected at 14C meccillinam concentration of 1 μg/ml.
precluded further measurements. The extent of residual division at this time was at least three times that obtained with cephalaxin or benzylpenicillin. High concentrations of morpholino compound therefore showed the lysis (4), but not the immediate inhibition of cell division (14), which is typical of other β-lactams. The different responses of E. coli to high concentrations of mecillinam and morpholino compound were paralleled by a difference in their binding properties to the PBPs. Mecillinam, at concentrations up to 1 mg/ml, bound virtually exclusively to PBP 2 and showed very little competition for [14C]benzylpenicillin binding to any of the other PBPs. The morpholino compound, however, showed (in addition to its binding to PBP 2) extensive competition for PBPs 1 and 4 at high concentrations.

Morpholino compound clearly functions identically to mecillinam at concentrations up to about 5 μg/ml; at higher concentrations the ability of the former compound to lyse E. coli correlates very well with its affinity for PBPs 1 and 4. This result is additional evidence for our suggestion (12) that lysis by β-lactams is caused by their binding to PBP 1. However, the correlation between lysis and binding to PBP 4 is also good and this protein cannot be excluded as the possible target at which β-lactams bind to cause lysis. Hopefully the isolation of a temperature-sensitive cell lysis mutant which has a thermolabile PBP 1 (or PBP 4) will definitively answer this problem. The failure of either amidinopenicillanic acid to show typical penicillin inhibition of cell division agrees well with the failure of these compounds to bind to PBP 3 since this protein has been shown to be involved in the inhibition of division by penicillins and cephalosporins (12). The eventual inhibition of division caused by mecillinam is thought to result from the inhibition of an early step in the division cycle of E. coli and is not thought to be related to the immediate inhibition of division caused by other β-lactams (5).

The selectivity of binding of mecillinam to PBP 2 is remarkable. Modification of the side chain of this compound though can result in derivatives (e.g., the morpholino compound) which still maintain their mecillinam-like properties but which also have the lytic ability of typical β-lactams. Since mecillinam and typical β-lactams show synergy, both in vivo (3) and in vitro (1, 16), a compound that combines both these types of activity in the same molecule might be of clinical importance.