FL-1060: A NEW PENICILLIN WITH A UNIQUE MODE OF ACTION.

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SUMMARY: FL-1060, in contrast to other penicillins, does not inhibit murein transpeptidase, D-alanine carboxypeptidase I, or murein endopeptidase.

Lund and Tybring (1) recently reported the synthesis of a new group of semi-synthetic penicillins and described the biological activity of one such compound, designated FL-1060 (Fig. 1). The compound was remarkably active against most gram negative bacteria and caused the gradual conversion of Escherichia coli cells to large spherical bodies. These observations led Lund and Tybring (1) to suggest that the mode of action of FL-1060 differed from that of conventional penicillins.

We have confirmed their observations using Escherichia coli and wish to report biochemical evidence that FL-1060 does not have the same mode of action as ampicillin, a typical penicillin effective against Gram-negative bacteria.

RESULTS

1. The minimum inhibiting concentration of FL-1060 vs. E. coli. Lund and Tybring (1) reported that as little as 0.015 μg/ml of FL-1060 in a nutrient broth inhibited growth of some strains of E. coli by 50% when tested by a serial dilution method. By following growth over a shorter period of time, we observe that very low concentrations of FL-1060 do affect the growth and morphology of E. coli. However, even in the presence of a 1000-fold excess of FL-1060, increase in cell mass and cell division continues for the equivalent of 4 or 5 generations. At levels greater than 0.1 μg per ml,

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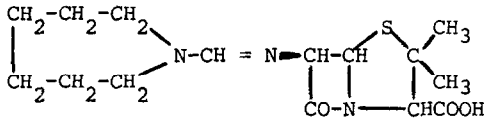

Fig. 1. Structure of FL-1060, 6β-[(hexahydro-1H-azepin-1-yl) methyleneamino]-penicillanic acid.

division is prevented and greatly enlarged, rounded cells are formed. With 0.025 μg FL-1060 per ml, the cells are small, almost spherical after 4 generations. On L-agar plates, nearly all cells form colonies in the presence of 0.05 μg FL-1060 per ml. In the presence of 0.1 μg/ml only 3% form colonies. E. coli β-lactamase hydrolyzes FL-1060 and ampicillin at about the same rate in a standard assay (2). Hence the high activity of FL-1060 is not due to unusual resistance to inactivation. Spontaneous mutants, with at least a 60-fold increase in resistance to FL-1060, occur with a frequency of about 1 in 10^5.

2. Effect of FL-1060 and ampicillin on cellular morphology. Figure 2 compares and contrasts the effects of FL-1060 and ampicillin on the morphology of E. coli D-11 during growth in L-broth. As can be seen, 0.02 μg/ml of FL-1060 causes the cells to change from rod-shaped to oval-shaped within 50 minutes. At higher concentrations (0.1 to 100 μg/ml) the cells enlarge and gradually become spherical. The loss of wall rigidity begins in the septal region so that after 50 minutes exposure many cells are egg-shaped. Low, but growth-inhibiting concentrations of ampicillin on the other hand, cause E. coli D-11 to form long rod-shaped cells in which bulges appear at the end or at the septum of the cell. The spheroplast then extrudes through the weakened region of the wall and eventually ruptures, leaving a rod-shaped ghost.

3. Effect of FL-1060 and ampicillin on murein synthesis in E. coli. Table I illustrates the effect of various concentrations of FL-1060 and ampicillin upon the amount of murein formed by growing cells and by non-growing cells. The results are similar under both conditions. FL-1060, at a relatively low concentration, inhibits murein synthesis by 60 or 70%. Increasing the

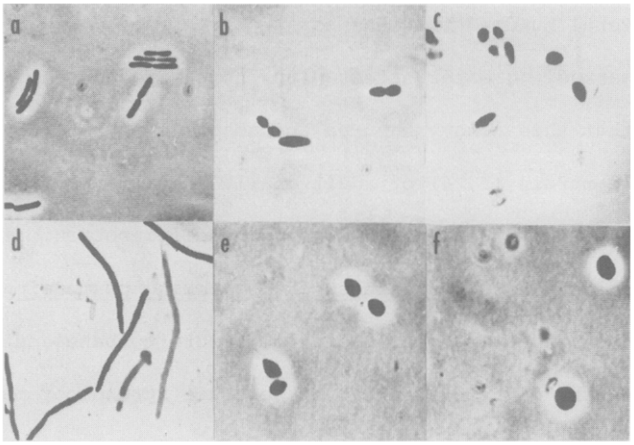

Fig. 2. Effect of FL-1060 on the morphology of E. coli grown in L-broth at 37°. a. control; b. 0.02 μg FL-1060/ml, 50 min.; c. 0.02 μg FL-1060/ml, 90 min.; d. 10 μg ampicillin/ml, 90 min.; e. 100 μg FL-1060/ml, 50 min.; f. 100 μg FL-1060/ml, 90 min.

TABLE I. Effect of FL-1060 and Ampicillin on Murein Synthesis by E. coli

	Murein Synthesis (% of Control)			
Antibiotic	Growing Cells[a]		Cells in Buffer[b]	
μg/ml	FL-1060	Ampicillin	FL-1060	Ampicillin
0.1	71	-	45	-
1.0	51	-	38	-
10	52	20	39	85
100	52	4	33	15
1000	48	-	34	6

a. About 2 x 10^9 log phase cells of E. coli W-7 were harvested from 5 ml of L-broth (supplemented with 1 mg L-lysine and 100 μg diaminopimelic acid (DAP) per ml) centrifuged, resuspended in 5 ml L-broth supplemented with 3.4 μg ^{3}H-DAP (≅2 μ C) and 1 mg L-lysine/ml plus antibiotics as indicated. The flasks were shaken 10 min at 37°. The cells were then collected by centrifugation, extracted for 3 min with boiling water, then with 80% ethanol, and counted.

b. About 3 x 10^9 log phase cells of E. coli D-11 were harvested, washed with 0.9% NaCl and suspended in 1 ml 0.05M tris buffer pH 7.6 containing 0.1 μ moles L-glutamic acid, 0.3 μ moles L-alanine, 2 μ moles L-lysine, 10 μ moles $MgCl_2$, 0.1 μ curies ^{14}C-DAP, 2 mg glucose, 200 μ g chloramphenicol and penicillin as indicated. Tubes shaken 30 min at 37°, centrifuged, and washed 2 times with 3 ml 0.9% NaCl containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The cells were resuspended in 50 μ l water, heated at 100° for 5 min, and the residue washed and counted.

concentration several hundred fold has no further effect. Ampicillin, in contrast, has a markedly greater effect with increasing concentration. It should be noted that this assay measures the amount of murein covalently bound to insoluble murein (3, 4) or additionally and alternatively, in the case of many Gram-negative bacteria, that bound to lipoprotein.

4. Effect of FL-1060 and ampicillin on cross-linking of murein. As was shown some years ago (5, 6) penicillin inhibits murein transpeptidase, thereby preventing the covalent linking of separate strands of murein to each other via peptide bonds between the carboxyl group of D-alanine on one strand and the available amino group on peptide of the other strand. Cross-linking by the murein transpeptidase of E. coli is also inhibited by penicillins (7, 8). We have measured the extent of cross-linking of newly synthesized murein in E. coli formed in the presence of FL-1060. As shown in Table II, FL-1060 had no effect on cross-linking of murein by membranes of E. coli at a concentration three hundred times higher than needed to inhibit growth or reduce the amount of murein formed. Ampicillin inhibited cross-linking by 66%. Similar results were obtained using as a source of enzymes, whole cells that had been frozen in 2M sucrose (9).

5. Effect of FL-1060 and ampicillin on other enzymatic activities sensitive to penicillin. Two other enzymatic activities, D-alanine carboxypeptidase I, measured by the release of the terminal D-alanine from uridine-5'-pyrophosphoryl-N-acetyl muramyl-L-alanyl- γ -D-glutamyl meso-diaminopimelyl-D-alanyl-^{14}C-D-alanine, and endopeptidase, which hydrolyzes the cross-link between D-alanine and diaminopimelic acid in E. coli have been shown to be sensitive to penicillin (8, 10). When tested at concentrations up to 1000 times the growth inhibiting concentration, these activities were completely insensitive to FL-1060, although in parallel experiments, 100 μ g/ml of ampicillin inhibited D-alanine carboxypeptidase I by 73% and markedly inhibited endopeptidase.

DISCUSSION

In the broad concentration range that causes a partial inhibition of

ABLE II. Effect of FL-1060 and Ampicillin on Extent of Cross-linking in Murein

	Murein Synthesized (cpm)	% Cross-linked
Control	1100	46
FL-1060 (10 µg/ml ≡ 300 x MIC[1])	1300	42
D-ampicillin (100 µg/ml ≡ 25 x MIC[1])	1700	16

[1]MIC = minimum growth inhibiting concentration.

Washed membranes from approximately 4 x 10^9 sonicated E. coli D-11 cells were incubated for 1 hr at 37° in 0.04 ml 0.04M tris buffer, pH8 containing 0.01M $MgCl_2$, 5 x 10^{-3}M ATP, 2 x 10^{-4}M UDPGlcNAc, and 1 x 10^{-4}M UDPMurNAc-L-ala-D-glu-meso DAP-^{14}C-D-ala-^{14}C-D-ala (68,000 cpm). After incubation, the reaction was stopped by boiling for 2 min. The reaction mixture was placed on Whatman 3MM paper and chromatographed in isobutyric acid-1M NH_4OH (5:3) overnight. The material at the origin was incubated overnight at 37° in 2 ml of ammonium acetate with 500 µg lysozyme. The lysozyme digest was chromatographed overnight as above and the components corresponding to cross-linked murein (C_3 + C_4) and uncross-linked murein (C_5 + C_6) were counted.
% cross-linked = C_3 + C_4/total x 100.

murein synthesis, FL-1060 does not inhibit murein transpeptidase. Although it is at least 300X more active than ampicillin in inhibiting growth of an E. coli, as an inhibitor of murein transpeptidase, FL-1060 is about 1 / 100 as active as ampicillin. FL-1060 is equally poor as an inhibitor of D-alanine carboxypeptidase I and endopeptidase. It is obvious that the effect of low concentrations of FL-1060 (0.02-100 µ g/ml) on the growth and morphology of E. coli is not due to inhibition of any of the enzymatic activities known to be sensitive to penicillin.

Nevertheless, from the effect of the antibiotic on the morphology of the cell and on the synthesis of murein, it can be deduced that the target for FL-1060 involves murein synthesis. Our results demonstrate that FL-1060, at a concentration approaching the minimum growth inhibiting concentration, significantly reduces the amount of insoluble murein formed by growing and non-growing cells of E. coli. The extent of inhibition has varied from 35 to 75% in different experiments. However, in any one experiment, the extent of inhibition is constant even when the concentration of FL-1060 is increased several hundred fold. This suggests that the low concentration has completely

inhibited or modified a specific and as yet unidentified reaction so as to partially reduce the amount of murein that is synthesized and attached to insoluble wall material. The high rate of spontaneous mutation to resistance to FL-1060 may suggest that a variety of ways exist for the cell to overcome the effect of FL-1060.

Since normal, cross-linked murein is made for extended periods of time in the presence of FL-1060, it would appear that the precursors of murein are made, assembled, polymerized, and cross-linked in the presence of the antibiotic. The reduced amount of murein formed in the presence of FL-1060 could be caused by inhibiting the reaction that covalently links murein to lipoprotein (11), or by altering the delicate balance between synthesis and breakdown of murein in a more subtle way. Whatever its action, FL-1060 is unique and should prove a useful probe to further elucidate the pathway of biosynthesis of murein in gram negative bacteria.

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