Ro 13-9904: AFFINITY FOR PENICILLIN BINDING PROTEINS AND EFFECT ON CELL WALL SYNTHESIS

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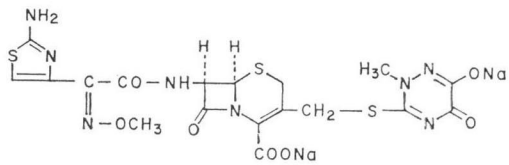
Studies on the binding of Ro 13-9904, a new broad-spectrum cephalosporin, showed that it had higher affinities for PBPs 1b, 2, and 3 of *Escherichia coli* (3>1b>2) than cefazolin, cephaloridine, cephalothin or cephalexin. With *Haemophilus influenzae*, Ro 13-9904 showed highest affinities for PBPs 4 and 5 followed by PBP 2. It inhibited total cell wall synthesis at lower concentrations than the other β-lactam antibiotics tested.

Recent investigations have compared the *in vitro* activities of several third generation β-lactam antibiotics including Ro 13-9904 (Fig. 1), a semi-synthetic cephalosporin, against Gram-negative bacteria[1~3]. We studied the mechanism of action of Ro 13-9904 from the viewpoint of affinity to penicillin-binding proteins (PBPs), morphological changes and inhibition of peptidoglycan synthesis.

Fig. 1. Chemical structure of Ro 13-9904.



Materials and Methods

Antibiotics, Labelled Compounds and Other Chemicals

The antibiotics used in this study included Ro 13-9904 (Hoffmann-La Roche, Nutley, N.J., U.S.A.); cefazolin, cephalexin, cephaloridine and cephalothin (Eli Lilly Co., Indianapolis, IN, U.S.A.); and penicillin G (pen G; Sigma, St. Louis, MO, U.S.A.). Radioactive compounds included [^{14}C]-pen G, specific activity of 50~60 mCi/mmol and UDP-N-acetyl-[^{14}C]-glucosamine, specific activity 323 mCi/mmol (Amersham Corp., Arlington Heights, IL, U.S.A.); and diamino [^{14}C]-pimelic acid (New England Nuclear, Boston, MA, U.S.A.), specific activity 105.26 mCi/mmol. Reagents for sodium dodecyl-polyacrylamide gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other chemicals were of reagent grade.

Bacterial Strains

Strains used included *Haemophilus influenzae* (ATCC 19418); *Escherichia coli* K12 strain KN126 (a gift from B. G. SPRATT); *Bacillus cereus* strain T (QMB 1590); *E. coli* Y-10, thr-1, leu B6, thi-1, ribf D1, sup E 44 (a gift from B. BACHMANN); *E. coli* K12 JE5707, Dap$^-$, Lys$^-$, Thi$^-$ (a gift from Y. HIROTA).

Binding of β-Lactam Antibiotics to the PBPs of *E. coli* and *H. influenzae*

Preparations of membranes and assay of PBPs of *E. coli* were performed according to the procedure reported by SPRATT[4]. A modification of that procedure, which was used to determine binding to *H. influenzae* PBPs, has been described recently[5]. The modified gel system, which separates PBPs 1a and 1b of *E. coli*[6], was used for the separation of the PBPs of *E. coli* and *H. influenzae*. Levels of [^{14}C]-penicillin G bound to PBPs were quantitated by densitometry of fluorograms (Zeiss PMQ II thin-layer chromatogram spectrophotometer coupled to a Columbia Scientific Industries CSI-38 integrator). Binding affinities are expressed in terms of I_{50} values, which are the concentrations (μg/ml) required to inhibit the

binding of [^{14}C]-penicillin G by 50%.

Morphological Studies

To determine effects on morphology, exponentially growing *H. influenzae* cells (5 ml; approx. 1×10^9 cells/ml) were further incubated after the addition of Ro 13-9904. At specified times, samples were removed and examined by phase-contrast microscopy.

Measurement of Inhibition of Peptidoglycan Synthesis

Incorporation of [^{14}C]-*meso*-diaminopimelic acid (DAP) into the cell wall of *E. coli* K12 JE5707 was measured according to NOZAKI *et al.*[7] with slight modifications. A synthetic medium (1 g $(NH_4)_2SO_4$, 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 0.1 g $MgSO_4$ per liter supplemented with 0.5% glucose, 10 μg/ml DAP, 200 μg/ml lysine and 0.5 μg/ml thiamine), which allowed a doubling time of 60 minutes, was used. After 2 hours at 37°C 0.5 ml samples (containing 0.25 μCi [^{14}C]-DAP) were processed.

Measurement of Inhibition of Peptidoglycan Transpeptidase

The procedure of MOORE *et al.*[8] was used to assay peptidoglycan transpeptidase activity of ether treated *E. coli* K12 Y-10 (ETB). Uridine 5'-diphospho-N-acetyl-muramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine (UDP-Mur-NAc-pentapeptide) was isolated from *B. cereus* strain T[8]. The concentration of pentapeptide was determined by ultraviolet adsorption at 262 nm relative to a uridine diphosphate (UDP) standard. An analysis of UDP-Mur-NAc-pentapeptide resulted in a ratio of alanine: glutamic acid: *meso*-DAP of 2.7: 1: 1.1, which is consistent with the theoretical value of 3: 1: 1.

Results

1. Affinity of Ro 13-9904 to PBPs of *E. coli* and *H. influenzae*

The affinities to PBPs were estimated in two ways: by measuring the competition of unlabelled Ro 13-9904 with [^{14}C]-pen G for binding to PBPs and by measuring the direct binding of [^{14}C]-Ro 13-9904 to PBPs. Profiles of competition of unlabelled Ro 13-9904 and other cephalosporin derivatives with [^{14}C]-pen G for binding to PBPs in *E. coli* and in *H. influenzae* are shown respectively in Figs. 2 and 3.

All the cephalosporins tested showed high affinity for PBP 1a of *E. coli*. Ro 13-9904 generally showed higher affinities for PBPs 1b, 2 and 3 than those of the reference cephalosporins (Table 1). With *H. influenzae*, Ro 13-9904 showed highest affinities for PBPs 4 and 5 (Table 2), whereas the other cephalosporins all showed highest affinity for PBP 2.

The binding of [^{14}C]-Ro 13-9904 to the PBPs of *E. coli* is shown in Fig. 4. We were unable to quantitate by densitometry the level of binding due to high background. Nevertheless, when bound

Table 1. Competition of β-lactam antibiotics for [^{14}C]-pen G binding to *E. coli* K12 KN126 PBPs.

Antibiotic	MIC[1] (μg/ml)	I_{50} for PBPs (μg/ml)					
		1a	1b	2	3	4	5/6
Ro 13-9904	0.125	<0.1	1.8	3.6	<0.1	>78	>78
Cefazolin	6.25	<1	17	7.5	9	11	>31
Cephaloridine	12.5	<1	8	130	13	15	>62.5
Cephalothin	12.5	N.D.[2]	>100	90	7.6	70	>62.5
Cephalexin	25.0	N.D.	N.D.[3]	N.D.	N.D.	N.D.	N.D.

[1] MIC is defined as the lowest concentration which still gives a zone of inhibition by the agar diffusion well technique.

[2] Not determined.

[3] Although I_{50}'s for cephalexin were not calculated, it is apparent from Fig. 2 that the I_{50} for PBP 1b is >125.

Fig. 2. Binding of [^{14}C]-pen G to the PBPs of *E. coli* K12 KN126.

Each lane represents a reaction containing 0.076 μmole/ml [^{14}C]-pen G (59.5 μCi/μmole) plus the following concentrations (μg/ml) of unlabelled cephalosporin derivatives: Ro 13-9904; A = 0, B~F = 0.125, 0.615, 3.125, 15.625, 78.12, respectively; cefazolin, G~I = 1.25, 6.25, 31.3, respectively; cephaloridine, J~L = 2.5, 12.5, 62.5, respectively; cephalothin, M~O = 2.5, 12.5, 62.5, respectively; and cephalexin, P, Q = 25, 125, respectively.

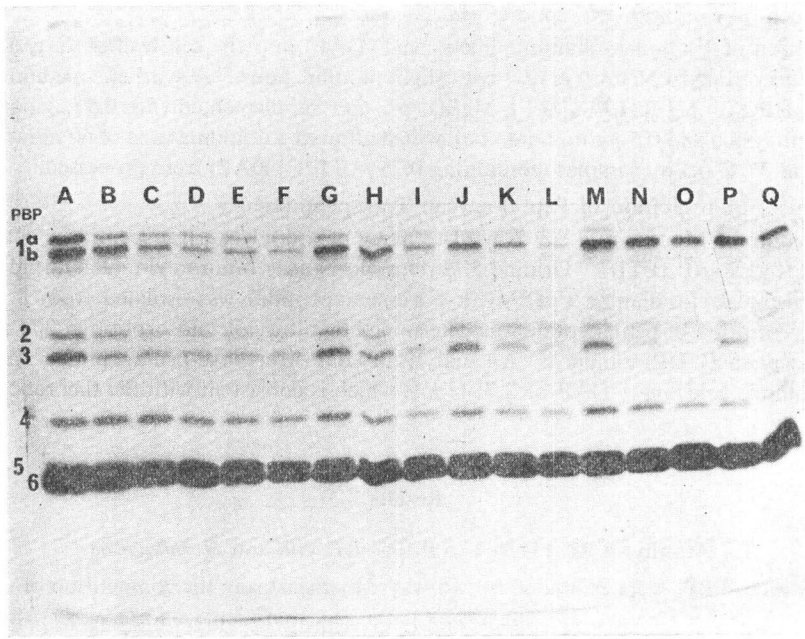


Fig. 3. Binding of [^{14}C]-pen G to the PBPs of *H. influenzae*.

Each lane represents a reaction containing 0.14 μmole/ml [^{14}C]-pen G (59.5 μCi/μmole) plus the following concentrations (μg/ml) of unlabelled cephalosporin derivatives: Ro 13-9904; A = 0, B~F = 0.01, 0.05, 0.25, 1.25, 6.25, respectively; cefazolin, G~I = 0.31, 1.6, 7.8, respectively; cephaloridine, J~L = 0.04, 0.19, 0.95, respectively; and cephalexin, P, Q = 12.5, 62.5, respectively.

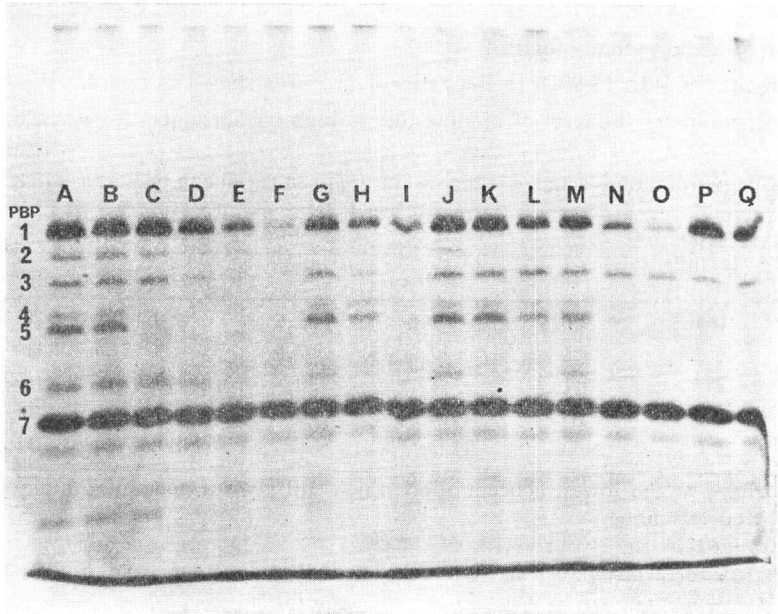
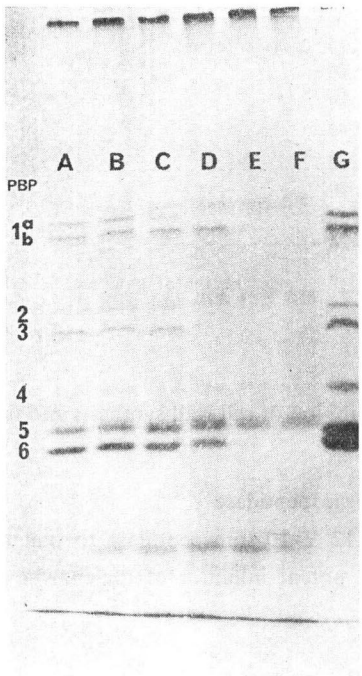


Table 2. Competition of β-lactam antibiotics for [^{14}C]-pen G binding to *H. influenzae* PBPs.

Antibiotic	MIC (μg/ml)	I_{50} for PBPs (μg/ml)						
		1	2	3	4	5	6	7
Ro 13-9904	0.06	1.2	0.15	0.6	0.017	0.038	1	>6.25
Cefazolin	1.57	<1.6	<0.15	1.4	1.7	2.7	N.D.	>7.8
Cephaloridine	0.19	>0.95	0.1	>0.95	>0.95	>0.95	0.85	>0.95
Cephalothin	0.79	0.75	<0.16	>1.95	N.D.	N.D.	N.D.	>1.95
Cephalexin	12.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Fig. 4. Binding of [^{14}C]-Ro 13-9904 to *E. coli* membrane fraction.

Each lane represents a reaction containing 0.09 μmole/ml [^{14}C]-Ro 13-9904 (48.2 μCi/μmole) plus the following concentrations (μg/ml) of unlabelled penicillin G: A = 0, B = 0.5, C = 2.5, D = 12.5, E = 62.4, F = 312. Lane G represents a reaction containing 0.09 μmole/ml [^{14}C]-penicillin G (48.2 μCi/μmole) plus H_2O.



3. Inhibition of Peptidoglycan Synthesis

The inhibitory effect of Ro 13-9904 and other β-lactam antibiotics on the incorporation of [^{14}C]-*meso*-diaminopimelic acid (DAP) into the cell wall of *E. coli* K12 JE5707 is shown in Fig. 5. Ro 13-9904 inhibited cell wall synthesis at concentrations considerably lower than were required for several other cephalosporins and for pen directly, [^{14}C]-Ro 13-9904 showed affinity for the same 6 PBPs as shown with [^{14}C]-pen G binding, although differences were observed in the relative distribution of label. In the case of *H. influenzae* (not shown), additional bands which did not compete with unlabelled pen G were detected. These may be artifacts for which we do not presently have an explanation.

2. Morphological Changes Induced in *H. influenzae* by Ro 13-9904

The morphological effects of some penicillins, cephalosporins and an amidino-penicillin on growing *H. influenzae* cells were previously reported[5]. In this study (data not shown) Ro 13-9904 produced slightly elongated cells after 30 minutes at a concentration as low as 0.005 μg/ml, whereas at 0.5 and 1 μg/ml cells with bulges appeared. Exposure to 1 μg/ml for 90 minutes resulted in spheroplasts and lysed cells. Upon prolonged incubation (5 hours) the cells exposed to 0.005 μg/ml Ro 13-9904 were 2~3 times longer than normal, and were 10~15 times longer at 0.05 and 0.1 μg/ml respectively.

Table 3. Inhibition of peptidoglycan synthesis in *E. coli* K12 JE5707.

Antibiotic	MIC (μg/ml)	Conc. required for 50% inhibition of [^{14}C]-DAP incorporation (μg/ml)
Ro 13-9904	0.2	0.3
Cefazolin	6.3	1.8
Cephaloridine	12.5	2.6
Cephalothin	6.3	13.5
Cephalexin	25.0	>100.0
Penicillin G	50.0	12.5

Fig. 5. Inhibition of peptidoglycan synthesis in *E. coli* K12 JE5707 by cephalosporins and pen G.

The amount of DAP incorporated in the control incubation mixture was 0.35 nmol. (See Table 3 for comparison of MIC values and concentration required for 50% inhibition of [^{14}C]-DAP incorporation).

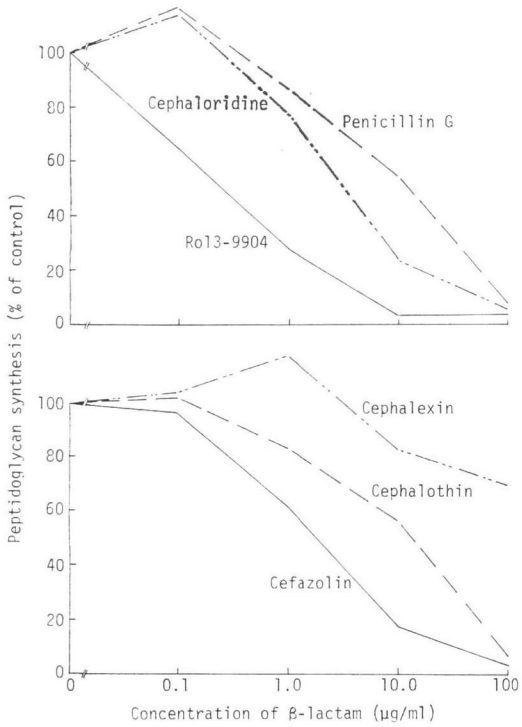
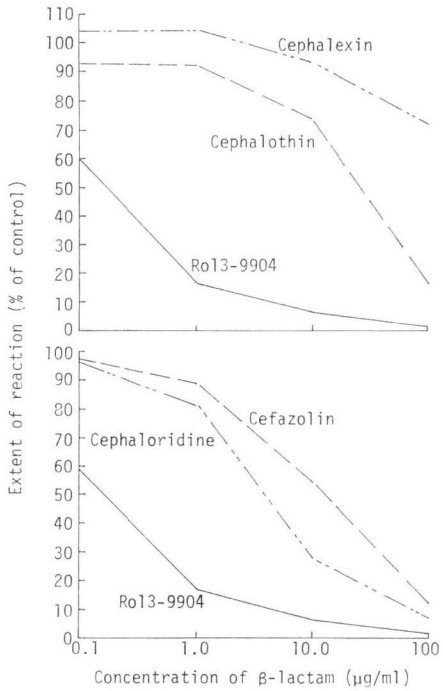


Fig. 6. Inhibition of transpeptidase activity by Ro 13-9904 and other cephalosporins in ETB of *E. coli* K12 Y–10.

The percent incorporation of N-acetyl-[^{14}C]-glucosamine at a range of antibiotic concentrations relative to the control reaction[8] is shown.



G. A comparison of the concentrations required for 50% inhibition of cell wall synthesis and the MICs for each of the β-lactams is shown in Table 3.

4. Inhibition of Peptidoglycan Transpeptidase

A comparison of the effect of Ro 13-9904 on *E. coli* K12 Y–10 transpeptidase to that of other cephalosporins is shown in Fig. 6. Ro 13-9904 is a more potent inhibitor of the enzyme than the other compounds tested.

Discussion

Our studies compared a few biochemical activities of Ro 13-9004 to those of several other β-lactam antibiotics and found it to be the most potent of those tested. Results of binding experiments revealed that it had higher affinity for PBPs 1b, 2 and 3 of *E. coli* (3>1b>2) than the other cephalosporins tested (Table 1). With *H. influenzae*, Ro 13-9904 differs from the other β-lactam antibiotics tested in that it has the highest affinity for PBPs 4 and 5 rather than PBP 2[5] (Table 2). The affinity of Ro 13-9904 for PBPs 4 and 5 is 9 and 4 times higher, respectively, than the affinity for PBP 2. It should be noted that the I_{50} of Ro 13-9904 for PBP 2 is similar to that of the reference cephalosporins. The high affinity for PBPs 4 and 5, may suggest that the mode of action of Ro 13-9904 against *H. influenzae* is different from that of other cephalosporins or other β-lactam antibiotics[6].

As with other β-lactam antibiotics which show high affinity for PBP 3 of *E. coli*[9], Ro 13-9904 causes filamentation of cells over a range of 1/4 to 4× MIC (personal communication, Dr. M. J. KRAMER). Our studies show that it also causes filamentation of *H. influenzae* cells.

Ro 13-9904 proved to be the most potent inhibitor of total cell wall synthesis (Table 3) and peptidoglycan transpeptidase (Fig. 6) when compared to other β-lactam antibiotics tested. Evidence that PBP 1b may be one of the essential enzymes involved in cross-linking of peptidoglycan was presented by TAMAKI *et al*[10]. Our results show that, of the cephalosporins tested, the order of transpeptidase inhibition followed their affinities for PBP 1b (Table 1 and Fig. 6).

Acknowledgement

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