[Chem. Pharm. Bull. 21(1) 144—148 (1973)]

UDC 547.722.5.09 : 542.98

Inhibition of the Synthesis of Macromolecules in *Escherichia coli* by Nitrofuran Derivatives. III.[1] Nifurpirinol

Yoshiyuki Takase, Shinichi Nakamura, Masamitsu Ishiyama, and Masanao Shimizu

Research and Development Division, Dainippon Pharmaceutical Co., Ltd.[2]

(Received June 21, 1971)

The effect of nifurpirinol on deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein syntheses was investigated in 2 strains of *Escherichia coli*. In the both strains these syntheses were all inhibited at the concentrations higher than the minimal inhibitory concentration against the strains. DNA and RNA syntheses were also inhibited at lower concentrations than the minimal inhibitory concentration where bacterial growth was temporarily suppressed. The most sensitive to nifurpirinol was DNA synthesis among the syntheses tested. The concentration and the time inhibiting DNA synthesis almost coincided to those inhibiting bacterial growth. The results indicate that the inhibition of DNA synthesis may be a mechanismof antibacterial action of nifurpirinol.

Nifurpirinol is a nitrofuran derivative which is used as an antibacterial agent against fish diseases.[3] As the mechanism of action of nitrofurans, various effects have been proposed.[4] However it is uncertain which effects are really responsible for antibacterial action of nitrofuran derivatives. Recently it was found that nitrofuran derivatives inhibited the synthesis of macromolecules of *Escherichia coli* and the inhibition of deoxyribonucleic acid (DNA) synthesis was closely correlated with antibacterial activity.[5]

To know whether nifurpirinol has such inhibitory actions, its effect on the synthesis of macromolecules of *Escherichia coli* was investigated in connection with its antibacterial activity.

Experimental

(a) Chemicals——Nifurpirinol, 6-hydroxymethyl-2-[2-(5-nitro-2-furyl)vinyl]pyridine, was synthesized in Research and Development Division, Dainippon Pharmaceutical Co.[6] ^{14}C-Labeled thymine, uracil and L-histidine were purchased from Radiochemical Centre, whose specific activities were 455, 535 and 373 mCi/mg respectively.

(b) Organisms and Media——*Escherichia coli* NIHJ JC-2, a standard strain for sensitivity test recommended by Japan Society of Chemotherapy, and *Escherichia coli* JG181-1, an auxotroph requiring uracil and L-histidine were employed. JG181-1 was a spontaneous revertant of JG181,[7] an auxotroph requiring thymine, uracil and L-histidine, and used in the present study to avoid thymine-less death. The media used were nutrient broth[8] or nutrient agar for the growth of the former and Davis' minimal medium (MM)[9] supplemented with uracil (U) and L-histidine(H) in 50 μg/ml each for that of the latter.

(c) Measurement of Antibacterial Activity of Nifurpirinol——Minimal inhibitory concentration (MIC) was determined by a serial 2-fold tube dilution method, where one loopful overnight broth culture (about 10^5 cells) was inoculated into 1 ml of the media containing nifurpirinol at graded concentrations and incubated

1) Part II: S. Nakamura and M. Shimizu, *Chem. Pharn. Bull.* (Tokyo), **21**, 137 (1973).
2) Location: *Enoki 33-94, Suita, Osaka, 564, Japan.*
3) M. Shimizu and Y. Takase, *Bull. Jap. Soc. Sci. Fish.*, **33**, 544 (1967).
4) H.E. Paul and M.F. Paul, the Nitrofurans-Chemotherapeutic Properties in "Experimental Chemotherapy," Vol. 2, Part 1 ed. by R.J. Schnitzer and F. Hawking, Academic Press Inc., New York, 1964, p. 307.
5) S. Nakamura and M. Shimizu, *Chem. Pharm. Bull.* (Tokyo), **21**, 130 (1973).
6) A. Fujita, M. Nakata, S. Minami, and H. Takamatsu, *Yakugaku Zasshi*, **86**, 1014 (1966).
7) M. Ishibashi, *Japan J. Genetics*, **41**, 75 (1966).
8) M. Shimizu, S. Nakamura, and Y. Takase, *Antimicrob. Ag. Chemother.*, **1970**, 117 (1971).
9) B.D. Davis and E.S. Mingioli, *J. Bacteriol.*, **60**, 17 (1950).

at 37° for 18—20 hours. The effect of nifurpirinol on the growth curve of the bacteria was determined by colony count following exposure of the bacteria to the drug at various concentrations. Initial bacterial concentrations were about 10^6 cells/ml and incubation was made at 37° with shaking followed by colony counting on nutrient agar.

(d) Chemical Analysis of Bacterial DNA, RNA and Protein——An overnight culture of the bacteria was diluted 10 times with the fresh medium and shaken at 37° for 3—5 hours until bacterial density reached to about 5×10^8 cells/ml. The culture was mixed with an equal volume of the medium containing nifurpirinol at various concentrations and incubated at 37° with shaking. After incubation for 2 hours, an equal volume of 10% cold trichloroacetic acid (TCA) was added to the culture and fractionated according to Schmidt-Thanhauser's procedure[10] to get DNA, RNA and protein fractions. DNA was colorimetrically determined by Burton's method,[11] RNA by Drury's method[12] and protein by Lowry's method.[13]

(e) Measurement of Inactivation of Nifurpirinol by Bacteria——The bacteria were grown at 37° for about 4 hours up to a density of around 10^9 cells/ml. The cultures were diluted 10 and 100 times with the fresh media, mixed with an equal volume of the media containing nifurpirinol at a concentration of 1 μg/ml and shaken at 37° for 60 minutes. At indicated times, 2 ml samples were taken, heated in boiling water for 10 minutes, cooled and assayed for residual nifurpirinol by the cup-plate method using *Bacillus subtilis*.[3]

(f) Incorporation Experiment of ^{14}C-Labeled Precursors——One half ml of the fresh culture of *Escherichia coli* JG181-1 in MM supplemented with U and H, containing about 6×10^7 cells/ml, was mixed with 0.3 ml MM, 0.1 ml ^{14}C-labeled precursor solution (^{14}C-thymine: *ca.* 5 μCi/ml, ^{14}C-uracil and ^{14}C-histidine: *ca.* 0.5 μCi/ml) and 0.1 ml nifurpirinol solution (graded concentrations). The mixtures were shaken at 37° and 1 ml of 10% TCA was added to them with 1 mg bovine serum albumin (Armour) as carrier 15, 30 and 60 minutes postincubation. The precipitate was washed 3 times with 5% cold TCA, dissolved in 0.5 ml of 0.2N NaOH, heated in boiling water for 5—10 minutes, mixed with 1 ml water and 10 ml dioxane scintillator solution[14] and counted for radioactivity by a TEN liquid scintillation counter, model GSL-161 (Kobe Kogyo).

Result

(a) Antibacterial Activity of Nifurpirinol

With the tube dilution method, the MIC of nifurpirinol against *Escherichia coli* used in this study was 0.78 μg/ml for NIHJ JC-2, and 1.56 μg/ml for JG181-1. The effect of nifurpirinol on the growth of these two strains was examined in more detail as shown in Fig. 1. Under the experimental conditions, a control showed exponential growth for 2 hours in NIHJ JC-2 and for 5 hours in JG181-1 after 1-hour lag period. Doubling times were approximately 35 minutes for NIHJ JC-2 and 76 minutes for JG181-1 in a logarithmic phase. In the presence of nifurpirinol, growth rates were markedly affected. At 1/4 MIC, the number of viable cells was almost unchanged during the incubation time of 6 hours. At higher concentrations than 1/2 MIC, the loss of viability was observed from 30 minutes or 1 hour and viable cells were reduced exponentially with time elapsed. The bactericidal action

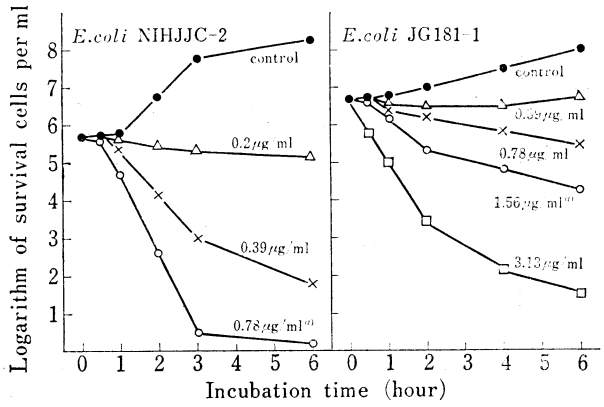

Fig. 1. Effect of Nifurpirinol on the Growth of *Escherichia coli* NIHJ JC-2 and JG181-1

a) MIC

10) E. Volkin and W.E. Cohn, "Method of Biochemical Analysis," Vol. 1, ed. by D. Glick, Interscience Publishers, Inc., New York, N.Y., 1954, p. 287.
11) K. Burton, *Biochem. J.*, **62**, 315 (1956).
12) G.L. Miller, R.H. Golder, and E.E. Miller, *Anal. Chem.*, **23**, 903 (1951).
13) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
14) G.A. Bray, *Anal. Biochem.*, **1**, 279 (1960).

of nifurpirinol in NIHJ JC-2 was remarkable compared with that in JG181-1. It was noted that antibacterial activity of nifurpirinol against the both strains was observed not only at ligher concentrations than MIC but also at lower concentrations within a limited period of 6 hours.

(b) Effect of Nifurpirinol on the Synthesis of Macromolecules of *Escherichia coli*

The two strains of *Escherichia coli* were grown in the presence or absence of nifurpirinol. The grown cells were fractionated and colorimetrically analyzed for macromolecules. Under the conditions employed, the increase of DNA, ribonucleicacid(RNA), and protein of a control culture was 2.3, 2.3 and 3.1 times initial amount respectively in JG181-1, and was 2.7, 3.6 and 2.6 times in NIHJ JC-2. In the presence of nifurpirinol at graded concentrations around MIC, the synthesis of these macromolecules was inhibited as shown in Fig. 2. The higher the concentration of the agent, the lower the relative increase of the macromolecules. Although the increase of RNA and protein was significantly inhibited by nifurpirinol at the concentrations higher than MIC, the most sensitive to the agent was the increase of DNA, which was evidently arrested at the concentrations higher than 1/2 MIC in the both strains.

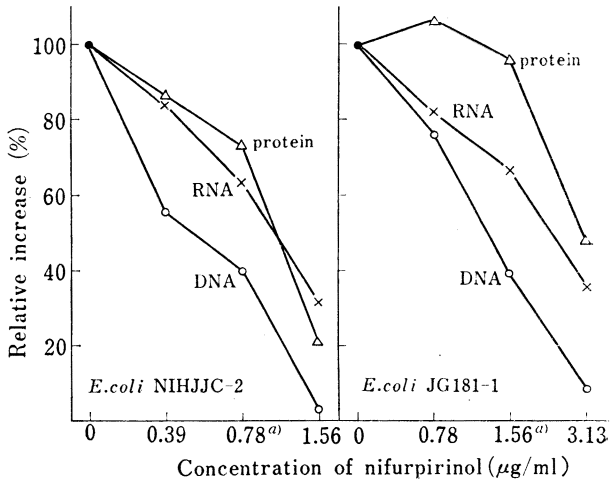

Fig. 2. Effect of Nifurpirinol on the Synthesis of Macromolecules of *Escherichia coli* NIHJ JC-2 and JG181-1
a) MIC

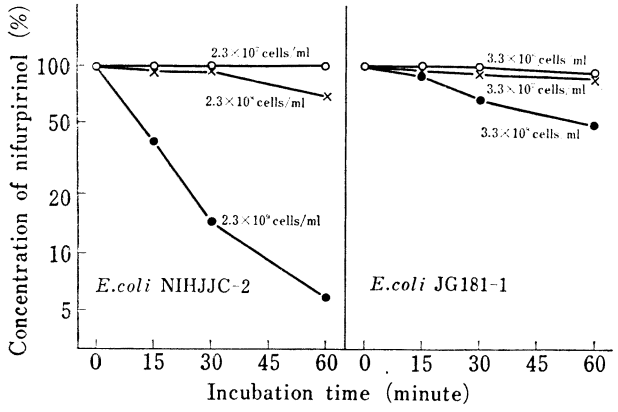

Fig. 3. Degradation of Nifurpirinol by the Cells of *Escherichia coli* NIHJ JC-2 and JG181-1

(c) Inactivation of Nifurpirinol by Bacterial Cells

It has been known that nitrofuran derivatives were decomposed by bacterial cells.[15] So, the concentration of nifurpirinol in the incubation media was determined to know whether the concentration had been kept constantly during the incubation period (Fig. 3). In NIHJ JC-2, slight descent of the drug concentration was seen at the bacterial density of 2.3×10^8 cells/ml and marked descent was detected at 2.3×10^9 cells/ml. In JG181-1, gradual inactivation was observed at the bacterial density of 3.3×10^8 cells/ml. However, inactivation was not seen below 3.3×10^7 cells/ml in the both strains. The inactivating activity of the strains was almost same each other. Under the conditions used

15) D.L. Cramer, *J. Bacteriol.*, **54**, 119 (1947); R.E. Asnis, F.B. Cohen, and J.S. Gots, *Antibiot. Chemotherapy*, **2**, 123 (1952); A.H. Beckett and A.E. Robinson, *J. Pharm. Pharmacol.*, **8**, 1072 (1956).

for colorimetrical determination of the bacterial macromolecules, the concentration of nifurpirinol seemed to drop gradually during the incubation period.

(d) Effect of Nifurpirinol on the Incorporation of ^{14}C-Labeled Precursors into Acid-insoluble Fraction

The effect of nifurpirinol on the synthesis of macromolecules in JG181-1 was investigated with the incorporation of ^{14}C-labeled thymine, uracil and histidine into acid-insoluble fraction. To avoid lowering the concentration of nifurpirinol during incubation period, the bacterial concentration used was finally made at 3×10^7 cells/ml. As shown in Fig. 4, ^{14}C-thymine incorporation was 467 cpm in a control for 60 minutes. Although no inhibition was detected at the concentration of 1.56 μg/ml (MIC) of nifurpirinol until 30 minutes of incubation, the incorporation was weakly inhibited at 60 minutes. The percent inhibition relative to the control was 25%. At the higher concentration such as 6.25 and 25 μg/ml, the incorporation was almost completely suppressed throughout the incubation period.

In the case of ^{14}C-uracil a control incorporated 1240 cpm during 60-minute-incubation. In the presence of nifurpirinol at 1.56 μg/ml, the incorporation was reduced by 16% during 60 minutes postincubation. The inhibition of the incorporation was complete at the concentration of 25 μg/ml, whereas it was incomplete at the concentration of 6.25 μg/ml. A similar result was obtained with ^{14}C-histidine incorporation, where a control incorporated 1040 cpm, although the inhibition was not seen at all at the concentration of 1.56 μg/ml. These results indicated that DNA synthesis is most sensitive to nifurpirinol. It was noted that the inhibition of DNA synthesis was seen within 30 minutes at the concentrations more than 6.25 μg/ml but was not at the MIC of 1.56 μg/ml.

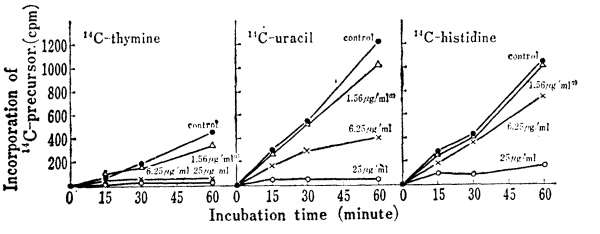

Fig. 4. Effect of Nifurpirinol on the Incorporation of ^{14}C-Thymine, ^{14}C-Uracil and ^{14}C-Histidine into Acid-insoluble Fraction in *Escherichia coli* JG181-1

a) MIC

Discussion

As shown in Result, nifurpirinol showed antibacterial activity on the 2 strains of *Escherichia coli*, NIHJ JC-2 and JG181-1, not only at MIC but also at 1/2 or 1/4 MIC. A similar result has been reported with *Escherichia coli* K-12 and *Vibrio anguillarum* K-3.[3]

By the colorimetrical analysis, nifurpirinol inhibited the DNA, RNA and protein syntheses of *Escherichia coli* NIHJ JC-2 and JG181-1. The most inhibited was the DNA synthesis. In NIHJ JC-2, the agent suppressed the increase of DNA to about 40% of a control at MIC and to about 60% at 1/2 MIC. In JG181-1, the DNA synthesis was inhibited to about 40% of a control at MIC and to about 80% at 1/2 MIC. It should be noted that the inhibition of DNA synthesis is observed below MIC as well as growth inhibiting action. Determination of nifurpirinol in the incubation media revealed that the concentration in the media gradually descended with such high bacterial concentrations as those used for colorimetrical analysis. The activity inactivating nifurpirinol was almost similar in the both strains. Under the conditions where the concentration of nifurpirinol should be constant during incubation period, nifurpirinol inhibited the incorporation of ^{14}C-labeled thiymine, uracil and histidine into acid-insoluble fraction of JG181-1 at the concentration above MIC. However, at MIC, only ^{14}C-thymine and ^{14}C-uracil incorporation was inhibited and the former was more strongly inhibited than the latter. The time when the inhibition of ^{14}C-thymine incorporation became observable, was 60 minutes postincubation at MIC though it was observed within

15 minutes at the concentrations more than 4 MIC. The similar phenomenon was seen in bactericidal action of nifurpirinol. The loss of viability in the presence of nifurpirinol was not seen within first 30 minutes around MIC whereas viable cells were rapidly reduced at a higher concentration than MIC. The reason why such lag time exists at MIC, is not clear at present. It has been reported that nitrofurazone is reduced in bacterial cells prior to its action.[16] Such lag may be due to the time required for metabolic conversion of nifurpirinol in bacterial cells. Aside from such intracellular events, the fact that DNA synthesis is most sensitive to nifurpirinol among the macromolecule syntheses tested and that the concentration inhibiting DNA synthesis almost coincides with that inhibiting bacterial growth, suggest the inhibition of DNA synthesis to be a mechanism of antibacterial action of nifurpirinol. This conclusion agree with previous one[5] drawn from the experiments using various nitrofuran derivatives.

Acknowledgement The authors wish to thank Dr. S. Ose, Managing Director of Dainippon Pharmaceutical Co. Ltd., Dr. T. Mizuma, Director of this laboratory, and Dr. H. Takamatsu, Vice-Director of this laboratory, for their encouragement throughout this work.

16) D.R. McCalla, A. Reuvers, and C. Kaiser, *J. Bacteriol.*, **104**, 1126 (1970).