

## Antibacterial Activity of Cinoxacin In Vitro

HELEN GIAMARELLOU AND GEORGE G. JACKSON\*

Department of Medicine, Abraham Lincoln School of Medicine, University of Illinois, Chicago, Illinois 60680

Received for publication 2 January 1975

Cinoxacin is a new synthetic compound similar chemically and in antimicrobial activity to oxolonic acid and nalidixic acid. It is most effective against *Escherichia coli* and *Proteus mirabilis*, but at concentrations expected in the urine it is inhibitory for all species of *Enterobacteriaceae*. Relative to nalidixic acid, cinoxacin has slightly greater inhibitory and bactericidal activity, less inoculum effect probably due to less heterogeneity in the susceptibility of bacterial cells, and less inhibition by high concentrations of serum protein. Both drugs are more active in an acid than an alkaline medium. Glucose can specifically antagonize the inhibitory effect against *P. mirabilis*. In urine the bactericidal rate and effect are decreased. Resistance to cinoxacin can be developed quickly by serial transfers in vitro. Some nonresistant organisms remained viable in bactericidal drug concentrations. The in vivo importance of the favorable features of cinoxacin must be determined by clinical trials.

Cinoxacin is a new synthetic compound with antibacterial activity (5). The name is derived from the basic ring structure which is a cinnoline (1-2 benzodioxine). Chemically, the compound is 1-ethyl-1,4-dihydro-4-oxo-(1,3)-dioxolo-(4,5-g)cinnoline-3-carboxylic acid. Cinoxacin belongs to the same class of antimicrobial drugs as nalidixic acid and oxolinic acid which are used in the treatment of urinary tract infections. Its antibacterial activity is similar to these drugs in vitro and perhaps superior in vivo against experimental pyelonephritis in rats (1, 5). In this study a series of observations were made to further evaluate the antimicrobial action of cinoxacin against recent clinical isolates and compare its activity with nalidixic acid.

### MATERIALS AND METHODS

Recent bacterial isolates (170) from patients at the University of Illinois Hospital were studied. Cinoxacin was provided by the Lilly Research Laboratories (compound 64716) and nalidixic acid by Sterling Winthrop Research Institute. Stock solutions of 2,000 µg of each drug per ml were prepared in phosphate buffer (pH 7), stored at -20 C, and used within 6 weeks. Minimal inhibitory concentrations (MIC) for cinoxacin and nalidixic acid were determined by the standard tube dilution or microtiter technique. The basic medium was tryptose phosphate broth which was modified for specific tests by adjustment of pH, or by the addition of defined amounts of horse serum, sodium chloride, sucrose, dextrose, or urea. Urine that was used as a bacteriologic medium was obtained from healthy adults, pooled, and filtered through a sterilizing filter. Serial twofold dilutions of drug standards were made in tryptose phosphate broth, yielding drug concentrations from 500 to 0.25 µg/ml. The standard bacterial

inoculum was a 1:1,000 dilution of a log-phase bacterial culture, yielding a final concentration of approximately 10<sup>6</sup> bacteria per ml. The MIC end point was determined by visual turbidity after 16 to 18 h of incubation at 37 C. Most of the strains tested by the broth dilution methods were retested by the agar disk diffusion method using 30 µg disks of each drug.

The minimal bactericidal concentration (MBC) was determined from the clear tubes in the sensitivity test by quantitative subculture onto semisolid media. The MBC end point was designated as that concentration of antibiotic that produced a reduction in viable organisms of at least 4 logs or 99.99%. Bactericidal rates were determined by counting the number of viable bacteria at different intervals after incubation in various drug concentrations. The drug carryover effect was reduced by dilutions in broth and use of alkaline medium.

Two strains of drug-susceptible *Escherichia coli* were serially passed in subinhibitory concentrations of cinoxacin and nalidixic acid. The strains were transferred 11 times using as inoculum the organism growing in the highest concentration of drug. To detect single-step-resistant mutants, a large inoculum of organisms was incubated in tryptose phosphate broth containing 100, 200, or 400 times the MIC of either antibiotic.

A strain of *E. coli* made resistant to cinoxacin in the laboratory (MIC = 1,000 µg/ml) was used as the donor strain in the attempted transfer of resistance to a recipient strain of *E. coli* K-12, which was susceptible to cinoxacin but able to grow in sodium azide.

### RESULTS

Of the 170 different bacterial strains tested for susceptibility to cinoxacin, the cumulative percentage of each species inhibited by increasing concentrations is shown in Fig. 1. *Proteus mirabilis* and *E. coli* were the most susceptible

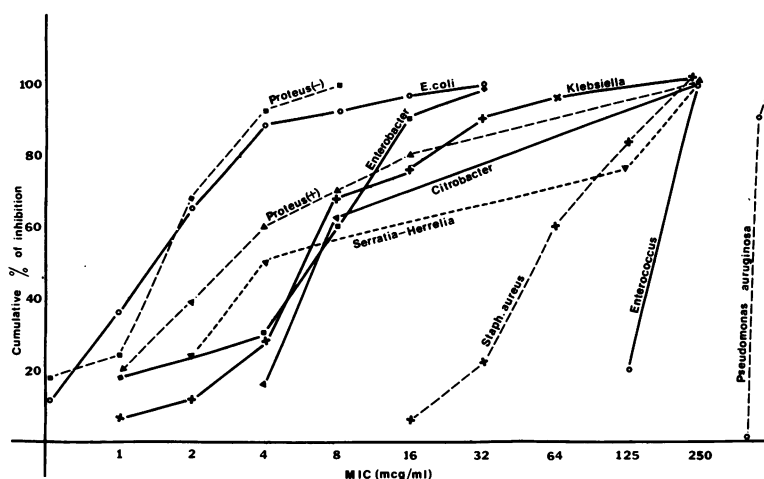


FIG. 1. The *in vitro* susceptibility of different genera of bacteria to cinoxacin. *E. coli* ( $N = 30$ ), *P. mirabilis* ( $N = 13$ ), indole-positive *Proteus* ( $N = 10$ ), *Enterobacter* ( $N = 14$ ), *Klebsiella* ( $N = 22$ ), *Citrobacter* ( $N = 6$ ), *Serratia-Herrelia* ( $N = 8$ ), *Staphylococcus aureus* ( $N = 26$ ), *Enterococci* ( $N = 13$ ), *Pseudomonas aeruginosa* ( $N = 12$ ).

species and, as shown by the steepness of the slopes, different strains were quite uniform in their susceptibility. These species had the smallest difference in the range of inhibition (MIC = 0.5 to 32  $\mu\text{g/ml}$ ). In contrast, indole-positive strains of *Proteus* bacilli, *Klebsiella* sp., *Citrobacter* sp., and *Serratia* sp. were nonuniform in the susceptibility of the different strains which had a range of inhibitory concentrations from 1 to 250  $\mu\text{g/ml}$ . However, between 50 and 70% of the strains of these species were inhibited by 8  $\mu\text{g/ml}$ . In contrast all strains of *Pseudomonas aeruginosa* were resistant to > 250  $\mu\text{g/ml}$  and staphylococci and enterococci were inhibited only at relatively high concentrations of cinoxacin, 32 to 250  $\mu\text{g/ml}$ .

The relation between the MIC in broth and the zone size in the agar diffusion sensitivity tests using a 30- $\mu\text{g}$  disk were analyzed. The results using cinoxacin and nalidixic acid were comparable. Although a general correlation was observed, the variation in zone size at each level of susceptibility was quite great. If 8  $\mu\text{g/ml}$  were designated as an inhibitory level for susceptible strains, only those with a zone size of 25 mm or more could reliably be considered susceptible. This would fail to identify a few susceptible strains of *Proteus* and *Klebsiella* (about 10%) and improperly include some resistant strains (about 20%), especially those of the *Klebsiella-Enterobacter* group. If urinary concentrations of cinoxacin (200 to 500  $\mu\text{g/ml}$ ) (2) were considered as the basis for susceptibility (3, 4) any zone around a 30  $\mu\text{-g}$  disk would indicate a susceptible strain.

Data on the bactericidal activity of cinoxacin

against gram-negative bacilli are shown in Table 1 and compared to nalidixic acid. On the basis of the end point for 50% of the strains, cinoxacin was generally more effective than nalidixic acid. The minimal concentration required to kill 90% of strains among *E. coli*, indole-positive *Proteus* bacilli, and *P. mirabilis* was significantly lower for cinoxacin than nalidixic acid.

The influence of the inoculum size in determining susceptibility to cinoxacin and nalidixic acid is shown in Table 2. As the inoculum of *E. coli* was increased from  $10^1$  to  $10^7$  bacteria per ml, the MIC to cinoxacin increased eightfold. With nalidixic acid under the same conditions the MIC increased 32-fold. A similar result was observed using a susceptible strain of *P. mirabilis* suggesting less heterogeneity in the susceptibility of cells to cinoxacin than nalidixic acid.

The effect of the pH of the medium on the bacterial inhibition by cinoxacin and nalidixic acid is shown in the upper portion of Fig. 2. Increasing alkalinity decreased the susceptibility of all species of gram-negative bacilli tested. With nalidixic acid, the critical pH range appeared to be narrower for *E. coli* and *P. mirabilis*, but otherwise the changes were similar to those observed with cinoxacin.

The effect of the serum concentration of the medium is shown in the lower panels of Fig. 2. Increasing the amount of serum from 0 to 75% had essentially no effect on the antibacterial activity of cinoxacin but a directly related four- to eightfold increase in the MIC of strains to nalidixic acid.

TABLE 1. MBC ( $\mu\text{g/ml}$ )

Strain	No. of strains	Cinoxacin		Nalidixic acid	
		50% of strains	90% of strains	50% of strains	90% of strains
<i>E. coli</i> .....	45	8	24	12	125
<i>Proteus</i> (indol positive) .....	12	8	16 <sup>a</sup>	8	125 <sup>a</sup>
<i>Proteus</i> (indol negative) .....	13	16	48	48	> 250
<i>Citrobacter</i> .....	6	16	250	16	500
<i>Klebsiella</i> .....	22	24	250	64	250
<i>Enterobacter aerogenes</i> .....	6	24	186	16	375
<i>Enterobacter cloacae</i> .....	8	64	500	64	500
<i>Herellea-Serratia</i> .....	8	64	> 500	125	> 500

<sup>a</sup> For 80%.

TABLE 2. MIC

Inoculum size	<i>E. coli</i>		<i>P. mirabilis</i>	
	Cinoxacin	Nalidixic acid	Cinoxacin	Nalidixic acid
10 <sup>1</sup>	0.5	0.5	0.5	0.5
10 <sup>2</sup>	0.5	1	0.5	1.0
10 <sup>3</sup>	2	2	2	4
10 <sup>4</sup>	2	2	2	4
10 <sup>5</sup>	2	4	2	4
10 <sup>6</sup>	2	4	4	4
10 <sup>7</sup>	4	16	32	125

Increasing concentrations of glucose had an antagonistic effect on the inhibitory activity of cinoxacin and nalidixic acid against strains of *P. mirabilis*. Typical results for two strains are given in Table 3. A seven-fold increase in the concentration of glucose from 0.2 to 1.4% was accompanied by a four- to eightfold increase in the MIC to cinoxacin and an eightfold increase in the MIC to nalidixic acid. Thus over this range there was an almost unit:unit relationship between the percentage increase in glucose and the MIC to cinoxacin. No such effect was observed when strains of indole-positive *Proteus* bacilli, *E. coli*, *Klebsiella* or *Enterobacter* bacilli were tested. That the effect was both specific and chemical rather than osmotic was demonstrated by the absence of such an effect when the same strains of *Proteus* were tested in the presence of 10% sucrose or 4% sodium chloride medium.

An unknown factor(s) in normal human urine at neutral pH was also antagonistic to the killing effect of both cinoxacin and nalidixic acid. In Fig. 3 the average log number of viable bacteria recovered after overnight exposure to various concentrations of cinoxacin in human

urine or tryptose phosphate broth is shown. As the drug concentration was increased the antagonistic effect of urine on drug action became increasingly apparent. Some viable cells persisted over the entire range of drug concentrations. The bactericidal effect was eight- to 16-fold less when the drug was in urine than in tryptose phosphate broth. If organisms were inoculated into urine or broth without drug, growth was well supported in either medium, although tryptose phosphate broth yielded greater growth of *P. mirabilis*. When subcultures of the persisting organisms were made and retested for their susceptibility, the MIC was the same as that observed for the original parent stains. When varying concentrations of urea were incorporated into tryptose phosphate broth to test its possible role in the persistence of susceptible viable cells in urine with drug, there was no demonstrable effect on the MBC of the strains tested.

The rate of bacterial killing was also decreased in urine. Figure 4 shows killing curves of *E. coli* and *P. mirabilis* when the organisms were inoculated into three or 50 times the MBC

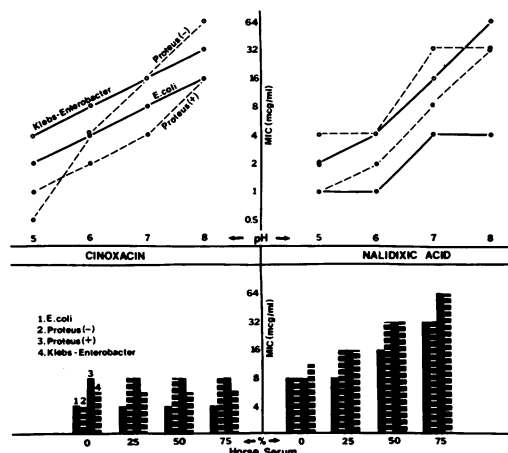


FIG. 2. A comparison of the effects of pH and protein content of the medium on the inhibition of gram-negative bacilli by cinoxacin and nalidixic acid.

TABLE 3. MIC

Dextrose Conc'n (%)	Cinoxacin ( $\mu\text{g/ml}$ )		Nalidixic acid ( $\mu\text{g/ml}$ )	
	Strain 3693H	Strain (4289)	Strain 3693H	Strain 4289
0.2	4	16	8	16
0.4	4	64	8	64
0.8	8	64	16	64
1.4	16	125	64	125

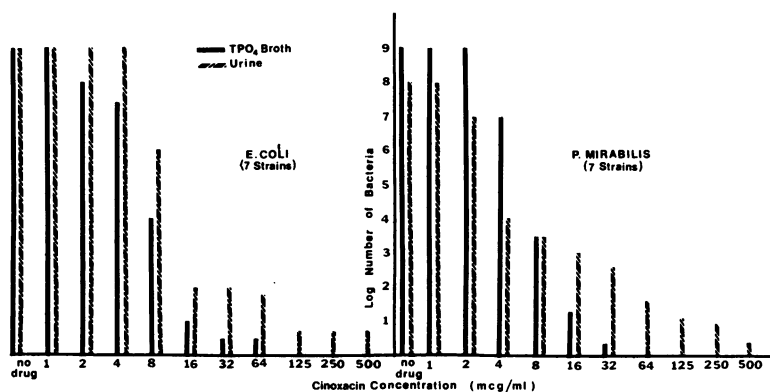


FIG. 3. A comparison of the heterogeneity of bacterial cells to inhibition by cinoxacin and nalidixic acid.

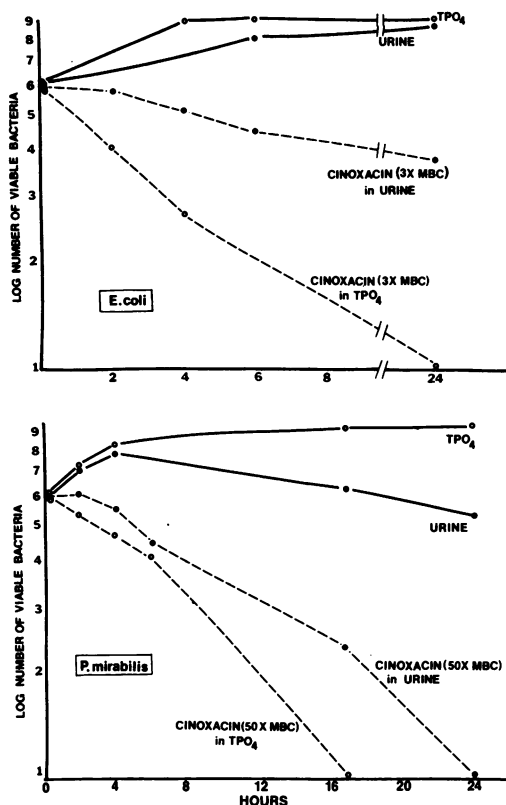


FIG. 4. Antagonism of the bactericidal effect of cinoxacin by human urine.

of drug in urine or tryptose phosphate broth. Both an early and a sustained decrease in the rate of killing in urine was observed for both species. It was not overcome by the high concentration of drug in excess of the MBC. In urine there was also a less rapid and lower replication rate of bacteria relative to broth which could be the cause of the slower killing rate.

The development of resistance of *E. coli* to cinoxacin or nalidixic acid occurred rapidly through multiple obligatory steps with serial transfers in the presence of subinhibitory concentrations of drug. The data are given in Table 4. In twelve passages there was a 1,000-fold increase in the MIC. No single-step mutants with facultative resistance were identified in the specimens studied. The induced resistance was non-transferable as an R-factor.

DISCUSSION

The in vitro antibacterial activity of cinoxacin is readily demonstrable against all of the species tested with the exception of *P. aeruginosa*. With that exclusion gram-negative bacilli are much more susceptible than gram-positive cocci. The activity of the drug closely resembles that of nalidixic acid (2a, 5). Only strains of *E. coli* and *P. mirabilis* may be appreciably more susceptible to cinoxacin. In this respect it is more like oxolinic acid which cinoxacin also resembles closely chemically. The differences in antimicrobial activity observed in vitro may not be apparent in the treatment of urinary tract infections because at concentrations expected to be achieved in the urine (250 to 500 µg/ml) (1, 2; J. L. Ott and W. E. Wick, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 14th, San Francisco, Calif., Abstr. 99, 1974) a bacterial inhibitory effect can be expected against almost all of the common causes of urinary tract infection other than *P. aeruginosa*.

Other factors related to the bactericidal activity of the drug and the development of resistant strains may be of more clinical importance. The marked effect of pH on the activity of cinoxacin can be expected to have an influence in the treatment of urinary tract infections. The influence of the protein concentra-

TABLE 4. *In vitro* development of resistance by *E. coli*

Transfer no.	Highest concn showing growth ( $\mu\text{g/ml}$ )	
	Cinoxacin	Nalidixic acid
1	0.5	0
2	1	1
3	2	2
4	4	4
5	8	8
6	16	16
7	32	32
8	64	64
9	125	125
10	250	250
11	500	500
12	1,000	1,000

tion which is much less in the case of cinoxacin than with nalidixic acid is probably of less importance, if any, in the treatment of urinary tract infections. Nevertheless, it is an index of difference in the biologic activity of cinoxacin and nalidixic acid. The size of the bacterial population may be a very relevant factor. With high numbers of bacteria cinoxacin is significantly more active than nalidixic acid at comparable drug concentrations. This greater homogeneity of bacterial cells and the higher urine concentration of cinoxacin could be of importance in suppressing the emergence of resistant strains (J. L. Ott and W. E. Wick, *Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother.*, 14th, San Francisco, Calif., Abstr. 99, 1974). In the laboratory the latter were shown to develop rapidly by the selection of resistant clones through multiple passages. The other observed phenomenon of persistence of bacteria in concentrations above the bactericidal level occurs by a mechanism unrelated to the development of resistance. The presumption is that these persistors are metabolically inactive cells. Clinically they may present a reason for prolonged treatment but they should not account for treatment failures nor the development of resistant strains.

The demonstration of susceptible bacterial

persistors suggests that the bactericidal effect of cinoxacin requires bacterial replication. The effect is probably exerted by alterations in DNA metabolism as it is with nalidixic acid. The general cross-resistance between these two drugs may mean that the site of action is also nearly the same. The antagonistic effect of glucose with *P. mirabilis* and the demonstration of drug antagonistic factor(s) in human urine indicate that there are species differences and species-specific drug inhibitors.

Several of these factors, including pH related activity, glucose antagonism (*P. mirabilis*), variations in strain susceptibility, metabolic persistors, the requirement for replication, possible biologic antagonists in urine and tissues, and the ease of selection and emergence of resistant clones, forbode difficulties in the successful treatment of patients with chronic and complicated urinary tract infections. However, cinoxacin appears to have some advantages over nalidixic acid on the basis of these *in vitro* observations and evaluation of the *in vivo* importance of the various factors will need to be made from clinical trials.

#### ACKNOWLEDGMENTS

The technical assistance of Victoria Zimelis is gratefully recognized. H.G. is an Eli Lilly International Medical Fellow.

#### LITERATURE CITED

- Holmes, D. H., P. W. Enswinger, and R. S. Gordee. 1974. Cinoxacin: effectiveness against experimental pyelonephritis in rats. *Antimicrob. Agents Chemother.* 6:432-436.
- Lilly Research Laboratories. 1974. Clinical pharmacology of cinoxacin. Lilly Research Laboratory, Indianapolis.
- Lumish, R. M., and C. W. Norde. 1975. Cinoxacin: *in vitro* antibacterial studies of a new synthetic organic acid. *Antimicrob. Agents Chemother.* 7:159-163.
- McCabe, W. R., and G. G. Jackson. 1965. Treatment of pyelonephritis: bacterial, drug and host factors in success or failure among 252 patients. *N. Engl. J. Med.* 272:1037-1044.
- Stamey, T. A., W. R. Fair, M. M. Timothy, M. A. Miltar, and G. Minhara. 1974. Serum versus urinary antimicrobial concentrations and urinary tract infections. *N. Engl. J. Med.* 291:1159-1163.
- Wick, W. F., D. A. Preston, W. A. White, and R. S. Gordee. 1973. Compound 64716, a new synthetic antibacterial agent. *Antimicrob. Agents Chemother.* 4:415-420.