Inhibition of DNA replication in vitro by pefloxacin

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Pefloxacin (a novel quinolone antibiotic) is demonstrated to be a drug inhibiting DNA replication 10-times more efficiently than oxolinic acid measured either in toluene-treated *E. coli* or in an in vitro replication system for *oriC* plasmids [6]. DNA repair synthesis is not inhibited by the drug.

Pefloxacin   Nalidixic acid   DNA replication   DNA Polymerase I   Toluenized cell

oriC *plasmid*

1. INTRODUCTION

Pefloxacin is a novel quinolone having a powerful antibacterial action [1] (fig.1). The quinolone drugs, like nalidixic acid, oxolinic acid, pipemidic acid or piromidic acid are specific inhibitors of bacterial type II DNA topoisomerases [2]; their primary target is one of the subunits of these enzymes (which bears a nicking–closing activity). Both in vivo and in vitro, the quinolone drugs block DNA replication at low concentrations; at higher concentrations DNA transcription is also affected [3]. For a long time, nalidixic acid has been the only well studied compound; more recently however, oxolinic acid, which is a more potent inhibitor both in vivo and in vitro has been preferentially used to study DNA replication [4,5]. Like oxolinic acid, pefloxacin is a stronger antibacterial agent than nalidixic acid in vivo. The inhibition of bacterial growth, DNA, RNA and protein synthesis by pefloxacin occurs at concentrations two orders of magnitude lower compared to nalidixic acid and one order of magnitude lower than pipemidic acid [1]. The results presented in this paper show that pefloxacin is also a much more potent inhibitor than nalidixic acid of *Escherichia coli* DNA replication in situ, in a toluenized cell system, and in vitro in the new system of initiation using a crude extract which replicates an exogenous *oriC* plasmid [6]. Furthermore, in both systems pefloxacin inhibits DNA replication at doses ten times lower than oxolinic acid itself.

Fig.1. Chemical structures of some quinolones.

2. MATERIALS AND METHODS

2.1. Drugs

Pefloxacin (1589RB) was obtained from Roger Bellon Laboratories; nalidixic acid was obtained from Winthrop and oxolinic acid from Dr Staudenbauer, Max Planck Institut (Berlin).
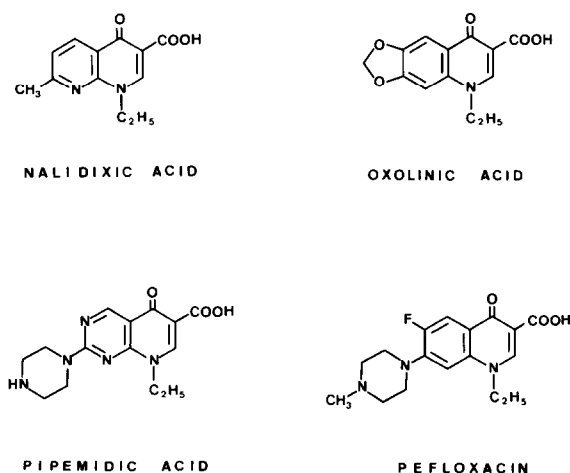
2.2. Bacterial strains

Escherichia coli K12H560 (F⁻*pol* A⁻, *endo* I⁻, *thy*⁻) and parental strain AB1157 were used in this study. Spontaneous *Nal*ʳ mutants were isolated by their ability to grow in the presence of 20 μg/ml nalidixic acid.

2.3. Methods

Cells were grown in Luria broth at 37°C to 5 × 10⁸ cells/ml and harvested at 4°C.

Toluenized cells: cells were resuspended to 10¹⁰ cells/ml in 100 mM phosphate buffer (pH 7.4), containing 2 mM MgCl₂ and 1% toluene, permeabilized for 2 min at room temperature by Vortex treatment and immediately assayed for DNA synthesis as previously described [7].

In vitro system: active enzyme extracts were prepared according to the procedure of Kornberg [6]. DNA replication was assayed in a 50 μl reaction mixture containing the following components: N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid (pH 7.6), 40 mM; ATP, 2 mM; GTP, CTP and UTP, each 500 μM; bovine serum albumin, 50

μg/ml; creatine kinase, 100 μg/ml; creatine phosphate, 21.6 mM; dATP, dCTP, dGTP and dTTP, each 100 μM with [*methyl-*³H]dTTP at 100 cpm per pmol of total deoxynucleotide; dithiothreitol, 4 mM; KCl, 0.1 M; Mg(OAc)₂, 11 mM and POC 42 supercoiled DNA, 8.6 μg/ml. Polyethyleneglycol 6000 was added to a final concentration of 5% to 6% (wt/vol). The reactions were initiated by addition of 200–300 μg of protein and incubated at 30°C for 30 min. Total nucleotide incorporation was measured by determining radioactivity after trichloroacetic acid precipitation.

3. RESULTS

3.1. Effect of pefloxacin on DNA synthesis in toluene treated cells

The experiment described in fig.2a shows the comparative effects of various concentrations of pefloxacin, oxolinic acid and nalidixic acid on DNA replication in toluenized *E. coli* cells carrying a deficient DNA polymerase I activity (*pol* A⁻). It can be seen that pefloxacin inhibits 50% of
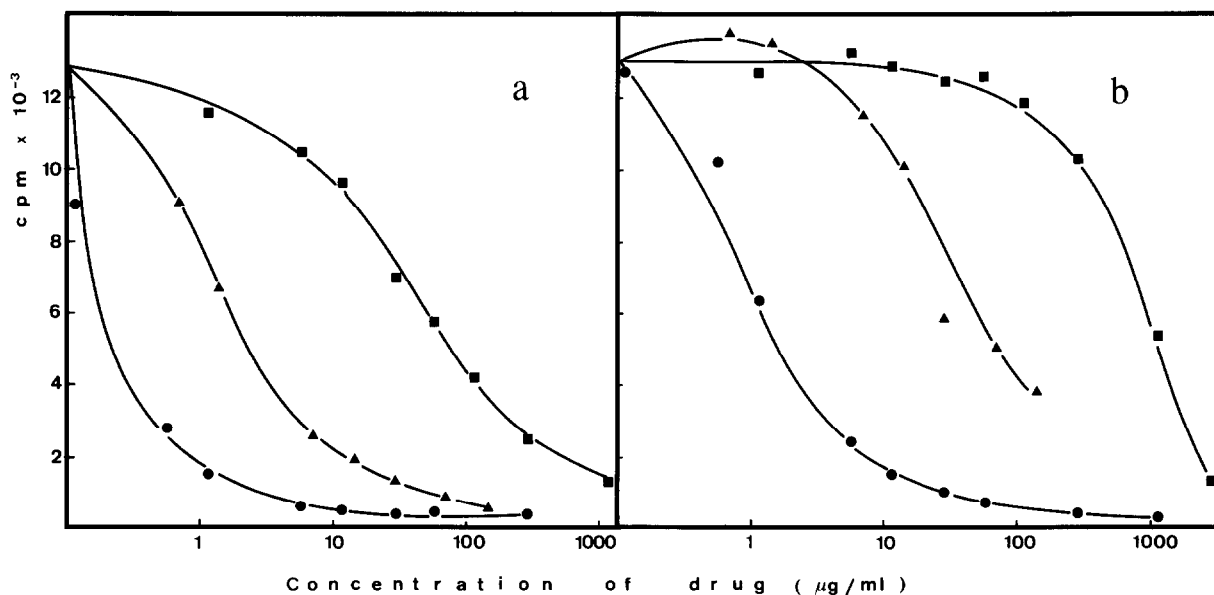

Fig.2. Effect of nalidixic acid (■) oxolinic acid (▲) and pefloxacin (●) on DNA synthesis in toluenized cells. 2.5 × 10⁸ toluenized cells of *E. coli* H560 *Nal*ˢ (a) or *Nal*ʳ (b) were incubated in 175 μl of incubation mixture (30 mM Tris · HCl (pH 7.5), 3.4 mM MgCl₂, 110 mM KCl, 0.7 mM β-mercaptoethanol, 1.1 mM ATP and 22 μM each dATP, dGTP, dCTP and [³H]dTTP (90 cpm/pmol) with the drug concentrations indicated. After 60 min at 30°C, acid insoluble radioactivity was determined.

replicative DNA synthesis at a drug concentration of 0.20 µg/ml whereas a 10-fold higher concentration of oxolinic acid and a 220-fold higher concentration of nalidixic acid are required to achieve the same effect. Furthermore, in contrast with the results obtained with nalidixic acid, absolutely no residual replication occurs at high concentration of pefloxacin.

The experiment described in fig.2b shows that pefloxacin interacts most likely with the same target as nalidixic acid. In this experiment, we compared the effects of pefloxacin and nalidixic acid on DNA replication in a toluene treated spontaneous mutant of *E. coli* that is resistant to high concentration of nalidixic acid (*Nal^r*). It can be seen from the comparison between the results of fig.2a and b that DNA replication in the *Nal^r* mutant becomes more resistant to both nalidixic acid and pefloxacin.

It was important to test if pefloxacin shared with nalidixic and oxolinic acid a complete specificity for DNA replication, i.e., no effect on repair DNA synthesis.

An extensive repair like type DNA synthesis performed by DNA polymerase I can be induced

Fig.3. Selective inhibition of ATP dependent DNA synthesis by pefloxacin. 2.5 × 10^8 toluenized cells of *E. coli* AB1157 were incubated as described in fig.2 for 60 min at 30°C (•) with different concentrations of pefloxacin. Repair synthesis (○) was assayed in the same incubation mixture without KCl and β-mercaptoethanol in the presence of 7.9 mM NEM and absence of ATP for 60 min at 40°C.

in *pol* A$^+$ toluene treated cells at 40°C, in the absence of ATP, and by the addition of N-ethyl maleimide (NEM) [8]. In these conditions the replicative DNA synthesis is completely inhibited both by the lack of ATP and by the sulfhydryl blocking agent (NEM). However, at 30°C in the presence of ATP and in the absence of NEM, DNA replication can be measured by adding ATP and precursors. Thus by changing these three factors, both DNA replication and repair can be measured in the same culture. As can be seen in fig.3, replicative DNA synthesis is completely inhibited by pefloxacin in the same way as in *pol* A$^-$ mutant (fig.2a). In contrast fig.3 shows that, under conditions which induce DNA repair like synthesis, the activity is completely resistant to even very high doses of pefloxacin. The same results are obtained with nalidixic and oxolinic acid (data not shown).

3.2. Effect of pefloxacin on in vitro DNA replication of an oriC plasmid

Recently, an enzyme system that replicates plasmids bearing the origin of the *E. coli* chromosome has been successfully devised [6]. This enzyme systems performs the physiological process of chromosomal initiation in vitro coupled with DNA elongation. The DNA gyrase inhibitors, nalidixic acid and novobiocin, block DNA replication of the *oriC* plasmid in vitro [6]. We have successfully repeated this system using the POC 42 plasmid DNA (a chimere of PBR 322 and a 1.9 kbp fragment containing *oriC* [9]). PBR 322 DNA is not replicated with this system, demonstrating the importance of *oriC*. Fig.4a shows that pefloxacin completely inhibits DNA replication of the *oriC* plasmid POC42 at doses similar to that required to inhibit DNA replication in toluene treated cells; also similar is the relative efficiency of the two inhibitors, nalidixic acid and pefloxacin. Fig.4b shows that when the enzyme system is prepared from a spontaneously isolated nalidixic resistant strain, DNA replication of the *oriC* plasmid becomes resistant to all three drugs. It can be seen as in fig.2, that cross resistance between pefloxacin and nalidixic acid is not complete. When the enzyme is prepared from a *pol* A$^+$ strains, it contains DNA polymerase I activity as revealed by additions of both poly(dAT) and NEM (10^{-3} M). This activity is completely resistant to pefloxacin. Pefloxacin appears therefore to be a novel and
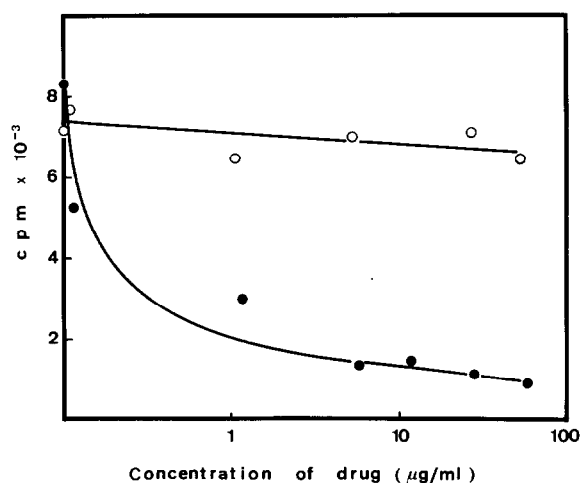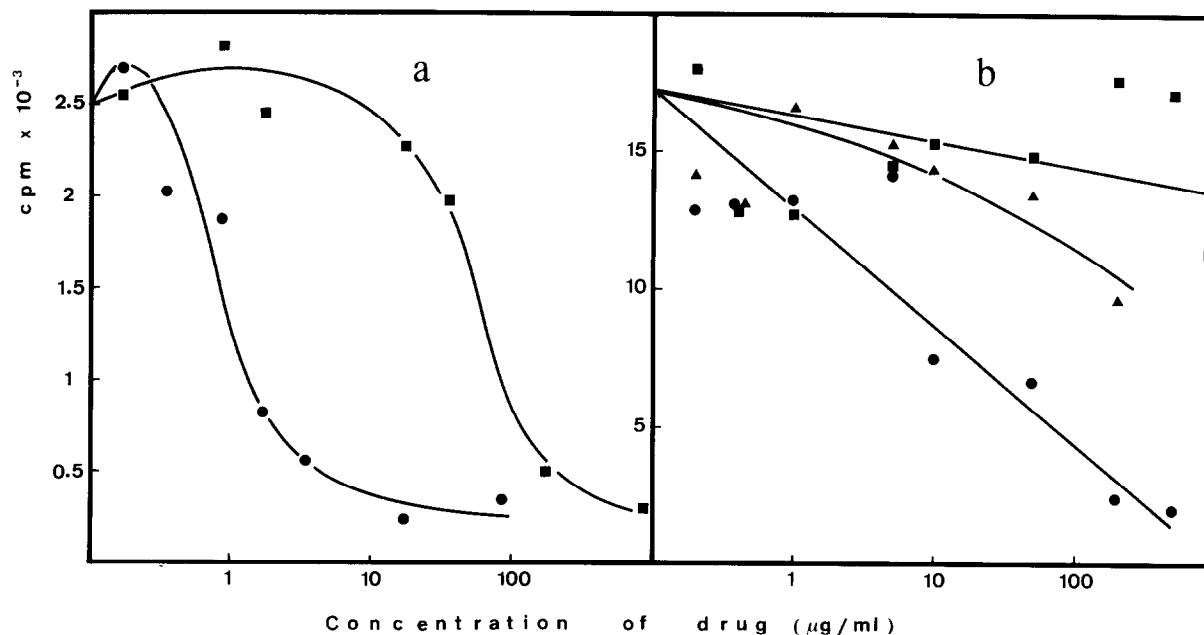
Fig.4. Effect of nalidixic acid (■) oxolinic acid (▲) and pefloxacin (●) on in vitro DNA synthesis. Protein extracts obtained from *E. coli* H560 (a) and H560 Nal^r (b) strain containing POC42 plasmid were incubated as described in Materials and Methods.

very strong inhibitor of DNA replication both in vivo and in vitro which interacts specifically with the same target as nalidixic and oxolinic acid but with better efficiency.

4. DISCUSSION

We have shown that pefloxacin is a more potent inhibitor than nalidixic acid and oxolinic acid of DNA replication in vitro both with a permeabilized cell system which performs only DNA elongation and in a crude enzyme system which performs both the initiation and elongation stage of *oriC* plasmid DNA replication. There is a good correlation between the antibacterial activity of these drugs and their capacity to block DNA synthesis in vitro. Pefloxacin is one hundred times more active than nalidixic acid and ten times more active than oxolinic acid. Doses of the drug as low as 5 μg/ml are sufficient to completely inhibit DNA replication in vitro. In contrast, even a very high dose of pefloxacin failed to repress repair type DNA synthesis carried out by DNA polymerase I. This antibiotic is therefore a specific in-

hibitor for DNA replication. Cross-resistance of spontaneously isolated nalidixic acid resistant mutants with pefloxacin have suggested that both drugs interact in vivo with the same target [1]. The situation observed in vitro indicates that pefloxacin must likely interact with the *gyrA* gene product both in vivo and in vitro. All these data made pefloxacin a very attractive compound to study the various roles of DNA topoisomerase II in bacterial life cycle. Pefloxacin may be also an important tool for the elucidation of the precise mode of action of quinolone antibacterial agents. Although the mode of action of nalidixic acid and its derivatives has been intensively studied for more than 15 years and in spite of the recent advances stimulated by the discoveries of their intracellular protein target, many questions remain to be resolved concerning the various mechanisms by which these drugs seem to alter DNA metabolism [3,7,10].

ACKNOWLEDGMENTS

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