Antiviral Research, 8 (1987) 103–113
Elsevier

AVR 00257

Antiviral activity and inhibition of topoisomerase by ofloxacin, a new quinolone derivative

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(Received 23 January 1987; accepted 13 July 1987)

Summary

The antiviral activity of ofloxacin, a new quinolone derivative, against vaccinia virus (VV), herpes simplex virus (HSV) and influenza virus (InfV) was evaluated in both in vitro and in vivo experiments. As a result, ofloxacin showed inhibitory activity against VV in cultured mammalian cells, and prevented formation of pox tail lesions in VV-infected mice. However, it was less effective against HSV and InfV than VV. The antiviral activity of ofloxacin assessed by VV tail-lesion test was strongest when administered to mice through the oral route daily for five consecutive days post-infection. Nalidixic acid and novobiocin, well-known gyrase inhibitors, showed only weak antiviral activity in both in vitro and in vivo tests against VV.

It was also demonstrated that ofloxacin inhibited virus-specific DNA and RNA syntheses. It was more inhibitory to VV topoisomerase than cellular topoisomerases. Thus, ofloxacin has selectivity for VV.

Ofloxacin; Vaccinia virus; Topoisomerase

Introduction

Ofloxacin ((±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido [1,2,3,-de] [1,4]benzoxazine-6-carboxylic acid) is a new quinolone derivative noted as an orally absorbable antimicrobial agent. It has broad-spectrum ac-

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tivity against bacteria and mycoplasms (Sato et al., 1982; Osada and Ogawa, 1983). In clinical trials, ofloxacin has proved to be an excellent antimicrobial agent against bacterial diseases in prostatic tissues and respiratory organs, particularly in urinary tract infections due to drug-resistant bacteria (Abe et al., 1984; Hasegawa et al., 1984; Odagiri et al., 1984).

In the present investigation, we showed antiviral activity of ofloxacin against vaccinia virus (VV) in cell culture and mice. The results also demonstrated that ofloxacin acts by inhibition of the VV topoisomerase, which initiates the replication and transcription of the VV genome.

Materials and Methods

Test compounds

The chemical structure of ofloxacin is shown in Fig. 1. Ofloxacin (MW=361), nalidixic acid (MW=232) and novobiocin (MW=612) were a gift from the Research Institute of Dai-ichi Seiyaku Co. Ltd., Tokyo.

Cells and viruses

VV (Lister strain) and herpes simplex virus (HSV) type 2 (UW strain) were propagated in either HEL cells, a human embryonic lung cell-line, or HeLa cells, a human cervix carcinoma cell-line. Influenza virus (InfV) (A/PR/8/34/H1N1) was propagated in allantoic fluids of fertile hen's eggs. The stock viruses were stored at −80°C until used. HeLa cells were used for titrations of VV and HSV. SK-K, a swine kidney cell-line, was used for InfV titration. The cells were cultivated in Eagle's minimum essential medium (EMEM) supplemented with 3 mM glutamine, 0.07% bicarbonate and either 10% fetal-calf serum or 5% newborn-calf serum depending on the cell-lines used. To replace maintenance medium after virus infection, 1% newborn-calf serum was added to the above EMEM.

Animals

Female ddY mice, 4 wks old, were obtained from Shizuoka Experimental Animals (Hamamatsu, Japan). The mice were bred for 1 wk under a specific pathogen-free environment before experimental use. Female, HRS/J hairless mice, 4 wks old, were obtained from Jackson Laboratory (Bar Harbor, ME, U.S.A.) and used for the HSV-skin lesion test (Ikeda et al., 1981).

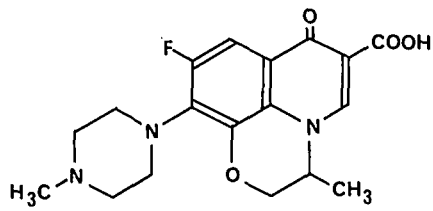

Fig. 1. Chemical structure of ofloxacin.

In vitro antiviral test of compounds

For in vitro antiviral test against VV and HSV, HeLa cells were infected with virus and cultivated in the presence of the test compounds. For antiviral test against InfV, SK-K cells sensitive to this virus were used in the same way. Briefly, cells were cultivated in 24-well plastic microplates (Termo Co. Ltd., Tokyo, Japan) for 24 h in a CO_2 incubator. After formation of monolayers, cells were infected with virus diluted in maintenance medium at a dose of 5 $TCID_{50}$/0.1 ml/well to reach a multiplicity of infection (moi) of 0.01. For the antiviral activity assays, the test compounds were diluted in maintenance medium and added to monolayers of infected cells. Four wells per dilution were used for each test compound. After 3 days cultivation at 37°C in a CO_2 incubator, virus-specific cytopathic effect (CPE) was observed microscopically and reduction of CPE in infected cells was expressed by MIC_{50} (50% minimum inhibitory concentration) as described in previous papers (Fiala et al., 1971; Ikeda et al., 1985). Similarly, MTC_{50} (50% minimum toxic concentration) of test compounds were determined in the cell lines used. Both cytotoxic CPE and inhibition of virus-specific CPE of the test compounds were observed on the 2nd or 3rd day. Observations were recorded for at least five different concentrations of test compounds to determine dose-response curves. The MIC_{50} and MTC_{50} of the compounds were then calculated according to the formula of Kärber (1931), expressed as the minimum concentration of compound required to achieve a 50% toxicity for the host cells or 50% protection against viral CPE, respectively. CPE (cell damage) was determined by microscopic observation of the cell monolayers. Antiviral indexes were then calculated as the ratio MTC_{50}/MIC_{50}. For virus yield reduction assays, VV-infected HeLa cells cultured in the presence of ofloxacin were harvested at several time intervals, and harvested cells were disrupted by freezing–thawing three times. Virus titers were determined by plaque formation on HeLa cell monolayers. The infectious virus yields are expressed as percentage of the virus yield measured for VV-infected cells cultivated for 48 h in the absence of ofloxacin. The experiments were repeated at least twice.

In vivo antiviral test of compounds

Groups of 7–14 mice were used in all experiments. The compounds were administered by different routes at different doses. For VV infection, $10^{4.8}$ $TCID_{50}$ of virus stock was injected intravenously into the tail vein of ddY mice. On the 7th day of infection, the numbers of lesions appearing on the tail were counted by staining with 1% fluorescein–0.5% Methylene Blue solution (Ikeda et al., 1985). For assessing anti-HSV activity groups seven HRS/J hairless mice were treated perorally with different doses of compounds, and then inoculated intradermally with 5×10^4 $TCID_{50}$ of HSV (Ikeda et al., 1981). Antiviral effects were assessed based on protection against skin lesions such as zosteriform, vesicle formation, and systemic symptoms such as paralysis and death due to herpes encephalitis. Acute toxic doses LD_{50} of ofloxacin for ddY mice were very low; e.g., orally 5290, intravenously 234, and subcutaneously >10,000 mg/kg. Test compounds were used at subtoxic concentrations.

Assay of virus-specific RNA and DNA synthesis in VV-infected cells

To study virus-specific RNA synthesis in VV-infected HeLa cells, cell monolayers in 24-well microplates were infected with VV at a high moi (2000). After a 30-min adsorption period, the monolayers were washed with PBS and 0.5 ml maintenance medium was added to the infected cells in the presence or absence of the test compounds. The cells were cultivated for 90 min at 35°C, and pulse-labeled for another 20 min with 3 μCi [^{3}H]uridine (48.7 Ci/mmol; Amersham, Buckinghamshire, England) per well. Control uninfected cells were treated in the same way. The pulse-labeled cells were washed twice with ice-cold PBS, and cytoplasm was extracted from monolayer cells with 0.2 ml of 0.1% Nonidet-P40, 140 mM NaCl, 10 mM Tris-hydrochloride buffer (pH 7.5), 1.5 mM $MgCl_2$ for 5 min at 37°C, leaving nuclei behind in the microplate wells (Goswami and Sharma, 1983). The cytoplasm solubilized with Nonidet P-40 was precipitated with 1 ml of 2% ice-cold perchloric acid. After keeping the mixture for 1 h in an ice bath, the precipitated cytoplasmic fractions were collected on a glass fiber filter, washed twice with 5% trichloroacetic acid (TCA) and once with cold ethanol, and radioactivity was counted by liquid scintillation spectrometer.

To assess viral DNA synthesis, HeLa cells were infected with VV at a moi of about 5, the other procedures being the same as for RNA synthesis assay. 6 h after infection, the cells were pulse-labeled with 1 μCi [^{3}H]thymidine (19.3 Ci/mmol; New England Nuclear Corp., Boston, MA, U.S.A.) per well. After 30 min pulse-labeling, the cells were washed twice with ice-cold PBS, then harvested with a rubber policeman. The cells were extracted with cold TCA, and TCA precipitates were collected on Whatman GF/C filter papers. The filter papers were washed with ethanol, and the radioactivity was determined in a liquid scintillation spectrometer (Hruby et al., 1980). Uninfected cells were treated in the same way and the increased radioactivity in infected, over uninfected cells, was defined as virus-specific DNA and RNA syntheses.

Viral and cellular topoisomerase assays

The procedure used for purification of viral topoisomerase is that of Bauer et al. (1977) with minor modifications. VV cores were prepared from 10 mg purified virus by resuspending 2×10^{11} particles per ml in 50 mM Tris-HCl (pH 8.5), 50 mM 2-mercaptoethanol, 0.2% Triton X-100. After incubation for 30 min at 37°C, naked cores were collected by centrifugation at 20,000 × *g* for 15 min and resuspended at 10^{12} particles per ml by sonication (150 W) in a buffer containing 0.25 M NaCl, 0.3 M Tris-HCl (pH 7.8) and 0.1 M dithiothreitol (DTT). The suspension was adjusted to contain 0.1% sodium deoxycholate and incubated for 45 min at 4°C. The freed DNA was then shared by 10 s sonication, and a supernatant fraction was obtained from 135,000 × *g* spin at 4°C for 60 min. This fraction was adjusted to contain 10% glycerol, 0.1% Triton X-100 and 1 mM EDTA. For purification of viral topoisomerase, DNA was removed from the preparations by passage through a 0.6 × 5 cm DEAE-cellulose column equilibrated with 0.2 M NaCl, 0.25 M Tris-HCl (pH 7.8), 10% glycerol, 0.1% Triton X-100, 1 mM EDTA and 2 mM DTT.

Purification of topoisomerase from HeLa cells was performed as described by Liu and Miller (1981) and by Miller et al. (1981). Washed HeLa cell nuclei from 7.5×10^8 cells were resuspended in 6 ml of nuclei wash buffer consisting of 5 mM KH_2PO_4 (pH 7.5), 1 mM $PhMeSO_2F$, 1 mM 2-mercaptoethanol and 1 mM EDTA. EDTA was then added to 4 mM. Nuclei were lysed by the slow addition of an equal volume of 2 M NaCl, 0.1 M Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol and 1 mM $PhMeSO_2F$. The DNA in the extract was precipitated by slow addition of 6 ml of 18% PEG6000 dissolved in 1 M NaCl and removed by centrifugation at $15,000 \times g$ for 25 min. This supernatant was loaded directly onto a hydroxylapatite column (0.6×10 cm) equilibrated with 1 M NaCl, 50 mM Tris-HCl (pH 7.5), 6% PEG6000, 10 mM 2-mercaptoethanol and 1 mM $PhMeSO_2F$. The column was washed with 2 ml of 0.2 M potassium phosphate (pH 7.0), 10% glycerol, 10 mM 2-mercaptoethanol and 1 mM $PhMeSO_2F$, and then developed with 30 ml of a 0.2–0.7 M potassium phosphate linear gradient in the same buffer. Topoisomerase I and II activities were eluted at positions of 0.3–0.4 M and 0.2–0.3 M of the eluting buffer, respectively.

Viral and cellular topoisomerases were measured by the relaxation of ^{3}H-labeled superhelical plasmid (pUC-9) DNA. For the viral topoisomerase assay the 12 μl-reaction mixture contained 10 mM Tris-HCl (pH 7.8), 30 mM NaCl, 0.3 mM EDTA, 0.6 μg [^{3}H]DNA, 24 U VV topoisomerase and various amounts of inhibitors. Cellular topoisomerase I reaction mixture contained 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 0.5 mM EDTA, 0.6 μg [^{3}H]DNA, 12 U HeLa cell topoisomerase I and various amounts of the inhibitors in the same assay system. For the cellular topoisomerase II assay, the reaction mixture contained Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM EDTA, 0.6 μg [^{3}H]DNA, 30 U HeLa cell topoisomerase II and various amounts of inhibitors. After incubation for 30 min at 37°C, reactions were stopped by chilling in an ice-bath. Then the samples were mounted on a 1% agarose slab gel and subjected to electrophoresis at 50 V for 90 min. The gel- and chamber-buffer consisted of 40 mM Tris-acetate (pH 8.3), 20 mM sodium acetate, 2 mM EDTA and 0.7 μg ethidium bromide. The DNA bands were visualized by illumination from below with a shortwave length UV light and photographed with Polaroid type 667 film. The extent of conversion from superhelical DNA to the relaxed form was then evaluated by determining radioactivity in DNA bands separated on agarose gel electrophoresis.

Results

In vitro antiviral activity of ofloxacin

First, to assess cell toxicity of ofloxacin and two gyrase inhibitors, nalidixic acid and novobiocin, we determined the 50% minimum toxic concentrations (MTC_{50}) of the compounds in HeLa and SK-K cells after 3 days cultivation. The MTC_{50} of ofloxacin for HeLa and SK-K cells was 375 μg/ml, i.e. higher than nalidixic acid and novobiocin as shown in Table 1. In vitro antiviral activity was examined against VV, HSV and InfV. Table 1 shows that ofloxacin was inhibitory to VV in HeLa

TABLE 1

Cytotoxicity and in vitro antiviral activity of ofloxacin[a].

Agents	MTC_{50} (μg/ml)		MIC_{50} (μg/ml)			Antiviral index[b]		
	HeLa	SK-K	VV	HSV	InfV	VV	HSV	InfV
Ofloxacin	375	375	37	250	>500	10.8	1.6	<0.8
Nalidixic acid	188	125	83	>250	>250	2.3	<0.8	<0.5
Novobiocin	28	31	25	25	>125	1.1	1.1	<0.3

[a] Cytotoxicity and antiviral activity were determined as described in Materials and Methods.
[b] Antiviral indexes were calculated by the ratios of MTC_{50} to MIC_{50}.

cells at an MIC_{50} of 37 μg/ml. An MIC_{50} of 250 μg/ml was demonstrated against HSV and no activity was seen against InfV. Ofloxacin showed an antiviral index of about 10 against VV. Nalidixic acid and novobiocin did not show a selectivity for any of the viruses tested (VV, HSV or InfV).

In vivo antiviral activity of ofloxacin

In vivo antiviral activity of ofloxacin against VV and HSV was determined by using the tail-lesion test for VV, and the skin-lesion and survival test for HSV. Formation of tail lesions in VV-infected mice was reduced by 50% upon a single oral administration of ofloxacin at a dose of 10 mg/kg, and reduced by 84% upon consecutive oral administrations of ofloxacin for 5 days at a dose of 10 mg/kg (Table 2). Of all administration routes, the oral route was the most effective (Table 3). Although oral administration of ofloxacin to mice demonstrated weak protection against HSV in the skin-lesion test, it could not protect HSV-infected mice from death due to herpes encephalitis (data not shown). Nalidixic acid did not protect mice and novobiocin afforded only weak protection against VV (data not shown).

TABLE 2

Antiviral activity of ofloxacin in tail-lesion test in mice infected with vaccinia virus.

Treatment[a]	Dose (mg/kg)	No. of lesions[b] (mean ± SD)	Inhibition (%)
Virus control	–	38.3 ± 9.6	–
Single dose	0.1	25.5 ± 7.8	34
	1	16.3 ± 4.4[c]	57
	10	15.4 ± 8.4[c]	59
Multiple doses	0.1 × 5	9.1 ± 4.3[c]	76
	1.0 × 5	8.8 ± 3.4[c]	77
	10 × 5	6.1 ± 5.5[c]	84

[a] Single oral administration of ofloxacin occurred simultaneously with the infection and multiple oral administrations of ofloxacin for 5 consecutive days started from the time of infection.
[b] The number of lesions formed on the tail were counted on the 7th day after virus infection.
[c] $P<0.05$ (Mann–Whitney U-test).

Mechanism of anti-vaccinia activity of ofloxacin

To define the mechanism of anti-vaccinia activity, we determined serum interferon (IFN) titers in mice treated orally with various amounts (1 to 100 mg/kg) of ofloxacin. Mice were bled at 6, 12, and 24 h after oral administration of ofloxacin. However, no IFN was detected in any mouse serum, suggesting that IFN does not contribute to the antiviral mechanisms of ofloxacin against VV infection in mice.

Another experiment carried out according to the method of Pompei et al. (1979) demonstrated that ofloxacin did not directly inactivate VV particles. Experiments were also carried out to investigate whether ofloxacin actually inhibited virus production. Fig. 2 shows that ofloxacin inhibited production of infectious particles from VV-infected HeLa cells in a dose-response manner.

To further examine the mechanism of ofloxacin inhibition of VV replication, we investigated the effects of ofloxacin on DNA and RNA syntheses in VV-infected cells. As shown in Fig. 3, the specific increases in viral DNA and viral RNA syntheses in VV-infected cells were inhibited in a concentration-dependent fashion by ofloxacin. Viral DNA polymerase induced in the cytoplasm of infected cells was also inhibited by ofloxacin in a concentration-dependent manner. The same was true for the viral RNA polymerase associated with the virion (data not shown).

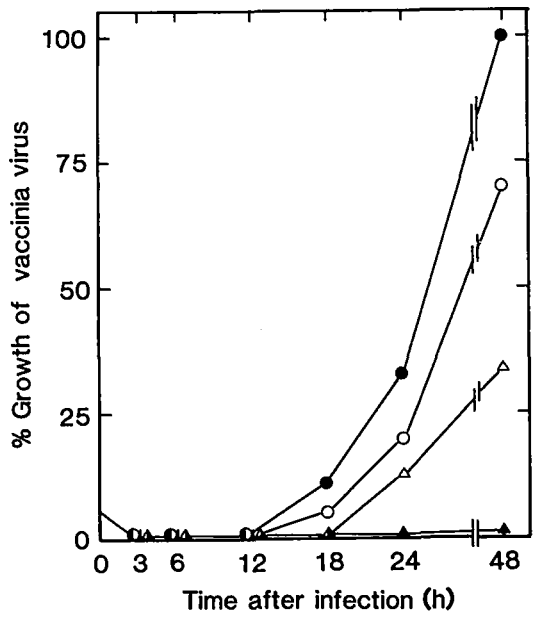

Fig. 2. Effect of ofloxacin on growth of vaccinia virus in HeLa cells. HeLa cells infected with VV at a moi of 0.1 were cultured in the presence or absence of ofloxacin. The infected cells were harvested at the time indicated and virus yields were determined by plaque assay. The ordinate indicates percent growth of virus to the virus control (3.6×10^5 PFU/0.1 ml) harvested at 48 h post-infection without ofloxacin. Virus control (●); ofloxacin 25 μg/ml (0.07 mM) (○); 100 μg/ml (0.28 mM) (△) and 400 μg/ml (1.1 mM) (▲).

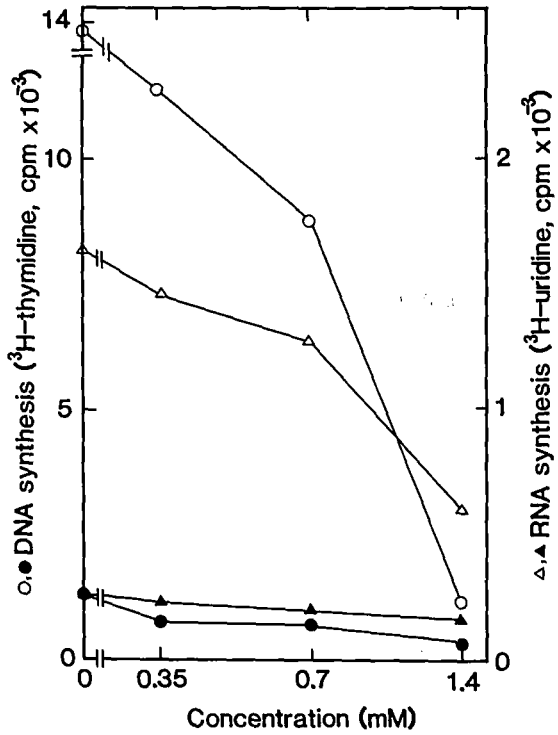

Fig. 3. Effects of ofloxacin on DNA and RNA syntheses in vaccinia virus-infected or uninfected HeLa cells. Assays of DNA and RNA syntheses are described in Materials and Methods. DNA synthesis in uninfected cells (•) and virus-infected cells (○); RNA synthesis in uninfected cells (▲) and in virus-infected cells (△).

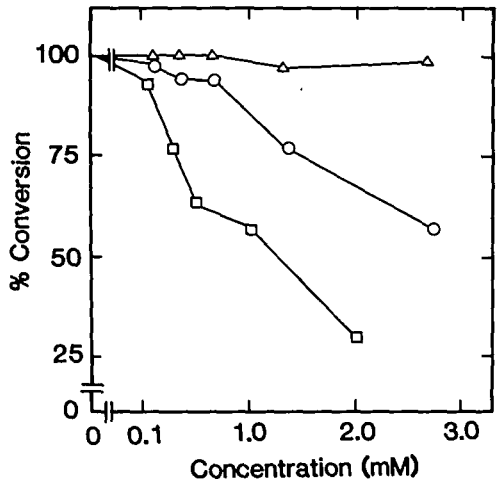

Fig. 4. Effects of ofloxacin, nalidixic acid and novobiocin on the activity of vaccinia virus topoisomerase I. Viral topoisomerase I activity in the purified virion was determined in the presence of various inhibitors by ethidium bromide-agarose gel electrophoresis. ○ = ofloxacin; △ = nalidixic acid; □ = novobiocin.

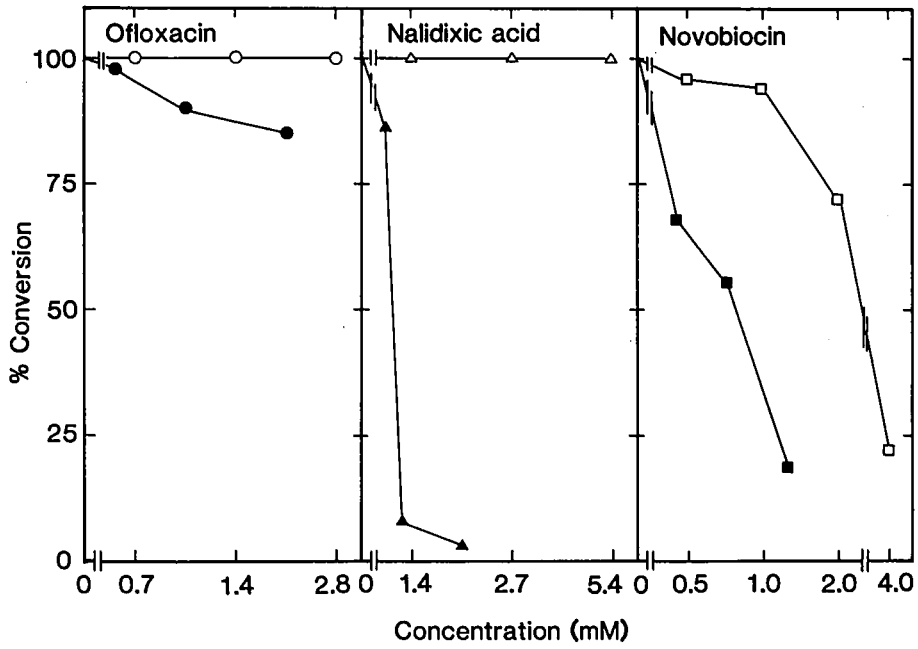

Fig. 5. Inhibitory effects of ofloxacin, nalidixic acid and novobiocin on HeLa cell topoisomerases I and II. Purified cellular topoisomerases I and II were assayed in the presence of inhibitors as described in the text. Open symbols represent cellular topoisomerase I (○ = ofloxacin; △ = nalidixic acid; □ = novobiocin), and closed symbols indicate cellular topoisomerase II (● = ofloxacin; ▲ = nalidixic acid; ■ = novobiocin).

There are reports that VV virions contain type I topoisomerase which promotes relaxation of superhelical DNA (Bauer et al., 1977; Foglesong and Bauer, 1984). Both the replication and transcription of the input VV-genome depend on the activity of this enzyme. Therefore, the effect of ofloxacin on the activity of purified VV topoisomerase was compared with that of other gyrase inhibitors, nalidixic acid and novobiocin. As shown in Fig. 4, ofloxacin inhibited the activity of VV topoisomerase, but the inhibition was less pronounced than that of novobiocin. Nalidixic acid, the parent compound of ofloxacin, did not inhibit VV topoisomerase. However, ofloxacin had no marked inhibitory effect on cellular topoisomerases I and II. Nalidixic acid strongly inhibited cellular topoisomerase II but not cellular topoisomerase I. Novobiocin markedly inhibited both types of cellular topoisomerase, as shown in Fig. 5.

Our results thus demonstrated that ofloxacin has some selectivity against vaccinia virus, and inhibits early nucleic acid syntheses, presumably by interfering with the viral topoisomerase.

TABLE 3

Anti-vaccinia activity of ofloxacin, administered by different routes, as assessed by tail-lesion test.

Treatment	Route of administration[a]	No. of lesions[b] (mean ± SD)	Inhibition (%)
Virus control		39.6 ± 7.9	-
Ofloxacin (10 mg/kg)	s.c.	32.3 ± 9.4	18
	i.m.	33.1 ± 8.8	17
	i.p.	30.9 ± 7.6	22
	i.v.	23.9 ± 6.9[c]	40
	p.o.	17.1 ± 8.7[c]	57

[a] Ofloxacin (10 mg/kg) was administered as a single dose by different routes (subcutaneously (s.c.), intramuscularly (i.m.), intraperitoneally (i.p.), intravenously (i.v.), or perorally (p.o.)) and, simultaneously, the mice were challenged intravenously with $10^{4.8}$ $TCID_{50}$ of vaccinia virus.

[b] The number of lesions formed on the tail were counted on the 7th day of infection.

[c] $P<0.05$ (Mann–Whitney U-test).

Discussion

The present study was aimed at investigating the activity of ofloxacin, a new quinolone derivative, against DNA and RNA viruses. The results show that ofloxacin possesses inhibitory activity against VV in cell culture and mice where it prevents formation of tail lesions. Moreover, characteristic of ofloxacin is that the oral route is the most effective of several administration routes evaluated (Table 3). Previous studies have established that orally administered ofloxacin is very well absorbed through the small intestine (Okazaki et al., 1984), not metabolized in the liver and retained for a long time in tissues (Sudo et al., 1984).

As to the anti-vaccinial mechanism of ofloxacin, our results indicate that the inhibitory effect of ofloxacin on DNA and RNA syntheses in VV-infected cells may be due to inhibition of viral topoisomerase. Ofloxacin preferentially inhibits viral topoisomerase, a key enzyme in the replication and transcription of viral genome; it is less, or not, inhibitory to cellular topoisomerase I and II. During the course of vaccinia infection the early enzymes involved in DNA metabolism must be synthesized from transcripts of the input viral genome. Gellert et al. (1977) and Higgins et al. (1978) reported that the biochemical target for the antibacterial activity of quinolone derivatives is the bacterial gyrase, an example of the type II topoisomerase class of enzymes. Since VV DNA is apparently a linear duplex with cross-linked termini, the molecules are subject to topological constraint. Although the biological function of VV topoisomerase is still open to question, a requirement for nicking–closing activity might well be exhibited by a spatially constrained system in vaccinia cores to relieve torsional stress for the initiation of DNA replication and transcription. Our results suggest that the principal site of ofloxacin action may be the VV topoisomerase.

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